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Sprouting as a biotechnological process to improve the functional properties of cereal-based products

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Gaetano Cardone
(R12153)

Advisor: Professor Alessandra Marti

Ph.D. Dean: Professor Antonella Pagliarini

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1. ABSTRACT

Although some studies showed that the use of flours from controlled sprouted grain can improve bread properties, the relationship between macromolecule functionality and bread features needs to be investigated, especially in the case of fiber-enriched products. In this context, the PhD project focused on the assessment of the sprouting effects on the functionality of the main macromolecules in grains (i.e., protein, starch, and fiber) and their impact on dough and bread-making properties.

In the introduction chapter (**Chapter 2**), a literature review has been carried out, where the main effects of sprouting on the nutritional profile of grains and their health benefits were summarized. Moreover, the scaling-up of the sprouting process from domestic to industrial level, the importance of monitoring processing parameters were addressed.

The first two sections (**Section 4.1** and **4.2**) of the Results and Discussion chapter assessed the effects of the sprouting process on technological/functionality of wholegrain flours from common wheat. In particular, wholegrain flours were obtained from single-stream milling (**Section 4.1**) and the recombination approach by including 20% bran to wheat flour (**Section 4.2**). Sprouting process caused a worsening in both gluten aggregation kinetics – suggesting a gluten weakening – and starch pasting and gelation properties. However, following the conditions obtained by the Farinograph test, it was possible to obtain a wholegrain bread with improved characteristics in terms of volume and crumb softness.

Considering the positive effect of sprouting on the bread-making performance of common wheat, the process was carried out on durum wheat (**Section 4.3**), with the aim of improving its gluten functionality for the production of durum wheat bread. For this reason, the effects of enzymatic activities developed during sprouting on durum wheat kernel characteristics,

starch and gluten behavior were investigated, as well as their relationship with the bread-making performance. Although the sprouting process caused a decrease in the gelatinization and retrogradation capacity of starch and gluten aggregation properties, bread with improved volume and crumb porosity was obtained. In particular, the best results were obtained using wheat sprouted up to 48 h.

The last part of the project focused on the sprouting of quinoa (**Section 4.4**) and sorghum (**Section 4.5**), whose consumption has been constantly increasing, since they are considered environmentally sustainable crops with high nutritional value. However, from the technological standpoint, the suitability of quinoa and sorghum for achieving good bread-making performance in wheat-based formulation still needs deep investigation. As regards enriched-bread, the presence of sprouted grains at 20% replacement level showed higher specific volume and lower crumb softness compared to control. In addition, in the case of sorghum, sprouting improved the *in vitro* protein digestibility.

In conclusion, this PhD project provides evidence of how protein and starch functionality is affected by both the type of grain used and sprouting duration. In addition, bread-making performance of wheat-based formulations can be enhanced by using sprouted grains or their main milling fractions (refined flour or bran), opening a new approach to the production of fiber-enriched baked goods.

2. INTRODUCTION & LITERATURE REVIEW

This chapter was partially published in: Marti, A., Cardone, G., Pagani, M.A., 2020. Sprouted cereal grains and products. In Pojić, M., Tiwari, U. (Eds.), Innovative Processing Technologies for Healthy Grains, John Wiley & Sons, New York, pp. 113-141.

2.1 Introduction

Enzymes have been used for years to improve the processing behavior of cereal and contribute to the quality of the final products (Fox and Mulvihill, 1982; Hamer, 1995; Kulp, 1993). Specifically in bread-making, exogenous enzymes represent a good strategy to improve the quality of bread in terms of dough handling, bread volume and crumb structure and softness (Poutanen, 1997). The following table shows the main enzymes exploited in bread-making, their substrate, mechanism of action, and the effect on dough and bread (Table 2.1).

In addition, these enzymes are used also as a substitute of chemical additives (e.g., bromate, sodium bisulphite), in order to have a clean-label product (Poutanen, 1997). On the other hand, many of these enzymes are present in low amounts in grains as endogenous enzymes. Their content can vary according to the cultivar considered and the climatic conditions during growth and harvesting. For instance, high humidity conditions following rainfall can trigger the synthesis of hydrolytic enzymes (e.g., amylase and protease), before the spike is harvested. This event is known as Pre-Harvest Sprouting (PHS) and over the years it had been exclusively associated with negative consequences, in terms of kernel properties (e.g., decreasing in grain yield and test weight) or macromolecule functionality (e.g., worsening of starch pasting and gelation, gluten aggregation). In the case of wheat, bread made of flour from pre-harvest sprouted wheat is unacceptable by consumers, since it is characterized by low volume, dark and non-regular crumb (Moot and

Every, 1990). Therefore, it is necessary to avoid the onset of PHS to avoid economic losses, problems during the raw material processing (e.g., milling) and quality decrement of both raw materials and finished products.

Table 2.1. Main enzyme classes, their substrates and their effects on dough and bread. (Adapted from van Oort, 2010).

Classes of enzymes	Substrate	Mechanism of action	Dough improvement	Bread improvement
α-amylases	Starch	Hydrolyze random α-1,4 bonds		- color and flavor - volume development - crumb structure and softness
Proteases	Protein	Hydrolyze peptide bonds	- gluten extensibility - water absorption - dough handling and machinability - gas retention	
Endo-xylanase	Arabinoxylans and hemicelluloses	not yet clearly elucidated	- gluten extensibility - gas retention	- crumb structure and softness - oven spring - volume development
Lipases	Lipids	Hydrolyzes triglycerides and alkyl esters	- gluten extensibility - dough strength and stability - machinability - gas retention	- flavor - oven spring - crumb structure and whiteness

Unlike PHS, several authors demonstrated that the use of flour from wheat partially sprouted under controlled conditions of time and temperature improved both volume and crumb texture of bread (Bellaio et al., 2013; Richter et al., 2014). These findings along with improvements in nutritional value (e.g., decreased amount of antinutrients in raw grains and legumes, increased bioavailability of vitamins and minerals, increased antioxidant compounds), flavor and taste of grains resulting from sprouting have led to increased interest in sprouting. A review of the scientific literature of the last 20 years using “sprouting/germination of pulses or cereals” as search terms resulted in the identification of about 1,416 scientific papers (Figure 2.1). On conducting a literature review, articles dealing with pre-harvest sprouting, which

negatively affect product functionality, were excluded. The number of contributions has doubled in the last 4 years, highlighting the growing interest in this topic (“sprouting/germination”).

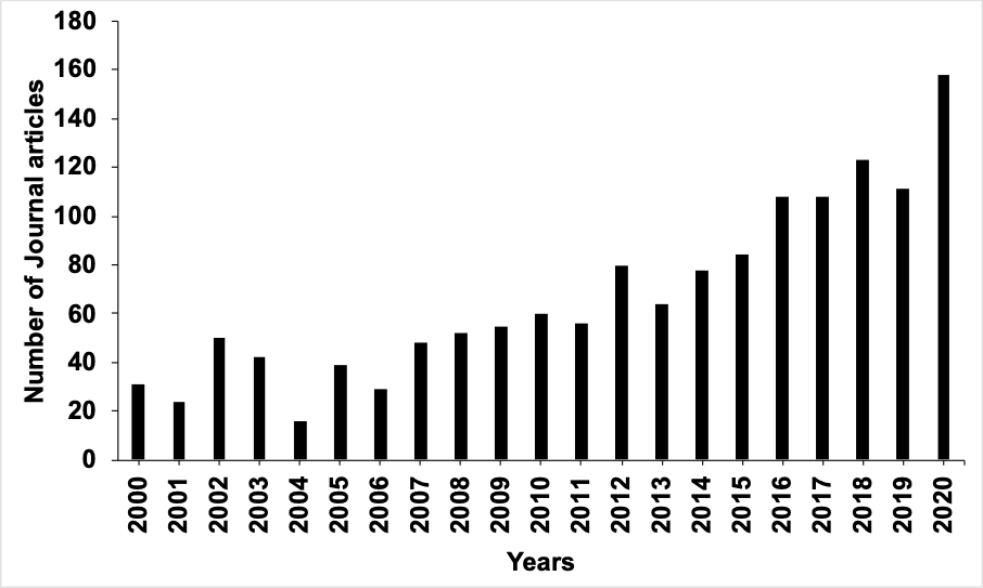

Figure 2.1. Number of scientific articles on sprouting (2000-2020) (December 2020; Source: Scopus; keywords: sprouting, germination, pulses, or cereals).

According to the report by trend agency Mintel, the number of products containing sprouted grains, which were launched on the market from 2008 to 2018, exploded with an annual growth rate of +23% (www.mintel.com). In particular, from January 2015 to April 2017, snacks represented 22% of all the products launched followed by meals (19%) and bakery products (15%) (Pagand et al., 2017). Health benefits, improvements in flavor and texture that sprouted grains bring to food products constitute the driving forces behind the growing interest in this product category.

2.2 Definition

In scientific literature, the term “sprouting” is often used loosely and sometimes incorrectly, and so it is important to clarify its meaning. The intent of the present paragraph is not to propose a definition, but to stimulate discussion with respect to the increasing interest in this topic.

Providing a generic definition of *sprouting* is quite difficult because the different actors along the chain (from physiologists to agronomists to seed testers, grain elevator technicians, food scientists and consumers) use different terms and meanings. The most common definition for this phenomenon is: “the process by which a plant grows from a seed” (Bewly and Black, 1994). According to physiologists, *sprouting* begins with water uptake by the seed (imbibition) and ends with the start of the elongation of the embryonic axis, usually the radicle. Therefore, germination promotes the *de-novo* synthesis of several hydrolytic enzymes necessary to depolymerize the cell-wall polysaccharides and mobilize the depolymerized storage macromolecules (Mares et al., 2016). Thus, germination does not include seedling growth, which commences when germination finishes (Bewly and Black, 1994). According to this definition, processes occurring in the nascent seedling, such as the mobilization of the major storage reserves, are also not part of germination; rather they are post-germination events. However, since breeders and agronomists are interested in monitoring the establishment of a vigorous plant of agronomic value, they refer to germination as seedling emergence from soil, even if germination ends sometime before the seedling is visible (Bewly and Black, 1994).

Much more difficult is to define germinated grains for the last link in the value chain of grains, i.e., food industries and consumers. Indeed, currently no regulated definition of “sprouted grain” is available. The Whole Grains Council (www.wholegrainscouncil.org) suggested to consumers who want to understand what they are eating, and companies who are considering manufacturing or marketing sprouted grains to start by reading how the

American Association of Cereal Chemists International (AACCI) defines sprouted grains. In early 2008, the AACC International Board approved the following statement regarding sprouted grains: "*Malted or sprouted grains containing all of the original bran, germ, and endosperm shall be considered whole-grains as long as sprout growth does not exceed kernel length and nutrient values have not diminished. These grains should be labeled as malted or sprouted whole-grain*" (https://www.aaccnet.org/initiatives/definitions/Pages/WholeGrain.aspx).

In addition to the lack of definition, the terms "sprouting", "germination" and "malting" are often used as synonyms, generating confusion in some cases. From a botanical standpoint, there is no difference between sprouting and germination, but for most people sprouting is the practice of germinating seeds to obtain sprouts to be eaten raw or cooked. The germination or sprouting process is somewhat like malting, which is used extensively in the brewing and distilling industries. However, in the malting process the rootlet emergence, called "chitting", occurs prior to the end of steeping (Pyler and Thomas, 2000) and the germination phase is typically allowed to proceed for 3-5 days, during which time approximately 40-50% of the proteins should have been solubilized and high levels of starch-degrading enzymes should have been released (Izydorczyk and Dexter, 2004).

Finally, the term "pre-harvest germination" or "pre-harvest sprouting" defines the biochemical changes that take place when cereals (mainly wheat) are exposed to prolonged wet or foggy conditions during their growth in the field. The consequent huge accumulation of hydrolytic enzymes (above all amylases) can impair the quantity and quality of wheat grains. For further details about the effects of pre-harvest sprouting on wheat kernel quality and bread-making performance, see the recent review by Olaerts and Courtin (2018).

2.3 Mechanisms of grain sprouting

After harvesting, sound grains are characterized by moisture values no higher than 14%, corresponding to water activity values less than 0.60 (at 25° C). In these conditions, grains are in a dormant/quiescent state as no depolymerization activities by hydrolytic enzymes have occurred (Delcour and Hoseney, 2010).

As mentioned earlier, the sprouting process begins with soaking or steeping the dry grains in water. During this phase, the water uptake is influenced by seed size, seed-coat permeability, quantity of available water, chemical composition of seeds and concentration of solutes in solution. For sprouting at a faster rate, most cereal seeds require 20-30° C as the optimal temperature range (Porter and Gawith, 1999). This depends on species, genetic differences, varietal variation, source of seeds and age. During this phase, an extensive physiological and biochemical process begins to support plant growth (Figure 2.2). Briefly, gibberellin hormone – that produces gibberellic acid – translocates from the embryo to the aleurone layer (1,2), promoting the synthesis and secretion of enzymes such as amylases and proteases to act on storage molecules in the endosperm, releasing simple sugars and amino acids, respectively (3). These products are used by embryo to support growth (4).

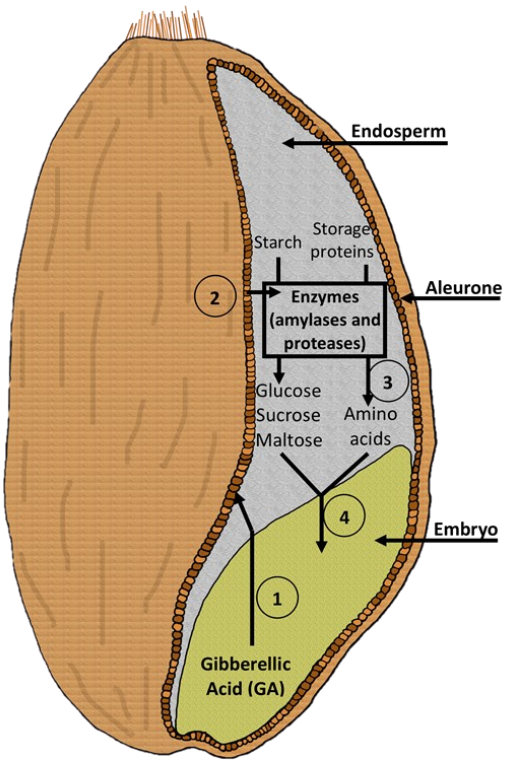

Figure 2.2. Sequence of main metabolic events during the sprouting process in the wheat kernel. 1,2) Release of gibberellic acid from embryo triggers the secretion of hydrolytic enzymes from aleurone layer and scutellum; 3) Amylases and proteases hydrolyze starch – to glucose, sucrose, and maltose – and proteins to amino acids; 4) These products are used by the embryo to support growth. (Adapted from Nelson et al., 2013).

α-amylases are endo-amylases and hydrolyses the α-(1,4)-linkages of starch, yielding soluble sugars and low molecular weight α-dextrins. β-amylases hydrolyze the α-(1,4)-linkages at the non-reducing ends of amylose and amylopectin molecules to produce β-maltose and β -limit dextrin (from amylopectin molecules). Sound wheat contains significant amounts of β-amylase but little α-amylase. However, the β-amylases have little action on undamaged, native starch granules, while their hydrolytic action is enhanced by that of α-amylase (Olaerts and Courtin, 2018).

Sprouting induces starch hydrolysis to yield simple sugars by the increased activity of amylases (Van Hung et al., 2011) while the extent of

starch degradation depends upon the length of sprouting (Lorenz and Valvano, 1981). Differences in the degree of starch degradation throughout the kernel were observed, with starch granules being more degraded near the aleurone layer and germ region, than in the inner endosperm (Faltermaier et al., 2015). Indeed, amylases are found principally in the pericarp layer and are responsible for the breakdown of starch during the early phases of development (Dedio et al., 1975). During sprouting, α-amylases are *de-novo* synthesized in the scutellum and aleurone. Linked to other seed proteins, β-amylases initially are only partially present in a free or soluble form, while during sprouting, they are progressively released in a soluble form, presumably due to proteases secreted by the aleurone and/or scutellum (Ziegler, 1995).

As the starch is degraded by amylases, increased sucrose occurs during early wheat sprouting with glucose and maltose predominating during later sprouting stages (Aoki et al., 2006).

Sprouting also induces the accumulation of proteolytic enzymes that catalyze the hydrolysis of peptide bonds in proteins. Proteases can be subdivided into two major groups according to their site of action: exopeptidases and endopeptidases. Exopeptidases, also referred to as proteinase, cleave the peptide bond proximal to the amino or carboxyl termini of the substrate, whereas endopeptidases cleave peptide bonds distant from the end of the substrate (Miguel et al., 2013).

Storage proteins such as globulins, prolamins, and glutelins after 2-3 days from the beginning of imbibition undergo varying degrees of proteolysis that frees stored nitrogen and carbon for the growing plant during sprouting (Muntz, 1996). In addition, endopeptidases might induce conformational changes that subsequently facilitate further breakdown by both endo- and exopeptidases (Muntz, 1996).

Conflicting results have been reported for the effect of sprouting on protein content (Nelson et al., 2013). Some studies reported the decrease in protein content associated with the hydrolytic action of proteases (Koehler et

al., 2007), while others reported the increase in protein content in whole-grain flours during sprouting associated with the loss of dry matter, mainly in the form of carbohydrates (Lorenz et al., 1981) or to the synthesis of enzymes during sprouting (Bau et al., 1997). However, a turnover of protein and non-protein nitrogen resulting in equilibrium of the degradation and synthesis processes during sprouting may account for the lack of changes (Bau et al., 1997). Thus, the effects of sprouting depend on seeds from different plant species, varieties, or cultivars as well as variations in sprouting conditions (temperature, light, moisture and time of sprouting) (Yang et al., 2001).

The products released by starch and protein hydrolysis (i.e., soluble sugars and amino acids) are then absorbed by the germ, thereby transitioning the grain from dormancy to active metabolism (Figure 2.2).

Such biochemical changes deeply affect the native grain composition to support the growth of a young plant, thus altering the nutritional (Danisova et al., 1994) and physicochemical (Noda et al., 2004) properties of grains.

2.4 Nutritional profile of sprouted cereal grains and their health benefits

Sprouting has been carried out for millennia to improve the nutritional properties of cereals and pulses. Most studies dealing with sprouting have focused on how sprouting affects the specific cereal components having positive or negative nutritional effects, as recently reviewed by several authors (Hübner and Arendt, 2013; Singh et al. 2015; Benincasa et al., 2019; Lemmens et al., 2019). A comprehensive review of current research leads to a general conclusion: caution must be applied when comparing the results of different studies, since the types and varieties of grains, soaking conditions (e.g., water quality, soaking time and temperature), sprouting conditions (e.g., duration and temperature), and measurements methods differ from one study to another. In addition, optimal sprouting conditions may vary for grain type and the compound of interest. Indeed, it has been reported that the concentration of the same compound may be ascribed as increased,

decreased, or unchanged within the same type of grain or for different grains (Nelson et al., 2013). Moreover, many nutrients and bioactive compounds increase and then decrease as they are utilized by the growing plant (Yang et al., 2001), making the sprouting time an important variable. In some cases, the increase in nutrients could be due to the loss of starch during sprouting, which results in a decrease with respect to the weight of grain, and thus in an increase of the other macromolecules (Van Hung et al., 2015).

Benincasa et al. (2019) and Lemmens et al. (2019) recently provided a comprehensive review of the chemical and health effects of sprouting on both cereals and pseudocereals. Most of the literature selected by the authors focused on wheat, which is the most common cereal for bread and baked products. The great attention paid to the nutritional effects of sprouted barley is probably due to the brewing industry's interest in malt (Hübner and Arendt, 2013). Likewise, sprouted brown rice also gained interest due to its high level of γ-aminobutyric acid (GABA) content (Patil and Khan, 2011). According to the authors, the amount of GABA in sprouted brown rice was doubled as compared to that of unsprouted brown rice. They also observed that soaking for 3 h and sprouting for 21 h proved to be the optimum method for reaching the highest GABA content in sprouted brown rice. Cáceres et al. (2014) identified the optimal time-temperature combination to be used during the soaking and sprouting process of brown rice through the use of advanced statistical techniques (e.g., response surface methodology). The highest GABA production and antioxidant activity in brown rice was reached when grains were soaked for 24 h at 28°C and sprouted at 34°C for 96 h. The numerous health benefits of GABA are the main reason for the popularity of sprouted brown rice. In this context, Okada et al. (2000) reported that a diet including GABA lowered blood pressure and decreased insomnia, and autonomic disorders observed during the menopausal or presenile periods. Similarly, Spanier et al. (2000) also showed that brown rice sprouts contain a potent inhibitor of the enzyme called protylendopetidase, which is involved in Alzheimer’s disease. In addition, the increase in GABA content during

sprouting was reported also in wheat and barley (Chung et al., 2009; Hung et al., 2012). However, GABA production in sprouted grains depends on both environmental (e.g., temperature, abiotic stresses) and soaking (e.g., temperature, time) conditions (Benincasa et al., 2019).

Unexpectedly, very few studies addressed the health benefits of sprouted sorghum and millet, although sprouting is commonly used in African countries to improve the protein digestibility of these less common cereals thanks to the degradation of storage proteins that become more easily available for pepsin hydrolysis (Sehgal and Kawatra, 2001). Among the various mechanisms proposed to explain the above-mentioned health benefits of sprouted sorghum and millet (Annor et al., 2017), the decrease in anti-nutrients such as phytic acid, tannins and other phenolics, as well as protease inhibitors, are also occurring (Sehgal and Kawatra, 2001).

The main effects of cereal sprouting, its mechanism effect, potential health benefits and the impact on product quality are summarized in Table 2.2. As discussed above (see paragraph 2.2), starch and protein are hydrolyzed during sprouting by the action of amylases and proteases, respectively.

Table 2.2. Effect, cause, and potential health benefits of cereal sprouting on selected nutrients.

Component		**Cereal**	**Effect ***	**Cause**	**Potential health effects**
Carbohydrate	Starch	Wheat	Decreased (> 1 day @ 25 °C, Swieca et al., 2017) (3 days @ 20 °C; Marti et al., 2017) (2 days @ 24 °C; Marti et al., 2018)	Hydrolysis by α-amylases	- Decrease in starch digestibility (Świeca et al., 2017) - Increase in slowly digestible starch fraction (Marti et al., 2018)
		Brown rice	Decreased (3 days @ 35 °C; Xu et al., 2012)		
		Barley	Decreased (4 days @ 17 °C; Vinje et al., 2015)		
		Oat	Decreased (6 days @ 16 °C; Peterson, 1998)		
	Sugars	Wheat	Increased (4 days @ 28 °C; Sibian et al., 2017) (3 days @ 20 °C; Marti et al., 2017) (2 days @ 24 °C; Marti et al., 2018)	Starch hydrolysis by α-amylases with production of simple sugars and oligosaccharides	- Decrease in postprandial glucose (Andersen et al., 2008; Mofidi et al., 2012) - Prebiotic effect (Hübner and Arendt, 2013)
	Total Fiber	Wheat	Decreased (4 days @ 15-20°C; Koehler et al., 2007)	Loss of starch	Delay in the increase of glucose in the blood (Anderson et al., 2004)
			Increased (2 days @ 15-20 °C; Koehler et al., 2007)	Hydrolysis by pentosanases and xylanases	
		Barley	Not change (3 days @ 15 °C; Teixeira et al., 2016)	-	

	Soluble Fiber	Wheat	Increased (> 4 days @ 20°C; Koehler et al., 2007) (> 6 h @ 30°C; Van Hung et al., 2015)	Hydrolysis by pentosanases and xylanases	
	Insoluble Fiber	Wheat	Decreased (> 7 days @ 20°C; Koehler et al., 2007) (> 6 h @ 30°C; Van Hung et al., 2015)	-	
Protein		Wheat	Decreased (> 2 days @ 25 °C; Koehler et al., 2007) (> 2 days @ 20-25 °C; Świeca et al., 2015)	Hydrolysis by proteases or leaching of water-soluble peptides during steeping	Decrease in gluten sensitivity (Hartman et al., 2006)
		Sorghum	Decreased (2 days @ 25°C; Elmaki et al.,1999)		
		Brown rice	(5 days @ 20 °C; Mohan et al., 2010)		
		Wheat	Increased (5 days @ 16.5 °C; Donkor et al., 2012) (> 1 days @ 30 °C; Van Hung et al., 2015) (> 4 days @ 28 °C; Sibian et al., 2017)	Decrease in starch content (Van Hung et al. 2015)	-
		Barley	Increased (5 days @ 16.5 °C; Donkor et al., 2012)		
		Oat	Increased (5 days @ 16.5 °C; Donkor et al., 2012) (3 days @ 17 °C; Teixeira et al., 2016)		
		Brown rice	Increased (5 days @ 16.5 °C; Donkor et al., 2012)		
		Wheat	Not change (3 days @ 25 °C; Zambiazi da Silva et al., 2014) (< 1 days @ 30 °C; Van Hung et al., 2015)	-	-

Lipid	Wheat	Decreased (5 days @ 16.5°C; Donkor et al., 2012) (2 days @ 30 °C; Van Hung et al., 2011)	Hydrolysis by lipases	Improvement of plasma free fatty acids (Andersen et al., 2008)
	Barley	Decreased (5 days @ 22 °C; Chung et al., 1989)		
	Oat	Decreased (6 days @ 16 °C; Peterson, 1998)		
	Brown rice	Decreased (1-5 days @ 25-30 °C; Watanabe et al., 2004)		
	Wheat	Increased (> 12 h @ 30°C; Van Hung et al., 2015)	Following the decrease in the content of starch (Lorenz et al., 1981; Van Hung et al., 2015)	-
	Wheat	Not change (48 h @ 30°C; Van Hung et al., 2011)	-	-
Total polyphenols	Wheat	Increased (2 days @ 30°C; Van Hung et al., 2011)	Hydrolysis by phenol oxidases and peroxidases (Barron et al., 2007)	Decrease in plasma polyphenols and antioxidants measured *in vivo* (Andersen et al., 2008)
	Barley	Increased (>1 day @ 28 °C; Ha et al., 2016)		
	Sorghum	Increased (2 days @ 20 °C; Hithamani and Srinivasan, 2014)		
	Brown rice	Increased (4 days @ 34 °C; Cáceres et al., 2014)		
Phytate	Wheat	Decreased (3 days @ 20 °C; Bartnik and Szafrańska, 1987)	Hydrolysis by phytases (Larsson	Increase in bio-accessibility of vitamins and minerals

	Barley	Decreased (3 days @ 20 °C; Bartnik and Szafrańska, 1987) (3 days @ 22 °C; Centeno et al., 2001)	and Sandberg, 1992)	
	Brown rice	Decreased (4 days @ 34 °C; Cáceres et al., 2014)		
	Oat	Decreased (6 days @ 10 °C, Hübner et al., 2010)		
Vitamin	Wheat	Decreased (vit. E) (4 days @ 28°C; Plaza et al., 2003)	-	-
		Increased (vit. E) (7 days @ 16.5 °C; Yang et al., 2001)	-	-
	Barley	Not change (vit. E) (4 days @ 15 °C; Haraldsson et al., 2004)	-	-
	Brown rice	Not change (vit. E) (1 days @ 28 °C; Watanabe et al., 2004)	-	-
		Increased (vit. E) (3 days @ 30 °C; Mohd. Esa et al., 2011)	-	-
	Wheat	Increased (vit. A, B1, B2, B6, C) (4 days @ 28°C; Plaza et al., 2003)	-	-
	Sorghum	Increased (vit. B1, B2, C) (3-4 days @ 25 °C; Asiedu et al., 1993)	-	-
	Rice	Increased (vit. B1, B2)	-	-

		(3-4 days @ 25 °C; Trachoo et al., 2006)		
		Decreased or not change (B1, B2, B3, B9) (1 day @ 28 °C; Watanabe et al., 2004)	-	-
Minerals	Wheat	Decreased (Fe; 3 days @ 25 °C; Zambiazi da Silva et al., 2014) (Ca, Mn, Na; 4 days @ 28°C; Plaza et al., 2003) (Mn; Pongrac et al., 2017)	-	-
	Sorghum	Decreased (Fe, Zn; 3 days @ 20 °C; Afify et al., 2011)	-	-
	Wheat	Increased (Na, P, S, Cl, K, Ca, Fe, Cu, Zn; Pongrac et al., 2017) (Cu, K, Mg, Z; 4 days @ 28°C; Plaza et al., 2003)	Hydrolysis by phytases (Larsson and Sandberg, 1992)	-
	Rice	Increased (Fe, Zn; 2 days @ 20 °C; Platel et al., 2010)		
	Barley	Increased (Fe, Zn; 2 days @ 20 °C; Platel et al., 2010)		
	Wheat	Not change (Fe; 4 days @ 28°C; Plaza et al., 2003)	-	-
	Brown rice	Not change (Zn; 3 days @ 30°C; Liang et al., 2008)		

*The sprouting conditions are reported in the brackets

The occurrence of starch degradation and the release of oligosaccharides and simple sugars suggest an increase in both starch digestibility and postprandial levels of blood glucose. On the contrary, Marti et al. (2018) showed that the amount of rapid (RDS) and slow (SDS) digestible starch in bread decreased and increased, as the percentage of sprouted wheat flour increased from 50% to 100%, respectively. The data of this study were obtained by *in-vitro* studies and are in partial agreement with those reported by Świeca et al. (2017), who attributed the decrease in starch digestibility of sprouted wheat-enriched bread to an increase in the aliquot of resistant starch and/or to the high phenolic content of sprouted wheat (Świeca et al., 2017).

Considering that the glycemic response appears to be directly related to the amount of RDS, while insulin demand is inversely correlated to the SDS fraction (Garsetti et al., 2005), data on sprouted wheat bread favor the use of sprouted wheat in new bread formulations aiming to reduce postprandial levels of blood glucose. Indeed, *in-vivo* studies on bread enriched with sprouted wheat reported favorable blood glucose effects compared with unsprouted wheat bread (Andersen et al., 2008), by reducing the glycaemic response to sprouted grain bread in both healthy subjects (Andersen et al., 2008) and in overweight or obese men (Mofidi et al., 2012). However, the above studies show that health benefits are not solely dependent on the sprouting process, but other factors as well. Therefore, further research would be required for assessing the beneficial health effects of sprouting and clarifying whether the potential health benefits of bread enriched in sprouted wheat can be really attributed to sprouting. Indeed, none of the above-cited studies investigated the characteristics of bread made from the same wheat variety before and after sprouting.

In regard to protein, protease activities result in an increase in peptides and/or free amino acids, including the essential amino acids. For example, lysine content - limiting amino acid in wheat - increased by 10% after sprouting (Sibian et al., 2017). An increase in digestibility of proteins has been observed

in sprouted wheat, in particular after 48 h of sprouting at 20° C, whereas after 96 h at 25° C it decreased (Świeca and Dziki, 2015). This decrease might be due to the accumulation of phenolic compounds. Indeed, there is evidence that the interactions between the phenolic compounds and the digestive enzymes and/or the food matrix could decrease, directly or indirectly, the bioavailability of nutrients (Świeca et al., 2013). Studies show that the gluten decreases in sprouted wheat (Koehler et al., 2007), likewise the proteases are responsible for the degradation of gliadin peptides (Hartmann et al., 2006). Thus, people suffering from gluten sensitivity may benefit from this kind of product. In the case of sorghum (Elkhalifa et al., 2010; Afify et al., 2012) and barley (Chung et al., 1989), the sprouting process led to an increase in protein digestibility by about 15%, when carried out for 72 to 144 h at 22 to 27° C.

The sprouting process also affects total lipids, resulting in an overall increase because of free lipids (Van Hung et al., 2015). On the contrary, bound lipids did not change, regardless of the sprouting time. Furthermore, the sprouting process does not seem to make any changes in the fatty acid composition (Van Hung et al., 2011; Van Hung et al., 2015). Lipid hydrolysis during wheat sprouting should be further investigated due to its potential role in affecting starch digestibility in other cereals (Annor et al., 2017).

The effect of sprouting on total fiber content is still unclear, with conflicting results available in the literature and strictly dependent on sprouting conditions (e.g., time and temperature) and grain genotypes (Nelson et al., 2013). In wheat, fiber changes are more pronounced when sprouting occurs at 15-20° C than at higher temperatures (25-30° C) (Koehler et al., 2007). At higher temperatures a distinct increase in total dietary fiber concentration (more than 25%) was found at prolonged time points (e.g., 102 h) (Koehler et al., 2007). Similar results were also observed in sprouted brown rice (Ohtsubo et al., 2005). Instead, no changes in total fiber content were found when barley was sprouted up to 72 h (Teixeira et al., 2016). Concerning fiber solubility, insoluble fiber generally decreased in wheat by 50% (Koehler et al., 2007), whereas soluble fiber values reported for wheat remained constant up to 96

h, and then increased steadily to 168 h (Koehler et al., 2007), likely due to the increase in cell-wall degrading enzymes, including endoxylanases (Olaerts and Courtin, 2018). This aspect is of great interest from a nutritional point of view as soluble dietary fiber has been associated with certain health benefits such as the maintenance of normal blood cholesterol levels due to its ability to form viscous solutions in the intestine (Kumar et al., 2012).

Increased polyphenol content (from 1.2 to 3.6-fold) has been reported in several *in-vitro* studies, carried out on wheat (Van Hung et al., 2011; Zilić et al., 2014; Świeca and Dziki, 2015; Van Hung et al., 2015), barley (Ha et al., 2016), sorghum (Hithamani and Srinivasan, 2014), and brown rice (Cáceres et al., 2014; Cornejo et al., 2015), when they were sprouted from 48 to 144 h at 15 to 28° C. Furthermore, an increase in free phenolics at the expense of bound phenolics has also been reported (Van Hung et al., 2011). Besides serving as antioxidants and protecting against lipid oxidation and the development of off-flavors (Maillard et al., 1996), increasing the intake of phenolic compounds can have positive health effects due to anti-carcinogenic, antioxidant, and anti-inflammatory effects for some of the compounds investigated (Santos-Buelga and Scalbert, 2000). Further studies are warranted to explore the transition from *in-vitro* findings to *in-vivo* effects (Nelson et al., 2013).

Results on the effects of the sprouting process on vitamins and minerals are conflicting. Most studies found an improvement of vitamin value as a result of sprouting (Yang et al., 2001; Plaza et al. 2003). However, drying conditions, especially if high drying temperatures are applied, may affect the loss of some vitamins.

Mineral content in sprouted cereals showed inconsistent results as well, depending on the applied methods of soaking and sprouting. During the process some minerals are leached or absorbed by the hydrating and germinating seeds (Finney, 1982). In general, if high mineral-containing water is used to steep and germinate, sprouted seeds will increase their uptake of

total minerals and ash with increasing sprouting. Conversely, if distilled water is used, minerals will invariably leach out (Omary et al., 2012).

The accessibility of minerals is the result of the interactions of many factors – such as the type of mineral, the composition and structure of grains and the processes used (Erba et al., 2018). Sprouting is generally reported as a process that improves mineral accessibility in grains by reducing anti-nutritional factors, including phytates, which form insoluble complexes not only with minerals, but also with proteins, carbohydrates, and lipids, thus reducing their bioavailability (Kumar et al., 2010).

Additionally, Hubner and Arendt (2013) suggested a potential prebiotic effect of sprouted grains as substrates for the growth of probiotic bacteria, providing high levels of fermentable sugars.

Despite the nutritional enhancement associated with sprouting, up to now, sprouted grains have typically been used in relatively small amounts when incorporated into bakery products. Thus, both industries and consumers should be aware that they are not going to reap all of the nutritional benefits.

2.5 From traditional to an industrial process

Sprouting of seeds for consumption has been practiced for thousands of years to improve their technological and nutritional properties. For example, sorghum and millet are commonly sprouted for improving their protein digestibility (Elmaki et al., 1999; Annor et al., 2017); whereas pulses are sprouted both to decrease anti-nutritional factors (Soetan and Oyewole, 2009) and to facilitate the dehulling and cooking process (Bellaio et al., 2011, Zamprogna et al., 2011). The basic sprouting process (Figure 2.3) consists of steeping grains in water for 8-24 h, until they reach the moisture content needed to start the growth of the seedling (30%) (Bewley and Black, 1985), after which the steeping water is drained, and the grains are left to germinate under controlled conditions. Aeration and mixing are advisable in this phase to allow the grain to use the nutrients to germinate and sprout. On the other

hand, time and temperature determine the retention of enzymatic activity and the development of color and flavor compounds. The sprouted grains are then consumed in the form of sprouts or further processed (i.e., dried or roasted).

Traditionally, the sprouting process has been conducted at home, neglecting the potential grain safety risk raised with regard to microbial growth that may be associated with the uncontrolled process. Controlling the process seems to be the unique way of decreasing the safety risk while preserving the nutritional and technological benefits of the product.

With the exception of the malting process, most of the studies are conducted on a laboratory scale, where the sprouting is carried out in thermostatic cells for a variable number of days, often without controlling relative humidity. Since both the mechanisms of grain sprouting and the related changes in chemicals are strongly dependent on temperature and relative humidity (apart from time and type of grains), an understanding of the phenomenon, a comparison of results, and the repeatability and reproducibility of the experiments is quite difficult to obtain.

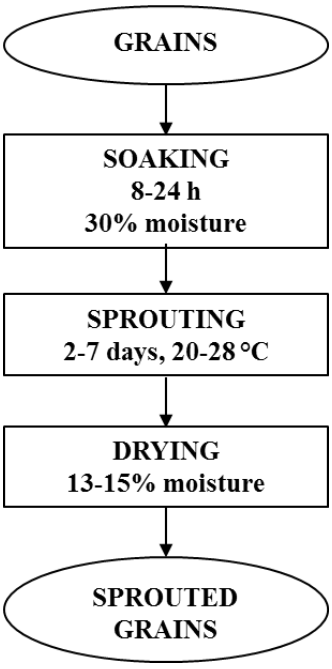

Figure 2.3. Flow-sheet of the controlled sprouting process.

After sprouting, seeds are stabilized by drying with hot air to extend their shelf-life. The thermal treatment stops the sprouting and lowers the moisture content from 30-50% to less than 12%. The drying temperature must be chosen carefully to keep the enzymatic pool developed during sprouting active and to improve the technological performance of flours. Nevertheless, the sprouted grains can be toasted to create additional flavors. Besides guaranteeing safety, the monitoring and controlling of the sprouting conditions standardize the nutritional and sensory properties of the final product, thus ensuring consistency in quality.

2.6 Utilization of sprouted cereal grains in different food products

Sprouting is of fundamental importance for the food industry. It has been applied for millennia to pulses to reduce their anti-nutritional components, such as trypsin inhibitors and phytic acid. At the same time, sprouting decreases the digestive discomfort caused by ROFs (raffinose family of oligosaccharides), while developing sweet taste notes.

In addition to the nutritional aspects listed above, sprouting affects the technological performance of grains and related flours. Surprisingly, sprouting facilitates the dehulling and cooking process of grains (Bellaio et al., 2011; Zamprogna Rosenfeld et al., 2011). This is of great interest since it would facilitate the consumption of wholegrains. Indeed, even while reducing the risk of cardiovascular disease and inflammation, the eating of grains is not so common in many countries, due to their long cooking times and bitter and pungent flavor notes (Heiniö et al., 2016). In the case of brown rice, sprouted kernels require less time for cooking and from the sensory standpoint taste sweeter and softer than regular brown rice (Patil and Khan, 2011).

Malt from barley is another example of using sprouting in the food industry. It is a special form of limited sprouting aimed at producing enzymes, which hydrolyze starch to make sugars available for fermentation. Although amylolytic enzymes are of prime importance, other enzymes play a key role

in the production of flavor compounds, contributing to the quality of the malt (Mäkinen and Arendt, 2015). However, reserve mobilization should not proceed to completion, to avoid losing potentially fermentable materials. This characteristic can be described by the diastatic power that measures the combination effect of starch-degrading enzymes. Low levels of hydrolytic enzymes create problems for fermentability and high wort viscosities (Mäkinen and Arendt, 2015). Other factors, such as complex cell-wall polysaccharides, which are not readily hydrolyzed, may also contribute to poor brewing performances (Mäkinen and Arendt, 2015). Although barley is the main malting cereal worldwide, other cereals, including sorghum, millets, and pseudocereals, can also be used (Hager et al., 2014). Malts produced from pseudocereals and some tropical grains can have low levels of amylolytic activity and often these malts have poor brewing performances, thus requiring exogenous enzymes when brewing (Hager et al., 2014).

In addition to brewing, barley and wheat malt are used to optimize and standardize the α-amylase levels in wheat flour, or as sources of color and flavor (Kulp, 1993). The amylase content in wheat flour can be variable, and low α-amylase activity results in low bread volume and quality. Thus, adding α-amylases or malt to the flour increases loaf volume and crumb softness during storage (Kulp, 1993). On the other hand, an excessive concentration of amylases results in sticky dough, which is difficult to handle, and in bread with irregular crumb structure (McCleary and Sturgeon, 2002).

Marti et al. (2017) explored the enzymatic activities developed during sprouting in bread-making, with the aim of decreasing or substituting the use of commercial enzymes, such as flour improvers that are commonly present in the formulation of baked products. The study incorporated 0.5% of sprouted wheat to a stiff refined flour, as an alternative to conventional flour improvers (i.e., malt, proteases, and an enzymatic improver based on xylanases). Small amounts of sprouted wheat flour were effective in increasing bread specific volume and crumb softness. Moreover, for up to 3 days of storage, sprouted wheat flour showed an effect similar to malt in lowering the staling process in

bread. The authors concluded that flour from sprouted wheat is a promising and interesting ingredient for formulating baked products, as it eliminates the need for enzymatic improvers, which is a plus for consumer acceptance and facilitates the adoption of clean labels (Marti et al., 2017). It is logical that such small amounts of sprouted wheat only play a technological role, with no nutritional benefits. At the same time, the amylases and proteases activities induced by sprouting – if excessive - negatively affect the technological performances of wheat (Morris and Rose, 1996), which then becomes unsuitable for baked foods, as shown in Figure 2.4.

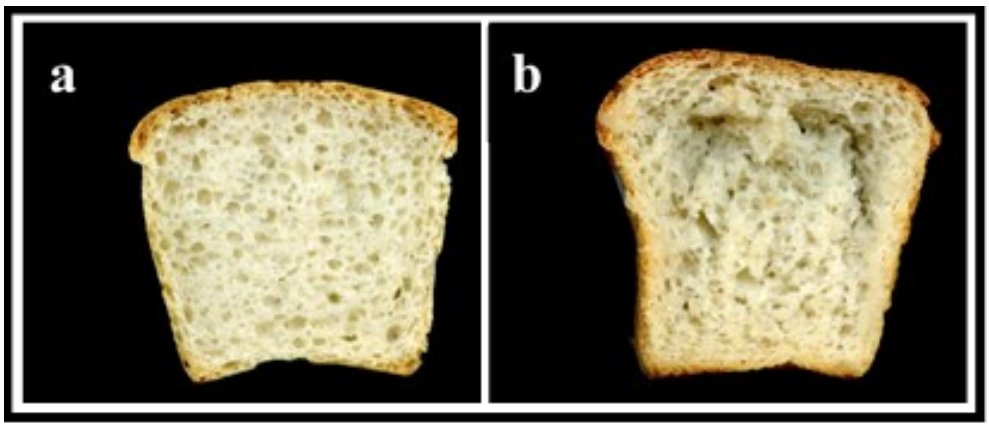

Figure 2.4. Slice of bread made with flour from unsprouted wheat (a) and sprouted wheat characterized by excessive amylase and protease activities (b).

This might occur directly in the field (i.e., pre-harvest sprouting) or when the sprouting is carried out under severe and uncontrolled conditions, whereby starch and protein are excessively hydrolyzed. This seems to be the case in most of the studies that highlighted decreases in bread volume when up to 20% of sprouted brown rice was added to wheat flour (Watanabe et al., 2004). Despite that, beneficial effects were more obvious for sprouted grain-enriched products rather than for unsprouted ones. Although the enzymatic activities of sprouted grains were not assessed by Watanabe et al. (2004), it is reasonable to assume that the processing conditions adopted by previous studies allowed high concentrations of hydrolytic enzymes to accumulate,

negatively affecting the technological performance of flour and making it unsuitable for baked foods. Both amylases and xylanases are responsible for dough stickiness. On one hand, high levels of α-amylases extensively degrade damaged starch during dough mixing and fermentation, generating high levels of sugars and dextrins, and releasing water that was previously bound by the starch (MacArthur et al., 1981). On the other hand, an excess of endoxylanases can cause extensive degradation of the arabinoxylans, releasing the water that was previously bound to them (Courtin et al., 2001).

In regard to protein, a marked decrease in insoluble residue protein in sprouted wheat samples has already been shown to compromise the baking performance of sprouted wheat (Koehler et al., 2007; Simsek et al., 2014). Moreover, the breakdown of gluten proteins by peptidases occurs even during dough processing. Experiments with the Farinograph (Brabender GmbH & Co., Duisburg, Germany) and Mixograph (National Manufacturing, Lincoln, USA) showed a reduction in dough development time and stability and in water absorption of dough as controlled sprouting time increases (Sekhon et al., 1995).

On the other hand, wheat sprouting carried out for 48 h at 20° C promoted a limited accumulation of proteases, so that gluten was still able to aggregate and form a network with good bread-making performance (Marti et al., 2017, 2018). At the same time, both pasting, and gelation properties of starch were not affected when sprouting was carried out for up to 72 h (Grassi et al., 2018). However, there is the risk that the amylases synthesized during sprouting could be activated during baking, thus promoting strong starch degradation that might negatively affect bread crumb structure and crust color.

By limiting both starch and gluten degradation, bread from 100% sprouted wheat can be made (Marti et al., 2018; Richter et al., 2014). The resulting dough was not sticky and required less time for leavening, resulting in a final product characterized by higher volume than control bread (Marti et al., 2018; Richter et al., 2014). Higher loaf volume is mostly explained by the greater CO_2 production due to increased amounts of fermentable sugars

released by higher α-amylase activity (Van der Maarel, 2009). In addition, bread from sprouted wheat was able to keep a soft crumb texture for up to 6 days of storage (Marti et al., 2018).

In addition to bread-making performance, sprouting influences the sensory properties of grains, giving them a typical flavor and odor generally perceived as pleasant. During sprouting, reducing sugars and amino acids are released, which subsequently react during heating, giving rise to Maillard reaction products (Hefni and Witthöft. 2011). Moreover, both sprouting and drying decrease the musty and earthy odor notes, favoring the perception of roasted, nutty, and intense flavor notes (Heiniö et al., 2001) and masking the typical bitterness of quinoa seeds (Suárez-Estrella et al., 2021) and whole-grain bread (Richter et al., 2014).

2.7 Monitoring of seed sprouting

The attempt to optimize the sprouting time leads to another issue, since no universally useful biochemical marker of the progress of sprouting has been found (Bewley and Black, 1994). The only stage of sprouting that we can determine precisely is its termination. The emergence of the radicle from the seed is normally used to define when sprouting has been completed. In this context, some companies and/or researchers use the radicle emergence as a marker, but this approach is often based on their own experience rather than on a scientific and systematic approach. Moreover, evidence of the sprouting process may occur in a seed where radical emergence does not occur. In cases where the radicle is not evident, different methods have been proposed, and their strengths and weaknesses are summarized in Table 2.3. These methods are based on the direct or indirect quantification of enzymes (mostly α-amylases) present in cereals such as wheat and barley. The presence of high α-amylase activity in wheat is generally associated with pre-harvest sprouting that also promotes the *de-novo* synthesis of proteolytic enzymes, which critically impair the commercial

quality of grains (Olaerts and Courtin, 2018). Among them, the Falling Number (FN) and the Stirring Number (SN) are the most used to measure the effect of amylase activity on flour quality. Neither test measures α-amylase activity directly but indirectly by quantifying the viscosity of the starch hydrolyzed by the enzymes during the test. They have been proposed as simple and rapid techniques and are performed according to international standards (AACC 56-81.03 and ICC 107-1 for the FN; AACC Method 22-08.01 and ICC 161 for the SN). The optimal value is 250 s; with FN < 250 s, the dough looks sticky while a FN > 300 s corresponds to some flour with almost no amylase activity (www.perten.com). Generally, increasing levels of α-amylase result in a decrease in FN down to 60 s, beyond which further increases in activity cannot be measured (MacArthur et al., 1981). A low FN value is generally associated with pre-harvest sprouting.

The SN is defined as the apparent viscosity in Rapid Visco Units (RVU) after 180 s of stirring a hot aqueous flour suspension undergoing liquefaction in the Rapid Visco Analyzer® (RVA, PerkinElmer, Inc., Spokane, USA). Due to the action of the hydrolytic α-amylase, viscosity decreases, and the stirring number decreases as well (Figure 2.5).

However, the limitation of FN and SN methods has turned out to be a reduced sensitivity to low levels of α-amylase activity, due to rapid heating during the analysis (Ross and Bettge, 2009). Despite that, this is a widely used method for wheat grading.

Similar to RVA, the presence of sprouting can also be detected using the Amylograph® (Brabender GmbH & Co., Duisburg, Germany), by measuring the activity of α-amylase in an aqueous suspension of flour while it gelatinizes during heating. The peak viscosity is inversely correlated to the integrity of the starch granules. When enzymatically weakened, starch granules lose their resistance to swelling; these structural changes result in the lowering of the paste viscosity of the sprouted grains (Simsek et al., 2014).

Although widely used to detect pre-harvest sprouting in wheat kernels, the FN and SN seem to overestimate the extent of starch hydrolysis in sprouted wheat under controlled conditions (Grassi et al., 2018). Indeed, running the test in the presence of an amylase inhibitor (i.e., $AgNO_3$) highlighted those changes in viscosity were caused by α-amylase activities during analysis and not by inherent changes in starch swelling, pasting, and gelation properties (Grassi et al., 2018). Hence, starch in sprouted wheat with a FN lower than 250 s is still of a good quality (Grassi et al., 2018).

Table 2.3. Main useful approaches for characterizing flour from sprouted wheat.

Index	Official method	Device	Principle of the method	Advantage	Disadvantage
Falling Number	AACC 56-81.03; ICC-standard 107/01	Falling Number system 1500 (PerkinElmer)	Indirect evaluation of α-amylases by evaluating the time of a plunger falling into a flour and water gel	- Rapid test (5-10 min) - Low influence of the analyst - Low sample size	- Not effective in determining the effect of low levels of α-amylase
Stirring Number	AACC 22-08.01; ICC-standard 161	Rapid Viscoanalyzer (RVA-4500, PerkinElmer)	Indirect evaluation of α-amylases by measuring the viscosity of a flour/water suspension during rapid gelatinization	- Rapid test (3 min) - Low influence of the analyst - Low sample size	- Not effective in determining the effect of low levels of α-amylase
α-amylase	AACC 22-02.01; ICC-standard 303	Enzymatic kit (Megazyme K-CERA 02/17)	Extraction of α-amylases from the flour made to act on a p-nitrophenyl-malto-heptoside substrate (BPNPG7), which possesses the non-reducing end blocked by the p-nitrophenol reactive chromophore	- Very low amount of sample (100 mg)	- Possibility of non-homogeneous sampling Long analysis times (2 hours) - Need for expert analysts
Viscosity	AACC 22-10.01; ICC-standard 162	Visco-Amilograph (Brabender)	Measurement of the viscosity of a suspension of mixing flour during programmed heating	- Sensitive to low levels of amylasic activity	- Use of a non-water solvent (aqueous solution consisting of anhydrous disodium phosphate and citric acid monohydrate) - Large amount of sample (65 g)

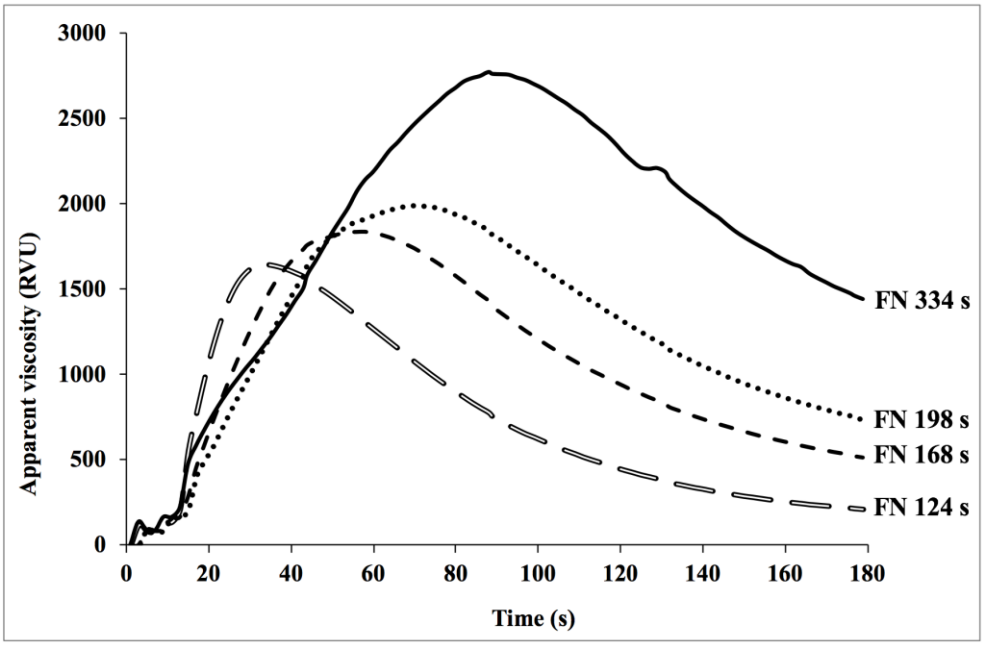

Figure 2.5. Examples of RVA plots of wheat flours with different values of Falling Number.

In addition, the above-mentioned methods use the starch as substrate, neglecting the effect of other hydrolytic enzymes such as protease on gluten proteins. Indeed, changes in protein aggregation properties during sprouting are worth investigating (Marti et al., 2018), since samples with similar FN or SN values can be quite different in composition and functionality (Kruger, 1994). As reported in Figure 2.6, semolina samples having the same FN value (about 62 s) showed different gluten aggregation kinetics that was measured by the GlutoPeak® Test (Brabender GmbH & Co., Duisburg, Germany).

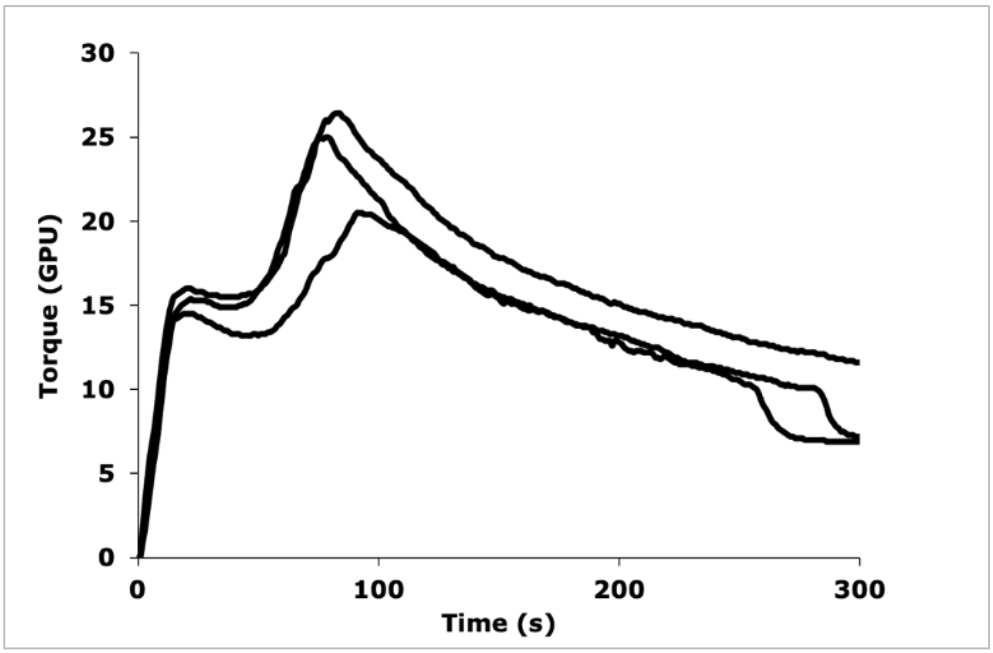

Figure 2.6. Flours with a similar Falling Number (about 62 s) but different gluten aggregation properties that were measured with the GlutoPeak® (Brabender GmbH & Co., Duisburg, Germany).

The enzymatic activities developed during sprouting can be directly quantified using standard methods such as those for α-amylase (AACC Method 22-10.01) and protease (AACC Method 46-12.01). These tests require laboratory equipment and operator expertise. Furthermore, the development of these approaches is a laborious process, involving the collaboration of different laboratories before proposing an official and internationally recognized method (Bridges and Wrigley, 2016). In fact, industries ideally need a rapid, non-destructive, and in-line approach to monitor the sprouting process. Spectroscopic techniques seem to answer this need. Infrared spectroscopy was applied on flour to detect grain sprouting and the starting time of the sprouting process in wheat and barley (Burke et al., 2016). Burke et al., (2016) obtained good Partial Least Squares models for FT-IR data (RCV2 of 0.75), but poor validation results by FT-NIR modelling due to high overfitting. As for the Near-Infrared Hyperspectral Imaging, this technique combined with different discriminant classification techniques

proved to be a useful tool to distinguish unsprouted from sprouted kernels (Singh et al., 2009). However, lack of information regarding the relation between wheat functionality and hyperspectral images makes it difficult to draw any conclusions useful for the technological development of robust simplified and cost-effective spectroscopic systems. More recently, Grassi et al., (2018) collected spectra - in the spectral range of 950–1650 nm - of both wet and dried kernels at different time intervals (from 24 h up to 72 h of sprouting) using a MicroNIR OnSite (VIAVI, California, USA), equipped with a shaker probe. The spectral profiles of sprouted grains different greatly when compared to those of unsprouted samples. The multivariate analysis of the spectra highlighted those differences between sprouted and unsprouted samples are ascribable to different absorptions in the range 1500–1626 nm, related to starch absorption signals (Juhász et al., 2005). Likewise, differences between early-stage sprouting (up to 36 h) and late-stage sprouting are influenced by absorption occurring in the range 1360–1440 nm, probably related to C-H combination bands (Workman and Weyer, 2008). In addition, data showed that the most interesting changes occurred in the first 48 h, whereas longer sprouting times generated no further relevant changes. Thus, a NIR portable device can predict the progress of controlled sprouting processes directly on wet kernels, thus skipping both the drying and refinement steps, providing information very similar to that obtained by complex and time-consuming analyses on refined flour (Grassi et al., 2018). The development of this approach could help food companies in standardizing and monitoring the sprouting process, as well as producing novel cereal-based foods with guaranteed and consistent characteristics. In addition, monitoring the sprouting process and defining when the process begins and when it ends it is of special interest when discussing the potential health benefits of sprouted grains. Thus, as stated above, the nutritional benefits of sprouting depend on many factors, including the type of grain, the variety of that grain, processing conditions, and processing time.

2.8 Conclusion and further remarks

The transformation of a grain into a new plant is based on complex and interdependent phenomena that, starting from the hydrolysis of storage macromolecules into soluble substances, permit the growth of the first sprout. Although the effects of sprouting on nutritional quality of grains have been shown, most of the nutritional benefits have been assessed *in vitro* and on raw material. Further *in vivo* studies on the final products as eaten are needed to determine the fate of chemical components during processing.

The nutritional improvement of sprouted grains was practically neglected by the Western consumers until a few years ago and the consumption of whole sprouted grains was almost non-existent in their diet. In fact, sprouting was judged negatively based on the poor technological characteristics of the resultant flour. Indeed, the loss of baking properties is normally observed in pre-harvest sprouted wheat. It is therefore easy to understand the efforts to develop and set up fast and reliable tests capable of recognizing so-called “sprout damage”. Indices related to this defect are included in the grading procedures of all countries, both exporters and importers, to check raw materials unsuitable for industrial transformation.

Conflicting results about the effects of sprouting suggest the need for more research to optimize cultivars and sprouting conditions of grains. Recent research carried out on wheat seems to indicate interesting developments that were unforeseeable a few years ago. Indeed, flour from wheat sprouted under controlled conditions, although rich in hydrolytic enzymes (amylases and proteases), could nonetheless be successfully transformed into bread with good quality characteristics. It is therefore essential to understand the molecular and structural differences between sprouting carried out under uncontrolled (pre-harvest sprouting) and controlled conditions. Only in this way, the parameters directly related to the maintenance of good cross-linking properties of proteins can be identified, thus replacing the present indirect indices, based on amylase activity.

Besides the positive effects of sprouting on nutritional and technological features, the conditions applied (i.e., high relative humidity) might favor the growth of pathogens, making the safety risk a critical point of the process. In this context, the treatment of seeds with either ozone or non-thermal technologies (i.e., cold plasma) needs to be further explored to potentially improve the microbiological quality of sprouted grains.

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3. AIMS AND OBJECTIVES

Sprouting is a process strongly associated with numerous nutritional and sensory properties due to the several biochemical changes that occur in the seeds because of the increased activity of hydrolytic enzymes (Benincasa et al., 2019; Lemmens et al., 2019). However, an excessive accumulation of hydrolytic enzymes leads to a worsening of the technological properties of the resulting flours. Indeed, a flour characterized by a high amylolytic activity will result in a bread characterized by low volume, darker and with irregular crumb structure. At the same time, flours characterized by a low amylolytic activity do not allow an optimal development of bread, making the use of enzymatic improvers needed (Poutanen, 1997). In this context, exogenous enzymes such as amylases, proteases, and xylanases are commonly exploited as flour improvers in bread-making (Poutanen, 1997; Whitehurst and van Oort, 2009). However, the use of enzymes can be expensive and not easy to be dosed, especially at artisan level. For this reason, the hypothesis of the present PhD project was to exploit the endogenous enzymatic activity accumulated during controlled sprouting to improve the bread-making performance of flours from wheat (both common and durum wheat) and alternative grains (sorghum and quinoa), thus avoiding the use of enzymatic improvers. To achieve this goal, the PhD project – whose outline is summarized in Figure 3.1 - aimed at assessing the effects of sprouting on starch, protein, and fiber functionality and their relationship with dough and bread-making performance.

Although replacing common wheat with sprouted wheat (up to 50%) improved the bread-making performance (Marti et al., 2017, 2018), knowledge about the effects of sprouting on macromolecule functionality and its relationship with bread features is still limited, especially in the case of whole wheat flour. The enrichment of food products in fiber has become increasingly important for its nutritional properties. However, the presence of fiber leads to a worsening of dough and bread properties (e.g., decrease in mixing and leavening stability, and bread volume, increase in crumb firmness and

bitterness) making wholegrain bread production difficult. Among the pre-treatments proposed to counter the negative effects of the presence of high amounts of fiber, the best results were reached when exogenous enzymes were used as such or produced by lactic bacteria (Katina et al., 2012; Zhang and Moore, 1999).

Starting from these findings, since sprouting promotes the accumulation of enzymatic activities, it might be proposed as a suitable process to improve the technological properties of wholegrain flours. Currently, wholegrain flour can be obtained by a single-stream milling or by a multiple-stream milling with a recombination process. In this context, the following sections focused on assessing the effects of the sprouting process on the chemical and technological/functional properties of wholegrain flour from a single-stream milling (**Section 4.1**) and recombination approach by including 20% bran to wheat flour (**Section 4.2**).

Although common wheat is the most suitable raw-material for bread production, durum wheat is also used in bread-making, above all in the Mediterranean area. However, the gluten network of durum wheat is characterized by high tenacity, resulting in a bread with low volume (Sissons, 2008). Indeed, the gluten network must guarantee elasticity and extensibility of the dough in such a way as to retain the gas produced during fermentation. Until now, this issue has been solved by exploiting protease activity developed during sourdough fermentation (Barber et al., 1992). In this regard, **Section 4.3** addressed the effects of sprouting duration on durum wheat gluten properties and its impact on bread-making attitude.

More recently, the use of less common grains (e.g., quinoa, sorghum) has been constantly increasing, because they are considered environmentally sustainable crops with high nutritional value (Teferra and Awika, 2019; Xiong et al., 2019). However, some aspects limit their use in food formulation. Specifically, saponins are responsible for the astringent and bitter taste of quinoa seeds/flour (Suárez-Estrella et al., 2018). A recent study showed that sprouting for 48 h was able to decrease saponin content, suggesting its

acceptability in food formulation (Suárez-Estrella et al., 2021). Despite that, the effects of sprouting on technological properties of quinoa-enriched dough and bread have not been studied yet. Thus, starting from this consideration, **Section 4.4** aimed at assessing the suitability of sprouted quinoa in bread-making, as well as the effect of sprouting on sensory traits.

In the case of sorghum, the presence of kafirins bodies stabilized by disulphide bonds together the tight starch-protein matrix in the endosperm result in low protein digestibility and starch gelatinization capacity (De Mesa-Stonestreet et al., 2010; Wong et al., 2010). Starting from this point, **Section 4.5** assessed the relationship between the changes in sorghum functionality – induced by sprouting time – and bread-making performance of composite flour (20% enrichment level).

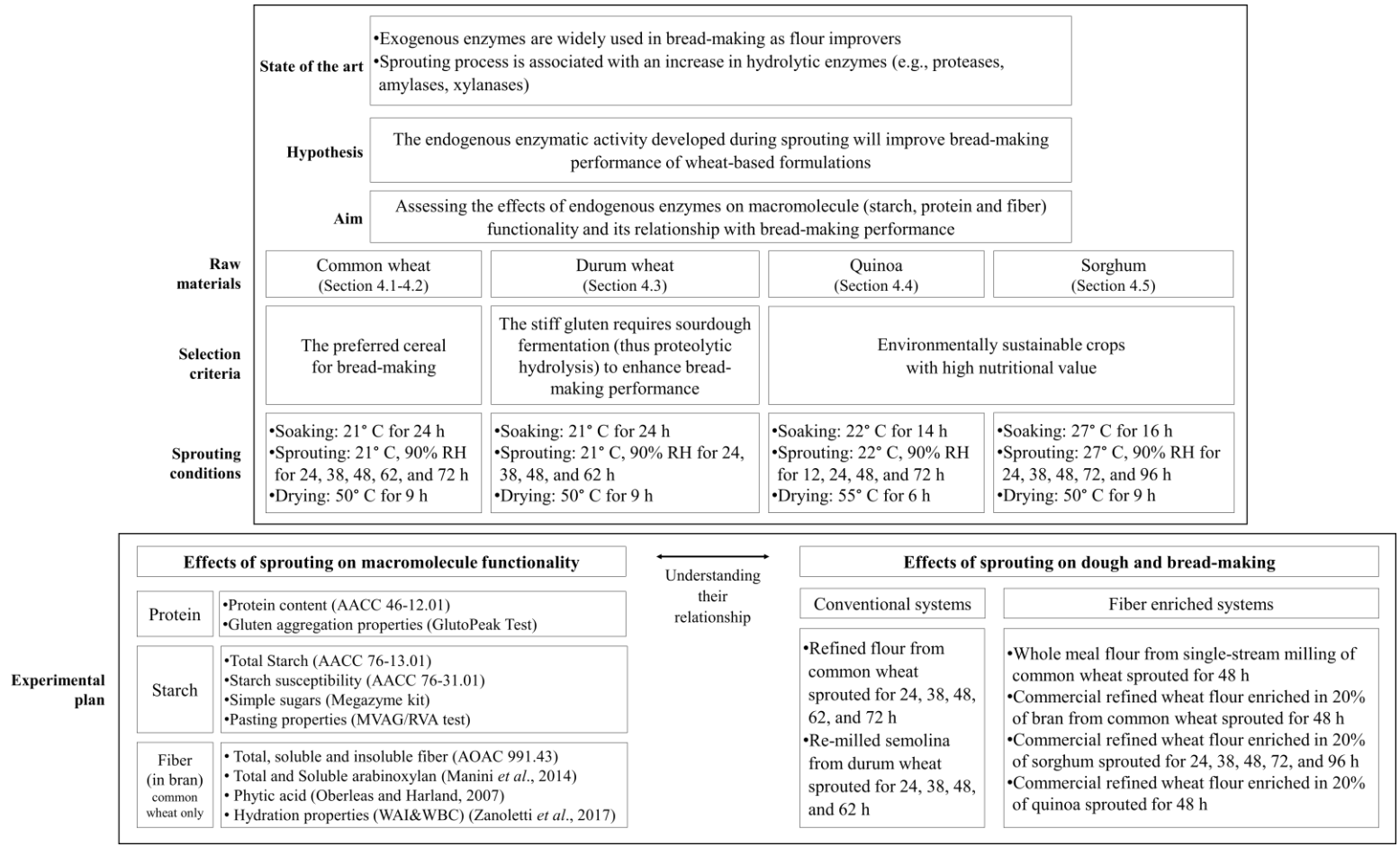

Figure 3.1. Outline of PhD project.

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4. RESULTS AND DISCUSSION

4.1. Sprouting improves bread-making performance of wheat (*Triticum aestivum* L.)

The results presented here below are partially published in: Cardone, G., D'Incecco, P., Pagani, M.A., Marti, A., 2020. Sprouting improves the bread-making performance of whole wheat flour (Triticum aestivum L.*). J. Sci. Food Agric. 100, 2453-2459.*

4.1.1. Abstract

The effects of sprouting on chemical and biochemical features of wheat and their impact on starch, gluten, and dough properties of both wholegrain and refined flours, were assessed. Bread-making performance was also investigated. Wheat sprouting was carried out at industrial scale for 48 h at 20° C and 90% relative humidity. Sprouting slightly affected starch pasting properties and did not prevent the gluten protein aggregation. However, changes in gluten aggregation kinetic suggested gluten weakening. On the other hand, sprouting led to an increase in gluten stretching ability, suggesting an increase in dough extensibility. Moreover, sprouting caused a decrease in water absorption, development time, and stability during mixing. However, following carefully the conditions obtained from the Farinograph test, sprouting improved bread height and volume and crumb softness even when wholegrain flour was used. Thus, sprouting can be exploited as a treatment to improve the bread-making attitude especially of wholegrain flour.

4.1.2. Introduction

Interest in enriching cereal-based products in sprouted grains is constantly increasing (Pagand et al., 2017), thanks to the improved nutritional and sensory profile associated with the chemical and biochemical changes promoted by sprouting. Such changes are strongly dependent on the

sprouting conditions (i.e., temperature and time) as well as grain species, varieties, and cultivars (Marti et al., 2020). Prolonged sprouting could represent a negative event since the high accumulation of hydrolytic enzymes developed during the process makes the wheat unsuitable for bread-making (Nelson et al., 2013). Consequently, the resulting bread could be characterized by a low volume and a sticky and gummy crumb (Olaerts and Courtin, 2018). Nevertheless, recent studies showed that it is possible to improve flour functionality by controlling the sprouting process conditions (Marti et al., 2018). Specifically, the most relevant functional changes in wheat have been shown within 48 h of sprouting (Grassi et al., 2018), so that refined flour from sprouted wheat at low level (≤1.5% level) might represent an optimal substitute of bread-making improvers (e.g., malt or enzymatic improvers) (Marti et al., 2018). On the other hand, using a high percentage of sprouted wheat (about 50%) improved bread volume was observed (Marti et al., 2018). On the contrary, using wholegrain sprouted wheat at 10% level negatively affected bread volume (Poudel et al., 2019). Conflicting results among studies might be due to different refinement levels, sprouting conditions and/or by the bread-making process (Świeca et al., 2017). Moreover, since previous studies have been carried out on blends, the characteristics of the commercial flours might mask the real role of the sprouting process. Thus, lack of information about changes in starch and protein behavior induced by sprouting and their relationship with rheological features and final product properties makes it difficult to understand if sprouting might improve the technological performance of either wholegrain or refined flours. In this context, this work aimed at assessing starch and gluten functionality before and after sprouting that was carried out on common wheat at industrial scale. The effects of sprouting on wholegrain and refined flours were explored by using ultrastructure techniques in combination with empiric rheology to elucidate the relationship between macromolecular features and bread-making performance.

4.1.3. Materials and methods

4.1.3.1. Sample preparation

Common wheat (*Triticum aestivum* L.) was kindly provided by Molino Quaglia S.p.A. (Vighizzolo d'Este, Padua, Italy). Kernels were divided into three aliquots. An aliquot was grinded into a M20 Universal Mill (IKA, Werke Staufen, Germany) to obtain a wholegrain (80% particle size ≤500 μm). Another aliquot was milled using a Bona laboratory mill (Labormill, Monza, Italy) obtaining a refined flour (95% particle size ≤250 μm; $W=280*10^{-4}$ J; P/L=1.16). The third aliquot (10 t) was soaked for 24 h at 20° C, 90% relative humidity, sprouted at industrial level (Bühler Pargem, Bühler AG, Uzwil, Switzerland) for 48 h at 20° C, 90% relative humidity, and dried for 9 h at 60° C. Sprouted grains were milled into wholegrain and refined flours as described for the unsprouted kernels.

4.1.3.2. Chemical composition and enzymatic activities

Protein, total starch, and damaged starch content were evaluated according to AACC methods 46-12.01, 76-13.01, and 76-31.01 (AACCI, 2001), respectively. Sugars were quantified by means of the Megazyme Maltose/Sucrose/D-Glucose Assay kit (NEO-GEN/Megazyme, Lansing, USA). α-amylase activity was determined according to AACC method 22-02.01 (AACCI, 2001), whereas β-amylases as reported by Mäkinen and Arendt (2012). Proteolytic activity was quantified by extracting proteases from flours (0.5 g) by using 5 mL of TRIS-HCl buffer (0.5 M, pH 7.5) in the presence of sodium hydroxide (0.1 M) for 2 h. The extracts were centrifuged (1500 *x g*) for 15 min and 1 mL of supernatant was incubated with 1 mL of azocasein (1% w/v, TRIS-HCl 0.5 M, pH 8) (1:1 ratio) as substrate for proteases for 1 h at 37° C. At the end, un aliquot of sample was added to trichloro acetic acid (TCA, 5% final volume) for 10 min and then centrifuged at 13,000 rpm for 10 min. 1 mL of supernatant was added to 80 μL of sodium hydroxide (0.5 M) to develop the color. The absorbance was read at 440 nm and the proteolytic

activity was expressed as mg of azocasein hydrolyzed in 1 h on 1 g of flour sample.

The Falling Number (FN) was determined according to AACC method 56-81.03 (AACCI 2001).

4.1.3.3. Pasting properties

Pasting properties were measured by using the Micro-Visco-AmiloGraph (Brabender GmbH & Co., Duisburg, Germany) device with 15 g of sample in 100 mL of either distilled water or 1 mM $AgNO_3$ (as an enzyme inhibitor) under stirring (250 min^{-1}). The temperature profile applied to the suspension ranged from 30° C up to 92° C (+7.5° C/min), holding at 92° C for 5 min, cooling from 92° C to 30° C (-7.5° C/min) and holding at 30° C for 1 min.

4.1.3.4. Visco-elasticity and aggregation properties of gluten

A creep-recovery test was carried out by using the Glutograph-E (Brabender GmbH & Co., Duisburg, Germany). The wet gluten obtained from 10 g of each sample was used to evaluate its stretching and elastic properties, following the procedure reported in the manufacturer's manual. Shear and relaxation angles were calculated from the curve.

Gluten aggregation kinetics were assessed on flours by using the GlutoPeak (Brabender GmbH & Co., Duisburg, Germany) device as reported by Marti et al. (2015a).

4.1.3.5. Mixing properties

Mixing properties were studied following the ICC method 115/1 (ICC, 1992), by means of the Farinograph (Brabender GmbH & Co., Duisburg, Germany) equipped with a 50 g bowl.

4.1.3.6. Micro-baking test

The bread was obtained by kneading flour (70 g) with compressed yeast (1.5%, Carrefour®) and salt (NaCl, 1%). The amount of water added to

the bread formulation was in accordance with the water absorption index previously determined by the farinographic analysis. The dough was prepared in a spiral mixer (KitchenAid® Artisan, Whirlpool, USA) for a time corresponding to the development time obtained from the farinographic test. After kneading, a portion of 80 g of dough was obtained, shaped in cylindrical forms, placed in a baking pan (length: 9 cm; height: 6; width: 4 cm) and left to rise at 31° C (70% relative humidity) for 90 min. Successively, the bread was baked in an oven (Self Cooking Center®, Rational Italia S.r.l., Mestre, Italy) at 220° C for 20 min. All the samples were characterized 2 hours after baking.

4.1.3.7. Bread properties

Specific volume was determined by the ratio between apparent volume - assessed by the sesame replacement method - and weight. Loaf height was determined by image analysis (Image ProPlus, v6; Media Cybernetics, Inc; Maryland, USA) measuring the highest point of the slices. Crumb firmness was measured 2 h (t_0) and 24 h (t_1) after baking as described by Marti et al. (2017).

4.1.3.8. Ultrastructure

Ultrastructure of kernels was investigated by scanning electron microscopy (SEM) at the end of soaking and after 48 h of sprouting. Kernels were air dried overnight on filter paper and cut/cracked with a razor blade to obtain a transversal section. Samples were mounted on circular specimen holders (Agar Scientific, Plain stubs 10*10 mm) with double carbon tape (Agar Scientific, Carbon Tabs 9 mm). Samples gold coated with a sputter coater (SEMPREP2; Nanotech) were observed with a Zeiss LEO 1430 SEM at 3 kV.

The microstructure of the samples collected from GlutoPeak at the maximum torque was analyzed by using an inverted confocal laser scanning microscope (CLSM, Nikon A1+, Minato, Japan). A concave microscope slide was filled with a sample that was directly stained by adding 15 µL of Fast Green FCF (0.1 mg/mL in water) (Sigma, Missouri, USA) for protein labelling.

The excitation/emission wavelengths were set at 638 nm/660–740 nm for Fast Green FCF and at 405 nm/440-530 nm to visualize auto-fluorescent bran particles in wholegrain samples. Images are presented as maximum projection of 150 layers of 512*512 pixel images that are stacked together with separation between layers set at 0.30 µm (ImageJ software Research Services Branch, National Institute of Health and Medicine, Maryland, USA).

4.1.3.9. Statistical analysis

Chemical composition, enzymatic activities, gluten visco-elasticity and aggregation properties were carried out in triplicate, instead pasting and mixing properties were determined in duplicate. As regards bread, for each type of sample two baking tests were performed and one loaf was obtained from each baking test. Specific volume was replicated two times, instead loaf height and crumb firmness were carried out on the two central slices of each loaf.

Statistical differences (t-Test; two-tailed distribution) were evaluated using Statgraphics Plus 5.1 (Statpoint Inc., Virginia, USA). Differences at *$p<0.05$, **$p<0.01$, and ***$p<0.001$ were considered significant.

4.1.4. Results

4.1.4.1. Ultrastructure of seeds

SEM pictures of the kernels after 48 h of sprouting showed the effects of enzymatic hydrolysis (Figure 4.1.1). The typical assembly of native starch granules was observed in the core of the endosperm (Figure 4.1.1a), whereas the characteristic pitting of granules was found only on those located at the periphery of the endosperm, close to aleurone cells (Figure 4.1.1b, red head-arrows). This structural scenario confirms the enzymatic hydrolysis during sprouting to be progressive from outside to inside.

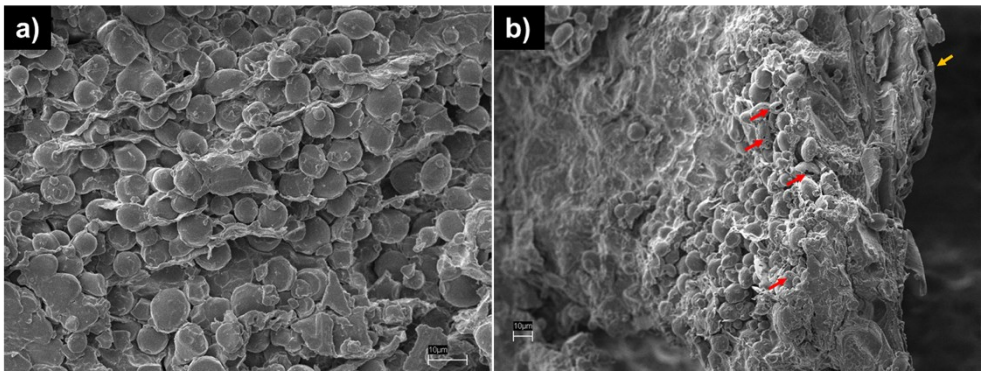

Figure 4.1.1. SEM images of the kernel after 48 h of sprouting showing starch granules in native conditions in the core of the endosperm (a), or partially hydrolyzed (red head-arrows) in the outermost portion of the endosperm in contact with bran layers (yellow arrow) (b). Scale bar is 10 μm.

4.1.4.2. Chemical composition and enzymatic activities

Sprouting up to 48 h did not significantly affect the protein content in wholegrain sample (Table 4.1.1), likely due to the protein and non-protein nitrogen turnover associated with sprouting (Bau et al., 1997). In the case of refined flour, proteins decreased (Table 4.1.1), as an effect of gluten proteins hydrolysis due a significant increase in protease activity (Table 4.1.1). As regards carbohydrates, total starch decreased while both damaged starch and simple sugars increased after 48h-sprouting. Nevertheless, the total starch decrease was significant only in wholegrain flour (Table 4.1.1), likely because enzymatic hydrolysis proceeds from the outside to the inner part of the kernel leaving intact the starch granules in the kernel core (Figure 4.1.1). The increase in simple sugars in sprouted samples can be also attributed to the α-amylase activity (Table 4.1.1). Compared to α-amylase, β-amylase activity increased at lower extent (Table 4.1.1).

Table 4.1.1. Effect of sprouting on chemical composition and enzymatic activities of wholegrain and refined flours.

	WHOLEGRAIN FLOUR		REFINED FLOUR	
	Unsprouted	**Sprouted**	**Unsprouted**	**Sprouted**
Protein	13.3±0.1	13.4±0.1^{ns}	12.65±0.03	11.7±0.2*
Total starch	65±1	63±1*	77±1	76±1^{ns}
Damaged starch	6.2±0.3	9.6±0.4***	7.2±0.2	9.9±0.4***
Maltose	0.5±0.1	3.41±0.01***	0.6±0.1	7.53±0.01***
Sucrose	0.9±0.1	2.6±0.1***	0.3±0.1	1.0±0.1***
D-glucose	0.2±0.1	0.85±0.04***	0.06±0.02	0.6±0.1***
α-amylase activity	0.110±0.001	70±1***	0.082±0.003	49±2***
β-amylase activity	28.6±0.4	29.5±0.3*	28±1	30±1*
Protease activity	0.12±0.02	0.24±0.01**	0.02±0.01	0.15±0.01***
Falling Number	334±9	62±0***	455±18	62±0***

The asterisks indicate significant differences between the means of the unsprouted and sprouted samples of each class (*p<0.05; **p<0.01; ***p<0.001; t-Test; n=3). ns: not significant differences. Compositional data are expressed as g/100g sample (d.b.). Damaged starch is expressed as g/100g of total starch (d.b.). α- and β-amylase activities are expressed as Ceralpha Units (CU)/g flour and protease activity is expressed as mg of azocasein/h*g of sample (d.b.). Finally, the Falling Number is expressed in seconds.

Overall, our results agree with those reported by Grassi et al. (2018) who monitored the chemical changes of common wheat upon sprouting time at pilot scale. This is of great interest since the scale-up of the process might represent a critical point at the industrial level.

4.1.4.3. Pasting properties

Neither wholegrain nor refined flours from sprouted wheat exhibited a typical viscosity peak, showing low viscosity values throughout heating and cooling (Figure 4.1.2a,b).

To better understand the actual effect of sprouting on starch properties, the test was also carried out in the presence of $AgNO_3$, an effective α-amylase inhibitor. Sprouting led to a slight but significant decrease in viscosity (Figure 4.1.2c,d; Table 4.1.2) compared to the unsprouted sample as a consequence of the presence of some starch granules already hydrolyzed. Similar trends were found even after 24 h, with no further changes

up to 72 h of sprouting (Grassi et al., 2018). The overall results suggest that *ex-novo* synthesis of amylases inside grains during the first days of sprouting is not yet associated with a relevant starch hydrolysis.

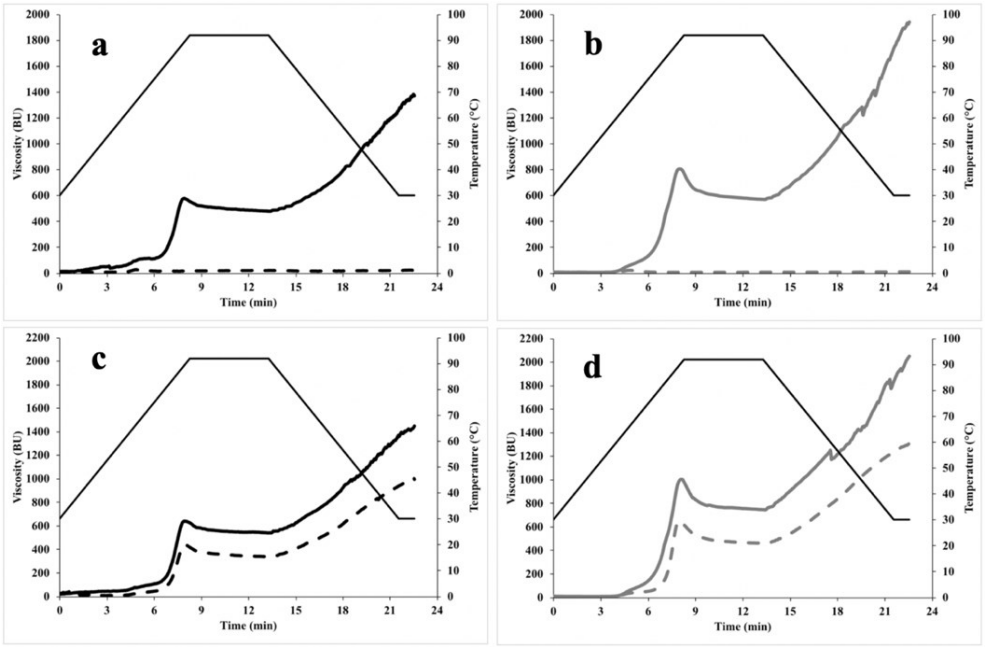

Figure 4.1.2. Micro-Visco-Amilograph profiles of wholegrain (black) and refined (grey) flours from unsprouted (solid lines) and sprouted (dash lines) wheat, in the presence of distilled water (a,b) and silver nitrate (c,d).

Table 4.1.2. Effect of sprouting on starch pasting properties of wholegrain and refined flours, in the presence of Silver Nitrate ($AgNO_3$).

	WHOLEGRAIN FLOUR		REFINED FLOUR	
	Unsprouted	**Sprouted**	**Unsprouted**	**Sprouted**
Beginning of gelatinization	61±1	66±1^{ns}	61±1	62.9±0.4^{ns}
Peak viscosity	588±16	447±11**	1014±17	653±2**
Peak temperature	89.6±0.1	89.1±0.4^{ns}	89.4±0.3	90.6±0.4^{ns}
Breakdown	101±2	102±1^{ns}	269±16	196±1*
Final viscosity	1338±42	1024±41*	2069±28	1302±11***
Setback	782±3	627±38*	1114±49	764±12*

The asterisks indicate significant differences between the means of the unsprouted and sprouted samples of each class (*p<0.05; **p<0.01; ***p<0.001; t-Test; n=3). ns: not significant differences. Beginning of gelatinization and peak temperature are expressed in degrees Celsius (° C), instead peak viscosity, breakdown, final viscosity, and setback are expressed in Brabender Units (BU).

4.1.4.4. Gluten properties

Washed gluten showed a typical profile of strong gluten when examined by GlutoGraph (Figure 4.1.3a,b), namely low stretch and relaxation angle (Table 4.1.3). The sprouting process led to a significant increase in stretch angle, whereas no differences in relaxation angle were found. The GlutoPeak test showed that the sprouting process caused a decrease in all parameters taken into consideration (Figure 4.1.3c,d; Table 4.1.3). Specifically, the sprouted samples showed a lower peak maximum time and maximum torque, resulting in lower aggregation energy.

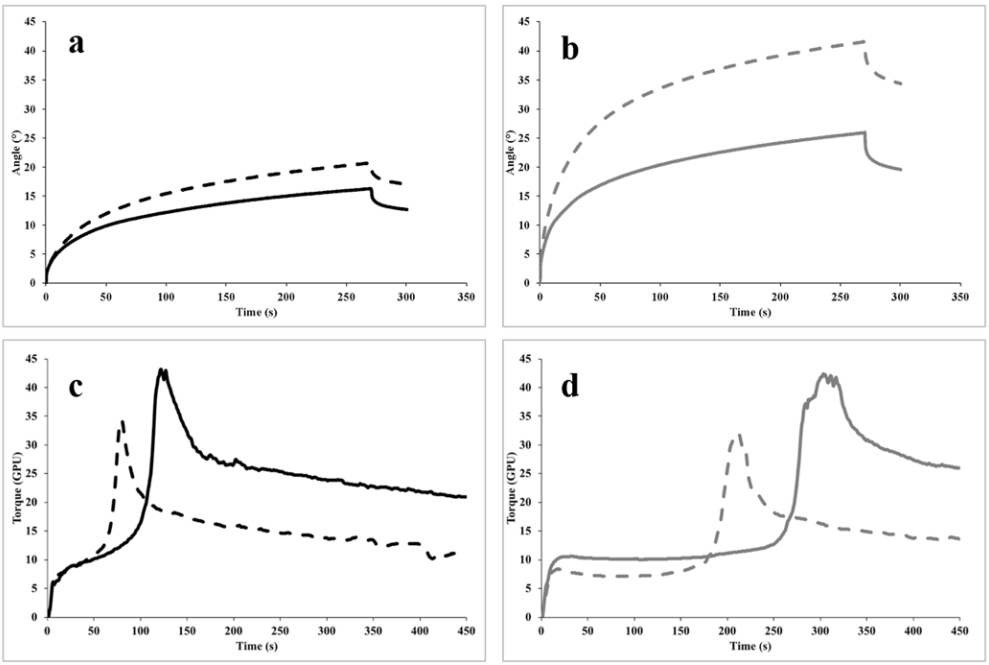

Figure 4.1.3. Profiles of wholegrain (black) and refined (grey) flours from unsprouted (solid lines) and sprouted (dash lines) wheat profiles obtained by GlutoGraph (a,b) and GlutoPeak (c,d) test.

Table 4.1.3. Effect of sprouting gluten properties of wholegrain and refined flours.

		WHOLEGRAIN FLOUR		REFINED FLOUR	
		Unsprouted	**Sprouted**	**Unsprouted**	**Sprouted**
GlutoGraph test	Stretch angle	16.3±0.1	20±1*	23±4	41±1*
	Relaxation angle	3.7±0.2	3.5±0.2^{ns}	6±1	7.5±0.4^{ns}
GlutoPeak test	Maximum torque	44±1	34±1**	43±1	32±1**
	Peak maximum time	123±1	80±1***	297±8	210±1**
	Aggregation energy	1130±11	797±10**	1247±21	858±10**

The asterisks indicate significant differences between the means of the unsprouted and sprouted samples of each class (*p<0.05; **p<0.01; ***p<0.001; t-Test; n=3). ns: not significant differences. Stretch and relaxation indices are expressed in angles (°). Maximum torque is expressed in GlutoPeak Units (GPU), peak maximum time in seconds (s) and aggregation energy in GlutoPeak Equivalent (GPE).

In the refined flour from unsprouted wheat, the protein matrix (Figure 4.1.4a, in green) was organized in thick strands giving rise to a network suitable to surround and contain the starch granules. Differently, few signs of fibrous protein organization were found in wholegrain flour from unsprouted wheat sample (Figure 4.1.4b). As regards the effect of sprouting, apparently, a more compact protein structure was observed in the refined sample (Figure 4.1.4c), while the ability to organize a network and form aggregates was almost absent in the wholegrain (Figure 4.1.4d). Indeed, in sprouted wheat flour, the protein matrix was mainly arranged into clumps which are homogeneously distributed and often connected to each other by short protein fibers (Figure 4.1.4e). In addition to proteins, CLSM images highlighted the presence of bran fragments as well as aleurone layer cells (Figure 4.1.4, in blue). Specifically, bran particles ~500 μm in size were detected in wholegrain from unsprouted wheat, while bran particles up to ~250 μm were observed after sprouting, suggesting weakening of bran layers in sprouted kernels during milling.

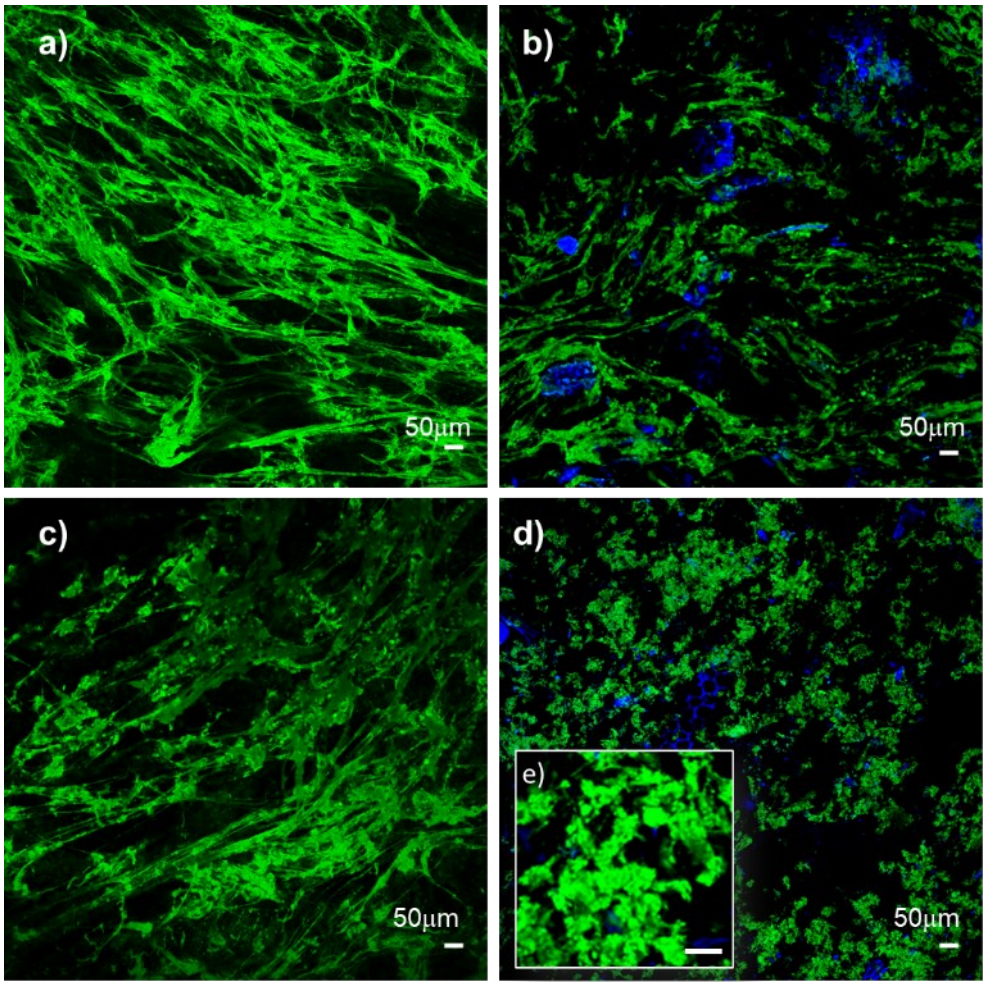

Figure 4.1.4. Microstructure of slurries from GlutoPeak test. Refined (a) and wholegrain (b) flours from unsprouted wheat; refined (c) and wholegrain (d) flours from sprouted wheat. Panel “e” is an enlarged frame of panel “d”. Protein is green and auto-fluorescent bran is blue. Scale bar is 50 µm.

4.1.4.5. Bread-making properties

Regardless of the refinement level (wholegrain vs refined flour), flours from sprouted wheat required less water to form a dough with optimal consistency (500 UF) (Table 4.1.4).

Table 4.1.4. Effect of sprouting mixing properties of wholegrain and refined flours.

	WHOLEGRAIN FLOUR		REFINED FLOUR	
	Unsprouted	**Sprouted**	**Unsprouted**	**Sprouted**
Water absorption	62.9±0.1	56.3±0.1***	55.3±0.1	52.7±0.1**
Dough development time	5.9±0.2	3.1±0.1**	20.3±0.1	1.1±0.1***
Stability	11±1	2.5±0.1**	29±1	1.0±0.1***
Degree of softening	8	39	8	28

The asterisks indicate significant differences between the means of the unsprouted and sprouted samples of each class (**p<0.01; ***p<0.001; t-Test; n=2). Water absorption and degree of softening are expressed in percentage (%), whereasdough development time and stability in minutes (min).

Furthermore, the sprouting process caused a significant decrease in both dough development time and stability (Table 4.1.4). Moreover, after sprouting, flours gave weaker dough with an increased degree of softening compared to reference samples. Similar results were shown in either pre-harvest (Dojczew and Sobczyk, 2007) and controlled (Shafqat, 2013) sprouted wheat.

Images of bread samples together with specific volume and height are shown in Figure 4.1.5a. Although the bread made from sprouted samples showed a significant increase in loaf height compared to the unsprouted samples, only the wholegrain bread from sprouted wheat showed a significant increase in specific volume (Figure 4.1.5a). In addition, the specific volume of this sample was similar to that of bread from refined flour from unsprouted wheat. Crumb bread from sprouted wheat exhibited a lower firmness than control samples, even after one day of storage (Figure 4.1.5b). In particular, the positive effect of sprouting on delaying crumb firmness during storage was more effective when wholegrain flour was used (-42% *vs* -36%, for wholegrain and refined flour bread).

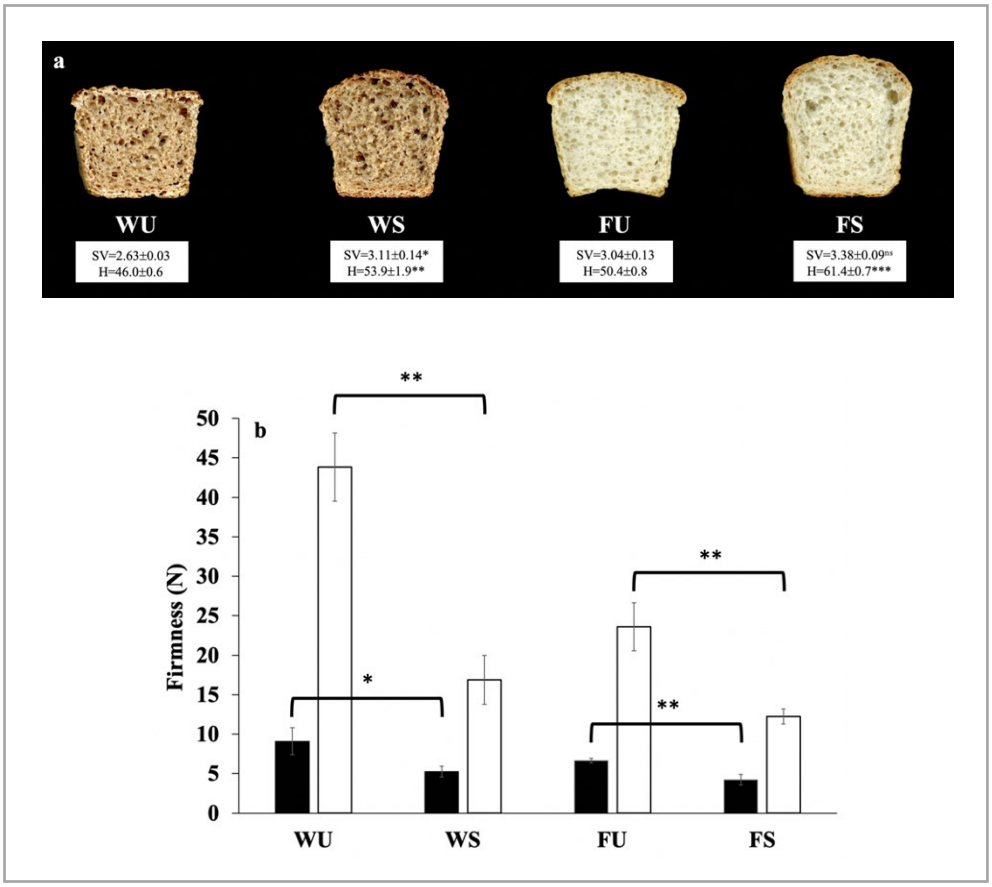

Figure 4.1.5. Bread crumb images, specific volume (SV), height (H) (a) and firmness after 2 h (black) and 24 h of storage (white) (b). The asterisks indicate significant differences between the means of the unsprouted and sprouted samples of each class (*p<0.05; **p<0.01; ***p<0.001; t-Test; n=2 for SV and H; n=4 for firmness). ns: not significant differences. WU: wholegrain flour from unsprouted wheat; WS: wholegrain flour from sprouted wheat; FU: refined flour from unsprouted wheat; FS: refined flour from sprouted wheat.

4.1.5. Discussion

The biochemical changes occurring during sprouting are the driving force for the well-documented enhancement in nutritional and sensory properties of sprouted grains (Lemmens et al., 2019). Besides that, high synthesis and accumulation of hydrolytic enzymes might lead to relevant changes in dough properties, responsible for an overall decrease in bread-making performance (Olaerts and Courtin, 2018). This behavior is typical of

wheat subjected to pre-harvest germination directly in field. In such conditions, the amount of α-amylase increases as much as several thousand folds (Kruger, 1994), due to the exposure of plants to the alternation of hot and humid weather conditions after maturity and before harvesting. On the contrary, the sprouting conditions applied in this study allowed the increase in α-amylase activity by about 600 times, while β-amylases increased less than one time (Table 4.1.1). The modest increase in β-amylases might be due to their conversion into a soluble form during sprouting (Ziegler, 1995) or by their inactivation during drying at 50° C (Grassi et al., 2018). Among the various enzymatic activities developed during sprouting, α-amylase activity is considered the most important in defining wheat quality (Dunn, 1974). The FN test is a rapid method widely used to indirectly evaluate the amylase activity, by exploiting the starch swelling during gelatinization. Generally, flours characterized by FN values under 250 s are considered raw materials of poor bread quality (Mares and Mrva, 2008). The FN of sprouted samples was 62 s (the lowest value that can be assessed by this test), suggesting they are potentially unsuitable for bread-making. However, by optimizing processing conditions - including the amount of water and the mixing time, as suggested by the farinograph test - bread with high specific volume was prepared from sprouted wheat (Figure 4.1.5a). It is worthy to mention that sprouting carried out in this study promoted only a limited hydrolysis of the storage macromolecules, as protein and starch content decreased only by 8% and 2%, respectively (Table 4.1.1). This result could be due to the short sprouting time applied, since protein and starch degradation depends on the duration of the process, achieving the maximum extent after more than 72 h (MacGregor and Matsuo, 1982; Grassi et al., 2018).

Despite the slight changes in starch content, sprouting modified the overall granule structure. Starch degradation, assessed as damaged starch (i.e., the fraction of starch readily hydrolyzed by α-amylases), was more intense in the wholegrain sample compared to the refined ones, because the

most hydrolyzed endosperm regions, which are very close to the bran layers, are maintained in the wholegrain flour (Figure 4.1.1b). The increase in the fast-hydrolysable starch resulted in the increase in simple sugars after sprouting (Table 4.1.1). The increase in simple sugars positively affects the bread-making performance of wheat being a substrate available for the growth of yeast and the production of CO_2, contributing to increase the specific volume of the corresponding bread loaves (Ranhotra et al., 1977; Ibrahim and Dappolonia, 1979; Lorenz et al., 1981).

To better elucidate the effects of sprouting on starch degradation and thus its functionality, the pasting properties were also evaluated. The pasting profile of sprouted wheat (Figure 4.1.2a,b) suggested a quick degradation by amylase on starch granules, which lost any gelatinization and retrogradation capacity. Interestingly, when the test was carried out in the presence of $AgNO_3$, to prevent the activation of α-amylases, the sprouted samples showed pasting profiles similar to those of the control samples (Figure 4.1.2c,d), suggesting that the capability of granules to gelatinize may have been masked by the presence of high levels of α-amylase during the test running rather than during the sprouting process. However, amylolytic enzymes might hydrolyze starch during kneading, leavening and the first stages of baking (Olaerts and Courtin, 2018). The lower retrogradation properties of sprouted flours – rich in α-amylases – accounted for the decrease in crumb staling (Figure 4.1.5b). A similar effect has been shown even at a low percentage (<2%) (Marti et al., 2017). Compared to white bread, the lower staling rate in wholegrain bread might be due to the higher α-amylase activity after sprouting (Table 4.1.1).

As regards proteins, protease activity increased by about 100 and 650% during 48 h of sprouting, seems to partially degrade gluten proteins (Marti et al., 2017), since sprouted wheat was still able to aggregate and create a network (see GlutoPeak pattern) and maintain viscoelastic properties (see GlutoGraph indices).

On the other hand, sprouting longer than 48 h caused gluten degradation due to a high accumulation of proteases (Koehler et al., 2007).

Specifically, glutenins are mainly hydrolyzed during the first 48 h of sprouting, while longer times are needed for gliadin hydrolysis, i.e., about 102 h (Koehler et al., 2007). Thus, the unexpectedly good baking performance observed in our study might be due to the unmodified gliadin fraction after 48 h of sprouting. According to Marti et al. (2015b), the maximum torque in the GlutoPeak profile is mainly related to the amount of gliadins, whereas both the aggregation time and energy are influenced by glutenin content and in particular by the glutenin macro polymer.

In accordance with empiric rheology, CLSM showed gluten weakening upon sprouting, since clumped proteins kept together by tiny fibrils are evident, especially in wholegrain flour (Figure 4.1.4). However, this peculiar and unusual protein organization was still able to assure volume development during baking (Figure 4.1.5a). Sprouting also affected dough mixing properties (i.e., decrease in water absorption, development time, and stability; Table 4.1.4) as the macroscopic effects of partial hydrolysis of starch and proteins. Specifically, the decrease in dough water absorption and mixing time could be due to the low molecular weight of the hydrolyzed gluten proteins (Delcour and Hoseney, 2010; Nelson et al., 2013). Another negative effect is the increase in dough softening (Table 4.1.2), usually related to dough stickiness (Caramanico et al., 2018). Even if, in this study, dough did not stick to hands or bowl during mixing and processing, evaluating the effects of sprouting on dough stickiness by an objective approach is worthy of interest. Finally, taking into consideration the information provided by the farinograph test (Table 4.1.4), it was still possible to prepare a dough and the related bread showed high volume and absence of collapse after baking (Figure 4.1.5a), concurring with rheological findings that showed sprouted wheat to be more extensible, without losing the ability to recover its initial structure following a deformation (Figure 4.1.3a,b; Table 4.1.3).

4.1.6. Conclusions

This study provides information about the effects of sprouting – obtained on industrial scale and controlled conditions – on starch and protein characteristics and their relation to dough rheological behavior as well as baking performance of wholegrain and refined flours. Despite the weakening of gluten, by optimizing the baking conditions, it is possible to obtain bread with improved volume and crumb softness even when wholegrain flour is used. Moreover, the overall results suggest that sprouting represents a biotechnological process able to improve the bread-making performance of fiber-rich flours.

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4.2. Exploiting milling by-products in bread-making: the case of sprouted wheat

The results presented here below are partially published in: Cardone, G., D'Incecco, P., Casiraghi, M. C., Marti, A., 2020. Exploiting milling by-products in bread-making: the case of sprouted wheat. Foods, 9, 260.

4.2.1. Abstract

The effect of sprouting on wheat bran was investigated. Bran from unsprouted (BUW) and sprouted (BSW) wheat were characterized in terms of chemical composition, enzymatic activities, and hydration properties. Furthermore, the rheological properties (GlutoPeak, Farinograph, Extensograph, and Rheofermentometer tests) and bread-making performance of dough enriched in bran (20% replacement level) were assessed. Sprouting process caused a significant decrease in phytic acid (~20%), insoluble dietary fiber (~11%), and water holding capacity (~8%), whereas simple sugars (~133%) and enzymatic activities significantly increased after processing. As regards the gluten aggregation kinetics, the BSW-blend profile was more similar to wheat than BUW-blend, indicating changes in the fiber and gluten interactions. BSW led to a worsening of the mixing and leavening properties; instead, no significant changes in extensibility were observed. Finally, BSW improved bread volume (~10%) and crumb softness (~52%). Exploiting bran from sprouted wheat might be useful to produce bread rich in fiber.

4.2.2. Introduction

Fiber-enrichment of food products has become increasingly important as a means to increase their nutritional properties. In this context, bran from cereals – with a total dietary fiber content of 30%-50% – is one of the most important sources of dietary fiber used in the bread-making industry (Sibakov et al., 2013). However, the inclusion of high levels of fiber in cereal-based

products remains a technological challenge, due to the need to maintain acceptable dough rheological properties as well as sensory attributes. Indeed, adding high levels of bran to dough leads to an increase in water absorption, a decrease in both mixing stability and leavening tolerance (Gan et al., 1992; Laurikainen et al., 1998). The most evident effects on the final baked product are the decrease in loaf volume, the increase in crumb firmness, the appearance of dark crumb, and, in some cases, the modification of taste with the appearance of bitterness (Pomeranz et al., 1977).

The detrimental effect of bran addition on bread-making cannot be solely attributed to the dilution of gluten proteins and to the physical disruption of the gluten network, but the physical, chemical, and biochemical properties of bran should be also considered (Hemdane et al., 2016). Besides specific physical properties – i.e., the strong tendency of bran to absorb water that might result in competition for water between bran and other key flour components like starch and proteins – bran seems to have a certain chemical reactivity (i.e., between ferulic acid and proteins) which might determine its functionality (Hemdane et al., 2016).

Several pre-treatments have been proposed to counter these negative effects, such as: (i) particle size reduction, which significantly influences the rheological properties of dough in terms of mixing time, stability and dough resistance to extension (Zhang and Moore, 1999), (ii) application of high-pressure (Marti et al., 2014a) and (iii) enzymatic treatment (Marti et al., 2014b), which alters the physical and structural properties of dough and its interaction with water and (iv) fermentation, which improves the bioactivity and baking properties of dough enriched with wheat bran (Katina et al., 2012). Of all these treatments, the best results were obtained when exogenous enzymes were used as such (Zhang and Moore, 1999) or produced by microorganisms (Katina et al., 2012).

In this context, sprouting can be proposed as a bio-technological process able to promote the accumulation of enzymatic activities. Indeed, as discussed in Section 4.1 wholegrain flour from sprouted wheat could be used

to produce bread with improved characteristics, in terms of volume and crumb softness, compared to conventional wholegrain flours. Enhancements in bread attributes were also found by using refined flour from sprouted wheat (Marti et al., 2017, 2018). Specifically, Marti et al. (2017) proposed the use of flour from sprouted wheat as an alternative to the conventional enzymatic improvers in bread-making. Indeed, a low amount of sprouted wheat flour (<2%) enhanced the bread-making performance of stiff flour, with the advantage of producing a clean label product.

Considering the potential use of flour from sprouted wheat, it remains to elucidate the physical, chemical, and structural characteristics of the wheat milling by-products (i.e., bran) and how these characteristics might affect the baking performance of bran-enriched wheat bread. Indeed, although in the literature there is a large amount of study available on the enrichment of baked products in bran, no information is available on the use of bran from sprouted wheat in bread-making. For this reason, the aim of this study was to investigate the features of bran obtained from sprouted wheat and how this ingredient affects both dough rheological properties and bread characteristics.

4.2.3. Materials and methods

4.2.3.1. Sample preparation

Wheat kernels (*Triticum aestivum* L.) were kindly supplied by Molino Quaglia (Molino Quaglia S.p.A., Vighizzolo d'Este, Italy) and a part of it was sprouted as previously reported in Section 4.1.3.1. Both samples, unsprouted and sprouted kernels, were milled using a laboratory mill (Labormill, BONA, Monza, Italy) to collect bran (bran yield: 20%).

Wheat brans (bran from unsprouted and sprouted wheat – BUW and BSW – respectively) were toasted (Self-Cooking Center – Rational AG, Landsberg am Lech, Germany) at 200° C for 120 s, in order to inactivate parts of the enzymes. Bran fractions with particle size >500 μm were further ground using the Cyclotec 1093 (FOSS, Höganäs, Sweden) to decrease their size

(<500 μm). BUW and BSW were used in replacement of the 20% of a commercial refined wheat flour (CTRL; W=280*10^{-4} J; P/L=1.16) provided by Molino Quaglia (Molino Quaglia S.p.A., Vighizzolo d'Este, Italy). In this way, two whole-wheat flours were obtained which differences were associated solely to bran type (BUW or BSW).

4.2.3.2. Microstructural evaluation

Kernels from either unsprouted or sprouted wheat were prepared for confocal laser scanning microscopy (CLSM) and light microscopy (LM) to specifically evaluate changes due to the sprouting process itself. Specimens were fixed and dehydrated according to Faltermaier et al. (2015) then embedded in a methacrylate resin (Technovit 7100, Wertheim, Germany). After resin polymerization, 10 μm-thick sections were obtained using a rotary microtome (Leitz 1512). Sections were treated, with 2,4-dinitrophenylhydrazine (20 min) followed by washing in tap water (30 min), then in 0.5% periodic acid (20 min) and again in tap water (30 min) (Faltermaier et al., 2015). This procedure is a modification of the periodic acid–Schiff's reaction that allows the dye acid fuchsin to be specific for protein without binding to other polysaccharides like starch.

The staining for protein and cell walls was performed by using 0.1% (*w*/*v*) water solution of acid fuchsin (Sigma-Aldrich, Missouri, USA) for 1 min and 10% (*w*/*v*) Calcofluor white for 1 min (fluorescent brightener 28, Sigma-Aldrich, Milan, Italy), respectively. Sections were inspected using an inverted CLSM (Nikon A1+, Minato, Japan). Acid fuchsin was excited at 560 nm and the emission filter was set at 630–670 nm while Calcofluor white was excited at 409 nm and the emission filter was set at 430–480 nm. Starch was stained by using Lugol's solution (5 g I2 and 10 g KI in 100 mL MilliQ water), and sections were examined after 5 min staining with an Olympus BX light microscope (Tokyo, Japan) equipped with QImaging Retiga camera (Surrey, Canada).

BUW and BSW were prepared for CLSM as mentioned above and observed exploiting the auto-fluorescence of the samples. Specimens for SEM observations were prepared as reported in Section 4.1.3.8 and observed using a Zeiss LEO 1430 SEM at 3 kV.

4.2.3.3. Chemical composition

Moisture content of the bran was evaluated at 130° C until the sample weight did not change by 1 mg for 60 s in a moisture analyzer (Radwag - Wagi Elektroniczne, Chorzòw, Poland). Total starch content was evaluated by standard method (AACC 76-13.01; AACCI, 2001). Sugars were assessed by HPLC by Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) (Zygmunt et al., 1982). Total (TDF), soluble (SDF), and insoluble (IDF) dietary fiber contents were quantified by an enzymatic-gravimetric procedure as reported by standard method (AOAC 991.43; AOAC, 2012).

Total and soluble arabinoxylans were determined as reported by Manini et al. (2014). The phytic acid content was determined by HPLC with spectrophotometric detection as previously reported by Oberleas and Harland (2007).

4.2.3.4. Enzymatic activities

α-amylase activity of the bran was evaluated by standard method (AACC 22-02.01; AACCI, 2001). The analysis of xylanase activity was performed by using the Azo-wheat arabinoxylan kit (K-AZOWAX 09/04; NEO-GEN/Megazyme, Lansing, USA) with some modifications (i.e., 1 h of incubation instead of 20 min). Finally, protease activity was assessed as reported in Section 4.1.3.2.

4.2.3.5. Hydration properties

The water holding capacity (WHC) of the bran was determined as reported by Lebesi and Tzia (2011) by suspending 0.5 g of each bran sample

with 45 mL distilled water. Instead, water binding capacity (WBC) was evaluated according to Zanoletti et al. (2017).

4.2.3.6. Rheological properties

The effects of bran on flour functionality and bread-making performances were determined on two blends prepared by substituting the 20% of commercial flour with BUW and BSW at the same level, respectively. Control flour without bran was also examined.

4.2.3.6.1. Gluten aggregation properties

The aggregation properties of gluten were investigated by means of the GlutoPeak (Brabender GmbH and Co., Duisburg, Germany) test, according to Marti et al. (2017). The major indices considered were: (i) Maximum Torque (MT, expressed in Brabender Equivalents, BE), indicating the peak following the aggregation of the gluten proteins; (ii) Peak Maximum Time (PMT, expressed in s), indicating the time to obtain the Maximum Torque; (iii) Total Energy (expressed in GlutoPeak Equivalent, GPE) indicating the area under the curve from the beginning of the analysis up to 15 s after the Maximum Torque.

4.2.3.6.2. Mixing properties

Mixing properties were evaluated following the standard method (ICC 115/1; ICC, 1992) in the 50 g kneading bowl of the Farinograph-E (Brabender GmbH & Co., Duisburg, Germany).

4.2.3.6.3. Extensibility properties

The extensograph test was carried out on a 20 g dough by means of the micro-Extensograph (Brabender GmbH & Co., Duisburg, Germany), at three resting times (45, 90, and 135 min). Dough samples were prepared by following the standard method (AACC 54-10.01; AACCI, 2001), in the 50 g kneading bowl of the Farinograph-E (Brabender GmbH & Co., Duisburg, Germany).

4.2.3.6.4. Leavening properties

The Rheofermentometer (Chopin, Tripette, and Renaud, Villeneuve La Garenne Cedex, France) was used to analyze dough development and carbon dioxide (CO_2) production and retention using the method described by Marti et al. (2018).

4.2.3.7. Baking test and bread properties

A straight-dough method was applied to produce bread according to the method reported in Section 4.1.3.6. The dough samples were leavened for 60 min in a thermostatic chamber at 30° C (70% relative humidity). To prevent the formation of crust too quickly, the leavened dough was baked in an oven (Self Cooking Center, Rational International AG, Landsberg am Lech, Germany) in two stages. Firstly, the samples were baked at 120° C with vapor injection (90% relative humidity) for 4 min and then the oven temperature was increased to 230° C for 11 min. The resulting loaves, two hours after baking, were analyzed or packaged in orientated polypropylene film for three days.

4.2.3.7.1. Crumb color

The evaluation of the browning (100-L*) and saturation of the color intensity (redness and yellowness, a* and b*, respectively) of bread crust and crumb were assessed with a reflectance color meter (CR 210, Minolta Co., Osaka, Japan).

4.2.3.7.2. Specific volume

The bread specific volume was performed through the ratio between volume and mass of bread and expressed in mL/g. The apparent volume was evaluated by the sesame displacement approach.

4.2.3.7.3. Crumb hardness

Crumb hardness was assessed by means of a dynamometer (Z005, Zwick Roell, Ulm, Germany), equipped with a 100 N load cell as previously described by Marti et al. (2014b). Bread samples were evaluated two hours

after baking (day zero), and after one and three days of storage. Crumb hardness was determined as the maximum compression force at a deformation of 30%. Three central slices (15 mm thick) of one loaf from each bread-making trial were analyzed. The values of hardness resulting from each slice of bread were analyzed by applying the Avrami Equation:

$$\theta = \frac{(A_\infty - A_t)}{(A_\infty - A_0)} = e^{-ktn}$$

where θ indicates the fraction of the total change in hardness still to occur, A_0, A_t, and A_∞ are experimental values of hardness at times zero, *t* and infinity respectively, *k* is a rate constant and *n* (n=1) is the Avrami exponent. All the parameters were obtained from the modelling process.

4.2.3.8. Statistical analysis

Chemical composition, enzymatic activities, hydration properties, and gluten aggregation properties were determined in triplicate, whereas mixing, extensional, and leavening properties were analyzed in duplicate. As regards the extensograph test, the determinations for each sample were made in duplicate and from each dough two subsamples were analyzed. For the bread production, two baking tests were carried out, and three loaves were prepared from each baking test. Color measurements were replicated five times. Bread specific volume, crumb porosity, and hardness were carried out on six loaves or slices.

To determine differences between means (for BUW and BWS) a paired t-test was applied. Analysis of variance (one-way ANOVA) was performed by utilizing the Fisher's Least (LSD) test. Data were elaborated by Statgraphics XV v. 15.1.02 (StatPoint Inc., Warrenton, VA, USA).

4.2.4. Results

4.2.4.1. Microstructure evaluation of kernels and brans before and after sprouting

Microstructural modifications due to the sprouting process were evaluated on the whole kernel to avoid interferences of mechanical breaks possibly brought by milling. Sprouting caused the hydrolysis of the subaleurone endosperm cell walls, that were no longer visible in sprouted kernels (Figure 4.2.1a,b), and the hydrolysis of some starch granules near the aleurone cells (head arrows in Figure 4.2.1b), consistently with the hydrolytic enzyme synthesis in the aleurone layer. Fluorescence of Calcofluor white, specific for cell walls especially β-1,4-glucans, considerably decreased in sprouted kernels (Figure 4.2.1c,d). At the same time, the decrease in fluorescence of acid fuchsin suggested a protein degradation in subaleurone region (Figure 4.2.1c,d).

CLSM of brans (Figure 4.2.2a,b) clearly show that BUW is almost composed of pericarp and aleurone layers, whereas BSW is richer in starch granules since large portions of starchy endosperm remained adherent to the aleurone layer. This difference may be a consequence of different break paths after sprouting and it explains the lower flour yield of BSW (51% *versus* 54%; data not shown). SEM analysis confirmed the relevant structural changes induced by sprouting and showed the hydrolysis of starch granules mainly to occur in the BSW as erosion pits (red arrow in Figure 4.2.2e).

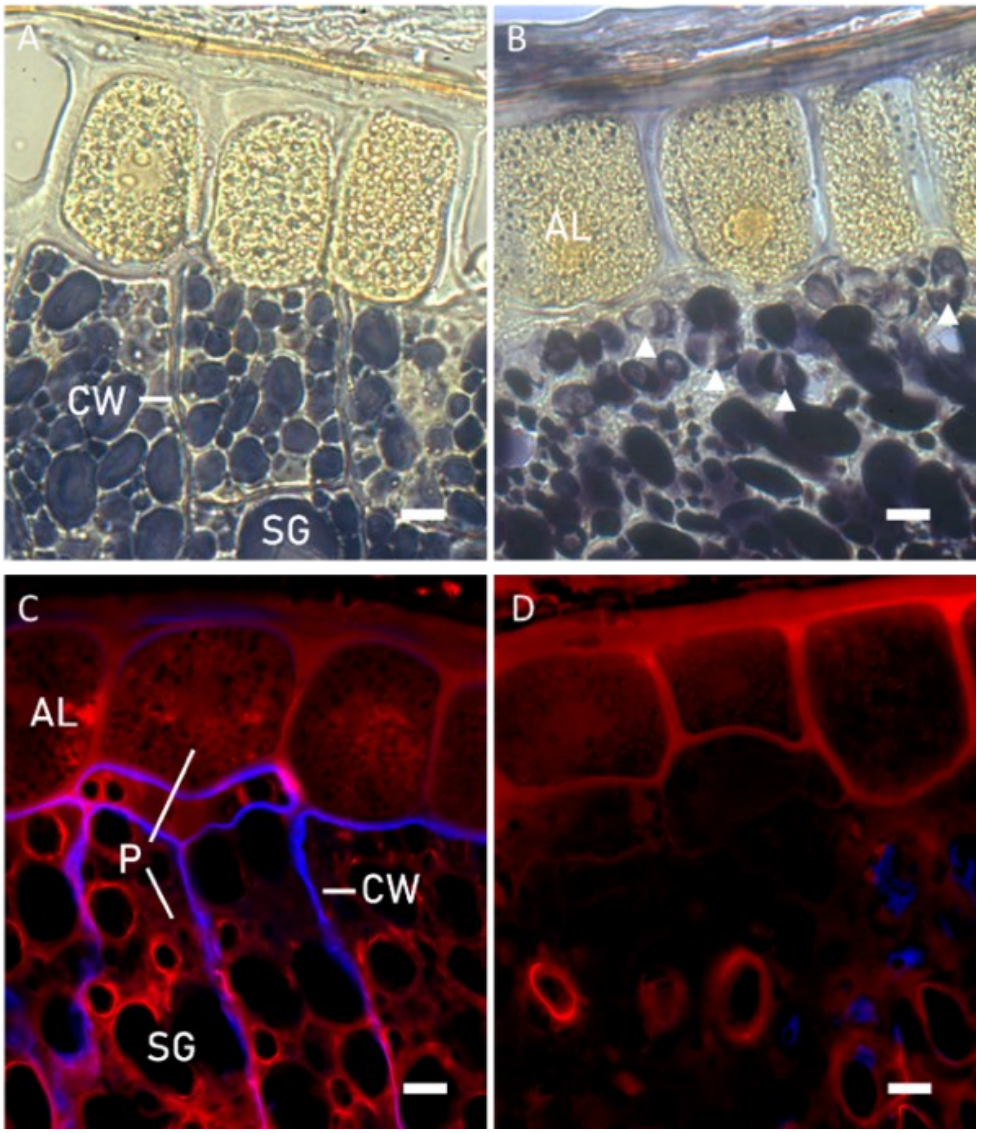

Figure 4.2.1. Light microscopy (a,b) and confocal laser scanning microscopy (c,d) of kernels before (a–c) and after (b–d) sprouting process. AL: aleuron layer, CW: cell wall, SG: starch granule, P: protein. White head arrows in panel B show hydrolyzed starch granules. Bars are 10 μm in length.

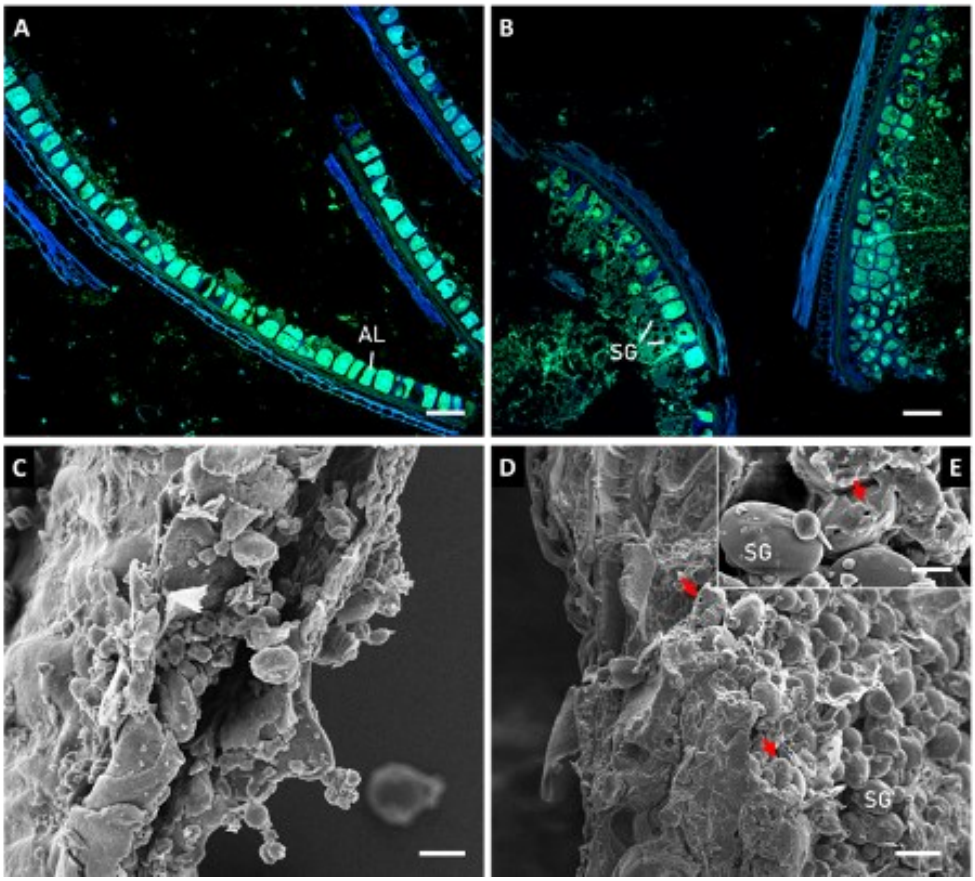

Figure 4.2.2. Confocal laser scanning microscopy (a,b) and scanning electron microscopy (c–e) of bran before (a–c) and after (b,d,e) sprouting process. AL: aleuron layer, SG: starch granule. Red arrows in panel “d” and “e” show the effect of amylolytic enzymes as “erosion pits” on the granule surface. Bars are 40 μm in length in panels “a,b”, 10 μm in length in panels “c-d” and 5 μm in panel “e”.

4.2.4.2. Effect of sprouting on bran features

Sprouting process increased the amount of total sugars in bran, and specifically glucose, fructose and maltose (Table 4.2.1). The higher starch content in BSW might be the consequence of the different compactness and, therefore, milling behavior of BUW and BSW, as shown in Figure 4.2.2. A significant decrease in IDF was found after sprouting (−11% in BSW with respect to BUW). Instead, the SDF content of BUW and BSW was not affected by the process. Moreover, sprouting did not induce a significant degradation

and solubilization of arabinoxylans. On the contrary, in the case of prolonged sprouting (i.e., four days), a significant decrease in the total content of arabinoxylans and an increase in water-extractable ones was reported in barley (Li et al., 2005). In contrast, the sprouting degraded antinutritive factors, such as phytic acid, confirming previous studies carried out on wholemeal flours (Hübner and Arendt, 2013).

Table 4.2.1. Effect of sprouting on wheat bran features.

	Bran from unsprouted wheat (BUW)	**Bran from sprouted wheat (BSW)**
Total starch	18±1	27±2**
Arabinoxylans		
Total	14±1	12±1^{ns}
Soluble	0.22±0.01	0.29±0.08^{ns}
Phytic acid	14.0±0.5	11.2±0.02*
Total sugar	3±1	7±2*
Glucose	0.14±0.01	0.53±0.02*
Fructose	0.05±0.01	0.20±0.01*
Sucrose	2.0±0.2	4.99±0.01*
Raffinose	0.79±0.02	n.d.
Maltose	n.d.	1.29±0.03^{ns}
Total dietary fiber	45±1	40±1*
Soluble	2.2±0.4	2.0±0.3^{ns}
Insoluble	43.1±0.2	38±1*
α-amylase activity	0.094±0.003	143±16*
Xylanase activity	0.16±0.04	0.35±0.03*
Protease activity	0.17±0.01	0.27±0.02*
Water holding capacity	4.9±0.1	4.5±0.1*
Water binding capacity	3.9±0.2	3.7±0.2^{ns}

Values associated with asterisks in the same row are significantly different (t-test, *p<0.05; **p<0.001; n=2); n.d.: not detectable; ns: not significant difference. Compositional and hydration property data are expressed as g/100g sample (d.b.). α-amylase activity and xylanase activity are expressed as Ceralpha Units (CU)/g flour and as activity/g flour, respectively. Protease activity is expressed as mg of azocasein/h*g of sample (d.b.).

As expected, sprouting promoted an accumulation of α-amylases, xylanases and proteases in the bran fraction (Table 4.2.1). Specifically, the activity of α-amylase, xylanase, and protease in BSW increased by about 1500-, 1.2-, and 0.6-fold, compared to BUW. BUW and BSW hydration properties were assessed in terms of their water holding (WHC) and water binding (WBC) capacity. The WHC significantly decreased after the sprouting process, by about 8% (Table 4.2.1). Instead, there were no significant differences between BUW and BSW in terms of WBC.

4.2.4.3. Gluten aggregation and mixing properties

During the GlutoPeak test, the speed of the rotating paddle allows the formation of gluten, and a rapid increase in the torque curve occurs. Additional mixing breaks down the gluten network and the torque curve declines (Marti et al., 2015). The gluten aggregation kinetics of CTRL was typical of a strong flour with good bread-making performance that is usually characterized by long aggregation time (i.e., PMT), high torque (i.e., MT) and high energy values (Figure 4.2.3; Table 4.2.2).

Replacing 20% of refined wheat flour with both types of bran, the PMT and MT indices significantly decreased and increased, respectively, resulting in a decrease in the energy value, suggesting gluten weakening (Malegori et al., 2018). Worsening in gluten aggregation properties were more evident when BUW was added to CTRL sample, instead of BSW.

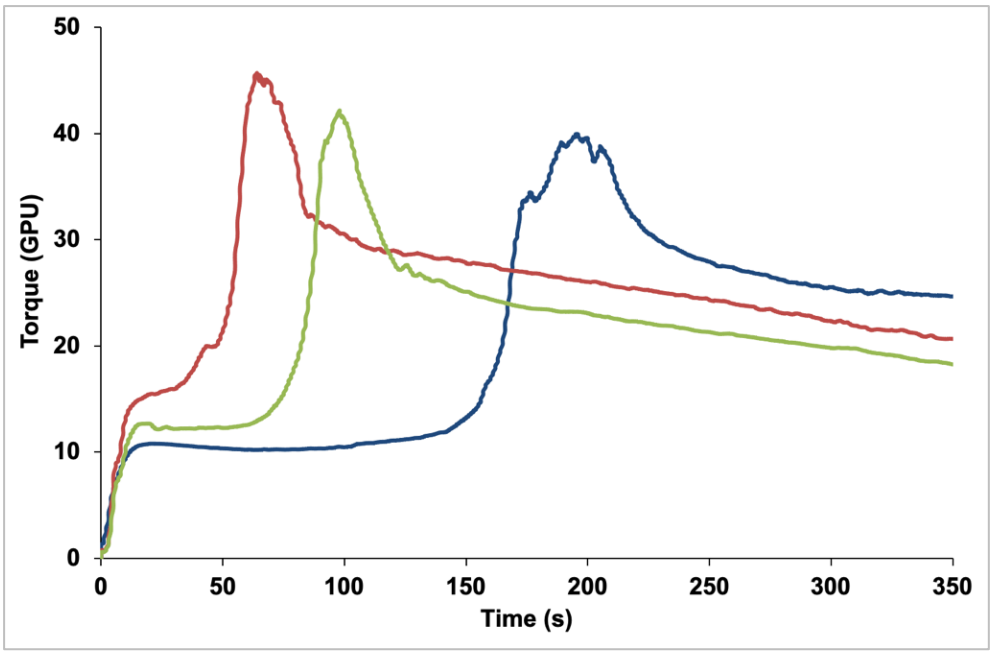

Figure 4.2.3. GlutoPeak profiles of refined wheat flour alone (blue line) and in the presence of bran from unsprouted wheat (red line) or bran from sprouted wheat (green line).

Table 4.2.2. Gluten aggregation and mixing properties of refined wheat flour alone, with bran from unsprouted wheat, or bran from sprouted wheat.

		CTRL	CTRL + BUW	CTRL + BSW
GlutoPeak Test	Peak maximum time	192±3^{c}	65±1^{a}	93±4^{b}
	Maximum torque	40±1^{a}	46±1^{c}	44±2^{b}
	Total energy	3567±112^{c}	1914±25^{a}	2059±35^{b}
Farinograph Test	Water absorption	58.1±0.2^{a}	66±1^{c}	62±1^{b}
	Dough development time	10.8±0.3^{c}	5.6±0.3^{b}	4.5±0.5^{a}
	stability	23±2^{c}	12±2^{b}	6.4±0.1^{a}
	Degree of softening	15±1^{a}	44±5^{b}	104±1^{c}

Different letters in the same row correspond to significant differences (one-way ANOVA, LSD test, $p<0.05$; n=3 for GlutoPeak test; n=2 for Farinograph Test). BUW: bran from unsprouted wheat; BSW: bran from sprouted wheat. Maximum torque is expressed in GlutoPeak Units (GPU), Peak maximum time in seconds (s) and aggregation energy in GlutoPeak Equivalent (GPE). Water absorption and degree of softening are expressed in percentage (%), whereas dough development time and Stability in minutes (min).

The CTRL flour used for making the blends was characterized by a long dough development time and high stability (Figure 4.2.4; Table 4.2.2), in agreement with the GlutoPeak test (Figure 4.2.3; Table 4.2.2). When 20% of flour was replaced by BUW or BSW, a significant increase in water absorption was observed (Table 4.2.2), with BUW-blend absorbing more water than BSW-blend, in agreement with the hydration properties of the related bran samples (Table 4.2.1). Regarding the development time, the presence of bran (BUW) decreased the time needed to achieve maximum consistency (−48%). This effect was even more evident when BSW was added (−58%). Dough stability, which in wheat dough indicates dough strength, decreased significantly by about 47% and 72% in the case of BUW- and BSW-blends, respectively. In contrast to the GlutoPeak test, the farinograph test showed the highest weakening of the dough when BSW was added.

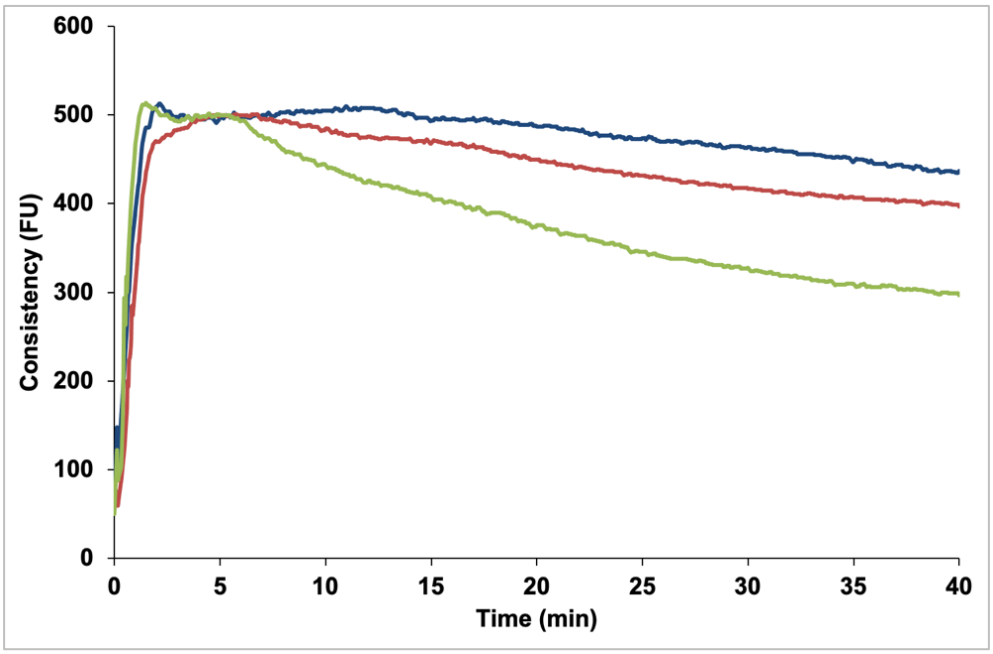

Figure 4.2.4. Farinograph profiles of refined wheat flour alone (blue line) and in the presence of bran from unsprouted wheat (red line) or bran from sprouted wheat (green line).

4.2.4.4. Dough extensibility

Dough extensibility significantly decreased when bran was added, with no significant differences according to the type of bran (Figure 4.2.5 a–c; Table 4.2.3). In addition, all samples showed a decrease in dough extensibility as the resting time increased, in particular when the resting time was extended from 45 min to 90 min. However, the extensibility for the CTRL flour remained practically unmodified between 90 and 135 min.

Bran-enrichment did not cause any significant modification regarding resistance at 45 and 90 min of resting time, while after 135 min of resting time, bran caused an increase in dough resistance, suggesting dough stiffness (Skendi et al., 2009). This phenomenon is more evident when BSW was included in the dough.

The ratio number, which indicates the ratio between the resistance to extension and extensibility, increased with the addition of bran, confirming the role of this milling fraction in inducing dough stiffness. This phenomenon is well shown by the high ratio number values at 135 min. The highest increase in ratio number was recorded when BSW was added to the CTRL flour. Finally, the energy required for deformation was decreased by the addition of bran, particularly when BUW was used.

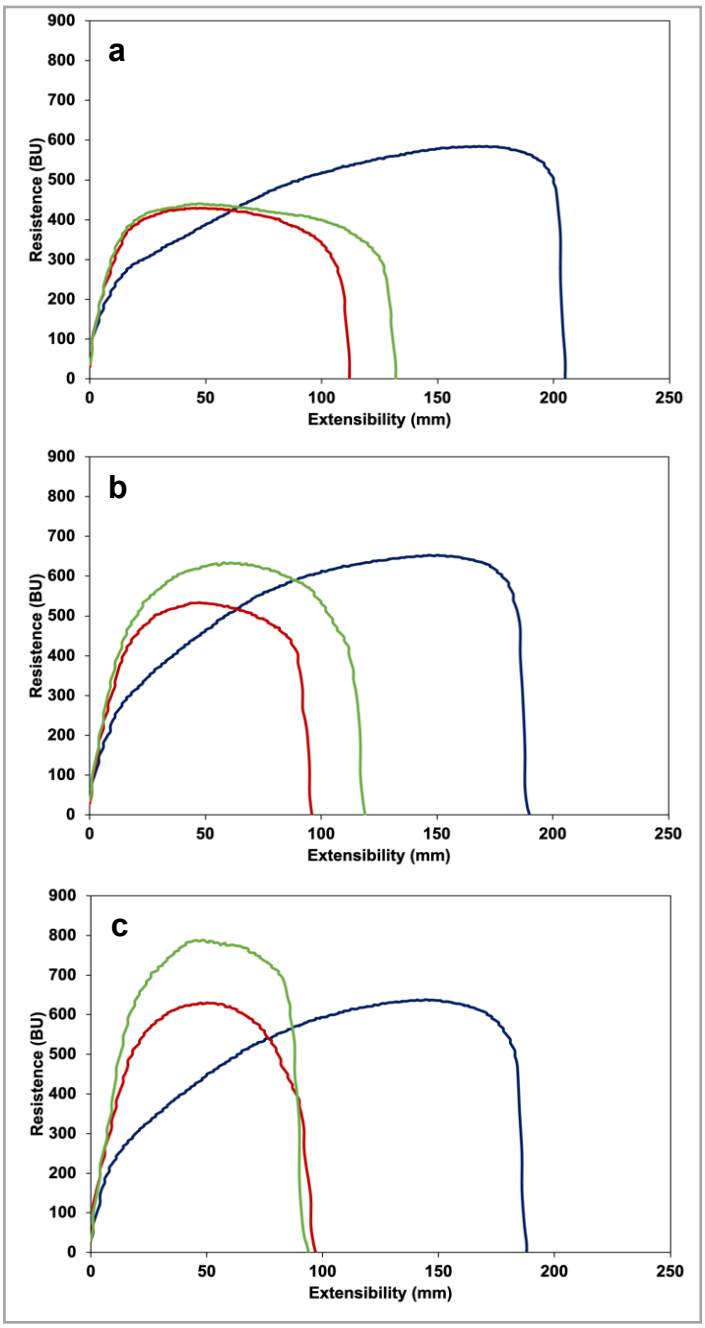

Figure 4.2.5. Micro-Extesograph profiles of refined wheat flour alone (blue line) and in presence of bran from unsprouted wheat (red line) or bran from sprouted wheat (green line), after 45 min (a), 90 min (b), and 135 min (c) of resting time.

Table 4.2.3. Extensibility properties of refined wheat flour alone, with bran from unsprouted wheat, or bran from sprouted wheat.

	Resting time	CTRL	CTRL + BUW	CTRL + BSW
Extensibility (E)	45 min	209±11^{b}	119±6^{a}	132±4^{a}
Resistance to extension (R)		353±46^{a}	423±29^{a}	431±1^{a}
R/E ratio		1.7±0.3^{a}	4.0±0.4^{b}	3.3±0.1^{b}
Energy		155±9^{b}	76±2^{a}	88±2^{a}
Extensibility (E)	90 min	182±7^{b}	104±6^{a}	109±3^{a}
Resistance to extension (R)		405±63^{a}	512±26^{a,b}	583±41^{b}
R/E ratio		2.2±0.3^{a}	4.9±0.5^{b}	5.4±0.2^{b}
Energy		147±31^{b}	77±4^{a}	94±11^{a,b}
Extensibility (E)	135 min	185±2^{b}	97±2^{a}	97±1^{a}
Resistance to extension (R)		430±45^{a}	609±32^{b}	721±7^{c}
R/E ratio		2.3±0.3^{a}	6.0±0.3^{b}	7.5±0.1^{c}
Energy		163±4^{b}	85±5^{a}	101±3^{a}

Different letters in the same row correspond to significant differences (one-way ANOVA, LSD test, $p<0.05$; n=2). CTRL: refined wheat flour; BUW: bran from unsprouted wheat; BSW: bran from sprouted wheat. Extensibility is expressed in millimeters (mm), resistance to extension in Extensograph Units (EU), and energy in squared centimeters (cm^2).

4.2.4.5. Leavening properties

Maximum dough height during leavening was not significantly altered by the addition of bran (Figure 4.2.6). On the other hand, the time of maximum dough development decreased, from 180 min in the control to 161 min and 131 min by adding BUW and BSW, respectively. The time of dough development and the loss in dough volume (weakening coefficient), was high for all bran-enriched samples, especially when BSW was added. The decrease in dough stability agrees with the farinograph index. The drop off after three hours of leavening ranged from 6% and 17.5% for BUW and BSW samples.

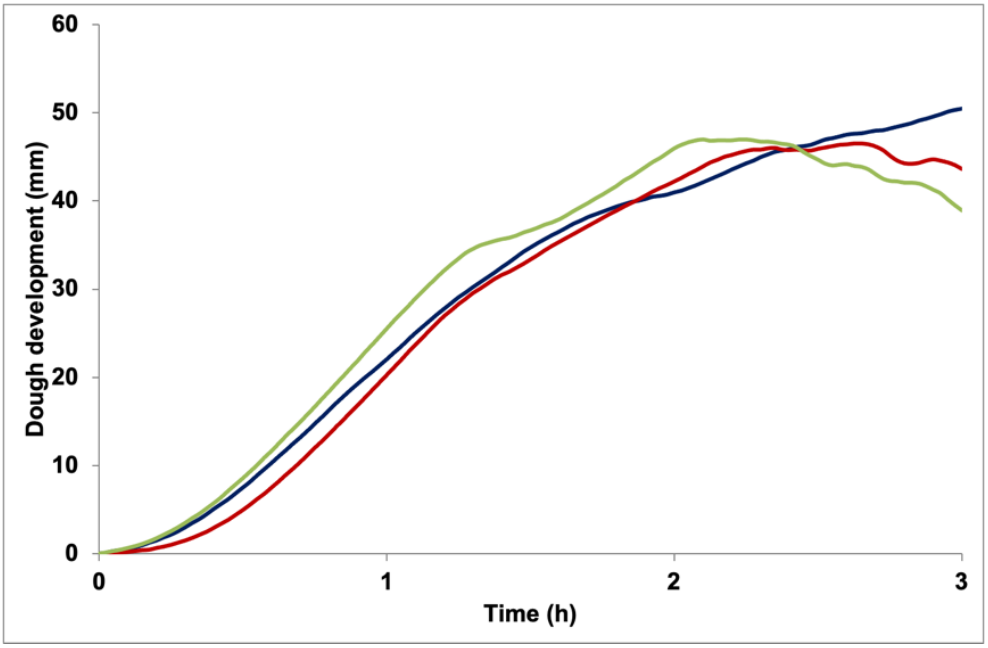

Figure 4.2.6. Dough development profiles of commercial wheat flour (blue line); with bran from unsprouted wheat (red line) or bran from sprouted wheat (green line).

Regarding gas production and retention (Table 4.2.4), dough with bran produced more gas than the CTRL samples and this is due to a higher content of simple sugars (Table 4.2.1), which are consumed faster by yeast compared to CTRL samples. This effect was more noticeable when BSW was used. As regards the gas retention coefficient (Table 4.2.4), the BUW addition did not induce a significant negative effect after three hours of leavening. In contrast, in the presence of BSW the gas retention capacity decreased from 94% (CTRL) to 86%, after the same time (i.e., three hours).

Table 4.2.4. Leavening properties of refined wheat flour alone, with bran from unsprouted wheat, or bran from sprouted wheat.

	CTRL	CTRL + BUW	CTRL + BSW
Maximum dough height	51±3^{b}	47±1^{a}	47±1^{a}
Time of maximum dough development	180±0^{c}	161±2^{b}	131±6^{a}
Dough height at 180 min	51±3^{b}	44±1^{a}	39±1^{a}
Weakening coefficient at 180 min	n.d.	6±1^{b}	17.5±0.4^{a}
Total CO_2	1129±52^{a}	1301±42^{a,b}	1476±84^{b}
Retained CO_2	1064±45^{a}	1197±29^{b}	1271±46^{b}
Released CO_2	65±7^{a}	104±12^{a}	205±38^{b}
CO_2 retention coefficient	94±1^{b}	92±0.1^{b}	86±2^{a}
Porosity time	n.d.	103±4^{b}	76±4^{a}

Different letters in the same row correspond to significant differences (one-way ANOVA, LSD test, $p<0.05$; n=2). n.d.: not detectable. CTRL: refined wheat flour; BUW: bran from unsprouted wheat; BSW: bran from sprouted wheat. Maximum dough height and dough height at 180 min indices are expressed in millimeters (mm); time of maximum dough development and porosity time are expressed in minutes (min); weakening coefficient at 180 min and CO_2 retention coefficient are expressed in percentage (%); total, retained and released CO_2 are expressed in milliliters (mL).

4.2.4.6. Bread properties

Bran-enriched bread resulted in a darker (increase in 100-L*) and redder (higher a*) crumb and crust than CTRL bread (Table 4.2.5). As expected, this phenomenon was more intense in BSW-enriched bread. When BSW was added instead of BUW, also the color crumb (Table 4.2.5) showed a significant increase in browning (100-L*) and in redness (a*) levels.

As expected, the addition of bran had a slight negative effect on specific volume, however no significant differences were found between CTRL and BSW.

The addition of BSW to wheat resulted in a relevant decrease in crumb hardness (3.1 N) compared to the other samples (6.0 N). During storage (up to 3 days), only BSW-samples exhibited higher softness than the CTRL (Figure 4.2.7). In addition, the softness of BSW-enriched bread after 3 days was similar in softness to the CTRL and BUW-enriched breads after one day.

Table 4.2.5. Properties of bread from refined wheat flour alone, with bran from unsprouted wheat, or bran from sprouted wheat.

		CTRL	CTRL + BUW	CTRL + BSW
Bread	Specific volume	2.7±0.6^{b}	2.2±0.1^{a}	2.4±0.1^{a,b}
Crust	Browning (100-L *)	53±4^{a}	60±4^{b}	60±1^{b}
	Redness (a *)	9±3^{a}	12±1^{b}	12±1^{b}
	Yellowness (b *)	25±2^{b}	20±5^{a}	19±1^{a}
Crumb	Browning (100-L *)	48±3^{a}	52±2^{b}	57±2^{c}
	Redness (a *)	-1.3±0.2^{a}	3.80±0.3^{c}	3.2±0.4^{b}
	Yellowness (b *)	18±1^{b}	16±1^{a}	16±1^{a}
Crumb firming kinetics	A_0	6.48	6.62	3.12
	A_∞	66.01	34.55	31.35
	A_∞-A_0	59.53	27.93	28.23
	k (h^{-n})	0.186	0.480	0.252

Different letters in the same row correspond to significant differences (one-way ANOVA, LSD test, $p<0.05$; n=5 for crumb and crust color; n=6 for bread specific volume and crumb firmness). CTRL: refined wheat flour; BUW: bran from unsprouted wheat; BSW: bran from sprouted wheat. A0: hardness at times zero; A∞: hardness at infinity; k: rate constant; n: Avrami exponent. Specific volume is expressed in mL/g; instead A0, A∞ and A∞-A0 are expressed in Newton (N).

To evaluate the kinetic attitude of starch during the retrogradation phenomena, the Avrami equation was applied, fixing the value of Avrami exponent (n=1) as proposed by Michniewicz et al. (1992). The estimated Avrami coefficient *k* for bran-enriched bread samples was higher than that for the CTRL sample. The high coefficient of starch retrogradation with BUW described a fast-firming rate. The BSW led to a decrease in the firming rate (Table 4.2.5) compared to the BUW and CTRL. Regarding the firmness at infinite time (A∞) the highest value was found for the CTRL sample, instead the lowest A∞ was observed in the presence of BSW. The intensity of staling evaluated as the total firmness increment (A∞–A0) decreased with the addition of bran, due to the high-water binding capacity of fiber. For this parameter, no difference was observed between bran-enriched breads.

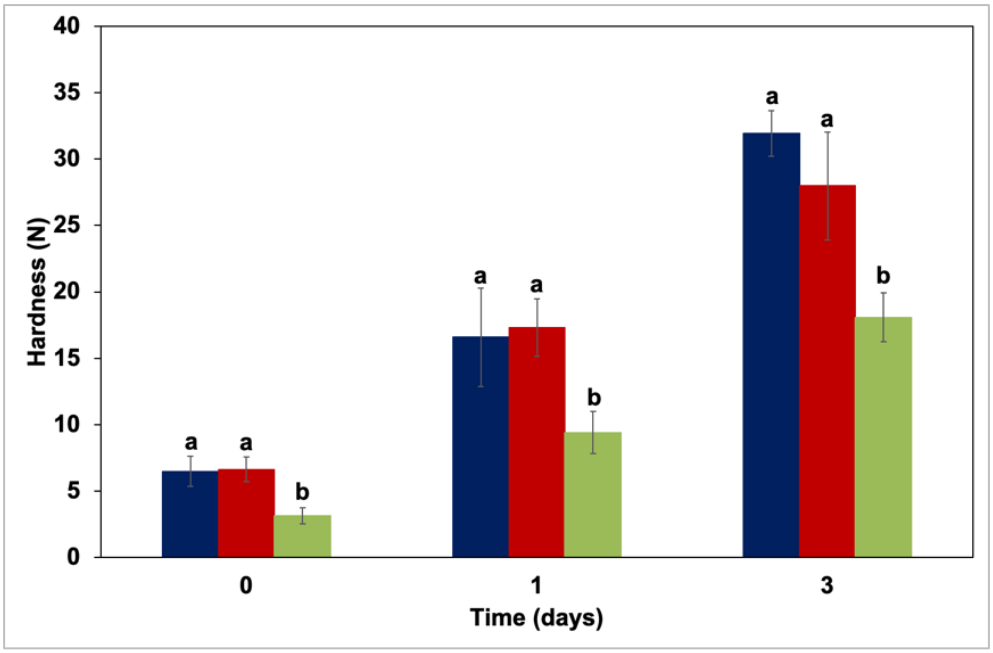

Figure 4.2.7. Crumb firmness properties of loaves obtained from refined wheat flour (black), with bran from unsprouted wheat (light grey), or bran from sprouted wheat (dark grey) during storage. Different letters on the same day correspond to significant differences (one-way ANOVA, LSD test, $p<0.05$; $n=6$).

4.2.5. Discussion

Due to the recent interest in using refined flour from sprouted wheat in bread-making as a flour improver or ingredient (Marti et al., 2017, 2018), it is worthy of interest to investigate the potential use of bran obtained from the milling of sprouted wheat. In this study, the effect of sprouting was assessed on both physical and chemical properties of wheat bran. Moreover, the effect of wheat replacement (at 20% level which is roughly the percentage of bran in wholegrain) with bran from sprouted wheat was studied on both dough rheology and bread-making performance.

Despite the well-known nutritional benefits of bran, the presence of antinutritional factors (i.e., phytic acid) inhibit the absorption of minerals and vitamins (Enneking and Wink, 2000). Several authors have reported the positive effects of the sprouting process on the degradation of phytates in

wheat and other cereals (Hübner and Arendt, 2013). As reported in Table 4.2.1, the sprouting conditions applied in the present study caused a decrease in phytic acid by about 110 folds.

From the technological standpoint, it is well known that the incorporation of fiber into flour negatively affects the textural and sensory properties of cereal-based products. Indeed, numerous negative effects on dough and bread properties have been attributed to bran, including increased dough stickiness, decreased mixing, fermentation tolerance, volume, and crumb softness (Pomeranz et al., 1977). For this reason, it is indispensable to modify the structural properties of fiber (i.e., by increasing the soluble fraction) to improve the quality of bran-enriched foods. Specifically, the soluble fraction of the dietary fiber contributes to the creation of the viscosity of the liquid phase of the system (Guillon and Champ, 2000). The sprouting effects on fiber content are reported to be varied and unclear, with increased, decreased and no change reported for several grains (Nelson et al., 2013). The sprouting process carried out under the conditions applied in this study determined a slight decrease in the TDF and IDF content, but no changes were observed in terms of SDF (Table 4.2.1). A similar trend in dietary fiber change was also found by Koehler et al. (2007) up to 4 days of sprouting at about 20° C. The decrease in TDF is likely due to the breakdown of the water extractable dietary fiber components to smaller molecules which are not precipitable in ethanol, thus not counted as dietary fiber by the method used (Hansen et al., 2002; Marti et al., 2014b). On the other hand, the decrease in IDF might be due to the xylanase activity developed during sprouting (Table 4.2.1). Indeed, such enzymes are the main responsible for the hydrolysis of xylan, the principal component of hemicellulose (about 30% of cell walls) (Shekiro et al., 2012). The changes in dietary fiber component ratio might affect the functional properties of foods, which are mainly related to fiber-water interactions (i.e., water holding capacity (WHC) and water binding capacity (WBC). The WHC indicates the amount of water that fibers can absorb in the absence of an external force, instead, WBC represents the amount of water that remains

bound to fiber after the application of an external force (Jacobs et al., 2015). Sprouting process affected fiber-water interactions as a result of the changes induced by the xylanase activity developed during the process (Table 4.2.1). Specifically, the decrease in WHC in BSW could be due to the decrease in IDF (Singh et al., 2018) and perhaps to the lower (even if not statistically significant) content of water-unsoluble arabinoxylans, compared to BUW (Table 4.2.1). Indeed, water-unsoluble arabinoxylans are characterized by a stronger WHC compared to the water-soluble ones (Foschia et al., 2013). The significant decrease in IDF and the slight increase in water-soluble arabinoxylans in BSW could also explain its lower ability to absorb water in the dough system (see farinograph index; Table 4.2.2) compared to BUW. Indeed, these two components are responsible for the WHC of the fiber-enrich foods (Lebesi and Tzia, 2012). The xylan-degradation might profoundly modify the bran attitude in bread-making, likely due to modification in water distribution caused by losing their strong water-holding capacity (Gruppen et al., 1993). In order to improve the bran enriched-bread acceptance, generally hemicelluloses (e.g., endoxylanase) are used in bread-making (Lebesi and Tzia, 2012), since the soluble arabinoxylans released contribute to forming a hydrated network, together with gluten-forming proteins (Katina, 2003).

To understand the effects of sprouting on fiber-protein interactions, the bran-enriched flours were assessed by means of the GlutoPeak test. Generally, flour with good bread-making quality is characterized by a much slower buildup in dough consistency, requiring more time to reach peak consistency and show high maximum torque (Marti et al., 2015). Regardless of the type of bran, the profile of bran-enriched flour samples suggested a weakening of the gluten network, since the presence of fiber causes deleterious effects on dough structure due to the dilution of the gluten matrix (Laurikainen et al., 1998). Several authors postulated that the worsening of dough attributes cannot be related to gluten dilution only (Pomeranz et al., 1977), but also to physical, chemical and/or biochemical bran properties (Hemdane et al., 2016). As reported in Table 4.2.2, BSW-blend profile showed

a longer peak maximum time than BUW-blend, suggesting that sprouting attenuated the negative impact on gluten aggregation properties. A possible reason is that the higher insoluble content of dietary fiber in the BUW-blend allowed the formation of a viscous film that hindered the interaction among flour particles (Bucsella et al., 2016), preventing the formation of a strong gluten network like that of BSW-blend. In addition, even the decrease in phytic acid content in the BSW-sample could play a role in determining a less negative impact on gluten aggregation. Indeed, phytic acid might react with gluten-forming proteins worsening their aggregation capacity (Hídvégi and Lásztity, 2003; Noort et al., 2010).

The gluten weakening resulting from the addition of bran was also observed in the dough system, evaluated as mixing properties (i.e., dough development time and stability, and degree of softening) (Table 4.2.2). In this system, the addition of BSW had a greater impact than BUW (Table 4.2.2). This result might be due to proteases developed in the bran layers during the sprouting process (Table 4.2.1), as also shown by CLSM (Figure 4.2.1d). The effect of proteases is evident during the farinograph test that provides for a long mixing time (about 20 min). In accordance, Marti et al. (2017) reported that after 48 h of sprouting, proteases in refined flour increased from 0.66 to 1.43 unit/g.

Despite the worsening of mixing properties, the enzymatic activity developed during sprouting did not further worsen the extensibility properties of the dough (Table 4.2.3). Indeed, differences between BUW- and BSW-blends were observed only after 135 min in terms of resistance to extension and energy (Table 4.2.3). The high capacity of the BSW-blend to maintain the extensibility properties could be attributed to the new interactions established between the gluten network and the fiber able to partially contrast the negative effect of the hydrolytic activity developed during the sprouting process and activated during the test (Table 4.2.1). Selinheimo et al. (2006) reported that proteases - specifically laccase which could play some role in the pre-harvest sprouting (Arif et al., 2012) - increase the maximum resistance and, at the

same time, decrease the extensibility of the dough. Moreover, it was reported that the use of cell-wall degrading enzymes (i.e., xylanases) increases the resistance to extension in wheat doughs (Hartikainen et al., 2014; Selinheimo et al., 2006). The parameter of resistance to extension is an indicator of dough-handling properties and it is positively correlated to dough volume, since doughs characterized by a high resistance to deformation show better performance in bread-making (van Vliet, 2008). In that respect, the more resistant gluten of BSW-blend led to a bread characterized by higher volume than BUW, although not statistically significant (Table 4.2.5). We expect that differences between the samples will be highlighted whenever the bread-making trials will be carried out on larger scale (i.e., >250g loaf), instead of micro-scale, as in this study, or using different leavening processes.

The improvement of bread in the presence of BSW (Table 4.2.5) can also be linked to the xylanase activity developed during the sprouting process (Table 4.2.1). Indeed, in bread-making, xylanases are commonly used to improve dough handling properties (Courtin et al., 2001), and loaf volume (Courtin et al., 1999, 2001).

As regards crumb firming rate, it was evaluated by using the Avrami equation (1). In general, samples with high firmness rate constant (k) values are characterized by fast crumb firming kinetics, whereas low k values lead to a slow firming kinetics. Both firmness rate constant and initial crumb firmness showed a decrease when BSW was used (see crumb firmness kinetic values; Table 4.2.5) instead of BUW. This result might indicate that the xylanase activity developed during the sprouting process improved the initial crumb texture and its firming rate of the resulting bread (Table 4.2.5). Indeed, the positive effects of xylanases on bread are related to the cleavage of the backbone of arabinoxylans, with the consequent release of water and decrease in water-insoluble pentosane (Rouau et al., 1994). In addition to xylanases, also α-amylases have positive effects on dough development and crumb staling. The greater presence of fermentable sugars in the BSW (Table 4.2.1) – consumable by yeasts for their growth and CO_2 production – might

have contributed positively to the decrease in leavening time (Table 4.2.4) and to the greater volume of the corresponding bread (Table 4.2.5) (Ibrahim and Dappolonia, 1979). The presence of BSW enhanced the textural properties of loaves, since the crumb hardness – already after two hours of baking – was significantly lower compared to the other samples. This difference is not due to either the crumb moisture, which was not statistically different among the samples (data not shown), or to the crumb porosity, as the BSW-bread was characterized by the lowest value of this index. On the contrary, the crumb texture improvement is due to the α-amylase activity, whose effects have been demonstrated in several studies (De Leyn, 2006; Goesaert et al., 2005, 2009). Furthermore, the high α-amylase activity of the BSW had positive effects on the firming rate of the corresponding bread crumb (Table 4.2.5).

4.2.6. Conclusions

This research provides information about the effects induced by the sprouting process on the chemical and physical properties of bran and how these features affect its interaction with water and gluten in both slurry (using the GlutoPeak test) and dough systems. Last but not least, the effect on bread was considered. Unlike the numerous studies present in the literature, where the enzymatic treatments were conducted directly on bran, isolated after milling (i.e., fermentation, addition of enzymes), in this study the bio-technological treatment was performed directly on the wheat kernels. Using bran from sprouted wheat in bread-making has led to positive effects in terms of gluten aggregation kinetic, bread volume and crumb softness, compared to the use of conventional bran. Thus, bran from sprouted wheat might represent a valid strategy to produce staple foods rich in fiber with high quality traits. Moreover, the use of milling by-products is a good approach to decrease food losses. Finally, results from this study might encourage the use of bran from sprouted wheat in small amounts in bread formulations to replace the use of exogenous enzymatic improvers and create clean-label products.

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4.3. Bread-making performance of durum wheat as affected by sprouting

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4.3.1. Abstract

The effects of sprouting time (from 24 h to 62 h) were assessed on durum wheat kernel characteristics, semolina chemical composition, rheological properties, and bread-making performance. Sprouting decreased both kernel hardness (~29%) and test weight (~19%). Starch gelatinization and retrogradation capability, as well as the gluten aggregation properties, decreased as sprouting time increased. The 62 h sample showed the worst aggregation properties leading to a bread with the lowest specific volume (2.69 mL/g). The best bread specific volume (3.08 mL/g) and crumb porosity distribution were obtained using semolina from sprouted wheat up to 38 h. Principal Component Analysis and clustering confirmed the relationships between all the considered variables and allowed to assess three sprouting levels: 24-38 h with improved bread-making performance; 48 h with decreased overall quality; 62 h with the worst quality. In conclusion, the sprouting of durum wheat up to 38 h could improve its bread-making attitude.

4.3.2. Introduction

Durum wheat (*Triticum turgidum* subsp. *durum*) is characterized by a peculiar hard and vitreous endosperm which influences its milling behavior, e.g., milling energy, yield and the starch damage (Turnbull and Rahman, 2002). The strength and poor extensibility of its gluten network makes durum wheat the ideal raw material for pasta-making but unsuitable for baked- goods (Ammar et al., 2000). Despite the enhanced nutritional traits thanks to the carotenoids (Pasqualone et al., 2004), using durum wheat in bread-making results in low loaf volume and dense crumb structure (Sissons, 2008).

However, dough extensibility and bread volume improved using sourdough fermentation, since the combination of acidity and hydrolytic activity of both lactic acid bacteria and yeasts positively affect durum wheat gluten functionality (Barber et al., 1992). Considering the above, this study investigated the exploitation of the enzymatic pattern developed throughout sprouting to improve the bread-making performance of durum wheat. Although an excessive accumulation of enzymes in wheat has always represented a negative event from a technological standpoint, recently it has been reported that sprouting improved the bread-making performance of common wheat (Marti et al., 2017, 2018). In the case of durum wheat, the sprouting process has been recently investigated in relation to bioactive compounds (Jribi et al., 2019b) and functional properties (Jribi et al., 2019a) of wholemeal semolina. To the best of our knowledge, no study has focused yet on the relationship between sprouting and bread-making performance of durum wheat. Since the understanding of flour functionality is a key element in the production of cereal-based products, the aim of this study was to evaluate the effects of sprouting time on durum wheat kernel characteristics, starch and gluten behavior, and their relationship with the bread characteristics also from a multivariate point of view, thus applying Principal Component Analysis and clustering.

4.3.3. Materials and methods

4.3.3.1. Sample preparation

Five aliquots (1 kg each) of durum wheat (*Triticum durum* Desf.), supplied by Molino Quaglia S.p.A. (Vighizzolo d'Este, Italy), were sprouted at 20° C for 24 h, 38 h, 48 h and 62 h and dried at 50° C for 9 h, as previously reported by Grassi et al. (2018). Unsprouted durum wheat was used as control (CTRL). Unsprouted and sprouted samples were conditioned until they reached 16.5% moisture and an aliquot was ground by using a laboratory mill (M20 Universal Mill; IKA, Werke Staufen, Germany; particle size <500 μm)

into wholegrain semolina flour, instead another aliquot was milled into refined semolina flour by using a laboratory mill (RM1300, Erkaya, Turkey), equipped with a 250 μm sieve.

4.3.3.2. Kernel hardness and test weight

Kernel hardness was assessed by NIR (6500, Foss, USA) following the AACC method 39-70.02 (AACCI, 2001). test weight was determined with a Grain Analysis Computer (2100b, DICKEY-john, USA).

4.3.3.3. Chemical composition and enzymatic activities of both wholegrain and refined semolina flour

Total and damaged starch content were evaluated according to AACC methods (76-13.01 and 76-31.01, respectively) (AACCI, 2001). Simple sugars were quantified by means of the Maltose/Sucrose/D-Glucose Assay kit commercialized by Megazyme (NEO-GEN/Megazyme, Lansing, USA). Protein content was quantified by following the ISO method 20483:2006 (ISO, 2006). α-amylase activity was determined according to the AACC method 22-02.01 (AACCI, 2001), whereas protease activity was evaluated according to the method reported in Section 4.1.3.2.

4.3.3.4. Pasting properties of refined semolina flour

Starch pasting properties by using the Rapid Viscoanalyzer (4500, PerkinElmer, Inc., Spokane, USA) according to the AACC method 76–21.01 (AACCI, 2001) in presence of either water or silver nitrate ($AgNO_3$; 1mM) as enzymatic inhibitor.

4.3.3.5. Gluten aggregation properties of refined semolina flour

Gluten aggregation kinetic by using the GlutoPeak (Brabender GmbH & Co., Duisburg, Germany) device. Flour (9 g) was dispersed in distilled water (9 mL), scaling both on a 14% sample moisture basis. The test was performed by setting the paddle speed at 2750 rpm and the circulating water bath at 35° C.

4.3.3.6. Dough preparation and leavening properties of refined semolina flour

Semolina flour was kneaded with fresh yeast (3%; Carrefour) and salt (NaCl, 1.5%; Candor) in an automatic mixer equipped with a spiral hook (KitchenAid 5KSM125EER, Whirlpool, USA) for 6 min, until a smooth and non-sticky dough was obtained. The amount of water used in the formulations has been added on the basis of preliminary tests. Specifically, 64.5% of water was added to CTRL and 24 h sample, 60.5% of water for 38 h and 48 h samples and, finally, 58.5% of water for 62 h sample. Three portions (5 g) of the resulted doughs were molded in a spherical shape and then placed in three Petri dishes, and subjected to leavening at 30° C. The Petri dishes were scanned at 300 dpi with a flatbed scanner (Epson Perfection 550 Photo, Seiko-Epson, Japan) at the beginning of the test, and after 15 min, 30 min, 45 min, 60 min, 90 min, 120 min and 180 min. The radial increase of the dough area (mm^2) was determined by image analysis using the Image Pro Plus software v. 6.0 (Media Cybernetics, USA) and it was used to determine the relative increase of dough surface (A_t/A_t_0), through the ratio between the area at time t (A_t) and the area of the dough at the beginning of the test (A_t_0), according to Caramanico et al. (2018).

4.3.3.7. Micro-baking test of refined semolina flour

Dough samples were obtained as reported in the previous paragraph. Samples were shaped, left to rise (90 min at 30° C) and baked (20 min at 200° C) as reported in Section 4.1.3.6. The obtained loaves were characterized 2 h after baking.

4.3.3.8. Bread properties

Each loaf was characterized for specific volume (SpV) through the ratio between the bread volume, evaluated by the seed replacement method (AACC 10-05.01) (AACCI, 2001) and the bread weight. Crumb porosity was assessed as described by Marti et al. (2017) with some modifications about pore dimensional classes (i.e., <0.09 mm^2; 0.10-0.99 mm^2; 1.00-2.99 mm^2;

3.00-9.99 mm^2; >10.00 mm^2). Crumb yellowness was evaluated by means of a digital colorimeter (Digital Color Meter, Apple Inc., USA).

4.3.3.9. Statistical analysis

Chemical composition, enzymatic activities, aggregation, and leavening properties were carried out in triplicate; instead, pasting properties were determined in duplicate. As regards bread, for each type of sample one baking test was performed and two loaves were obtained. Specific volume was replicated two times, instead crumb porosity, and crumb yellowness were carried out on the three central slices of each loaf.

Data were elaborated by one-way analysis of variance using sprouting time as factors (Statgraphics Plus 5.1; Statpoint Inc., USA). When a factor was found significant ($p<0.05$), the differences among the mean values were determined by the Tukey HSD test. Data were also explored by Principal Component Analysis (PCA) after data mean centering by means of Matlab software (v. 2016a, Mathworks, Inc., USA). Samples grouping was confirmed by K-Nearest Neighbor cluster analysis (PLS toolbox, v. 8.5, Eigenvector Research, Inc., USA).

4.3.4. Results

4.3.4.1. Kernel characteristics

The sprouting process caused a significant decrease in both kernel hardness (from 112 to 78 after 24 h of sprouting) and test weight (from 80 kg/hL to 69 kg/hL after 24 h of sprouting). However, both the indices seem not to be affected by the sprouting time (Figure 4.3.1).

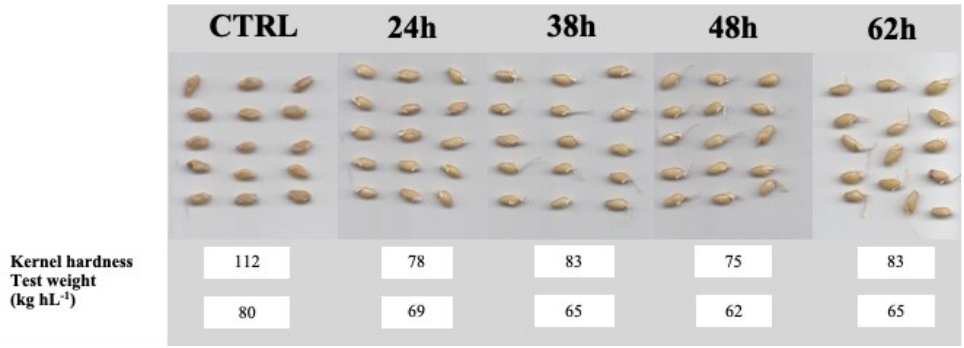


Figure 4.3.1. Kernel hardness and test weight of durum wheat kernels during sprouting, from 24 h to 62 h.

4.3.4.2. Chemical composition and enzymatic activities of both wholegrain and refined semolina flour

Regardless of the type of semolina flour, sprouting did not affect the starch content, instead the damaged starch fraction increased after 38 h of sprouting (Table 4.3.1). As the damaged starch increased also simple sugars increased; in particular, maltose increased after 24 h, instead sucrose and glucose after 38 h of sprouting (Table 4.3.1), due to the increase in the α-amylase activity (by about 260 folds), already after 24 h of sprouting (Table 4.3.1).

Sprouting time affected the protein content of wholegrain and refined semolina flour with different trends (Table 4.3.1). Specifically, sprouting led to an increase and a decrease in the protein content of wholegrain (by about 10%) and refined (by about 6%) semolina flour, respectively (Table 4.3.1). The increase in protein content could be associated with the increase in the enzyme amount in the scutellum layer, instead the decrease in the refined flours might be explained by the increase in the protease activity, starting from 24 h of sprouting (Table 4.3.1).

Table 4.3.1. Chemical characteristics and enzymatic activity of wholegrain and refined semolina flour from unsprouted (CTRL) and sprouted durum wheat at different times (24 h, 38 h, 48 h and 62 h).

		CTRL	24 h	38 h	48 h	62 h
Wholegrain semolina	Total starch	61±2^{a}	69±2^{b}	62±2^{a}	60±2^{a}	60±1^{a}
	Damaged starch	5.2±0.3^{a}	5.0±0.3^{a}	6.9±0.2^{b}	7.9±0.3^{c}	8.4±0.3^{c}
	Maltose	0.4±0.1^{a}	0.8±0.1^{b}	1.6±0.1^{c}	1.9±0.1^{d}	2.3±0.1^{e}
	Sucrose	1.8±0.1^{a}	1.9±0.1^{a}	2.2±0.1^{b}	2.3±0.3^{b}	2.6±0.1^{c}
	D-glucose	0.2±0.1^{a}	0.2±0.1^{a}	0.3±0.1^{b}	0.5±0.1^{c}	0.6±0.1^{c}
	Protein	14.5±0.1^{a}	15.9±0.1^{b}	15.9±0.1^{b}	16.0±0.1^{b}	16.0±0.1^{b}
	α-amylase activity	0.10±0.01^{a}	3.2±0.2^{b}	9.8±0.5^{c}	19.8±0.3^{d}	27±1^{e}
	Protease activity	0.5±0.3^{a}	0.8±0.2^{b}	1.7±0.8^{c}	2.0±0.5^{c}	2.8±0.3^{d}
Refined semolina	Total starch	71±2^{a}	71±1^{a}	72±1^{a}	71±1^{a}	69±2^{a}
	Damaged starch	10.3±0.2^{a}	9.7±0.2^{a}	13.2±0.3^{b}	13.4±0.5^{b}	15.9±0.3^{c}
	Maltose	0.3±0.2^{a}	2.1±0.4^{b}	4.7±0.3^{c}	5.4±0.5^{c}	6.6±0.5^{d}
	Sucrose	1.5±0.3^{a}	1.9±0.2^{ab}	2.0±0.1^{b}	2.0±0.4^{b}	2.1±0.2^{b}
	D-glucose	0.2±0.1^{a}	0.2±0.1^{a}	0.4±0.1^{ab}	0.41±0.03^{b}	0.4±0.1^{b}
	Protein	14.1±0.1^{c}	14.11±0.02^{c}	13.80±0.02^{b}	13.9±0.1^{b}	13.3±0.1^{a}
	α-amylase activity	0.09±0.01^{a}	3.8±0.3^{b}	9.9±0.5^{c}	21.6±0.9^{d}	24.3±0.8^{e}
	Protease activity	0.09±0.03^{a}	0.21±0.06^{b}	0.30±0.03^{c}	0.30±0.05^{c}	0.35±0.04^{c}

Different letters in the same row correspond to significant differences (Tukey HSD test; $p<0.05$; n=3). Compositional data are expressed as g/100g sample (d.b.). α-amylase and protease activities are expressed as Ceralpha Units (CU)/g flour and as mg of azocasein/h*g of sample (d.b.), respectively. Damaged starch is expressed as g/100g total starch.

4.3.4.3. Pasting properties of refined semolina flour

Regardless of the sprouting time, in the presence of water, sprouted samples showed low viscosity values (<100 cP), in both heating and cooling stages (data not shown). Inhibiting the amylase activity with a solution of silver nitrate ($AgNO_3$; 0.1M) all samples showed a higher viscosity, indicating that the pasting and gelation properties of sprouted samples were not drastically affected by sprouting (Figure 4.3.2).

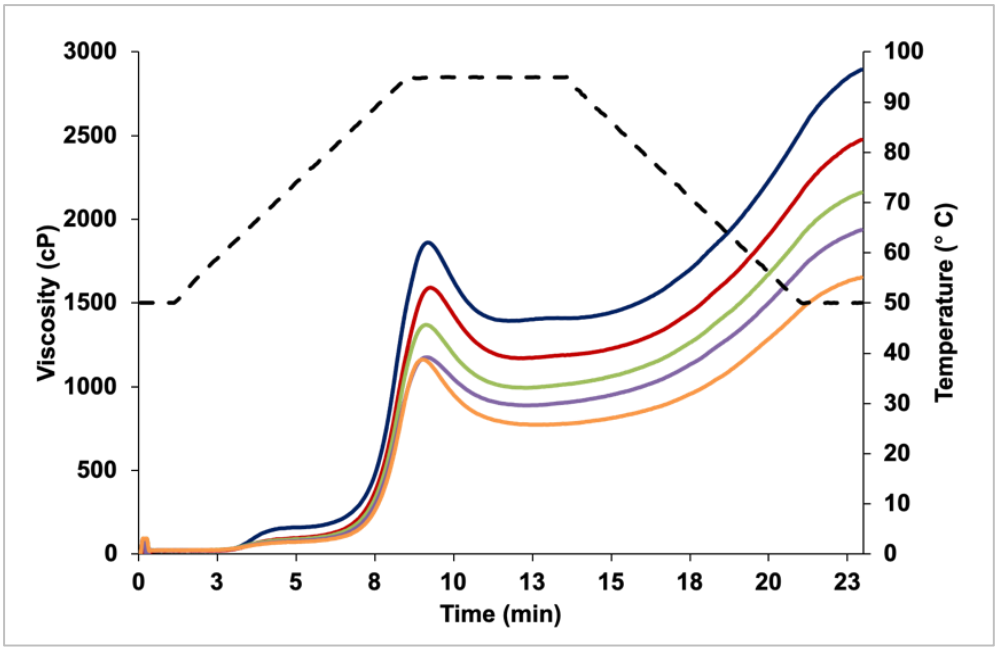


Figure 4.3.2. Profiles of pasting and gelation properties of semolina from unsprouted (CTRL) and sprouted durum wheat. CTRL: blue line; 24 h: red line; 38 h: green line; 48 h: purple line; 62 h: orange line.

Specifically, the peak viscosity and the breakdown index (i.e., resistance of the gel to mechanical stress) significantly decreased after 48 h of sprouting (Figure 4.3.2; Table 4.3.2). Moreover, the final viscosity and the setback index (i.e., the tendency of starch to retrograde) decreased as sprouting time increased, up to 62 h of sprouting (Figure 4.3.2; Table 4.3.2).

Table 4.3.2. Pasting properties of semolina flour from unsprouted (CTRL) and sprouted durum wheat at different times (24 h, 38 h, 48 h and 62 h).

	CTRL	24 h	38 h	48 h	62 h
Peak viscosity	1866±8^{d}	1576±24^{c}	1342±40^{b}	1176±1^{a}	1156±8^{a}
Breakdown	443±11^{e}	391±28^{d}	335±45^{c}	275±6^{a}	360±40^{b}
Final viscosity	2924±43^{e}	2471±8^{d}	2163±2^{c}	1948±15^{b}	1708±78^{a}
Setback	1501±23^{e}	1285±11^{d}	1155±3^{c}	1047±10^{b}	912±46^{a}

Different letters in the same row correspond to significant differences (Tukey HSD test; $p<0.05$; n=3). Peak viscosity, breakdown, final viscosity, and setback are expressed in Centipoise (cP).

4.3.4.4. Gluten aggregation properties of refined semolina flour

As regards changes in gluten aggregation kinetics (Figure 4.3.3), sprouting led to a significant increase in the peak maximum time starting from 38 h of sprouting, and a decrease in both maximum torque and aggregation energy (i.e., energy required for gluten aggregation) (Table 4.3.3), already after 24 h of sprouting.

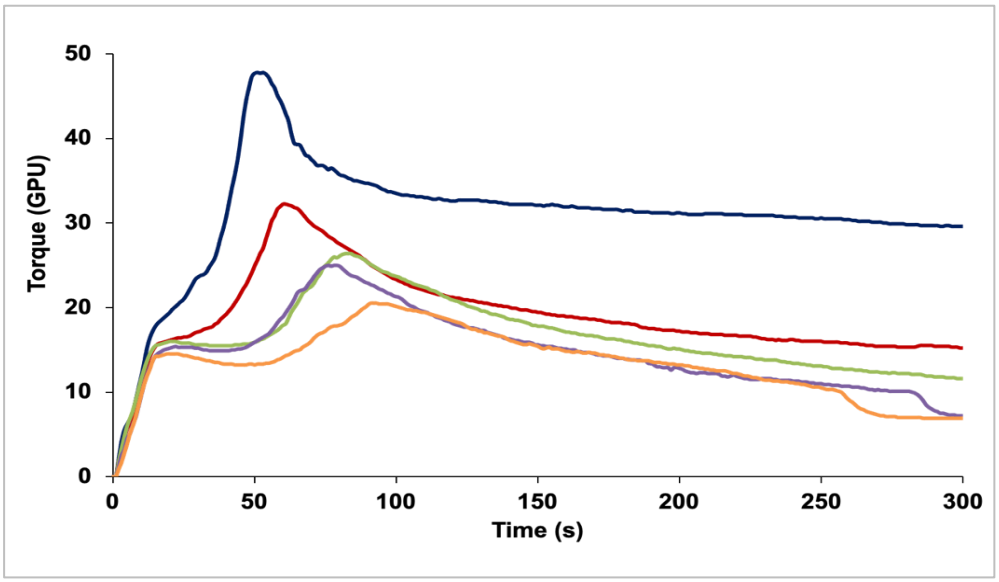


Figure 4.3.3. Profiles of gluten aggregation properties of semolina from unsprouted (CTRL) and sprouted durum wheat. CTRL: blue line; 24 h: red line; 38 h: green line; 48 h: purple line; 62 h: orange line.

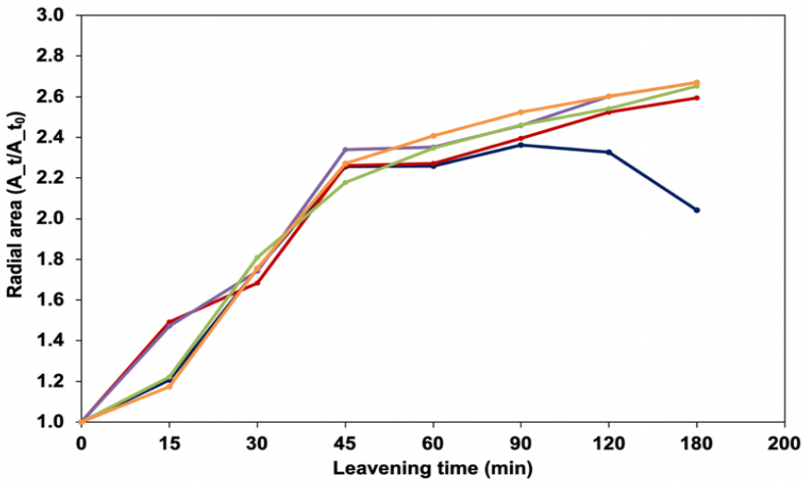
Table 4.3.3. Gluten aggregation properties of semolina flour from unsprouted (CTRL) and sprouted durum wheat at different times (24 h, 38 h, 48 h and 62 h).

	CTRL	24 h	38 h	48 h	62 h
Maximum torque	47.0±0.8^{e}	31.8±0.9^{d}	26.4±0.1^{c}	24.2±0.9^{b}	20.5±0.7^{a}
Peak maximum time	60±2^{a}	62±3^{a}	83±2^{b}	77±2^{b}	98±6^{c}
Aggregation energy	1239±47^{e}	887±15^{d}	758±9^{c}	694±21^{b}	592±15^{a}

Different letters in the same row correspond to significant differences (Tukey HSD test; $p<0.05$; n=3). Maximum torque is expressed in GlutoPeak Units (GPU), peak maximum time in seconds (s) and aggregation energy in GlutoPeak Equivalent (GPE).

4.3.4.5. Dough leavening properties of refined semolina flour

Dough leavening properties were evaluated by monitoring changes in the radial area. CTRL reached the maximum development in 45 min (A_t_{45}/A_t_0=2.3) and no longer increased up to 120 min of leavening; after that, it decreased (A_t_{180}/A_t_0=2.0) (Figure 4.3.4). In contrast, the radial area of sprouted wheat dough constantly increased until the end of the test period (A_t_{180}/A_t_0=2.7) (Figure 4.3.4). The fastest area expansion was observed after 24 h and 36 h of sprouting, subsequent to leavening for 15 min.



Leavening time (min)	CTRL	24 h	38 h	48 h	62 h
15	1.21±0.01^a	1.49±0.05^b	1.47±0.05^b	1.22±0.04^a	1.17±0.01^a
30	1.75±0.02^a	1.68±0.03^a	1.74±0.07^a	1.81±0.07^a	1.76±0.03^a
45	2.26±0.03^a	2.26±0.06^a	2.34±0.08^a	2.18±0.06^a	2.27±0.03^a
60	2.3±0.1^a	2.27±0.08^a	2.4±0.1^a	2.4±0.1^a	2.41±0.06^a
90	2.3±0.1^a	2.39±0.04^a	2.46±0.03^a	2.46±0.01^a	2.5±0.1^a
120	2.33±0.09^a	2.52±0.03^a	2.60±0.09^a	2.6±0.1^a	2.60±0.07^a
180	2.0±0.2^a	2.59±0.05^b	2.67±0.09^b	2.65±0.03^b	2.67±0.06^b

Figure 4.3.4. Increasing the radial area (A_t/A_t_0) of the dough during leavening. CTRL: blue line; 24 h: red line; 38 h: green line; 48 h: purple line; 62 h: orange line. Different letters in the same row correspond to significant differences among the samples at the same leavening time (Tukey HSD test, $p<0.05$; n=3). A_t_0, radial area of the dough at the beginning of the leavening; A_t, radial area of the dough at time t.

4.3.4.6. Bread-making properties of refined semolina flour

Using sprouted wheat did not lead to a drastic worsening of bread properties, in terms of volume, not even after 62 h of sprouting (Table 4.3.4).

Table 4.3.4. Crumb yellowness and specific volume of bread prepared from semolina flours from unsprouted (CTRL) and sprouted durum wheat.

	CTRL	24 h	38 h	48 h	62 h
Volume	178±4^{a}	173±4^{a}	180±1^{a}	180±1^{a}	178±4^{a}
Specific volume	2.88±0.03^{b}	3.05±0.05^{c}	3.08±0.01^{c}	2.76±0.04^{ab}	2.69±0.03^{a}
Crumb yellowness	29±2^{a}	37±1^{b}	35±2^{b}	37±2^{b}	34±1^{b}

Different letters in the same row correspond to significant differences (Tukey HSD test; $p<0.05$; n=3). Volume and specific volume were expressed in milliliters (mL) and milliliters/gram (mL/g), respectively.

Samples from 24 h and 38 h sprouted wheat showed the best bread-making performances, in terms of specific volume (Table 4.3.4). Instead, the 62 h sprouted sample showed the worst crumb structure that appeared sticky and irregular (Figure 4.3.5).

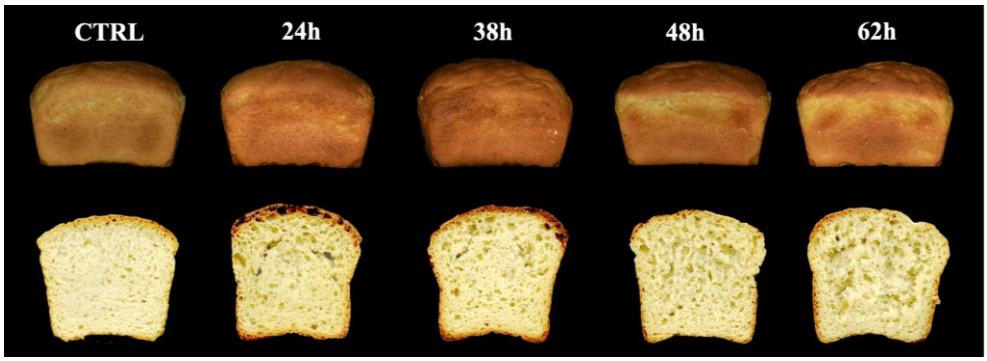


Figure 4.3.5. Pictures of the bread loaves prepared from semolina flours from unsprouted (CTRL) and sprouted durum wheat.

As regards crumb yellowness, loaves from sprouted wheat showed a more intense yellowness (Table 4.3.4). No significant differences were observed among the samples in terms of number of cells (data not shown). Unlike that, differences were observed in the cell area (Figure 4.3.6). Specifically, CTRL bread showed a crumb characterized by about 70% of small cells (<1 mm^2), instead this pore class represented about 50% of the

total in loaves from sprouted wheat. Moreover, large pores (>10 mm^2) were found only in bread from sprouted wheat whose area accounted for 10% of the total for 24 h bread, instead about 5% for 38 h and 48 h loaves.

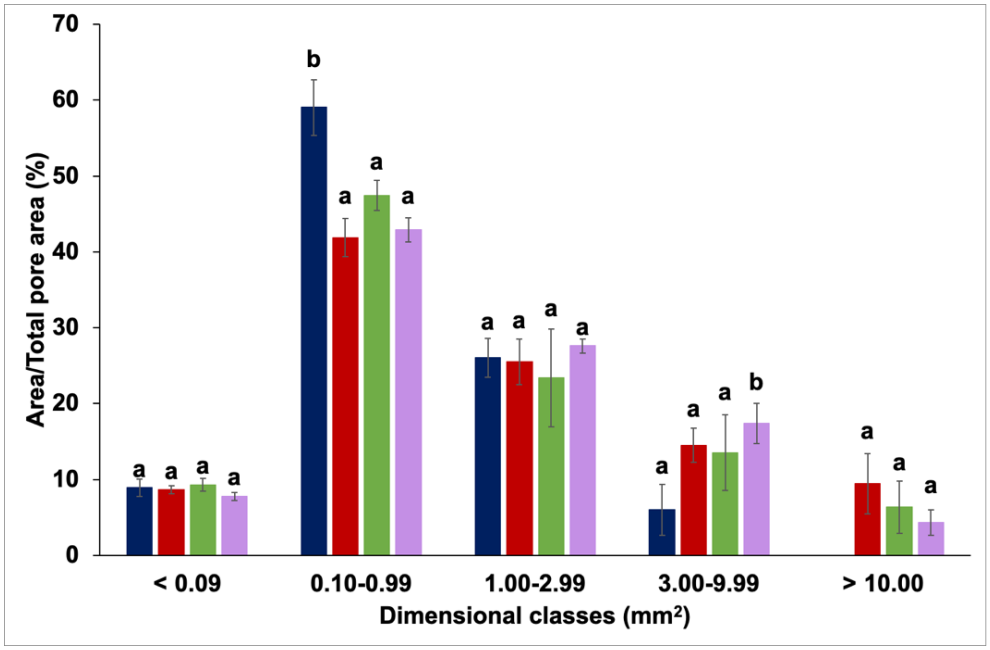


Figure 4.3.6. Dimensional classes of pores. CTRL: blue bars; 24 h: red bars; 38 h: green bars; 48 h: purple bars. Different letters in the same dimensional class correspond to significant differences (one-way ANOVA, Tukey HSD test, $p<0.05$; n=3).

4.3.4.7. PCA and cluster analysis

PCA results showed sample distribution according to chemical composition, enzymatic activities, dough leavening properties and bread-making properties. Since sprouting-related changes were similar in wholegrain and refined flours, only the data on refined flours were considered for the PCA analysis. The biplot defined by the first PCs describes almost 83% of the data variability (PC1=55.87%; PC2=27.11%) and showed a clear separation of CTRL samples from sprouted samples (Figure 4.3.7a). Indeed, CTRL samples assumed highly positive PC1 and PC2 values, being in the I quadrant of the biplot. 24 h sprouted sample is located in the IV quadrant (bottom right corner), assuming the lowest PC2 value; 38 h sprouted samples

is well separated in the III quadrant (bottom left corner); finally, 48 h and 62 h samples are grouped in the II quadrant (upper left corner). Most of the chemical indexes and α-amylase drive the separation of CTRL sample from sprouted samples along PC1, together with gluten aggregation properties; whereas leavening properties and bread characteristics result relevant in the discrimination among samples subjected to different sprouting times (24 h, 38 h, 48 h and 62 h).

The cluster analysis confirmed the sample grouping observed by PCA by identifying four clusters based on the whole results collected. From the dendrogram (Figure 4.3.7b), the first cluster, i.e., the group that differs the most from the others, is the one formed by CTRL which resulted highly (7) different from the sprouted samples, no matter the sprouting degree. By reducing the distance to 5, the analysis identified three sprouting levels: a cluster consisting of 24 h and 38 h sprouted samples and other two separated clusters for 48 h and 62 h sprouted samples.

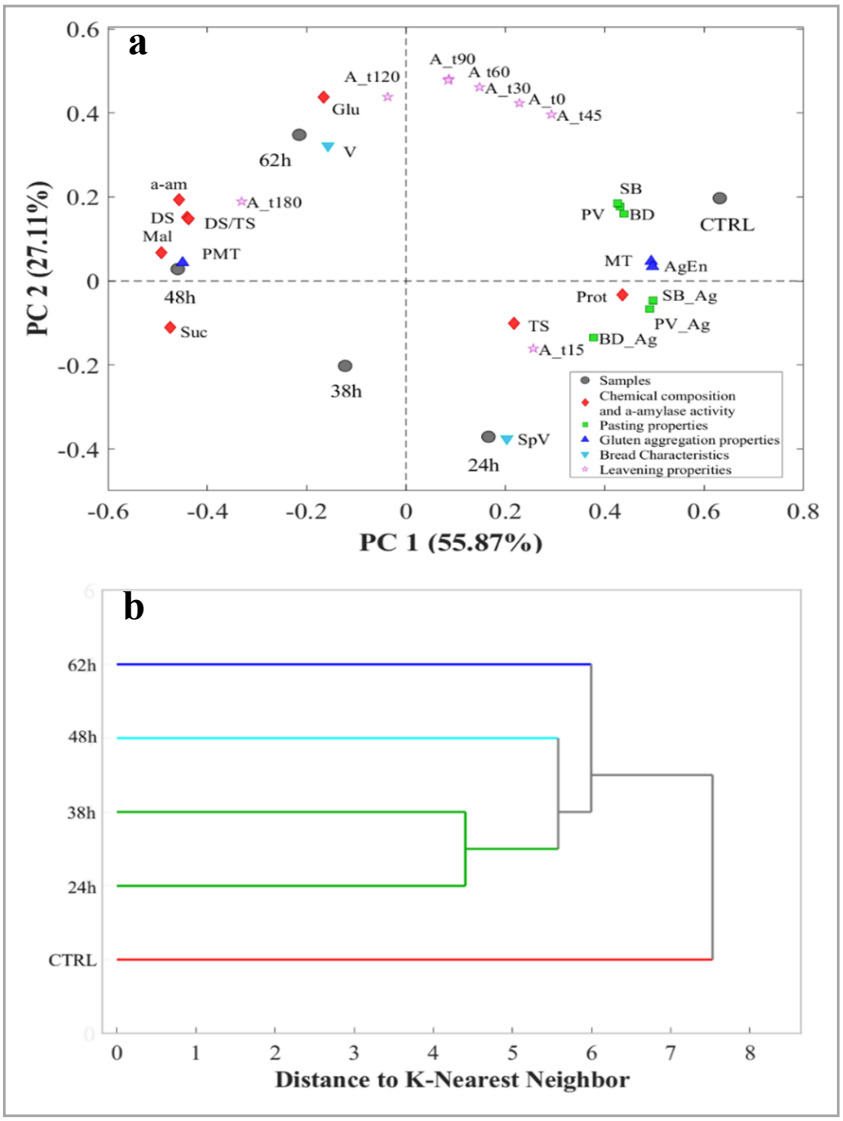


Figure 4.3.7. Multivariate data analysis on data collected for chemical composition, α-amylase activity, dough leavening properties and bread-making properties: biplot for Principal Component Analysis (a), dendrogram for cluster analysis by K-Nearest Neighbor (b). Chemical composition and enzymatic activity: A-am, α-amylase activity; TS, Total Starch; DS, Damaged Starch; Mal, Maltose; Suc, Sucrose; Glu, D-glucose; Prot, Protein. Pasting properties: PV, Peak Viscosity; BD, Breakdown index; FV, Final Viscosity. Gluten aggregation properties: PMT, Peak Maximum Time; MT, Maximum Torque; AgEn, Aggregation Energy. Leavening properties: relative increase of dough surface at 15 min (A_t_{15}), 30 min (A_t_{30}), 45 min (A_t_{45}), 60 min (A_t_{60}), 90 min (A_t_{90}), 120 min (A_t_{120}) and 180 min (A_t_{180}). Bread characteristics: SpV, Specific Volume; V, Bread Volume.

4.3.5. Discussion

Compared to common wheat, durum wheat is characterized by high kernel hardness, high gluten tenacity and intensive yellowness – due to its high carotenoid content. All these characteristics are used to evaluate the grain quality on the market. As regards the kernel characteristics, the sprouting process led to a significant decrease in hardness (Figure 4.3.1), with the greatest changes occurring at 48h sprouting time (Figure 4.3.1). The decrease in kernel hardness might positively affect the milling behavior. Indeed, hard kernels, such as durum wheat, require more energy to be milled than both soft and hard kernels (Różyło et al., 2003). Specifically, the decrease in kernel hardness might be attributed to the decrease in starch-protein matrix density in the endosperm. This hypothesis has been confirmed by the decrease in test weight (i.e., index related to the kernel density; Figure 4.3.1) due to the high α-amylase activity associated with sprouting (Table 4.3.1). The effect of enzymatic activity on decreasing the endosperm density has previously been reported in common wheat (Section 4.2.4.1). Moreover, the decrease in the kernel hardness and test weight are in line with previous studies carried out on sprouted common wheat (Miś and Grundas, 2002; Różyło et al., 2003).

In addition to milling energy, hardness also affects the milling yield and the damaged starch content of flours (Turnbull and Rahman, 2002). In this study, the milling yield does not appear to be affected by the sprouting time within 48 h, ranging from 49% for CTRL, to 48, 46, 47 and 38% for 24 h, 38 h, 48 h and 62 h, respectively (data not shown). The low yield ratio obtained could be due to the use of a laboratory mill that allowed the extraction of mainly the innermost regions of the endosperm, at the expense of the yield. The decrease in milling yield might be related to the decrease in test weight (Figure 4.3.1), with evidence of prolonged sprouting times. Indeed, after 62h the rootlet was quite evident (Figure 4.3.1), suggesting an intense hydrolysis of the storage macromolecules, as confirmed by the increased α-amylase

activity. It is generally recognized that the sprouting process is considered concluded when the rootlet reaches the kernel length, in order to avoid strongly negative effects on the kernel properties and flour functionality (Marti et al., 2020). During sprouting, high levels of hydrolytic enzymes – specifically α-amylases – are released and create some holes on the surface of the starch granules (see Section 4.1.4.1), making them more accessible to a further enzymatic action. Thus, the level of damaged starch (which is defined as the amount of starch readily accessible to α-amylase) might provide information about the intensity of the sprouting process. In general, high damaged starch content adversely affects the dough handling (e.g., greater water absorption and dough stickiness) and the bread characteristics (e.g., lower development in volume and darker crust color) (Sapirstein et al., 2007). Under the condition applied in this study, the damaged starch content increased as the sprouting time increased too (Table 4.3.1), as an effect of the increased α-amylase activity (Table 4.3.1), rather than exclusively as mechanical damage of the starch granules during milling. These findings are confirmed by the multivariate exploration by PCA, indeed damaged starch and α-amylase activity are close to each other and located in the II quadrant (upper left corner) of the biplot (Figure 4.3.7a) affecting the separation of samples sprouted 48h and 62h from lower germination exposure (24 h and 38 h) and CTRL. As regards the results about starch content, they partially agree with previous studies carried out on common wheat (Grassi et al., 2018; Świeca et al., 2017), likely due to differences in either raw material (common *vs* durum wheat) or sprouting conditions.

Sprouting resulted in lower pasting and gelation properties (Figure 4.3.2), because of the lower gelatinization and retrogradation ability of the smaller starch polymers accumulated in sprouted samples than CTRL. These changes are in line with other studies on sprouted durum (Jribi et al., 2019a) and common (see Section 4.1.4.3) wheat. This result is also remarked by the PCA biplot (Figure 4.3.7a), in which the pasting and gelation indexes calculated from the analysis performed in presence of water or silver nitrate

assumed positive PC1 scores, thus separating the CTRL from the sprouted samples. Furthermore, the lower ability to retrograde of the sprouted samples might lead to obtaining a fresh bread with a softer crumb, compared to the CTRL one, as shown in common wheat (see Section 4.1.4.5 and 4.2.4.7).

As regards the proteins, the decrease (Table 4.3.1) might be attributable to their hydrolysis into soluble peptides due to the proteolytic activity (Mbithi-Mwikya et al., 2000). On the other hand, it has been reported that changes in protein content of less than 10% indicates that the sprouting process did not significantly affect the protein content of grains (Lemmens et al., 2019). Similar changes in protein content were reported in previous studies on sprouted durum (Jribi et al., 2019b) and common (see Section 4.1.4.2; Grassi et al., 2018; Koehler et al., 2007; Marti et al., 2017) wheat.

Moving to gluten properties, the sprouting time negatively affected the aggregation properties of the gluten-forming proteins (Figure 4.3.3), in terms of peak maximum time (increased by ~63% after 62 h of sprouting), maximum torque (decreased by ~56% after 62 h of sprouting) and aggregation energy (decreased by ~52% after 62 h of sprouting), suggesting a weakening of the gluten network (Grassi et al., 2018; Marti et al., 2015a,b), as a consequence of the proteolytic activity (Table 4.3.1). Actually, the aggregation properties of the gluten-forming proteins resulted in the ones most affecting the separation between CTRL and the highly sprouted samples along the PC1 of the PCA biplot (Figure 4.3.7a), being the peak maximum time highly negative and maximum torque and aggregation energy highly positive. A possible explanation of the maximum torque and the peak maximum time shifts is that sprouting induced changes in the profile of gluten proteins (i.e., gliadin and glutenin fractions) (Koehler et al., 2007). Although the sprouted samples showed a different gluten aggregation profile that would suggest gluten weakening, they were still able to aggregate and form a gluten network with good performance in bread-making (Figure 4.3.3), confirming previous findings on common wheat (see Section 4.1.4.4). The only exception is the 62

h sample that lost its ability to form gluten (Figure 4.3.3), likely due to the stronger intensity of the sprouting process (Figure 4.3.1; Table 4.3.1).

In comparison with common wheat, durum wheat is characterized by a very strong and not very extensible gluten, making it suitable for the pasta-making but unsuitable for leavened baked-goods (Ammar et al., 2000). Indeed, the resulting bread will be characterized by a high density and a hard texture (Sissons, 2008). The interest in durum wheat bread lies in the fact that this raw material is richer in carotenoids (i.e., antioxidant compounds) compared to common wheat. Generally, to overcome the negative technological properties (i.e., low volume and high crumb density) of durum wheat bread, sourdough fermentation is used as a leavening agent. Indeed, the low pH and the enzymatic activities of lactic bacteria and yeasts enhanced bread-making performance, in terms of bread volume (Barber et al., 1992; Pagani et al., 2014). In this context, the increased enzymatic activity developed during the sprouting process might represent a good strategy to improve the bread-making attitude of durum wheat.

Thanks to the correlations between dough tenacity and strength and maximum torque and aggregation energy (Marti et al., 2015a; Rakita et al., 2018), it is possible to hypothesize that sprouting could represent a good way to decrease dough tenacity and consequently improve its bread-making performance. Despite the gluten weakening (Figure 4.3.3), the dough from sprouted durum wheat was able to withstand the leavening stresses, expanding itself without collapsing (Figure 4.3.4). The increased CO_2 production during leavening - thanks to the increased amount of fermentable sugars by yeasts, resulting from the α-amylase activity (Table 4.3.1) – increased loaf specific volume, mainly for the 24 h and 38 h samples (Figure 4.3.5). Similar results were reported for common wheat (see Section 4.1.4.5; Marti et al., 2018). The worsening of crumb structure in bread from 62 h sprouted wheat (Figure 4.3.5) agrees with the excessive gluten weakening (Figure 4.3.3). Indeed, the poor gluten aggregation properties and its gas retention capacity resulted in the lowest specific volume (Figure 4.3.5). As

regards the pore distribution, large pores (> 10 mm^2) were found only in bread from sprouted wheat, probably due to the coalescence of the gas cells, favored by α-amylase activity (Lagrain et al., 2008). In addition, bread from sprouted wheat resulted in a higher crumb yellowness (Figure 4.3.5). Yang et al. (2001) reported that the β-carotene content increased upon sprouting, and the color intensity of the carotenoid extract increased as the sprouting time increased too. Although this aspect needs to be further investigated, the finding suggests that the sprouting process might have a positive effect on the carotenoid content in bread from sprouted durum wheat.

All the considered chemical composition, α-amylase activity, dough leavening properties and bread-making properties do not act separately but are interconnected and correlated. Thus, the multivariate approach led us to confirm the relationships between all the considered variables and to define which of them contribute most to the sample distribution, i.e., in assessing the sprouting influence in the final product, as already hypothesized by Grassi et al. (2018). Indeed, the dendrogram obtained by the cluster analysis (Figure 4.3.7b) confirmed that samples sprouted up to 38 h have similar and improved bread-making performance. The two distinct clusters for 48 h and 62 h sprouted samples (Figure 4.3.7a) indicate a progressive and significant decrease of the overall quality.

4.3.6. Conclusions

Changes induced by sprouting strongly depended on the process time. Specifically, sprouting under controlled conditions (i.e., up to 48 h) did not strongly compromise the functional properties of starch (i.e., gelatinization and retrogradation phenomena). As regards proteins, despite the sprouting process caused a weakening of the gluten network, gluten proteins were still able to aggregate and retain gas during leavening, resulting in bread with improved volume. Specifically, the best bread-making performance was achieved using durum wheat that was sprouted for 38 h.

Overall results suggest that sprouting carried out under controlled conditions could improve the bread-making attitude of durum wheat and produce a more attractive product (i.e., improved bread volume and crumb porosity) for the consumer and with high carotenoid content compared to common bread. However, the effects of the sprouting process on gliadin and glutenin fractions need to be studied in depth.

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4.4. Sprouting as a pre-processing for producing quinoa-enriched bread

The results presented here below are published in: Suárez-Estrella, D., Cardone, G., Buratti, S., Pagani, M.A., Marti, A., 2020. Sprouting as a pre-processing for producing quinoa-enriched bread. J. Cereal Sci., 96, 103111.

4.4.1. Abstract

The relation among sprouting time (from 12 up to 72 h), changes in protein and starch components, and flour functionality in quinoa were considered. Changes related to the activity of proteases were observed after 48 h of sprouting in all protein fractions. Progressive proteolysis resulted in relevant modification in the organization of quinoa storage proteins, with a concomitant increase in the availability of physiologically relevant metals such as copper and zinc. Changes in the protein profile upon sprouting resulted in improved foam stability, but in impaired foaming capacity. The increased levels of amylolytic enzymes upon sprouting also made starch less prompt to gelatinize upon heating. Consequently, starch re-association in a more ordered structure upon cooling was less effective, resulting in low setback viscosity. The nature and the intensity of these modifications suggest various possibilities for using flour from sprouted quinoa as an ingredient in the formulation of baked products.

4.4.2. Introduction

Quinoa is a gluten-free grain from both an agronomic and nutritional standpoint. Specifically, quinoa is particularly high in lysine, which is the limiting amino acid in cereals, it is a good source of minerals, phenolic compounds, dietary fiber, and polyunsaturated fatty acids (Tang and Tsao, 2017). All these compositional traits account for the potential health benefits of quinoa seeds in contributing to the prevention of various diseases such as cancer, diabetes, cardiovascular diseases, and aging (Tang and Tsao, 2017). Thus, these characteristics are the driving force for enhancing the

consumption of quinoa not only as seeds but also as an ingredient in various food applications, including both enriched wheat-based goods and gluten free products.

Despite the well-known nutritional features of quinoa, its consumption is limited by the bitter and astringent taste, due to saponin compounds (Suárez-Estrella et al., 2018). Nowadays, pearling is one of the main processes applied to quinoa to improve its acceptability in food formulation; it consists in the removal of the seed external layers, which are rich in saponins (Suárez-Estrella et al., 2018). On the other hand, a significant loss of bioactive compounds occurs during the pearling process (Suárez-Estrella et al., 2018). Nowadays, quinoa is proposed in bread-making only as flour from pearled grains. Specifically, in wheat-based bread, 25% of pearled quinoa seems to be the threshold level in terms of dough rheological properties and sensory acceptability (Rosell et al., 2009); conversely, bitter aftertaste was detected at higher quinoa enrichment levels (Lorenz and Coulter, 1991).

Recently, several authors reported the possibility to exploit sprouted grains to enhance the bread-making attitude of wholewheat (see Section 4.1 and 4.2), brown-rice (Watanabe et al., 2004), and pulses (Hallén et al., 2004; Marengo et al., 2017b). The improved bread characteristics (i.e., high specific volume and crumb softness) are mainly attributable to the activity of the hydrolytic enzymes (e.g., α-amylase) developed during sprouting (Goesaert et al., 2009). Moreover, the sprouting process is associated with several grain nutritional and sensory improvements, in terms of increasing mineral and vitamin bio-availability and of decreasing antinutritional factors (e.g. phytic acid, trypsin inhibitors) (Lemmens et al., 2019). Thus, the sprouting of quinoa might be considered a useful approach to improve both its nutritional value and its bread-making attitude. Recent studies on sprouted quinoa have been pointed out the process significantly increases proteins, total phenolic content, fiber, minerals (Ca, Fe, K, Mg, P, and Zn), and decreases the phytic acid amount (Demir and Bilgiçli, 2020; Bhathal et al., 2017).

Up to now, the effects of sprouting on technological and sensory properties of quinoa dough and bread have not been extensively reported. In this context, Park and Morita (2005) studied the effects of the enrichment level of wheat flour with sprouted quinoa (up to 72 h), at 10% replacement level only. More recently, gluten-free bread with improved loaf volume and crumb softness was prepared using sprouted quinoa at 5% replacement level (Horstmann et al., 2019). Starting from the consideration above, the aim of this research was to assess the maximum enrichment level of sprouted quinoa suitable for achieving good bread-making performance. Once the optimal replacement level of sprouted quinoa was identified, the bread-making performance of this ingredient was compared with those of pearled quinoa, in order to assess the potential use of sprouted quinoa in bread-making.

4.4.3. Materials and methods

4.4.3.1. Sample preparation

Quinoa seeds (*Chenopodium quinoa* Willd. var. *Titicaca*) were provided by Quinoa Marche s.r.l. (Ancona, Italy), who also carried out the pearling process on the seeds. The untreated seeds (5 kg) were sprouted at lab scale (Memmert GmbH Co. KG, Schwabach, Germany) at 22° C for 48 h and dried (Self Cooking Center®, Rational International AG, Mestre, Italy) at 55° C for 6 h, as previously reported by Suárez-Estrella (2019). Sprouting time was selected based on preliminary results: the maximum intensity of the macromolecular modifications can be seen at 48 h of sprouting, without compromising functionality, in terms of starch gelatinization and foaming capacity and stability (Suárez-Estrella, 2019).

Pearled (PQ) and sprouted (SQ) quinoa seeds were ground by means of a Cyclotec 1093 (Foss Sample Mill, Höganäs, Sweden) lab-scale mill, to obtain flours with particle size < 250 μm.

Commercial wheat flour (WF; protein: 12.3% d.b.; W: $290*10^{-4}$ J) was provided by Molino Quaglia S.p.A. (Vighizzolo D'Este, Italy) and it was used

alone or in a mixture with either PQ or SQ flours. In particular, three sprouted quinoa:wheat blend ratios were investigated: 10:90 (10SQ), 20:80 (20SQ), and 30:70 (30SQ). In the second part of this study, the pearled quinoa:WF blend (20:80; 20PQ) was considered.

4.4.3.2. Pasting properties

Starch pasting properties were investigated by means of the Micro Visco-Amylograph (Brabender GmbH & Co. KG, Duisburg, Germany) as reported by Elkhalifa et al. (2017) with a modification (i.e., 3 min of pre-treatment at 30° C).

4.4.3.3. Gluten aggregation properties

The aggregation kinetics of gluten protein were studied by using the GlutoPeak device (Brabender GmbH & Co. KG, Duisburg, Germany), according to Section 4.3.3.5.

4.4.3.4. Mixing properties

Mixing properties were performed by means of the Farinograph-E (Brabender GmbH & Co. KG, Duisburg, Germany) with a 50 g kneading bowl following the ICC method 115/1 (ICC, 1992).

4.4.3.5. Leavening properties

Dough samples were prepared using commercial baker's yeast (2.5%; Carrefour®) and salt (1.5%; Candor®). The bread-making conditions (i.e., amount of water and mixing time) were previously determined by means of the farinographic test. Dough samples were prepared with a lab-scale kneading (Artisan 5KSM150PS KitchenAid, Whirlpool, USA) equipped with a hook. At the end of mixing, an aliquot of 315 g of the dough was placed in the Rheofermentometer F4 device (Chopin, Tripette & Renaud, Villeneuve La Garenne, France) for 3 h at 30° C, to measure dough development and gas production and retention during leavening.

4.4.3.6. Bread-making

The dough – prepared in the conditions reported in Section 4.4.3.5 – was left to rest for 10 min at room temperature (20±1° C), divided in three portions of 250 g each, modelled into cylindrical shapes, placed into baking pans (length: 12.5 cm, width: 7.5 cm; height: 5 cm), and leavened at 30° C (70% relative humidity) in a climate chamber (Self Cooking Centre®). The time necessary for leavening varied from 75 to 85 min, until the dough exceeded the top of the pans by 2.5 cm. Samples were baked at 220º C for 25 min (Self Cooking Center®), with steam injection for 5 s.

4.4.3.7. Bread characterization

Bread loaves were analysed 2 h after baking. Specific volume was obtained by the ratio between the apparent volume of bread, by sesame replacement method, and loaf weight. Crumb color profile was determined by means of a reflectance colorimeter CR 300 (Minolta Co., Osaka, Japan). The results were expressed in CIE L*a*b* colour space. Crumb softness was measured according to the Approved Method AACC 74-09.01 (AACCI, 2001) by using a Texture Analyzer TA.XT plus C (Stable Micro Systems, Surrey, UK), equipped with a 100 N load cell. Specifically, an aluminium probe (36 mm Radiused Cylinder Probe) and a test speed of 100 mm/min were used. Samples were analysed after 2, 24 and 72 h from baking, keeping the loaves in a plastic bag at room temperature until test.

4.4.3.8. Electronic tongue assessment

Electronic-tongue (e-tongue) assessment was performed on whole breads enriched in either sprouted or pearled quinoa at 20:80 replacement level, as well as on crusts and crumbs separately. Bread samples were freeze-dried (-80° C for 72 h; Alpha 1-2 LD plus; Deltek s.r.l., Naples, Italy) and milled in a lab scale mill (IKA M20, Staufen, Germany). Analyses were performed with the Taste-Sensing System SA 402B (Intelligent Sensor Technology Co. Ltd, Atsugi, Japan) according to Marengo et al. (2017a), with some

modifications. Briefly, 10 g of samples were suspended in 150 mL of distilled water and centrifuged at 5,000 *x g* for 10 min at 20° C. After centrifugation, the supernatants were tested.

4.4.3.9. Statistical analysis

All the rheological analyses were carried out in triplicate. As regards bread-making, three baking tests were performed for each sample and three loaves were obtained from each baking test. Thus, specific volume was replicated nine times while crumb color and firmness were measured on the three central slices of each bread obtained from each baking trial.

Analysis of variance (one-way ANOVA) was assessed by Statgraphics Plus 5.1 (StatPoint Inc., Warrenton, USA) using the samples as factors. The significant differences ($p < 0.05$) were determined by using the Tukey HSD test. A t-test was applied for comparing sprouted and pearled samples. Data from e-tongue measurements were elaborated by Principal Component Analysis (PCA) using MINITAB 14 (v.12.0; Minitab Inc, State College, USA) software package.

4.4.4. Results

4.4.4.1. Pasting properties

As the level of SQ increased, no significant differences were measured in terms of pasting temperature (Table 4.4.1), instead, viscosity during both heating and cooling steps decreased (Figure 4.4.1; Table 4.4.1).

Also, breakdown values decreased, suggesting an increase in heating stability in the presence of quinoa. This behavior is due to the quinoa starch granules that did not show a sharp peak but a plateau (Suárez-Estrella, 2019). Moreover, quinoa starch granules might be modified by sprouting, inducing a lower intensity of gelatinization (Suárez-Estrella, 2019). The decrease in viscosity during cooling resulted in a decrease in setback values, which seems to be related to the starch retrogradation tendency.

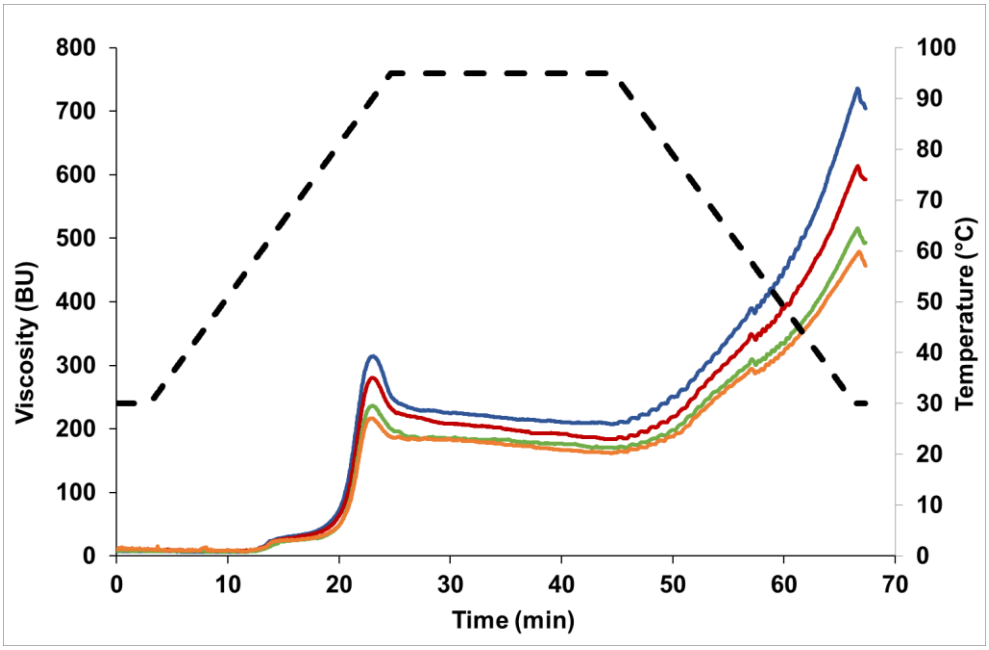


Figure 4.4.1. Profiles of pasting and gelation properties of wheat (CTRL), and with increasing replacement levels of sprouted quinoa (10SQ, 20SQ, 30SQ). CTRL: blue line; 10SQ: red line; 20SQ: green line; 30SQ: orange line. 10SQ, blend composed of sprouted quinoa and wheat flour at 10:90 ratio; 20SQ, blend composed of sprouted quinoa and wheat flour at 20:80 ratio; 30SQ, blend composed of sprouted quinoa and wheat flour at 30:70 ratio.

Table 4.4.1. Pasting properties of semolina flour from unsprouted (CTRL), and with increasing replacement levels of sprouted quinoa (10SQ, 20SQ, 30SQ).

	CTRL	10SQ	20SQ	30SQ
Pasting temperature	62.2±1.2^{a}	64.0±0.1^{a}	64.7±1.5^{a}	63.6±1.2^{a}
Peak viscosity	320±5^{d}	278±5^{c}	238±3^{b}	217±2^{a}
Breakdown	128±8^{c}	93±5^{b}	71±6^{a}	56±4^{a}
Final viscosity	712±15^{d}	578±13^{c}	493±7^{b}	449±11^{a}
Setback	505±11^{d}	393±14^{c}	326±5^{b}	288±7^{a}

Different letters in the same row correspond to significant differences (Tukey HSD test; $p<0.05$); n=3). Pasting temperature is expressed in ° C, whereas peak viscosity, breakdown, final viscosity, and setback are expressed in Brabender Units (BU).

4.4.4.2. Gluten aggregation and mixing properties

The gluten aggregation kinetics of WF was typical of a strong flour with good bread-making performance that is usually characterized by long aggregation time, high maximum torque, and energy (i.e., the area under the curve till 15 s after the maximum torque) values (Figure 4.4.2; Table 4.4.2).

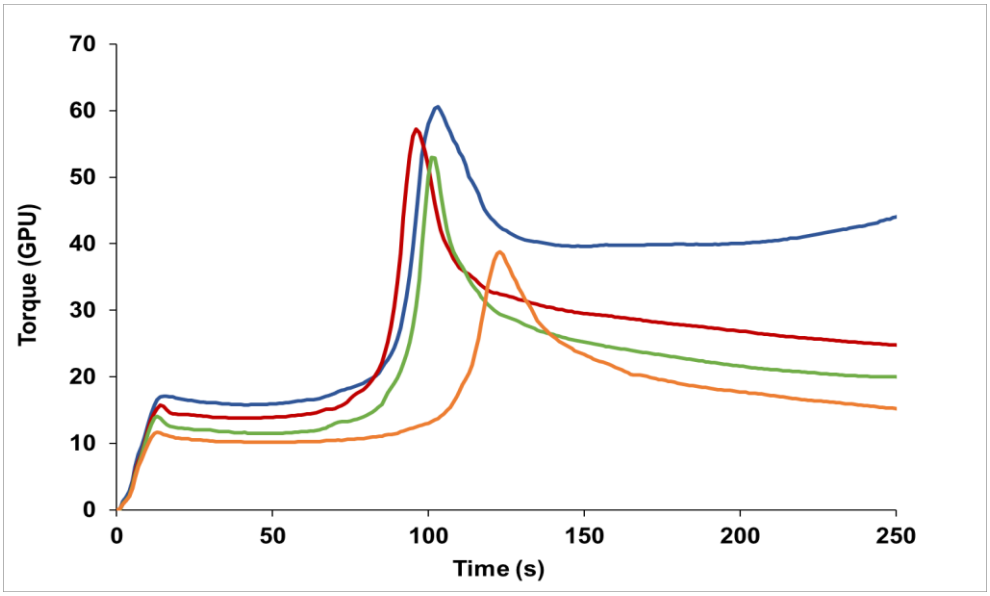


Figure 4.4.2. Profiles of gluten aggregation properties of wheat (CTRL), and with increasing replacement levels of sprouted quinoa (10SQ, 20SQ, 30SQ). CTRL: blue line; 10SQ: red line; 20SQ: green line; 30SQ: orange line. 10SQ, blend composed of sprouted quinoa and wheat flour at 10:90 ratio; 20SQ, blend composed of sprouted quinoa and wheat flour at 20:80 ratio; 30SQ, blend composed of sprouted quinoa and wheat flour at 30:70 ratio.

Replacing CTRL with SQ promoted a significant decrease in maximum torque, and energy values. This trend suggested gluten weakening as the amount of quinoa increased. As regards the time required for gluten aggregation, the value did not follow a consistent trend. Specifically, the peak maximum time decreased in 10SQ, did not change in 20SQ blend and increased in 30SQ. As regards dough mixing properties, CTRL showed a long dough development time and high stability (Figure 4.4.3; Table 4.4.2), which is a common characteristic for strong flours.

Table 4.4.2. Gluten aggregation properties of wheat (CTRL), and with increasing replacement levels of sprouted quinoa (10SQ, 20SQ, 30SQ).

	CTRL	10SQ	20SQ	30SQ
Maximum torque	61.1±0.3^{c}	60.1±0.6^{c}	53.3±2.3^{b}	39.6±1.3^{a}
Peak maximum time	104±3^{b}	96±2^{a}	102±1^{b}	123±2^{c}
Aggregation energy	1480±4^{d}	1239±25^{c}	1105±34^{b}	916±16^{a}
Water absorption	55.5±0.4^{a}	57.2±0.1^{b}	58.0±0.1^{c}	58.2±0.2^{c}
Development time	6.8±0.3^{c}	6.1±0.1^{b}	5.6±0.1^{ab}	5.5±0.3^{a}
Stability	23.8±1.2^{c}	5.6±0.2^{b}	3.5±0.1^{a}	3.3±0.2^{a}
Degree of softening	17±2^{a}	93±4^{b}	132±5^{c}	152±3^{d}

Different letters in the same row correspond to significant differences (Tukey HSD test; $p<0.05$; n=3). 10SQ, blend composed of sprouted quinoa and wheat flour at 10:90 ratio; 20SQ, blend composed of sprouted quinoa and wheat flour at 20:80 ratio; 30SQ, blend composed of sprouted quinoa and wheat flour at 30:70 ratio. Maximum torque is expressed in GlutoPeak Units (GPU), peak maximum time in seconds (s) and aggregation energy in GlutoPeak Equivalent (GPE); water absorption and degree of softening are expressed in percentage (%), instead dough development time and stability in minutes (min).

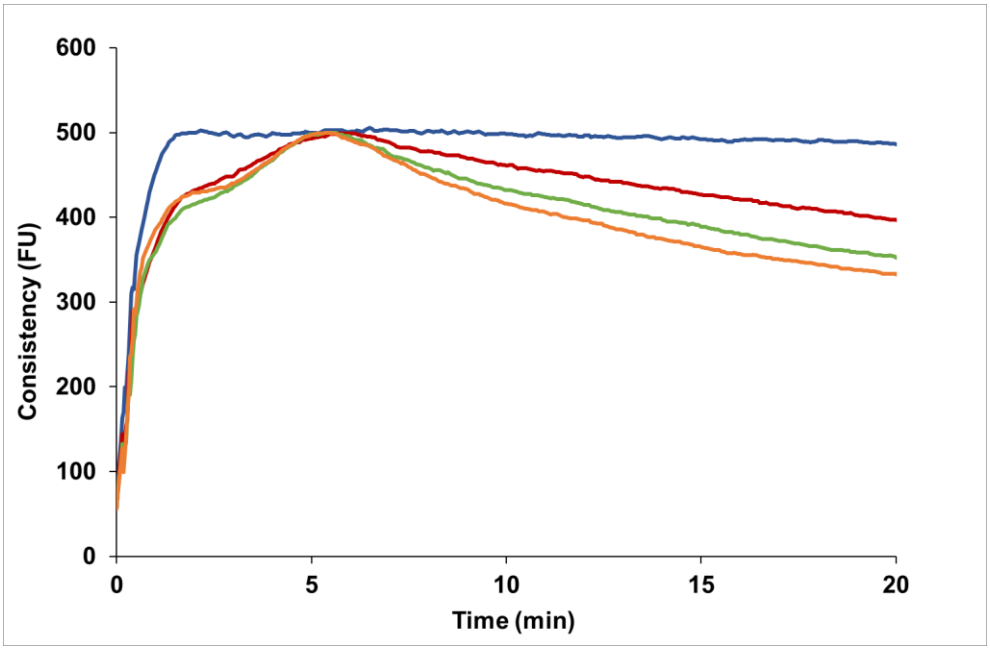


Figure 4.4.3. Profiles of mixing properties of wheat (CTRL), and with increasing replacement levels of sprouted quinoa (10SQ, 20SQ, 30SQ). CTRL: blue line; 10SQ: red line; 20SQ: green line; 30SQ: orange line. 10SQ, blend composed of sprouted quinoa and wheat flour at 10:90 ratio; 20SQ, blend composed of sprouted quinoa and wheat flour at 20:80 ratio; 30SQ, blend composed of sprouted quinoa and wheat flour at 30:70 ratio.

Replacing CTRL up to 20% significantly increased the amount of water to achieve the optimal dough consistency (i.e., 500 FU). The further increase in SQ did not result in a significant increase in water absorption. Up to 20% of the enrichment level, adding SQ to WF decreased the development time needed to reach optimal consistency, with no further decreasing at 30% of the replacement level. The same trend was registered for stability time, whose decrease agreed with the increase in the degree of softening.

4.4.4.3. Dough leavening properties

At the beginning of the leavening phase (up to 1 h), the sprouted quinoa-enriched dough exhibited a rapid dough development, regardless of the quinoa enrichment (Figure 4.4.4).

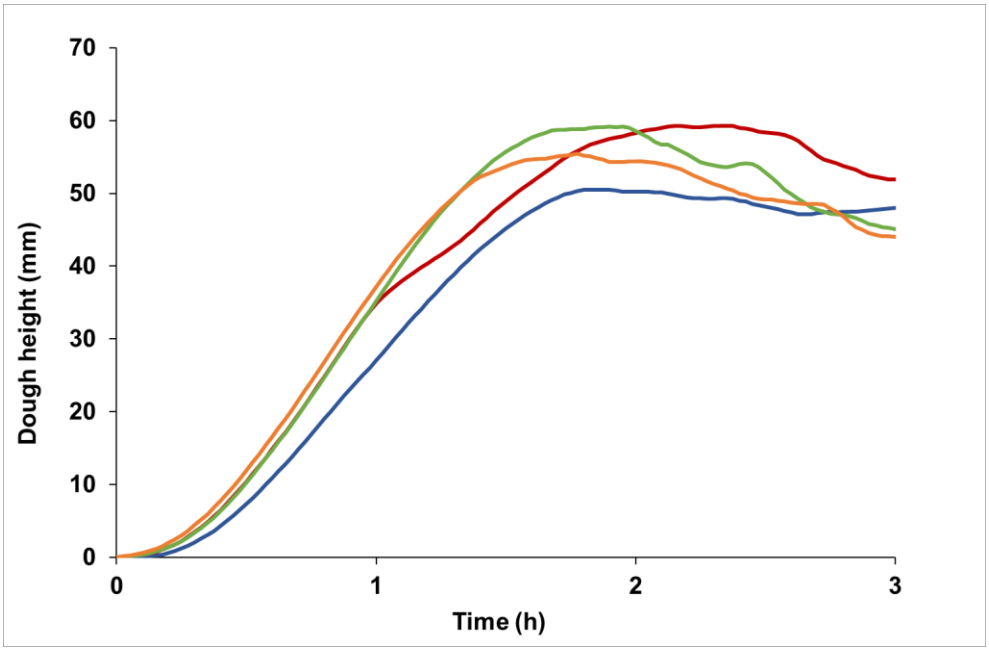


Figure 4.4.4. Dough development profiles of wheat (CTRL), and with increasing replacement levels of sprouted quinoa (10SQ, 20SQ, 30SQ). CTRL: blue line; 10SQ: red line; 20SQ: green line; 30SQ: orange line. 10SQ, blend composed of sprouted quinoa and wheat flour at 10:90 ratio; 20SQ, blend composed of sprouted quinoa and wheat flour at 20:80 ratio; 30SQ, blend composed of sprouted quinoa and wheat flour at 30:70 ratio.

Replacing CTRL up to 20% led to an increase in dough development (Table 4.4.3).

Table 4.4.3. Leavening properties of wheat (CTRL), and with increasing replacement levels of sprouted quinoa (10SQ, 20SQ, 30SQ).

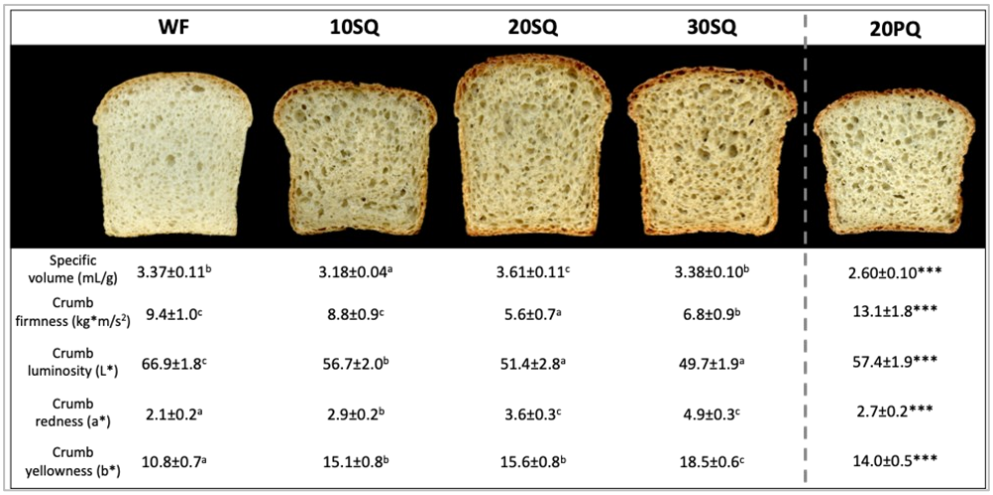
	CTRL	10SQ	20SQ	30SQ
Maximum dough height	51±1^{a}	60±1^{c}	60±1^{c}	56±1^{b}
Time of maximum dough development	138±6^{a}	138±6^{a}	114±5^{a}	114±7^{a}
Dough height at 180 min	48±2^{ab}	52±3^{b}	45±2^{a}	44±14^{a}
Weakening coefficient at 180 min	9±2^{a}	13±3^{b}	25±1^{c}	25±3^{c}
Total CO_2	1250±54^{a}	1426±26^{b}	1469±10^{b}	1464±9^{b}
Retained CO_2	1157±41^{a}	1286±14^{b}	1315±9^{b}	1294±17^{b}
Released CO_2	93±13^{a}	141±11^{b}	153±1^{bc}	169±8^{c}
CO_2 retention coefficient	93±1^{c}	90±1^{b}	90±1^{ab}	88±1^{a}
Porosity time	90±6^{b}	66±12^{a}	84±6^{ab}	72±6^{a}

Different letters in the same row correspond to significant differences (Tukey HSD test; $p<0.05$; n=3). 10SQ, blend composed of sprouted quinoa and wheat flour at 10:90 ratio; 20SQ, blend composed of sprouted quinoa and wheat flour at 20:80 ratio; 30SQ, blend composed of sprouted quinoa and wheat flour at 30:70 ratio. Maximum dough height and dough height at 180 min indices are expressed in millimeters (mm); time of maximum dough development and porosity time are expressed in minutes (min); weakening coefficient at 180 min and CO_2 retention coefficient are expressed in percentage (%); total, retained and released CO_2 are expressed in milliliters (mL).

Moreover, both CTRL and 10SQ dough samples required longer leavening time to reach the maximum dough height, compared to 20SQ and 30SQ samples (Table 4.4.3). After 2 h of leavening, either dough with 20SQ or 30SQ were not able to hold gas inside the dough, resulting in a decrease in dough height, as a consequence of the weakening of the gluten network. Dough weakening was less dramatic in 10SQ and therefore no loss in maximum height was detected up to 2.5 h of leavening. Finally, gas production increased, whereas the dough retention capacity slightly decreased in the presence of SQ, with no significant differences according to the enrichment level (Table 4.4.3).

4.4.4.4. Bread characteristics

Replacing WF with SQ led to a decrease in luminosity (L*) and an increase in both redness (a*) and yellowness (b*) of crumb (Figure 4.4.5).



	WF	10SQ	20SQ	30SQ	20PQ
Specific volume (mL/g)	3.37±0.11^{b}	3.18±0.04^{a}	3.61±0.11^{c}	3.38±0.10^{b}	2.60±0.10***
Crumb firmness (kg*m/s^2)	9.4±1.0^{c}	8.8±0.9^{c}	5.6±0.7^{a}	6.8±0.9^{b}	13.1±1.8***
Crumb luminosity (L*)	66.9±1.8^{c}	56.7±2.0^{b}	51.4±2.8^{a}	49.7±1.9^{a}	57.4±1.9***
Crumb redness (a*)	2.1±0.2^{a}	2.9±0.2^{b}	3.6±0.3^{c}	4.9±0.3^{c}	2.7±0.2***
Crumb yellowness (b*)	10.8±0.7^{a}	15.1±0.8^{b}	15.6±0.8^{b}	18.5±0.6^{c}	14.0±0.5***

Figure 4.4.5. Specific volume, crumb firmness (2 h after baking) and crumb color of bread from wheat flour (WF) and increasing replacement level of sprouted quinoa (SQ) or pearled quinoa (PQ). 10SQ, blend composed of sprouted quinoa and wheat flour at 10:90 ratio; 20SQ, blend composed of sprouted quinoa and wheat flour at 20:80 ratio; 30SQ, blend composed of sprouted quinoa and wheat flour at 30:70 ratio; 20PQ, blend composed of pearled quinoa and wheat flour at 20:80 ratio. Different letters in the same row correspond to significant differences among samples (Tukey test HSD; $p<0.05$; n=9). The asterisks indicate a statistically significant difference between the mean values of 20SQ and 20PQ (t-Test; ***$p<0.001$).

The presence of SQ did not cause negative effects on bread-making properties, except for 10SQ sample. Indeed, at this replacement level, the resulting bread was characterized by the lowest specific volume and the highest crumb firmness (Figure 4.4.5). 20SQ showed the best baking performance in terms of specific volume, whose value was even higher compared with WF and 30SQ loaves (Figure 4.4.5).

Unlike 10SQ bread, high replacement levels (20SQ and 30SQ) significantly decreased crumb firmness, contributing to high crumb softness,

not only in fresh bread (Figure 4.4.5) but also during storage (up to 72 h; data not shown).

4.4.4.5. Comparison between sprouted and pearled quinoa

Compared to using PQ, the blend enriched in SQ was characterized by a higher water absorption (~3%), shorter development time (-20%), lower stability (-49%), and higher degree of softening (76%) (Table 4.4.4).

Table 4.4.4. Mixing and leavening properties of enriched dough enriched in sprouted or pearled quinoa.

		20SQ	**20PQ**
Mixing properties	Water absorption	58.0±0.1	56.3±0.1***
	Dough development time	5.6±0.1	7.0±0.1***
	Stability	3.5±0.1	6.8±0.7**
	Degree of softening	132±5	75±5***
Leavening properties	Maximum dough height	60±1	49±2***
	Maximum height time	1.9±0.1	2.0±0.2 n.s.
	Porosity time	1.4±0.1	1.0±0.1***
	Total CO_2	1469±10	1900±20***
	CO_2 retained	1315±9	1475±15***
	CO_2 released	153±1	424±13***
	CO_2 retention coefficient	90±1	78±1***

The asterisks indicate significant differences between the mean values of the sprouted and pearled quinoa samples (***p<0.001; t-Test). n.s. indicates no statistical difference. 20SQ, blend composed of sprouted quinoa and wheat flour at 20:80 ratio; 20PQ, blend composed of pearled quinoa and wheat flour at 20:80 ratio. Water absorption is expressed in g/100 g of flour d.b.; dough development time and stability are expressed in min; degree of softening is reported in Farinograph Units (FU); maximum dough height and dough height at 180 min indices are expressed in millimeters (mm); time of maximum dough development and porosity time are expressed in minutes (min); weakening coefficient at 180 min and CO_2 retention coefficient are expressed in percentage (%); total, retained and released co_2 are expressed in milliliters (mL).

As regards dough performance during leavening, 20SQ dough showed a higher maximum dough height (~22%) and retention capacity coefficient (~15%) than 20PQ dough (Table 4.4.4). In addition, the best leavening performance accounted for the highest specific volume of SQ-enriched bread (Figure 4.4.5). The presence of SQ led to a darker (lower L*), redder and

yellower (higher a* and b*) crumb compared to PQ (Figure 4.4.5). Moreover, SQ also improved crumb softness not only of fresh bread (2 h after baking) but also during storage (up to 72 h), compared to PQ-enriched bread (Figure 4.4.6).

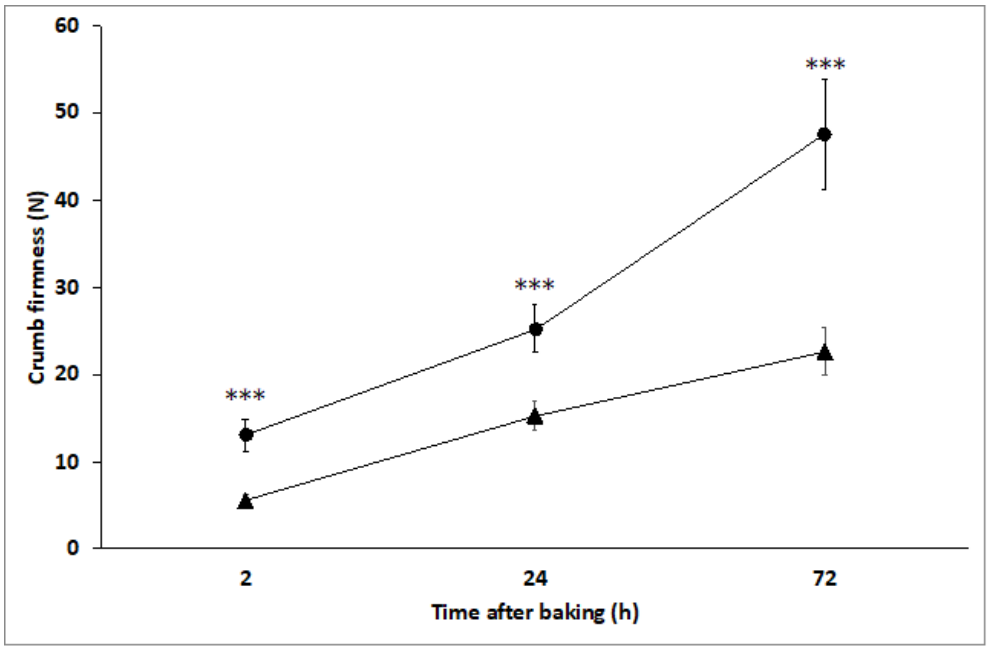


Figure 4.4.6. Crumb firmness of wheat bread enriched in sprouted (triangle) or pearled (circle) quinoa. The asterisks indicate a statistically significant difference between the mean values (t-Test; ***p<0.001).

The sensory traits of quinoa-enriched bread obtained from e-tongue measurement and elaborated through the Principal Component Analysis (PCA) are shown in Figure 4.4.7.

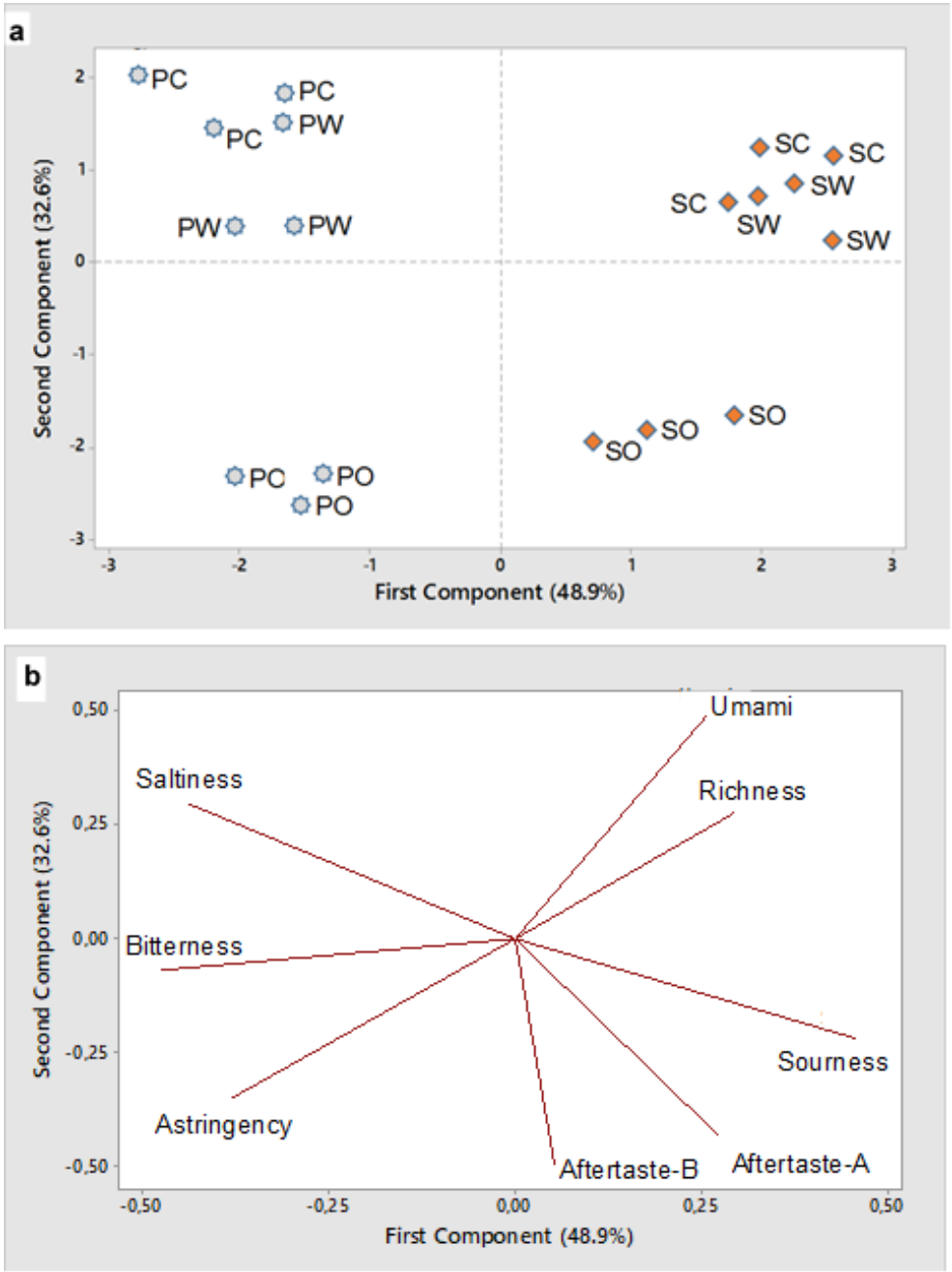


Figure 4.4.7. Score plot (a) and loading plot (b) from e-tongue PCA of bread with pearled (circle) or sprouted (diamond) quinoa. P: Pearled; S: Sprouted; W: whole bread; C: crumb; O: crust. Aftertaste-A: aftertaste-astringency; Aftertaste-B: aftertaste-bitterness.

The two main components accounted for 81.5% of the total variance. As shown in the score plot (Figure 4.4.7a), samples were clearly discriminated on PC1 (48.9% of the total variance) based on the treatment applied to seeds before milling (pearling or sprouting). In fact, the sprouted samples (S) were located on the right side (positive) of PC1. On the contrary, samples with pearled quinoa (P) were located on the left side (negative) of PC1. PC2 discriminated the samples (32.6% of the total of the variance) according to the assessed bread sections (whole bread, crumb, or crust). In particular, whole bread (W) as well as crumb (C) were located on the upper (positive), without great differences between them. Indeed, crumb represents more than 90% of the whole bread (data not shown). Whereas bread crust (O) was located on the lower (negative) of PC2.

4.4.5. Discussion

The effects of replacing wheat flour with quinoa on dough and bread properties have been shown in previous studies (Chauhan et al., 1992; Lorenz and Coulter, 1991). Briefly, when quinoa is blended with wheat, the dough water absorption and mixing tolerance index (or degree of softening) increased, whereas dough development time and loaf volume decreased (Chauhan et al., 1992; Lorenz and Coulter, 1991). At the same time, a worsening in crumb softness and overall acceptability have been reported (Lorenz and Coulter, 1991). To the best of our knowledge, most of the studies have been carried out on pearled quinoa, since pearling has been shown to improve product acceptability by decreasing the amount of saponins (Gómez-Caravaca et al., 2014). Beside pearling, sprouting has been shown to enhance the sensory profile of grains mainly due to the production of simple sugars (Heiniö et al., 2001). However, till now, the effects of sprouting on quinoa acceptability have not been yet addressed. On the other hand, from a technological standpoint, sprouted quinoa showed enhanced functional

properties (i.e., increased foam stability, decreased retrogradation degree) encouraging its use as an ingredient in bread-making (Suárez-Estrella, 2019).

The impact of sprouted quinoa was assessed in bread-making in light of results previously reported in this study. Specifically, sprouted quinoa was added to wheat at different enrichment levels (i.e., 10:90, 20:80, and 30:70, sprouted quinoa:wheat ratio).

The first part of the study focused on starch and protein functionality of sprouted quinoa blends. Understanding the effect on starch is important because this component is responsible for bread staling. Instead, gluten properties are important because gluten plays a key role in leavened products by retaining the gas produced during fermentation. The pasting profile of quinoa blends suggested a gradual loss of the ability to gelatinize and retrograde up to 20:80 substitution level (Figure 4.4.1). Changes in starch properties could be due to various factors: (1) the dilution effect, since the starch content in sprouted quinoa is lower than in wheat (Suárez-Estrella, 2019); (2) presence of fiber that restricts starch swelling during the initial stages of gelatinization (Collar et al., 2009); (3) starch hydrolysis by the amylases developed during sprouting, and formation of small glucose polymers that are less prompted to absorb water and gelatinize (Suárez-Estrella, 2019). The lower retrogradation tendency of 20SQ and 30SQ blends might account for the decrease in bread staling and the preservation of crumb softness even during storage (Figure 4.4.5). A similar effect has been shown in wheat bread (see Section 4.1 and 4.2).

Regarding proteins, gluten protein aggregation in different conditions of hydration and shear stress, namely in slurry (i.e., GlutoPeak test) and in dough (i.e., Farinograph test) systems, was addressed. The former measures the gluten aggregation kinetic which is solely affected by gluten quality (Goldstein et al., 2010); the latter measures the dough formation which is affected by other components, including starch and fiber (Ahmed et al., 2013; Soh et al., 2006). Replacing WF, up to 20:80 replacing level, seems to have only a partial effect on gluten aggregation behavior, mainly affecting maximum

torque rather than peak maximum time (Figure 4.4.2). Since the maximum torque is correlated to gluten content (Marti et al., 2015a), its decrease upon quinoa enrichment might be related to gluten dilution. Similar trends have already been observed in previous studies where flours high in fiber and low in gluten-forming proteins were added to wheat (Marti et al., 2015b). Increasing the amount up to 30:70 substitution level, the maximum torque decreased while the peak maximum time increased (Figure 4.4.2), resulting in a decrease in the aggregation energy, suggesting extensive gluten weakening, unsuitable for bread-making. Indeed, usually flours for bread-making exhibit faster gluten formations and higher peaks compared to those for cookies or cakes (Lu and Seetharaman, 2014). Regardless of the enrichment level, the GlutoPeak profile of quinoa-enriched flours showed a sharper peak compared to the WF profile (Figure 4.4.2), suggesting low resistance to intense shear stresses.

Findings on gluten weakening were confirmed on the dough system by using the farinograph test. Specifically, the worsening of dough mixing properties was evaluated by the decrease in stability and the increase in softening degree (Figure 4.4.3). Gluten dilution, together with fiber enrichment, might account for such modification at high levels of quinoa enrichment (20:80 and 30:70). Moreover, the increasing replacement level caused an increase in water absorption, likely due to the higher fiber content present in the quinoa flour. It is well known the great ability of fibers to bind a high amount of water leading to a higher water absorption index, thanks to the presence of its large number of hydroxyl groups able to establish interactions with water through hydrogen bonds. However, the water absorption of 30SQ dough was not different from the value of 20SQ. Our results partially confirmed the previous study of Park et al. (2005) who reported that replacing 10% of wheat with 48 h sprouted quinoa did not result in any modification of the dough development time, while it caused an increase in the water absorption and a decrease in the stability indices. Differences in sprouting conditions (i.e., temperature, relative humidity) and grain variety might account for different

results. The gluten dilution in SQ samples also affects the dough capacity to maintain its shape during proofing (Figure 4.4.2). However, by carefully following the results provided by the farinograph test (i.e., water absorption, mixing time) (Figure 4.4.3 and 4.4.4), the production of wheat bread enriched in sprouted quinoa was possible even at the highest replacement level (30:70). The best result in terms of specific volume was obtained by using 20SQ (Figure 4.4.5), in agreement with the results on dough properties during both mixing and leavening (Figure 4.4.3 and 4.4.4). Dough development increased, as well as the leavening rate, likely due to the higher presence of simple sugars (i.e., maltose, sucrose, and D-glucose) in sprouted quinoa (Suárez-Estrella, 2019), usable by the yeasts for CO_2 production. Indeed, the presence of sprouted quinoa also led to high gas production during leavening, in agreement with bread volume (Figure 4.4.5). The high bread volume might account for the crumb softness of sprouted quinoa-enriched bread (Figure 4.4.5). The positive effect of sprouted quinoa on bread features was evident only at high enrichment levels (20SQ and 30SQ).

Taking into consideration both the dough and bread features, results showed that the 20SQ blend is the most suitable for bread-making. For this reason, the second part of the study focused on the comparison between sprouting and pearling as pre-processing for producing quinoa-enriched bread.

Despite the dilution of gluten proteins, the enrichment in sprouting quinoa was associated with the best leavening properties, in terms of dough development and gas production and retention, in comparison with pearled quinoa (Table 4.4.4). As stated above, the best dough leavening performance in sprouted quinoa was due to the higher sugar content (Suárez-Estrella, 2020). Specifically, using sprouted quinoa improved bread volume and crumb softness in both fresh (2h after baking) and stored (upon 72 h) bread (Figure 4.4.5), thanks to the increased α-amylase activity during sprouting. The positive effects of α-amylase activity in bread-making have already been reported (Goesaert et al., 2009; De Leyn, 2006). As regards crumb color

profile, the darker crumb of sprouted quinoa-enriched bread might be associated with the presence of higher amount of the brown external layers (e.g. pericarp regions) in flour from sprouted quinoa, compared to ones from refined wheat or pearled quinoa. Indeed, the pericarp regions are removed during the milling and pearling processes.

Sprouting should be preferred to pearling also in relation to the sensory properties, as assessed by e-tongue (Figure 4.4.7). The loading plot (Figure 4.4.7a) evidenced the tendency of bread made with sprouted quinoa to umami, richness, sourness and astringency and bitterness aftertastes; while pearled quinoa samples were located on the left side of PC1, in correspondence of saltiness, bitterness and astringency. The location of sprouted samples at the opposite side of bitterness is an indicator of the suitability of the sprouting process to decrease the bitter perception of quinoa enriched bread.

4.4.6. Conclusions

Using sprouted quinoa at 20:80 replacement level in wheat formulation, it was possible to produce enriched bread with high specific volume, keeping the crumb firmness low even during storage (up to 72 h). Therefore, sprouting could be a suitable strategy for producing quinoa-enriched bread in order to increase the production and consumption of fiber-rich products, together with proteins characterized by high biological value.

Although pearling is - nowadays - the main pre-treatment of quinoa, sprouting might represent a valid alternative to this process to increase the use of quinoa in bread and other baked products. Moreover, sprouting is a quite simple process, requiring non technologically-advanced plants and easily transferable in low-income countries, as the world main producers are. Finally, the effect of sprouting on the actual saponins content – the main cause of quinoa bitterness – is worthy of interest.

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4.5. Sprouting time affects sorghum (*Sorghum bicolor* [L.] *Moench*) functionality and bread-baking performance

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4.5.1. Abstract

Despite being considered a climate-resilient crop, sorghum is still underutilized in food processing because of the limited starch and protein functionality. For this reason, the objective of this study was to investigate the effect of sprouting time on sorghum functional properties and the possibility to exploit sprouted sorghum in bread making. In this context, red sorghum was sprouted for 24, 36, 48, 72, and 96 h at 27° C. Sprouting time did not strongly affect the sorghum composition in terms of total starch, fiber, and protein contents. On the other hand, the developed proteolytic activity had a positive effect on oil-absorption capacity, pasting, and gelation properties. Conversely, the increased α-amylase activity in sprouted samples (≥36 h) altered starch functionality. As regards sorghum-enriched bread, the blends containing 48 h-sprouted sorghum showed high specific volume and low crumb firmness. In addition, enrichment in sprouted sorghum increased both the *in vitro* protein digestibility and the slowly digestible starch fraction of bread. Overall, this study showed that 48 h-sprouted sorghum enhanced the bread-making performance of wheat-based products.

4.5.2. Introduction

Although sorghum (*Sorghum bicolor* [L.] *Moench*) is a staple food for the populations of the sub-Saharan regions, it is becoming an interesting ingredient in those formulations which are typical of the Western countries

(Hugo et al., 2003; Schober et al., 2007; Renzetti et al., 2008; Renzetti and Arendt, 2009; Phattanakulkaewmorie et al., 2011; Marengo et al., 2015). Sorghum has been defined as the “crop of the future” thanks to its high resistance to semi-arid soils and its low water requirements (Teferra and Awika, 2019). In addition to the agronomic traits, from a nutritional standpoint, sorghum is a good source of dietary fiber, vitamins, minerals, and phenolic compounds (Xiong et al., 2019). Moreover, being a gluten-free cereal, sorghum is also suitable for the diet of people suffering from celiac disease. On the other hand, sorghum is characterized by low protein digestibility, due to the presence of protein bodies formed by kafirins (i.e., storage proteins with high hydrophobicity) stabilized by disulphide bonds (Wong et al., 2010). In addition, these structures form a tight starch–protein matrix that leads not only to a decrease in starch and protein digestibility (Oria et al., 2000; Wong et al., 2010), but also to a decrease in starch gelatinization properties (De Mesa-Stonestreet et al., 2010; Zhu, 2014). This is critical from a technological standpoint because starch pasting and gelation properties represent a key aspect in food products by affecting their final characteristics such as viscosity, structure, and texture. For these reasons, the use of sorghum in food production is still limited. As regards wheat-based bread, the presence of sorghum (from 10%) de-creases bread volume and increases dry mouthfeel and crumb firmness (Anglani, 1998). For this reason, sorghum should be treated in a way that improves its functionality, to obtain baked goods with satisfactory attributes for consumers (i.e., high volume and crumb firmness). In this context, sprouting has been proposed as a useful bio-process to modify the structure of sorghum, enhancing its functionality, in terms of oil absorption capacity, emulsion, and foam stability (Elmaki et al., 1999; Correia et al., 2008; Elkhalifa and Bernhardt, 2010; Ocheme et al., 2015; Yi et al., 2017; Marchini et al., 2021b). At the same time, sprouting is associated with a decrease in starch swelling and pasting properties, and cross-linked kafirins (Elmaki et al., 1999; Correia et al., 2008; Elkhalifa and Bernhardt, 2010; Afify et al., 2012; Marengo et al., 2015; Ocheme et al., 2015; Yi et al., Marchini et al., 2021a),

with a positive effect on the in vitro protein digestibility (Elmaki et al., 1999; Correia et al., 2008; Afify et al., 2012). Furthermore, sprouting is related to an increase in mineral bioavailability, polyphenol content, and antioxidant capacity, as well as to a decrease in antinutritional factors (e.g., condensed tannins and trypsin inhibitors) (Singh et al., 2015; Lemmens et al., 2019). Although several researchers have already investigated the effects of sprouting on chemical composition and functional properties of sorghum (Elmaki et al., 1999; Correia et al., 2008; Elkhalifa and Bernhardt, 2010; Afify et al., 2012; Marengo et al., 2015; Ocheme et al., 2015; Yi et al., 2017; Marchini et al., 2021a), to the best of our knowledge, the relation between these changes and the properties of sprouted sorghum-enriched bread have not been studied yet.

Considering the aspects reported above, the purpose of this research was to assess the relationship between the changes in flour functionality—induced by sprouting time—and bread-making performance of bread enriched with sprouted sorghum.

4.5.3. Materials and methods

4.5.3.1. Sample preparation

De-husked and tannin-free sorghum (*Sorghum bicolor* [L.] *Moench*; Armorik *cv*.) was purchased from Caj. Stobl Naturmühle (Linz-Ebelsberg, Austria). Grains were grown and harvested on an experimental field in Hörsching (Oberösterreich, Austria) in 2019. Six aliquots (1 kg each) of grains were sprouted in a climate chamber (Model 60/rW, MANZ Backtechnik GmbH, Creglingen, Germany). Specifically, seeds were soaked (1:3 w/w) for 16 h at 27±2° C (90% Relative Humidity, RH) and sprouted for 24, 36, 48, 72, and 96 h, at 27±2° C (90% RH). After sprouting, seeds were dried at 50 °C for 9 h (Self Cooking Center, Rational International AG, Landsberg am Lech, Germany). Untreated sorghum was used as control (CTRL). All samples were ground by means of the Retsch® ZM 200 Mill (Verder Scientific GmbH & Co.

KG, Golling, Austria) equipped with a 500 μm screen. Wholegrain flours were stored at 4° C for 7 days before using.

Commercial wheat flour (Fini's Feinstes; protein content: 14 g/100 g of flour; W = 290*10^{-4} J) was used as the base for sorghum replacement at 20% level.

4.5.3.2. Chemical composition and enzymatic activities

Moisture (AACC 44-15.02; AACCI 2001), total (AACC 76-13.01; AACCI 2001) and damaged (AACC 76-31.01; AACCI 2001) starch, protein (AACC 46-12.01; AACCI 2001), total, insoluble, and soluble dietary fiber (AOAC 991.43; AOAC 2003) contents, as well as α-amylase (AACC 22-02.01; AACCI 2001) and protease (AACC 22-62.01; AACCI 2001) activities, were determined according to the official methods. Simple sugars (i.e., maltose, sucrose, and D-glucose) were quantified by means of enzymatic kit (K-MASUG; NEO-GEN/Megazyme, Lansing, MI, USA).

4.5.3.3. Sorghum flour functionality

4.5.3.3.1. Water (WAC) and oil (OAC) absorption capacity

WAC and OAC were evaluated following the method reported by Marchini et al. (2021a). Briefly, 1 g of flour was weighted in a 50 mL plastic tube and vortexed for 1 min with water or sunflower oil (1:10 w/v), respectively. Tubes were left to decant at 21° C for 30 min. Finally, samples were centrifuged at 4,000 × *g* for 20 min and the supernatants were discarded. WAC and OAC were expressed as g of water or oil absorbed per 100 g of flour d.b.

4.5.3.3.2. Swelling power (Sp) and pasting properties

Sp was determined according to Zhang and Hamaker (1998). Pasting properties were evaluated by using the Rapid Visco Analyzer (RVA® 4500; PerkinElmer, Inc., Spokane, WA, USA), by dispersing 3.5 g (14% dry matter) of flour in 25 g of distilled water or silver nitrate solution (1 mM; $AgNO_3$)—as a strong α-amylase inhibitor. The temperature profile applied was in accordance with the standard method (ICC, 1992).

4.5.3.4. Protein features

4.5.3.4.1. Kafirin extraction and electrophoresis analysis (SDS-PAGE)

The extraction of kafirins and their electrophoretic profiles (i.e., SDS-PAGE) were carried out according to the method proposed by Carter and Reck (1970) with slight modifications undertaken by Espinosa-Ramírez and Serna-Saldívar (2016).

The SDS-PAGE was performed by using a Mini-Protean II cell (Bio-Rad) at 200 V by using 4–15% Mini-PROTEAN® TGX™ precast gels. Extracted kafirins were dissolved in the SDS sample buffer (62.5 mM Tris/HCl, 2% SDS, 25% glycerol, 0.01% bromophenol blue). Electrophoretic analysis was carried out also under reducing conditions by adding 1.4% (v/v) of β-mercaptoethanol at SDS sample buffer. After the samples were boiled for 5 min, 10 µg of protein were loaded. A broad unstained protein ladders (10–250 kDa) was used (BioRad, Richmond, CA, USA). Protein bands were fixed by using 10% (v/v) acetic acid solution (for 30 min), stained with brilliant blue G250 (Sigma-Aldrich, St. Louis, MO, USA) (for 30 min) and distained with 20% ethanol and 10% acetic acid solution (for 15 h).

4.5.3.4.2. Protein solubility and thiol accessibility

Protein solubility under different conditions (i.e., native, reducing and/or denaturing) and thiol accessibility were assessed by following the methods previously reported by Marengo et al. (2015).

4.5.3.5. Sorghum-wheat blend functionality

The gluten aggregation kinetics of blends were evaluated by means of the GlutoPeak (Brabender GmbH & Co., Duisburg, Germany) test, according to Section 4.3.3.5, by using 10 g of distilled water instead of 9 g.

The mixing properties of the blends were determined according to the ICC official method (ICC 115/1; ICC, 1992), by using the Farinograph (Brabender GmbH & Co., Duisburg, Germany) device, equipped with 50 g mixing bowl.

4.5.3.6. Bread-making

Bread doughs were prepared according to the ICC official method (ICC 131; ICC, 1992). Specifically, bread doughs were made as follows: flour, fresh baker's yeast (2% of flour; Hagold Hefe GmbH, Austria), salt (2% of flour; Salinen Co., Ebensee, Austria), and tap water (65%) at 25° C. Flour and salt were mixed for 1 min using an automatic mixer (Varimixer Teddy, Varimixer, Denmark). After that, yeast was dissolved in water and added into the mixing bowl. Dough was kneaded for 6 min and left to rest in a leavening chamber (BS60/3, Manz Co., Creglingen-Münster, Germany) for 30 min at 30° C (85% RH). The dough was divided into three sub-samples (300 g each), shaped, and put into the baking pans (length: 12.5 cm; width: 6 cm; height: 5 cm). After that, sub-samples were left to proof for 50 min (30° C and 85% RH) and then baked for 35 min at 180° C (BS60/3, Manz Co., Creglingen-Münster, Germany), with vapor injection.

4.5.3.7. Bread properties

Bread volume (mL) was evaluated by means of the VolScan Profiler (Stable Micro Systems, Surrey, UK) and bread specific volume (mL/g) was calculated through the volume/mass ratio. Crumb firmness was determined according to the AACC official method (AACC 74-09.01; AACCI 2001). Crumb color profile was determined through the digital colorimeter (Digital Color Meter, Apple Inc, Cupertino, USA) and expressed according to the CIE-L*a*b* color space.

In vitro starch and protein digestibility of bread was performed by following the Englyst (2006) and Hsu et al. (1977) methods, respectively. *Streptomyces griseus* (Type XIV, ≥3.5 units/mg solid, Merk) was used instead of protease from porcine intestinal, as suggested by Vilakati et al. (2015).

4.5.3.8. Statistical analysis

All analyses were replicated three times. Three baking tests were performed, and three loaves were obtained from each test (n=9). Bread

volume was measured from each loaf and the crumb firmness was evaluated on the three central bread slices of each bread, for a total of 27 measurements. Crumb color profile was replicated three times on the central slice from each loaf. In vitro starch and protein digestibility were determined on one slice from each bread of each baking trial, for a total of 9 slices. All data were subjected to analysis of variance (one-way ANOVA; $\alpha = 0.05$) by using Statgraphics XV version 15.1.02 (StatPoint Inc., Warrenton, VA, USA). When a factor resulted significantly different, the difference was determined through the Tukey HSD test. In addition, data were processed by Principal Component Analysis (PCA) by using Unscrambler version 9.7 (CAMO Software AS, Oslo, Norway).

4.5.4. Results

4.5.4.1. Chemical composition and enzymatic activities

Total starch and protein contents were slightly affected by sprouting time, although hydrolytic activities (i.e., α-amylase and protease) significantly increased during the process (Table 4.5.1). Similar results were reported in Section 4.3.4.2 on durum wheat sprouting that applied similar conditions to those reported here. On the other hand, Elmaki et al. (1999) reported that total starch content decreased from 30% to 50%, when sorghum was sprouted at 30° C from 24 to 72 h, whereas Marchini et al. (2021b) reported a smaller decrease in starch content (by about 5%) when sorghum was sprouted at 25° C for 72 h. Different results among the studies could be related to differences in varieties, as well as in sprouting conditions.

Table 4.5.1. Chemical composition and enzymatic activities of unsprouted (control - CTRL) and sprouted sorghum at different times (24 h, 36 h, 48 h, 72 h and 96 h).

	CTRL	24 h	36 h	48 h	72 h	96 h
Total starch	77.5±1.9^{b}	73.2±1.2^{a}	75.0±2.1^{ab}	75.2±2.5^{ab}	73.7±0.4^{a}	74.3±1.4^{ab}
Damaged starch	9.4±0.5^{a}	9.6±0.2^{a}	9.6±0.3^{a}	11.5±0.3^{b}	12.7±0.5^{c}	13.6±0.3^{d}
Maltose	0.2±0.1^{a}	0.12±0.03^{a}	0.54±0.09^{b}	1.31±0.03^{c}	2.23±0.03^{d}	3.5±0.3^{e}
Sucrose	0.64±0.05^{d}	0.25±0.05^{a}	0.24±0.01^{a}	0.22±0.08^{a}	0.36±0.05^{b}	0.45±0.03^{c}
D-glucose	0.24±0.01^{a}	0.25±0.01^{a}	0.29±0.08^{a}	0.47±0.02^{b}	0.58±0.01^{c}	0.70±0.01^{d}
Protein	8.8±0.1^{a}	9.0±0.1^{ab}	9.1±0.1^{b}	9.1±0.1^{b}	9.0±0.1^{ab}	8.9±0.1^{ab}
Total dietary fiber	7.2±0.2^{a}	6.9±0.4^{a}	6.8±0.5^{a}	6.7±0.2^{a}	7.0±0.5^{a}	6.9±0.1^{a}
Insoluble	82	78	85	82	89	90
Soluble	18	22	15	18	11	10
α-amylase	0.07±0.02^{a}	2.7±0.6^{b}	3.0±0.4^{bc}	3.7±0.4^{c}	4.5±0.4^{d}	6.2±0.6^{e}
Protease	1.3±0.1^{a}	2.5±0.1^{b}	2.7±0.1^{bc}	3.0±0.1^{c}	3.9±0.2^{d}	4.3±0.2^{e}

Different letters in the same row correspond to significant differences among samples (one-way ANOVA; Tukey HSD test; p≤0.05; n=3). Compositional data are expressed as g/100g of flour d.b. Insoluble and soluble dietary fiber were expressed as g/100g of total dietary fiber. Damaged starch is expressed as g/100g of total starch. α-amylase and proteolytic activities are expressed as Ceralpha Units (CU/g flour d.b.), and as the activity/g of flour d.b., respectively.

In contrast, the sprouting process did not strongly affect the total dietary fiber of sorghum (Table 4.5.1), in accordance with Marchini et al. (2021b). The changes in the insoluble and soluble fiber could be related to the fact that fiber components do not precipitate upon ethanol addition but remain in solution, resulting in an underestimation of the soluble fraction (Ku et al., 2003). On the other hand, damaged starch (~22%), maltose (~170%), and glucose (~96%) increased starting from 48 h of sprouting (Table 4.5.1), due to the increase in α-amylase activity (Table 4.5.1). Indeed, simple sugars are necessary to provide energy for the development of the new plant (Elmaki et al., 1999). In contrast, sucrose decreased during the early stages of sprouting as seeds use it as a primary source of energy (Hager et al., 2014), whereas its increase after 72 h of sprouting was attributed to the higher enzymatic activity synthesizing sucrose (Rosa et al., 2004).

As regards the enzymatic activities, sprouting (at all times) increased both amylolytic and proteolytic activities. In particular, the increase in α-amylase activity was more intense than proteolytic ones (~37- *vs.* ~2-folds).

4.5.4.2. Functional properties

Sprouting caused a significant decrease (~5%) in WAC from 48 h, while OAC increased (~20%) from 24 h (Table 4.5.2), as an effect of amylase and protease activity (Table 4.5.1). However, at 72 h and 96 h, the sprouted sample did not show differences with CTRL in terms of OAC. In contrast, sprouting for 96 h significantly decreased Sp, regardless of the temperature considered (85 and 100° C).

Table 4.5.2. Functional properties of unsprouted (control, CTRL) and sprouted sorghum at different times (24 h, 36 h, 48 h, 72 h, and 96 h).

	CTRL	24 h	36 h	48 h	72 h	96 h
Hydration Properties						
WAC	1.50±0.01^{d}	1.48±0.03^{cd}	1.50±0.01^{d}	1.42±0.03^{bc}	1.38±0.04^{ab}	1.33±0.02^{a}
OAC	1.07±0.02^{a}	1.28±0.02^{b}	1.29±0.04^{b}	1.31±0.04^{cd}	1.08±0.05^{a}	1.03±0.03^{a}
Swelling Power (Sp)						
85° C	7.2±0.4^{c}	6.7±0.1^{bc}	6.3±0.2^{bc}	6.3±0.2^{bc}	6.4±0.1^{bc}	5.4±0.3^{a}
100° C	10.2±0.1^{bc}	10.8±0.6^{bc}	10.2±0.5^{bc}	9.7±0.2^{c}	5.7±0.4^{b}	3.5±0.4^{a}
Pasting properties in water						
Viscosity peak	1858±21^{e}	2131±7^{f}	1593±44^{d}	945±4^{c}	306±11^{b}	187±1^{a}
Peak temperature	77.8±0.5^{a}	81.2±0.6^{c}	79.3±0.4^{b}	78.0±0.5^{ab}	78.0±0.5^{ab}	77.5±0.1^{a}
Breakdown	739±8^{d}	706±10^{c}	826±11^{e}	720±6^{cd}	276±13^{b}	166±2^{a}
Final viscosity	2392±75^{d}	2509±65^{d}	1606±67^{c}	602±22^{b}	70±2^{a}	43±1^{a}
Setback	1273±58^{e}	1084±55^{d}	838±35^{c}	378±14^{b}	40±1^{a}	23±1^{a}

Different letters in the same row correspond to significant differences among samples (one-way ANOVA; Tukey HSD test; p≤0.05; n=3). WAC and OAC are expressed as g/g of flour d.b. Sp is expressed as g/100g of sample d.b. Viscosity peak, breakdown, final viscosity, and setback are reported in cP, while peak temperature in °C. WAC: Water Absorption Capacity; OAC: Oil Absorption Capacity.

As regards the starch pasting properties using water as solvent, the 24 h sample showed a higher viscosity value than CTRL (Table 4.5.2; Figure 4.5.1a). However, as sprouting time increased (>24 h), viscosity decreased, as a consequence of increased α-amylase activity (Table 4.5.1). In particular, the 72 h- and 96 h-sprouted samples showed the lowest values throughout the duration of the test (Figure 4.5.1a). Moreover, peak temperature increased in the first 36 h of sprouting.

Inhibiting the α-amylase activity during the test – by using silver nitrate as solvent – all the samples showed viscosity values higher or comparable to CTRL (Figure 4.5.1b). In particular, samples sprouted up to 36 h showed a significant higher peak and final viscosity compared to CTRL; on the other hand, no significant differences were observed among CTRL and the samples that were sprouted from 48 to 96 h (Figure 4.5.1b).

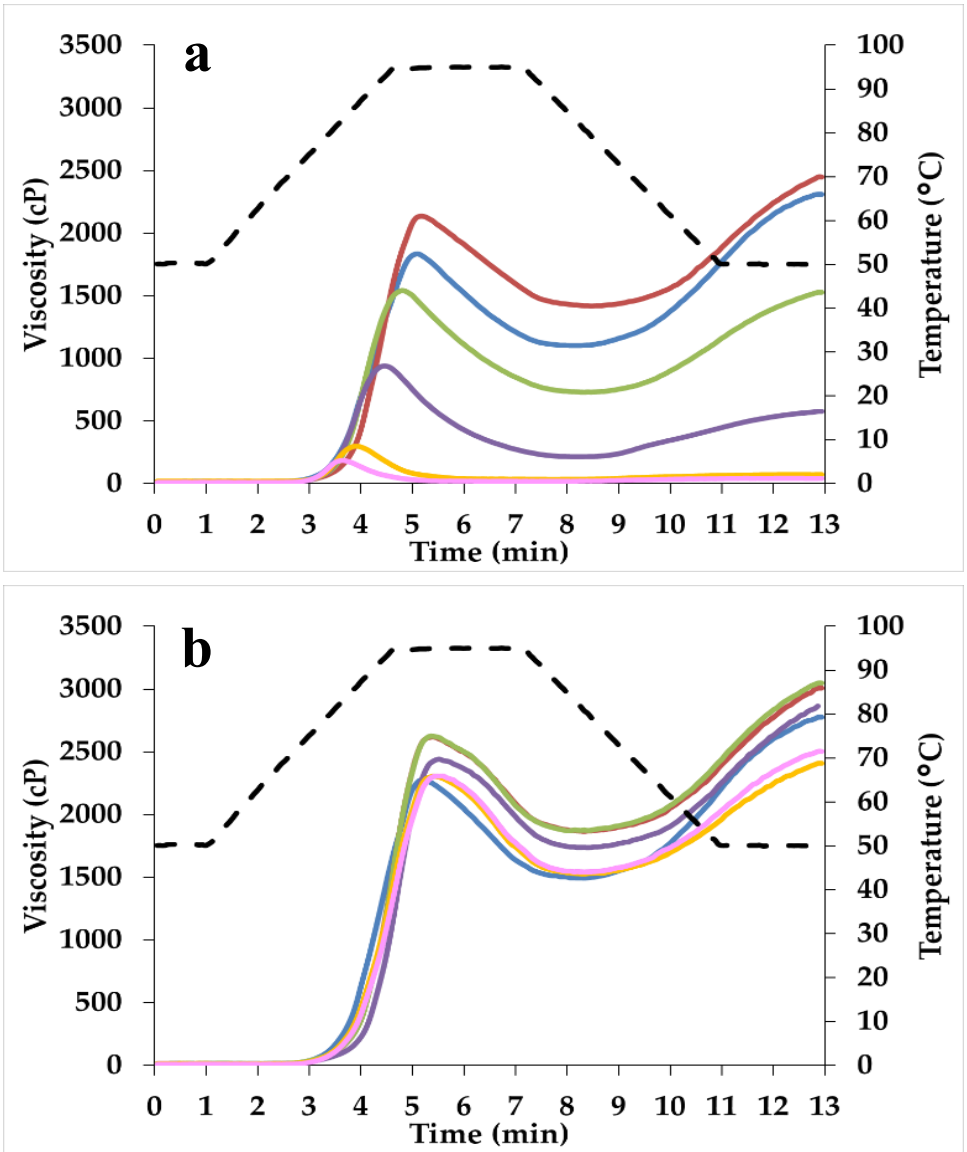


Figure 4.5.1. Profiles of pasting and gelation properties of flours from unsprouted (CTRL) and sprouted sorghum at different times (24 h, 36 h, 48 h, 72 h and 96 h), by using (a) water or (b) $AgNO_3$, as solvent. CTRL: blue line; 24 h: red line; 36 h: green line; 48 h: purple line; 72 h: yellow line; 96 h: pink line.

4.5.4.3. Protein features

The content of soluble proteins in the phosphate buffer +NaCl, significantly decreased from 24 h to 48 h of sprouting (~44%) (Figure 4.5.2).

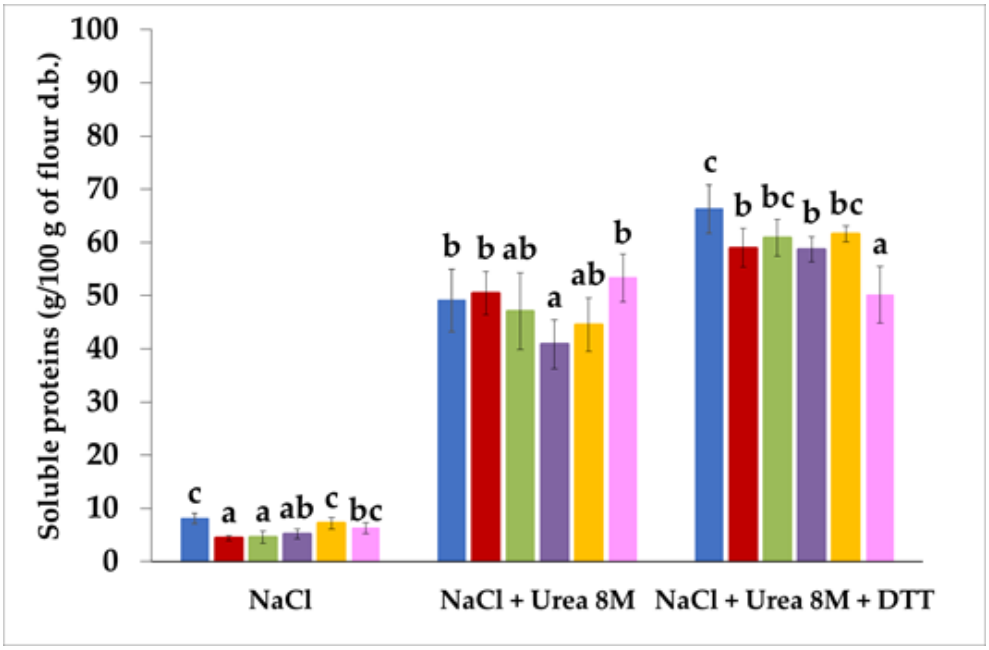


Figure 4.5.2. Amount of proteins of unsprouted (CTRL) and sprouted sorghum at different times (24 h, 36 h, 48 h, 72 h and 96 h), solubilized in various conditions. Different letters in the same condition indicate a significant difference among samples (one-way ANOVA; Tukey HSD test; $p<0.05$). CTRL: blue bars; 24 h: red bars; 36 h: green bars; 48 h: purple bars; 72 h: yellow bars; 96 h: pink bars.

As the sprouting progressed, the protein solubility increased again, showing no differences with the CTRL anymore. The addition of the chaotropic agent (i.e., Urea 8 M) did not strongly affect the solubility of the proteins stabilized by hydrophobic interactions. In contrast, the content of proteins stabilized by both hydrophobic interactions and disulfide bonds – evaluated by adding DTT – slightly decreased starting from 24 h of sprouting, reaching the minimum value after 96 h.

The content of free accessible thiols underwent a significant decrease during sprouting (1.5 µmol/g of flour d.b. for CTRL and, 1.1, 1.2, 1.1, 1.1 µmol/g of flour d.b. for 24 h-, 36 h-, 48 h-, and 96 h-sprouted samples), except

for the 72 h-sprouted sample (1.3 μmol/g of flour d.b). The addition of chaotropic agent, to evaluate the total accessible thiols, led to an increase in their content by about two times (2.6 μmol/g of flour d.b. for CTRL, and 1.8, 2.0, 2.1, 1.9, and 2.7 μmol/g of flour d.b. for 24 h-, 36 h-, 48 h-, 72 h-, and 96 h-sprouted samples, respectively), compared to free accessible ones, regardless of the sprouting time. Interestingly, 96 h-sprouted sorghum showed a similar value to the CTRL sample for this index.

The electrophoretic analysis (SDS-PAGE) of kafirins in absence and in presence of a reducing agent (β-mercaptoethanol) are shown in Figure 4.5.3a and b, respectively.

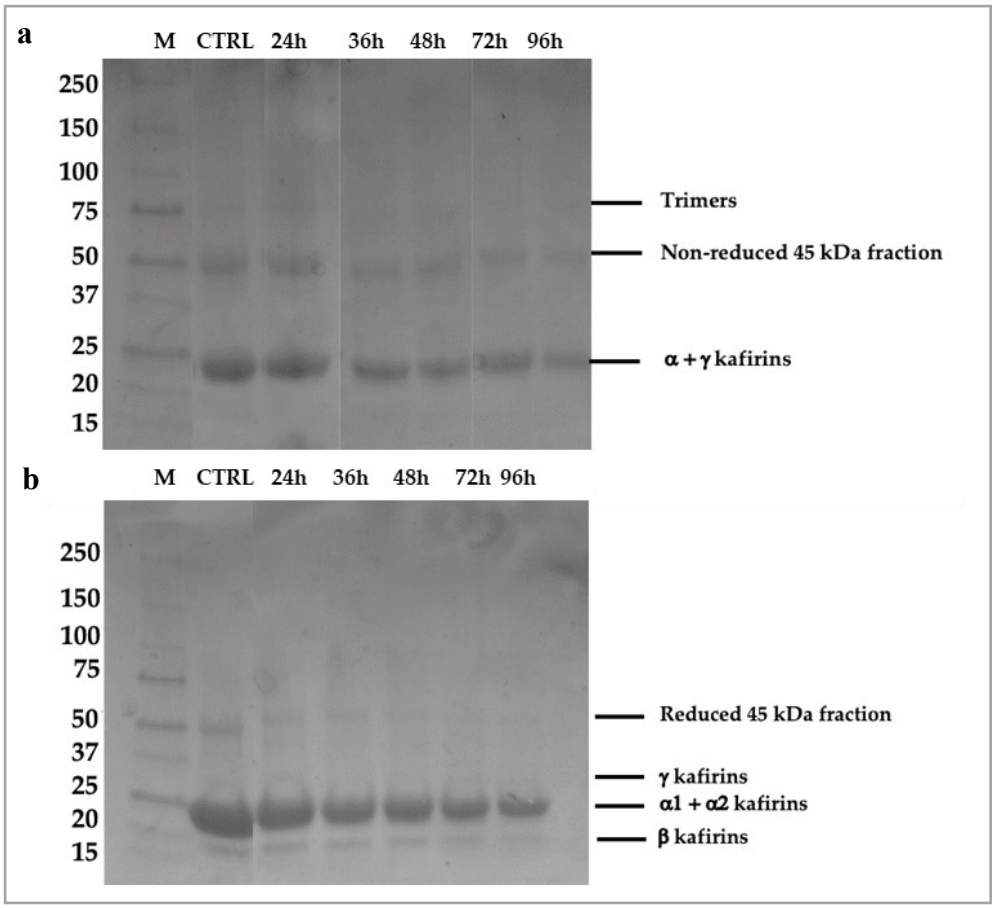


Figure 4.5.3. Electrophoretic profile of extracted kafirins in absence (a) and in presence (b) of reducing agent (β-mercaptoethanol). M: Marker.

Under unreduced conditions, samples might be identified into two groups on the base of the band density. Specifically, CTRL and 24 h-sprouted samples, as well as 36 h-, 48 h-, 72 h-, and 96 h-sprouted samples showed the same band density. Moreover, under these conditions, trimers (~75 kDa) and non-reduced fractions (~50 kDa) were found (Figure 4.5.3a). In contrast, under reducing conditions, the electrophoretic analysis did not show trimers bands but showed the presence of γ-, α1- + α2, and β-kafirins at 28–30, 21–23, and 17–18 kDa, respectively (Figure 4.5.3b). Since β- and γ-fractions are located on the outer part of the protein bodies, they are the first to be hydrolyzed by proteases during sprouting. Instead, the α1- and α2-fraction of kafirins are present in the inner part of the protein body (De Mesa-Stonestreet et al., 2010). The main kafirin fraction was represented by α-kafirins, since their bands are composed of the overlapping of α1- and α2-kafirin subunits. Indeed, these subunits are characterized by low molecular weight (Figure 4.5.3b), and consequently slightly different mobility. Moreover, faint bands related to γ-kafirins were shown only in CTRL and dried samples, instead α1-, α2- and β-kafirins were shown in all samples, even if their density decreased upon sprouting.

4.5.4.4. Effects of sprouting on sorghum-wheat blend functionality

As regards the gluten aggregation kinetics of composite flours (Figure 4.5.4; Table 4.5.3), the presence of sprouted samples caused a significant decrease in all the indices considered (Figure 4.5.4; Table 4.5.3). Specifically, sorghum sprouted from 72 h determined a significant decrease in the maximum torque (~12%) and in the aggregation energy (~63%), while the peak maximum time was already affected starting from 24 h of sprouting (~38%).

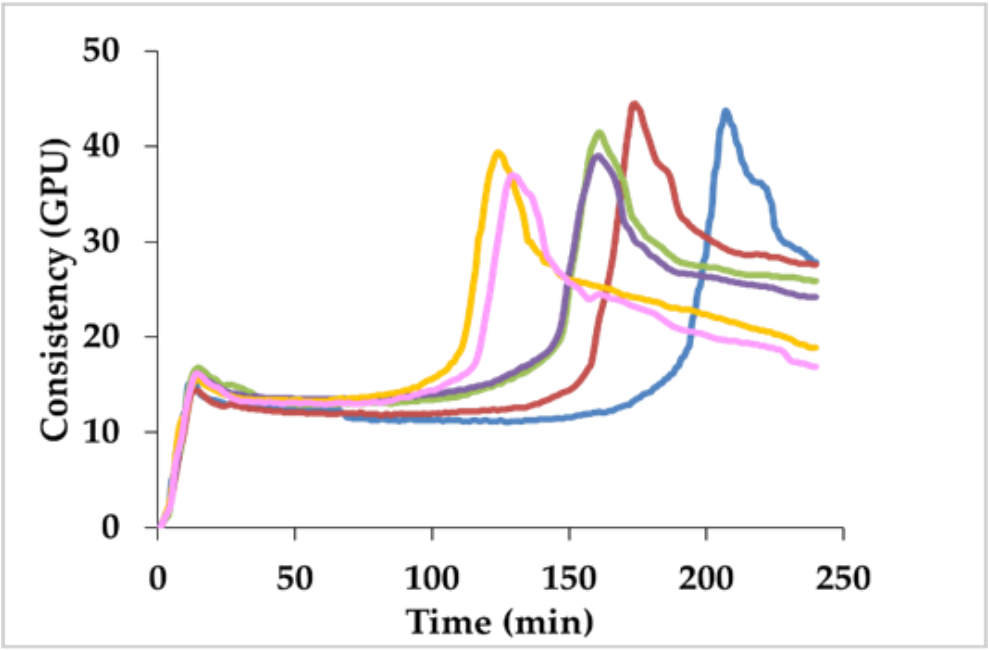


Figure 4.5.4. Gluten aggregation profiles of wheat flour containing 20% unsprouted (CTRL) or sprouted sorghum at different times (24 h, 36 h, 48 h, 72 h and 96 h). CTRL: blue line; 24 h: red line; 36 h: green line; 48 h: purple line; 72 h: yellow line; 96 h: pink line. GPU: GlutoPeak Units.

Table 4.5.4. Gluten aggregation and mixing properties of wheat flour containing 20% unsprouted (control - CTRL) or sprouted sorghum at different times (24 h, 36 h, 48 h, 72 h and 96 h).

	CTRL	24 h	36 h	48 h	72 h	96 h
Gluten aggregation properties						
Maximum torque	42.9±1.0^{b}	43.5±1.5^{b}	43.0±1.3^{b}	40.3±1.2^{ab}	39.5±0.1^{a}	37.6±1.5^{a}
Peak maximum time	217±14^{c}	180±8^{b}	166±5^{b}	167±6^{b}	125±3^{a}	135±6^{a}
Aggregation energy	1063±8^{c}	1075±26^{c}	1073±22^{c}	1029±15^{bc}	978±5^{ab}	935±21^{a}
Mixing properties						
Water absorption	61.6±0.3^{b}	61.7±0.3^{b}	61.5±0.2^{b}	61.9±0.1^{b}	61.6±0.3^{b}	61.4±0.2^{b}
Dough development time	3.0±1.0^{b}	3.0±0.1^{b}	2.7±0.2^{ab}	2.1±0.2^{ab}	2.0±0.1^{ab}	1.9±0.2^{a}
Stability	9.5±0.1^{cd}	9.0±0.6^{cd}	7.3±0.4^{c}	5.3±0.4^{b}	2.9±0.6^{a}	2.2±0.3^{a}
Degree of softening	57±4^{a}	62±6^{a}	84±1^{b}	108±1^{c}	167±8^{d}	191±5^{e}

Different letters in the same row correspond to significant differences among samples (one-way ANOVA; Tukey HSD test; $p<0.05$; n=3). Maximum torque is expressed in GlutoPeak Units (GPU); peak maximum time is expressed in s; aggregation energy is expressed in GlutoPeak Equivalent (GPE); water absorption is expressed in g/100 g of flour d.b.; dough development time and stability are expressed in min; degree of softening is reported in Farinograph Units (FU).

As regards the effects of sprouting time on the mixing properties (Table 4.5.3; Figure 4.5.5), the presence of sprouted sorghum did not affect the water absorption of dough, but it led to a decrease in both dough development time (~37%) and stability (from ~44% to ~77%), when samples sprouted for 96 and 48 h were used, respectively. The decrease in both indices was an effect of the proteolytic activity developed during sprouting (table 4.5.1). In addition, a great increase in the degree of softening was observed starting from 36 h of sprouting compared to CTRL (Table 4.5.3).

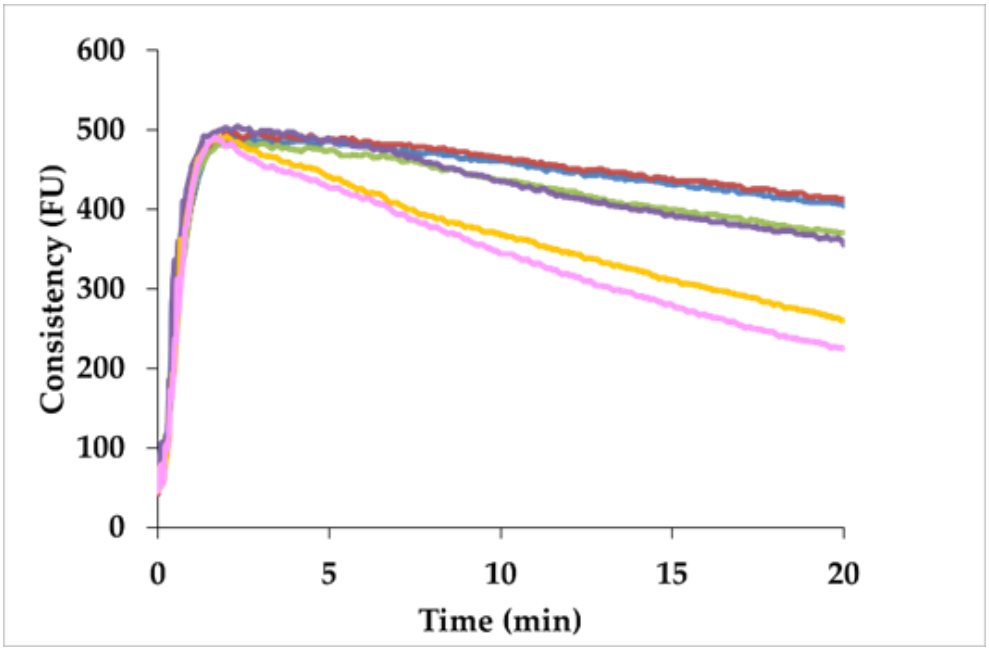
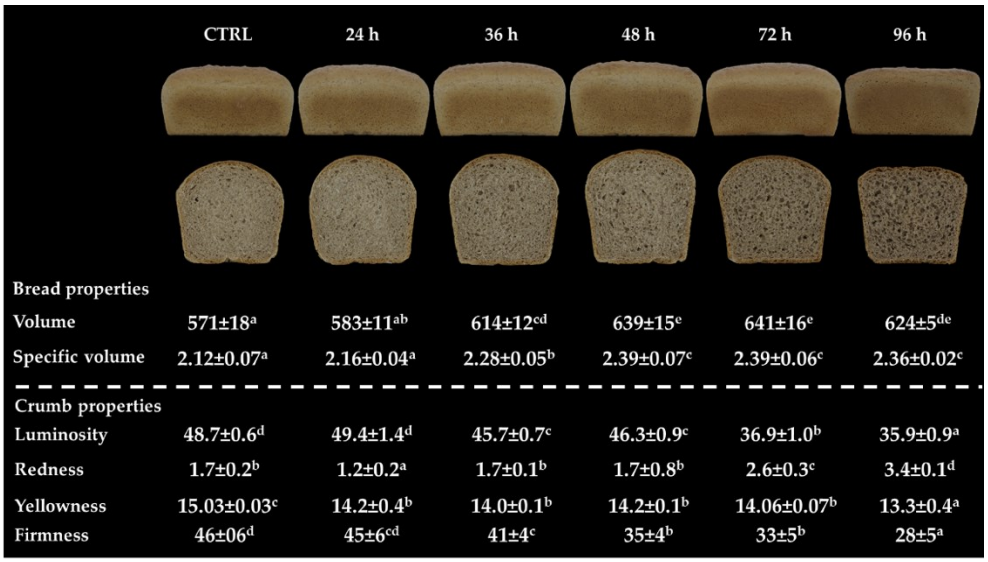


Figure 4.5.5. Mixing profiles of wheat flour containing 20% unsprouted (CTRL) or sprouted sorghum at different times (24 h, 36 h, 48 h, 72 h and 96 h). CTRL: blue line; 24 h: red line; 36 h: green line; 48 h: purple line; 72 h: yellow line; 96 h: pink line. FU: Farinograph Units.

4.5.4.5. Bread properties

The addition of sorghum sprouted for 36 h and longer resulted in a higher volume and specific volume of bread (~12%), compared to bread from wheat flour alone (610 mL and 2.30 mL/g, respectively; data not shown) and the CTRL sample (Figure 4.5.6).



	CTRL	24 h	36 h	48 h	72 h	96 h
Bread properties						
Volume	571±18^{a}	583±11^{ab}	614±12^{cd}	639±15^{e}	641±16^{e}	624±5de
Specific volume	2.12±0.07^{a}	2.16±0.04^{a}	2.28±0.05^{b}	2.39±0.07^{c}	2.39±0.06^{c}	2.36±0.02^{c}
Crumb properties						
Luminosity	48.7±0.6^{d}	49.4±1.4^{d}	45.7±0.7^{c}	46.3±0.9^{c}	36.9±1.0^{b}	35.9±0.9^{a}
Redness	1.7±0.2^{b}	1.2±0.2^{a}	1.7±0.1^{b}	1.7±0.8^{b}	2.6±0.3^{c}	3.4±0.1^{d}
Yellowness	15.03±0.03^{c}	14.2±0.4^{b}	14.0±0.1^{b}	14.2±0.1^{b}	14.06±0.07^{b}	13.3±0.4^{a}
Firmness	46±06^{d}	45±6^{cd}	41±4^{c}	35±4^{b}	33±5^{b}	28±5^{a}

Figure 4.5.6. Properties of bread from wheat containing 20% unsprouted (CTRL) or sprouted sorghum at different times (24 h, 36 h, 48 h, 72 h and 96 h). Different letters in the same row correspond to significant differences among samples (one-way ANOVA; Tukey HSD test; $p<0.05$; n=27 for volume and specific volume; n=9 for crumb color and firmness). Volume and specific volume are expressed in mL and mL/g, respectively. Crumb firmness is expressed in N.

The positive effects of sprouting on crumb firmness were evident when sorghum sprouted from 36 h was used. In particular, the lowest firmness was observed in the 96 h-enriched bread (Figure 4.5.6). The replacement of wheat flour with sorghum decreased luminosity (up to ~26% for the 36 h sample) and increased the redness of crumb (up to ~100% for the 72 h sample). Moreover, using sprouted sorghum caused a slight decrease in crumb yellowness (up to ~10% for the 72 h and 96 h sample).

As regards in vitro digestibility, the rapid digestible starch (RDS) significantly decreased from 36 h (~9%) up to 96 h (~75%) of sprouting. Unlike RDS, no difference was observed between CTRL and 96 h-enriched bread in terms of slowly digestible starch (SDS) (Figure 4.5.7a).

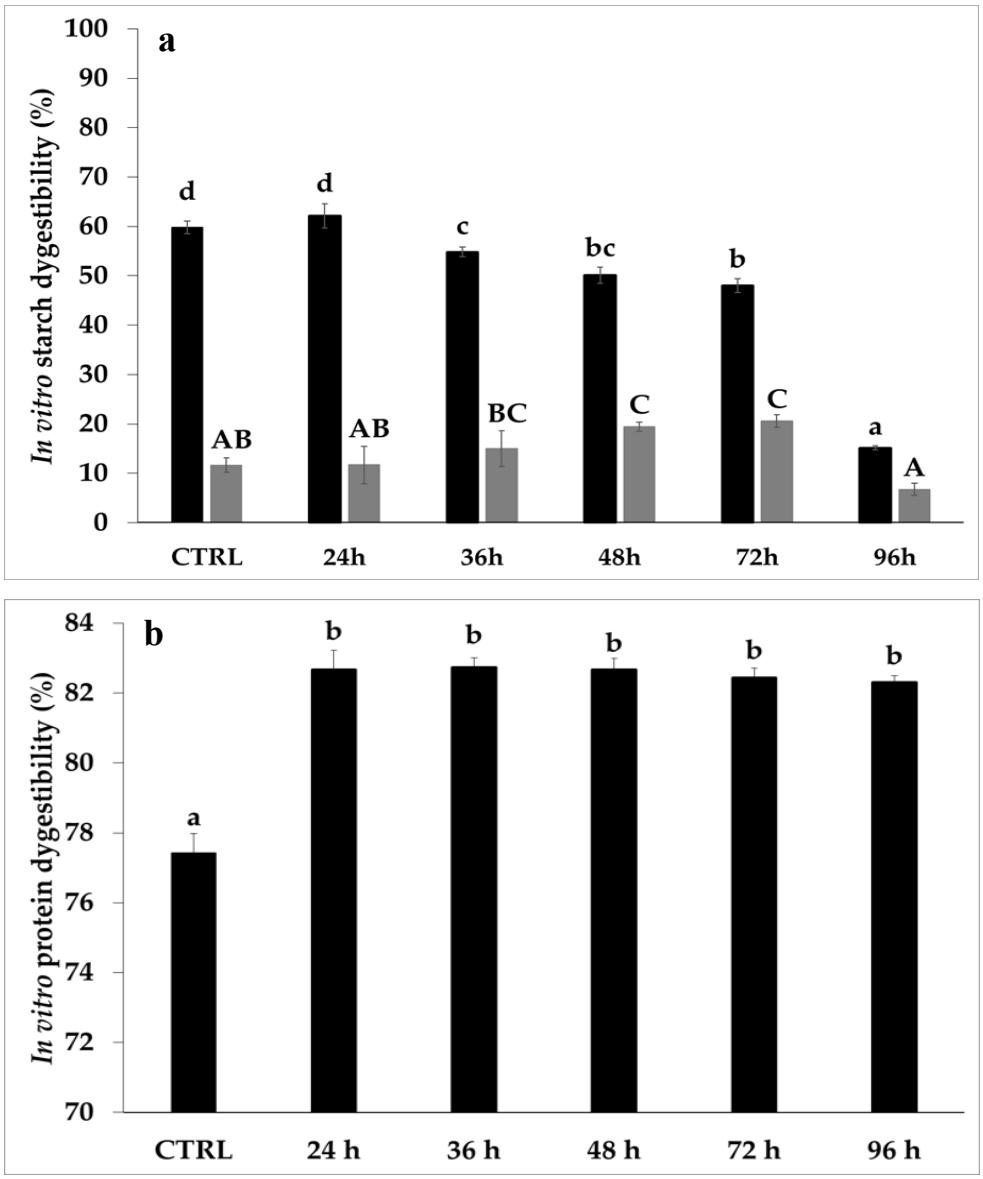


Figure 4.5.7. Rapidly (black bars; RDS) and slowly (grey bars; SDS) digestible starch (a), and protein digestibility (b) of wheat bread containing 20% unsprouted (CTRL) or sprouted sorghum at different times (24 h, 36 h, 48 h, 72 h and 96 h). Different letters (lower case for rapidly digestible starch and protein digestibility; uppercase for slowly digestible starch) correspond to significant differences among samples (one-way ANOVA; Tukey HSD test; $p<0.05$; n=3).

These results partly agreed with data reported by Swieca et al. (2017), when a commercial wheat flour was replaced with 20% of flour from wheat sprouted for 96 h. These authors related the decrease in RDS to the increase in the resistant starch fraction and/or in the polyphenol content of the sprouted material (Świeca et al., 2017). Interestingly, the trend followed by the SDS fraction of 96 h-enriched bread was not the same as other samples, likely related to the different bread structure; however, this aspect needs to be further investigated.

Concerning the *in vitro* protein digestibility, sprouting caused an increase in this index, regardless of sprouting time (Figure 4.5.7b).

4.5.4.6. Principal Component Analysis (PCA)

The results of PCA reported the distribution of samples in accordance with chemical composition, enzymatic activities, protein solubility, thiols, gluten aggregation kinetics, mixing, and bread properties (Figure 4.5.8). The scores plot – which described about 82% of the variability of the data (PC1: ~52%; PC2: ~30%) – highlighted a separation of samples based on sprouting duration (Figure 4.5.8a). Indeed, the CTRL sample is in the bottom right corner, assuming highly positive and negative values for PC1 and PC2, respectively. The 24 h- and 36 h-sprouted samples are in upper right corner assuming positive values for both PC1 and PC2, while 48 h-sprouted sample is in upper left corner assuming the highest value of PC2; 72 h- and 96 h-sprouted samples are in the bottom left corner, tending to the negative values of both PC1 and PC2. Furthermore, the loading plot identified the variables that determine sample grouping (Figure 4.5.8b). Most of the indices of chemical composition, enzymatic activities, functional properties, along with gluten aggregation and mixing properties, and bread characteristics determined the separation of samples along PC1, whereas indices mainly related to the protein features were responsible for the separation of sprouted samples along PC2.

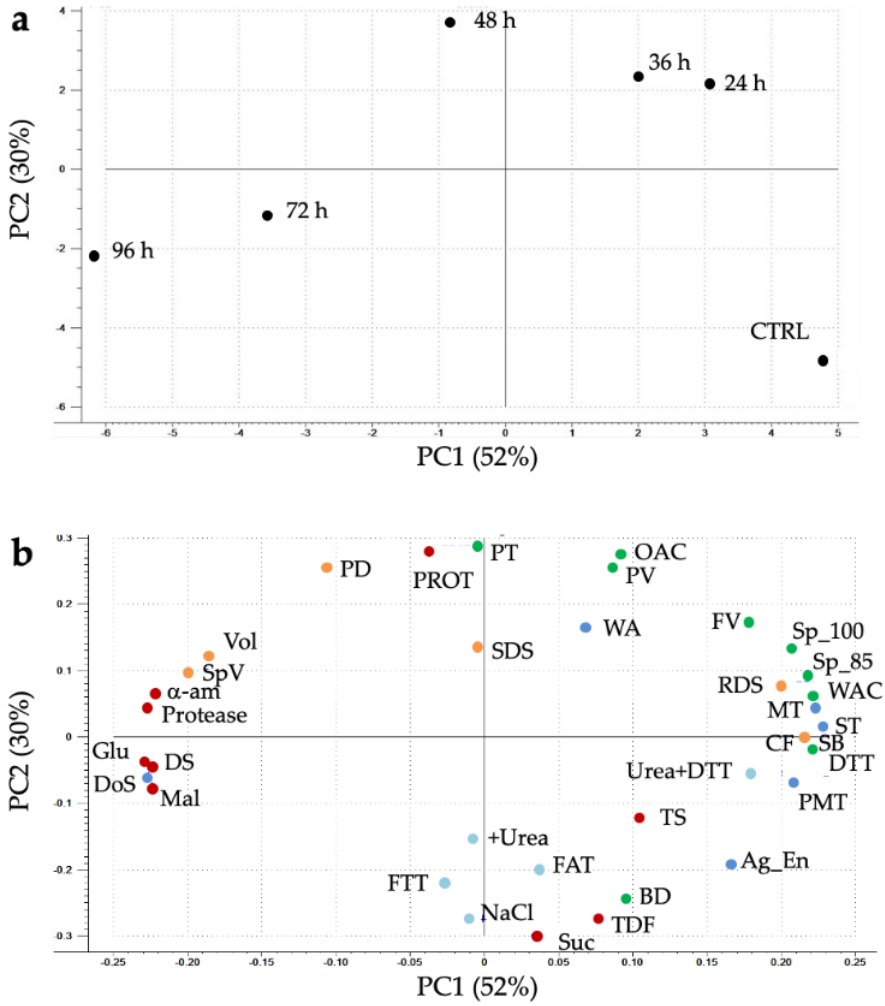


Figure 4.5.8. Score (a) and Loading (b) plots for Principal Component Analysis on chemical composition and enzymatic activities, protein solubility and thiols, gluten aggregation, mixing properties, and bread properties. α-am, α-amylase activity; DS, Damaged Starch; Glu, D-glucose; Mal, Maltose; Prot, Protein; Suc, Sucrose; TDF, Total Dietary Fiber; TS, Total Starch. Protein solubility: DTT, Dithiothreitol. Thiols: FAT, Free Accessible Thiols; FFT, Free Total Thiols. Functional properties: BD, Breakdown; FV, Final Viscosity; OAC, Oil Absorption Capacity; PT, Peak Temperature; PV, Peak Viscosity; SB, Setback; Sp_85, Swelling Power at 85° C; Sp_100, Swelling Power at 100° C WAC, Water Absorption Capacity. Gluten aggregation properties: PMT, Peak Maximum Time; MT, Maximum Torque; Ag_En, Aggregation Energy. Mixing properties: DDT, Dough Development Time; DoS, Degree of Softening; ST, Stability; WA: Water Absorption. Bread properties: CF, Crumb Firmness; PD, Protein Digestibility; RDS, Rapidly Digestible Starch; SDS, Slowly Digestible Starch; SpV, Specific Volume; Vol, Bread Volume.

4.5.5. Discussion

In recent times, sprouted grains, such as wheat (Marti et al., 2018; Grassi, et al., 2020), brown rice (Watanabe et al., 2004; Charoenthaikij et al., 2010a,b), quinoa (Park and Morita, 2005; Mäkinen and Arendt, 2012; Horstmann et al., 2019), oat (Mäkinen and Arendt, 2012; Mäkinen et al., 2013), finger millet (Bhol and John Don Bosco, 2014), and pulses (Hallén, İbanoğlu and Ainsworth, 2004; Bhol and John Don Bosco, 2014; Marengo et al., 2017), have already been exploited in bread-making to improve the features of composite bread. As regards sorghum, the effect of sprouting process on chemical composition and/or functional properties has already been reported in sever-al studies (Elmaki et al., 1999; Correia et al., 2008; Elkhalifa and Bernhardt, 2010; Afify et al., 2012; Marengo et al., 2015; Ocheme et al., 2015; Yi et al., 2017; Marchini et al., 2021b). But until now, to the best of our knowledge, no studies have elucidated the relation between flour functionality and bread-making performance of breads enriched in sorghum sprouted till 96 h.

Comprehending the effect of sprouting on starch and protein is important since these components are responsible for the properties of the final products, such as dough proper-ties, bread staling, and digestibility. In this context, in the first part of this study the changes in the chemical and functional properties induced by sprouting duration were assessed. Then, the effect of such changes on dough and bread properties were studied. Specifically, sprouted sorghum was used at 20% level in a wheat-based formulation.

From a compositional standpoint, the greatest effect of sprouting time was observed for sugar and damaged starch content, as an effect of the increased α-amylase activity (Table 4.5.1). These results were supported also by PCA, since these indices were in upper and bottom left quadrants of the loading plot (Figure 4.5.8b) influencing the separation of samples according to the sprouting time along the PC1. In a recent study, the increase in

damaged starch (i.e., starch susceptibility to α-amylase) was confirmed by observing some holes on the surface of starch granules of sprouted sorghum for 72 h (Marchini et al., 2021b). In addition, increased proteolytic activity may also have played a role in increasing starch susceptibility to the α-amylases. Indeed, as hydrolysis of the proteins surrounding the starch granules proceeds, the starch granules are more easily accessible to the amylolytic enzymes. The hydrolysis of protein bodies during sprouting was confirmed by SDS-PAGE (Figure 4.5.3), in accordance with previous reports (Correia et al., 2010; Marchini et al., 2021b). Consequently, the hydrolysis of protein bodies improved the in vitro starch and protein digestibility of bread (Figure 4.5.7). In addition, the hydrolysis of protein bodies at the initial stage of sprouting (24 h) make starch granules more available to gelatinize (Figure 4.5.1). The role of protein in decreasing the starch gelatinization ability is also supported by the different Sp of unsprouted and sprouted sorghum, found in this study (Table 4.5.2). In this regard, similar results were reported by Elkhalifa and Bernhardt (2013), who demonstrated that sorghum Sp decreased by about 23% after 72 h of sprouting when evaluated at 85° C, and about 39% at 100° C.

Changes in protein structure might have increased the amount of lipophilic amino acids on the protein surface (Elkhalifa and Bernhardt, 2010; Singh et al., 2015), with consequent increase in the flour ability to absorb oil (Table 4.5.2). The increased ability to absorb oil makes sprouted sorghum a suitable raw material for formulating products where the ability to absorb oil is crucial, such as in baby-foods and energy-dense snacks (Singh and Sharma, 2017). The decrease in the OAC shown by sorghum sprouted from 72 h (Table 4.5.2) might be due to the intense proteolytic activity (Table 4.5.1) leading to excessive protein hydrolysis, resulting in loss of OAC again. Elkhalifa and Bernhardt (2010) observed an increase in sorghum OAC in the first 72 h of sprouting, and a decrease after 96 h. On the other hand, protein solubility was not strongly affected by proteolytic activity (Figure 4.5.2), likely due to the hydrolyzed products that remained associated with the original protein, as previously suggested by Suárez-Estrella et al. (2020).

Moving to starch functionality, the changes in the protein–starch matrix, together with the increased enzymatic activities, resulted in a decrease in the pasting and gelation properties of sprouted samples for 36 h or longer (Table 4.5.2; Figure 4.5.1a), since the hydrolyzed starch is no longer able to form a rigid gel and simple sugars cannot absorb a high amount of water (Žilić et al., 2016). These results were in accordance with previous studies (Phattanakulkaewmorie et al., 2011; Xu et al., 2012; Marengo et al., 2015), and they were confirmed also by the PCA loading plot (Figure 4.5.8b), where the indices related to pasting and gelation properties were characterized by positive PC1 values, discriminating the CTRL sample from the sprouted ones. Moreover, the decrease in the retrogradation tendency of sprouted sorghum led to the production of bread with a softer crumb compared to CTRL-enriched bread (Figure 4.5.6), after one day of storage, regardless of crumb moisture (data not shown). Similar results were reported also when quinoa sprouted for 48 h was added at 20% replacement level to wheat bread (see Section 4.4.4.5).

Refined wheat flour was replaced with unsprouted and sprouted sorghum at 20% level, to produce sorghum-enriched bread. The gluten properties of the blends were evaluated both in slurry (i.e., GlutoPeak test) and dough (i.e., Farinograph test) systems, with different hydration and shear stress conditions. In this context, to the best of our knowledge, no study has previously evaluated the influence of sprouted sorghum on wheat flour properties, making it difficult to compare our results with the literature. The GlutoPeak test suggested a weakening of the gluten matrix when sprouted sorghum was used instead of CTRL (Table 4.5.3; Figure 4.5.4). This behavior might have been caused by the structural changes induced by sprouting on the sorghum components (i.e., protein and fiber) (Figure 4.5.2 and 4.5.3), which might have interacted differently with wheat gluten proteins during gluten formation. In addition, the lower maximum torque of the sprouted samples compared with CTRL could be explained by the decrease in the

accessible free thiols available to interact with wheat thiols to form a gluten matrix.

The results obtained by means of the GlutoPeak test were confirmed also in the dough system, with a decrease in both, the dough development time and stability, and the increase in the degree of softening (Table 4.5.3; Figure 4.5.5). Specifically, the worsening of mixing properties was related to sprouting time (Table 4.5.3), likely due to the increase in the proteolytic activity developed during the sprouting process (Table 4.5.1). Indeed, during farinograph test proteases are able to hydrolyze gluten proteins due to the long time of the test (20 min), resulting in a gluten weakening (Ahmed et al., 2015). Similar results were also reported when 48 h-sprouted quinoa was used at 20% replacement level (see Section 4.4.4.2). Despite the worsening in gluten aggregation and dough mixing properties, the dough was able to resist the stress during baking and did not collapse, except for the 96 h-enriched bread sample likely due to its high proteolytic activity and consequent higher gluten weakening. In fact, it was characterized by a lower height compared to samples sprouted between 36 and 72 h (data not shown). The increase in bread volume might also be related to the increase in simple sugars (Table 4.5.1), available for yeasts to produce CO_2 during leavening (Marti et al., 2018). Improvements in bread characteristics were also reported in previous studies carried out on sprouted common (see Section 4.1.4.5) and durum (see Section 4.3.4.6) wheat, and quinoa (see Section 4.4.4.5).

Finally, regarding starch digestibility, the increase in SDS (Figure 4.5.7a) in samples starting from 36 h of sprouting (Figure 4.5.7a) was associated with the increase in resistant starch (data not shown). In this context, there are controversial opinions in the literature on the sprouting effect on the in vitro starch digestibility of bread (Lemmens et al., 2019). For instance, an increase in SDS fraction was observed in wheat bread enriched in sprouted wheat (Marti et al., 2018), while a decrease in SDS was measured in sprouted brown rice-based bread (Cornejo et al., 2015). Different results

might be explained by different analytical methods, as well as sprouting and bread-making conditions used.

4.5.6. Conclusions

In conclusion, the present research highlighted the relationship between chemical-functional properties induced by sprouting and bread properties. Specifically, sprouting resulted in the most intense changes in the functional properties starting from 36 to 48 h of the process, although increased hydrolytic activity occurred as early as 24 h of sprouting. As regards bread, by using sorghum sprouted between 36 and 72 h in a composite flour (20% replacement level) it was possible to obtain a bread with increased specific volume and decreased crumb firmness, stimulating the possibility of using sprouted sorghum in baked goods. Therefore, sprouting could represent an interesting strategy to fully exploit the potential of sorghum to be used in cereal-based products and ensure wide consumer attraction for this sustainable crop. Further studies will address the relationship between flour functionality and starch and protein structure, as affected by sprouting process.

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4.5.7. References

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5. GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

Assessing the effects of sprouting on macromolecule (i.e., starch, protein, and fiber) functionality of grains and their relationship with dough and bread-making performance represent one of the most important driving forces in the development of cereal-based products with improved characteristics. This PhD thesis showed that sprouting grains could be a useful approach if the process is carried out under controlled conditions of time, temperature, and relative humidity. Indeed, monitoring the sprouting conditions allows control of enzymatic activities, limiting the worsening of starch and gluten protein functionality. Overall, the findings of this PhD thesis highlighted that:

- the sprouting process under controlled conditions allowed the accumulation of enzymatic activities, mainly in the first 36-48 h of the process. In particular, the highest α-amylase activity was shown by durum wheat, instead the highest accumulation of proteases was observed for quinoa and sorghum;

- the accumulation of hydrolytic activities that occur during controlled sprouting did not result in a remarkable change in either protein or starch content, regardless of the grain considered;

- the molecular changes due to controlled sprouting affected the functional properties of the related flours, regardless of the flour type (i.e., wholegrain or refined). However, sprouting improved volume and specific volume, and crumb softness of bread, even when wholegrain flours were used, suggesting new potential application of sprouting as a pre-treatment of wholegrain flours;

- controlled sprouting could be proposed as an alternative method to improve gluten functionality of semolina flour;

- sprouted quinoa and sorghum can be exploited in wheat-based formulations as new ingredients thanks to their ability to improve bread volume and crumb softness. Moreover, sprouting can be exploited to obtain quinoa-enriched bread characterized by decreased bitterness and sorghum-enriched bread with improved protein digestibility.

Future studies will include the assessment of sprouting effects on other less common cereals (e.g., millet, rye, oats), pseudo-cereals (e.g., buckwheat, amaranth) and pulses (e.g., chickpeas, beans) and in other foods, including cookies and pasta. Moreover, studies about product acceptability through panel test will be carried out.

6. SCIENTIFIC PRODUCTION

6.1. Publications

Research Article

SCI

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Sprouting improves the bread-making performance of whole wheat flour (*Triticum aestivum* L.)

Gaetano Cardone, Paolo D'Incecco, Maria Ambrogina Pagani and Alessandra Marti*

Abstract

BACKGROUND: Pre-harvest sprouting of wheat is viewed negatively because of the high level of enzymatic activity, which leads to a deterioration in the bread-making performance of the related flours. On the other hand, improvements in bread properties (i.e. volume and crumb softness) are reported when sprouted wheat under controlled conditions is used in mixtures with a conventional unsprouted flour. However, knowledge about the effects of sprouting on gluten functionality and its relationship with bread features is still limited, especially in the case of whole wheat flour.

RESULTS: Under the conditions applied in this study (48 h, 20 °C and 90% relative humidity), proteins of sprouted wheat were still able to aggregate, even if changes in gluten aggregation kinetics suggested gluten weakening. On the other hand, sprouting led to an increase in gluten stretching ability, suggesting an increase in dough extensibility. In the dough system, sprouting was responsible for a decrease in water absorption, development time, and stability during mixing. However, when the values for development time and water absorption indicated by the Farinograph® were followed carefully, sprouting improved bread height (~20%), specific volume (~15%), and crumb softness (~200% after 24 h of storage), even when whole wheat flour was used.

CONCLUSION: It is possible to produce bread with improved volume and crumb softness using whole wheat flour from sprouted kernels. Thus, sprouting can be exploited as a pre-treatment to improve the bread-making performance of fiber-enriched systems.

Supporting information may be found in the online version of this article.

Keywords: sprouting; gluten; whole wheat flour; bread-making; ultrastructure

INTRODUCTION

Interest in enriching cereal-based products in sprouted grains is constantly increasing[1] because of the improved nutritional and sensory profile associated with the chemical and biochemical changes promoted by sprouting. Such changes depend strongly on the sprouting conditions adopted (i.e. temperature and time) as well as grain species, varieties, and cultivars.[2] However, prolonged and uncontrolled sprouting could have negative consequences because the accumulation of large amounts of hydrolytic enzymes developed during the process makes the flour unsuitable for bread making. Consequently, the resulting bread will be characterized by low volume and sticky and gummy crumb.[3] Controlled sprouting might thus be a useful process to achieve the perfect balance between nutritional advantages and technological performance.[4] In this context, Grassi *et al.* proposed the use of a portable Micro Near Infrared (NIR) device to monitor the sprouting process.[5] Although the study was carried out at lab scale (1 kg of kernels), the analysis of the spectra suggested that the greatest changes in both starch (1480–1526 nm) and protein (1500–1530 nm) fractions occurred in the first 48 h, whereas longer germination time generated no further relevant changes.[5] Regarding the nutritional traits, Poudel *et al.* highlighted the positive effects of sprouting time (up to 72 h) in increasing γ-aminobutyric acid, asparagine, and lysine, and decreasing thiamine and phytic acids.[6]

Apart from the nutritional features, the relation between changes induced by sprouting on starch and protein functionality and the quality of the product have been poorly studied so far. The absence of such information makes it difficult to elucidate whether sprouting may improve the technological performance of wheat. This aspect is worthy of interest, especially in the case

* *Correspondence to: A Marti, Department of Food, Environmental, and Nutritional Sciences (DeFENS), Università degli Studi di Milano, Milano, via G. Celoria 2, 20133 Milan, Italy, E-mail: alessandra.marti@unimi.it*

Department of Food, Environmental, and Nutritional Sciences (DeFENS), Università degli Studi di Milano, Milan, Italy

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Article

Exploiting Milling By-Products in Bread-Making: The Case of Sprouted Wheat

Gaetano Cardone, Paolo D'Incecco, Maria Cristina Casiraghi and Alessandra Marti *

Department of Food, Environmental and Nutritional Sciences (DeFENS), Università degli Studi di Milano, 20133, Italy; gaetano.cardone@unimi.it (G.C.); paolo.dincecco@unimi.it (P.D.); maria.casiraghi@unimi.it (M.C.C.)

* Correspondence: alessandra.marti@unimi.it

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Abstract: This research investigated the effect of sprouting on wheat bran. Bran from un-sprouted (BUW) and sprouted (BSW) wheat were characterized in terms of chemical composition, enzymatic activities, and hydration properties. In addition, the rheological properties (using GlutoPeak, Farinograph, Extensograph, and Rheofermentometer tests) and bread-making performance (color, texture, volume of bread) of wheat doughs enriched in bran at 20% replacement level were assessed. Sprouting process caused a significant decrease in phytic acid (~20%), insoluble dietary fiber (~11%), and water holding capacity (~8%), whereas simple sugars (~133%) and enzymatic activities significantly increased after processing. As regards the gluten aggregation kinetics, the BSW-blend profile was more similar to wheat than BUW-blend, indicating changes in the fiber and gluten interactions. BSW led to a worsening of the mixing and leavening properties, instead, no significant changes in extensibility were observed. Finally, BSW improved bread volume (~10%) and crumb softness (~52%). Exploiting bran from sprouted wheat might be useful to produce bread rich in fiber with enhanced characteristics.

Keywords: bran; cell walls; sprouting; dough rheology; bread-making; microstructure

1. Introduction

Fiber-enrichment of food products has become increasingly important as a means to increase their nutritional properties. In this context, bran from cereals-with a total dietary fiber content of 30%–50%—is one of the most important source of dietary fiber used in the bread-making industry [1]. However, the inclusion of high levels of fiber in cereal-based products remains a technological challenge, due to the need to maintain acceptable dough rheological properties as well as sensory attributes. Indeed, adding high levels of bran to dough leads to an increase in water absorption, a decrease in both mixing stability and leavening tolerance [2,3]. The most evident effects on the final baked product are the decrease in loaf volume, the increase in crumb firmness, the appearance of dark crumb, and, in some cases, the modification of taste with the appearance of bitterness [4].

The detrimental effect of bran addition on bread-making cannot be solely attributed to the dilution of gluten proteins and to the physical disruption of gluten network, but the physical, chemical, and biochemical properties of bran should be also considered [5]. Besides specific physical properties—i.e., the strong tendency of bran to absorb water that might result in competition for water between bran and other key flour components like starch and proteins—bran seems to have a certain chemical reactivity (i.e., between ferulic acid and proteins) which might determine its functionality [5].

Several pre-treatments have been proposed to counter these negative effects, such as: (i) particle size reduction, which significantly influences the rheological properties of dough in terms of mixing time, stability and dough resistance to extension [6], (ii) application of high-pressure [7], and (iii)

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Bread-making performance of durum wheat as affected by sprouting

Gaetano Cardone, Silvia Grassi, Anna Scipioni, Alessandra Marti *

Department of Food, Environmental, and Nutritional Sciences (DeFENS). Università Degli Studi di Milano, Via G. Celoria 2, 20133, Milan, Italy

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Bread

ABSTRACT

The effects of sprouting duration (24 h, 38 h, 48 h, and 62 h) were assessed on durum wheat kernel characteristics (hardness, test weight), semolina chemical composition, pasting and gluten aggregation properties, and leavening and bread-making performance (bread volume and crumb porosity). Sprouting decreased both kernel hardness (~29%) and test weight (~19%). Starch gelatinization and retrogradation capability, as well as the gluten aggregation properties, decreased as sprouting duration increased. The 62 h sample showed the worst aggregation properties leading to a bread with the lowest specific volume (2.69 mL/g). The best results in terms of bread specific volume (3.08 mL/g) and crumb porosity distribution were obtained using semolina from sprouted wheat up to 38 h. A multivariate approach by Principal Component Analysis and clustering confirmed the relationships between all the considered variables and allowed to assess three sprouting levels: 24–38 h with improved bread-making performance; 48 h with decreased overall quality; 62 h with the worst quality. In conclusion, the sprouting of durum wheat up to 38 h could improve its bread-making attitude.

1. Introduction

Durum wheat (*Triticum turgidum* subsp. *durum*) is characterized by a peculiar hard and vitreous endosperm which influences its milling behavior, e.g., milling energy, yield and the starch damage (Turnbull & Rahman, 2002). The strength and poor extensibility of its gluten network makes durum wheat the ideal raw material for pasta-making but unsuitable for baked-goods (Ammar, Kronstad, & Morris, 2000). Despite the enhanced nutritional traits thanks to the carotenoids (Pasqualone, Caponio, & Simeone, 2004), using durum wheat in bread-making results in low loaf volume and dense crumb structure (Sissons, 2008). However, dough extensibility and bread volume improved using sourdough fermentation, since the combination of acidity and hydrolytic activity of both lactic acid bacteria and yeasts positively affect durum wheat gluten functionality (Barber, Ortolá, Barber, & Fernández, 1992). Considering the above, this study investigated the exploitation of the enzymatic pattern developed throughout sprouting to improve the bread-making performance of durum wheat. Although, an excessive accumulation of enzymes in wheat has always represented a negative event from a technological standpoint, recently it has been reported that sprouting improved the bread-making performance of common wheat (Cardone, D'Incecco, Pagani, & Marti, 2020a; Marti, Cardone, Nicolodi, Quaglia, & Pagani, 2017; Marti, Cardone, Pagani, & Casiraghi, 2018). In the case of durum wheat, the sprouting process have been recently investigated in relation to bioactive compounds (Jribi, Sahagùn, Debbabi, & Gomez, 2019a) and functional properties (Jribi, Sahagùn, Debbabi, & Gomez, 2019b) of wholemeal semolina. To the best of our knowledge, no study has focused yet on the relationship between sprouting and bread-making performance of durum wheat. Since the understanding of flour functionality is a key element in the production of cereal-based products, the aim of this study was to evaluate the effects of sprouting duration on durum wheat kernel characteristics, starch and gluten behavior, and their relationship with the bread characteristics also from a multivariate point of view, thus applying Principal Component Analysis and clustering.

Abbreviations: A_0, radial area of the dough at the beginning of the leavening; A-am, α-amylase activity; AgEn, Aggregation Energy; A_t, radial area of the dough at time t; BD, Breakdown index; CTRL, unsprouted durum wheat; DS, Damaged Starch; FV, Final Viscosity; Glu, D-glucose; GPE, GlutoPeak Equivalent; GPU, GlutoPeak Unit; Mal, Maltose; MT, Maximum Torque; PCA, Principal Component Analysis; PMT, Peak Maximum Time; Prot, Protein; PV, Peak Viscosity; SpV, Specific Volume; Suc, Sucrose; TS, Total Starch; V, bread volume.

* Corresponding author. address: via G. Celoria 2, 20133, Milan, Italy.
E-mail address: alessandra.marti@unimi.it (A. Marti).

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Sprouting as a pre-processing for producing quinoa-enriched bread

Diego Suárez-Estrella [a, b, 1], Gaetano Cardone [a, 1], Susanna Buratti [a], Maria Ambrogina Pagani [a], Alessandra Marti [a, *]

[a] Department of Food, Environmental and Nutritional Sciences (DeFENS), Università degli Studi di Milano, via G. Celoria 2, 20133, Milan, Italy
[b] Grupo de Investigación en Quimiometría y QSAR, Facultad de Ciencia y Tecnología. Universidad del Azuay, Av. 24 de Mayo, 7-77 y Hernán Malo, Cuenca, Ecuador

ARTICLE INFO

Keywords:
Chenopodium quinoa
Germination
Dough rheology
Electronic sensing

ABSTRACT

The impact of 48 h sprouted quinoa (SQ) was assessed in bread-making. Wheat flour (WF) was replaced with SQ at different levels (i.e., 10:90, 20:80 and 30:70, SQ:WF ratio). Once the optimal replacement level of SQ was identified, the bread-making performance of this ingredient was compared with those of pearled quinoa (PQ), commonly used in bread-making.

Starch pasting properties and gluten aggregation behavior were not strongly affected at 20:80 level. Regardless the replacement level, SQ caused an increase in dough water absorption and in softening degree, and a decrease in stability, suggesting weakening of the gluten network. During leavening, SQ improved dough development and gas production, due to increased sugar content (i.e. maltose, sucrose and D-glucose). The best bread-making performance (highest bread specific volume and lowest crumb firmness) was obtained at 20:80 replacement level. Compared to PQ, SQ exhibited the best leavening capacity (high dough development, gas production and gas retention) and bread properties (high specific volume and low crumb firmness), likely due to its higher sugar content. Moreover, 20SQ bread was characterized by a decreased bitterness assessed by electronic-tongue. In conclusion, sprouting might be considered a valid alternative to pearling to improve the characteristics of quinoa enriched bread

1. Introduction

Quinoa is a gluten-free grain from both agronomic and nutritional standpoint. Specifically, quinoa is particularly high in lysine, which is the limiting amino acid in cereals, it is a good source of minerals, phenolic compounds, dietary fiber and polyunsaturated fatty acids (Tang and Tsao, 2017). All these compositional traits account for the potential health benefits of quinoa seeds in contributing to the prevention of various diseases such as cancer, diabetes, cardiovascular diseases, and aging (Tang and Tsao, 2017). Thus, these characteristics are the driving force for enhancing the consumption of quinoa not only as seeds but also as an ingredient in various food applications, including both enriched wheat-based goods and gluten-free products.

Despite the well-known nutritional features of quinoa, its consumption is limited by the bitter and astringent taste, due to saponin compounds (Suárez-Estrella et al., 2018). Nowadays, pearling is one of the main processes applied to quinoa to improve its acceptability in food formulation; it consists in the removal of the seed external layers, which are rich in saponins (Suárez-Estrella et al., 2018). On the other hand, a significant loss of bioactive compounds occurs during the pearling process (Suárez-Estrella et al., 2018). Nowadays, quinoa is proposed in bread-making only as flour from pearled grains. Specifically, in wheat-based bread, 250 g/kg of pearled quinoa seems to be the threshold level in terms of dough rheological properties and sensory acceptability (Rosell et al., 2009); conversely, bitter aftertaste was detected at higher quinoa enrichment levels (Lorenz and Coulter, 1991).

Recently, several authors reported the possibility to exploit sprouted grains to enhance the bread-making attitude of wholewheat (Cardone et al., 2020b), brown-rice (Watanabe et al., 2004), and pulses (Hallén et al., 2004; Marengo et al., 2017b). The improved bread characteristics

Abbreviations: 10SQ, blend composed of sprouted quinoa and wheat flour at 10:90 ratio; 20PQ, blend composed of pearled quinoa and wheat flour at 20:80 ratio; 20SQ, blend composed of sprouted quinoa and wheat flour at 20:80 ratio; 30SQ, blend composed of sprouted quinoa and wheat flour at 30:70 ratio; BU, Brabender Unit; FU, Farinograph Unit; GPE, GlutoPeak Equivalent; GPU, GlutoPeak Unit; PQ, pearled quinoa; SP, sprouted quinoa; SV, specific volume; WF, wheat flour.

* Corresponding author.
E-mail address: alessandra.marti@unimi.it (A. Marti).
[1] The authors contributed equally to this work.

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Article

Sprouting Time Affects Sorghum (*Sorghum bicolor* [L.] Moench) Functionality and Bread-Baking Performance

Gaetano Cardone [1,2], Rubina Rumler [2], Sofia Speranza [2], Alessandra Marti [1,*] and Regine Schönlechner [2]

[1] Department of Food, Environmental and Nutritional Sciences (DeFENS), University of Milan, Via G. Celoria 2, 20133 Milan, Italy; gaetano.cardone@unimi.it
[2] Department of Food Science and Technology, Institute of Food Technology, University of Natural Resources and Life Sciences, Muthgasse 18, A-1190 Vienna, Austria; rubina.rumler@boku.ac.at (R.R.); sofia.speranza@boku.ac.at (S.S.); regine.schoenlechner@boku.ac.at (R.S.)
* Correspondence: alessandra.marti@unimi.it

Abstract: Despite being considered a climate-resilient crop, sorghum is still underutilized in food processing because of the limited starch and protein functionality. For this reason, the objective of this study was to investigate the effect of sprouting time on sorghum functional properties and the possibility to exploit sprouted sorghum in bread making. In this context, red sorghum was sprouted for 24, 36, 48, 72, and 96 h at 27 °C. Sprouting time did not strongly affect the sorghum composition in terms of total starch, fiber, and protein contents. On the other hand, the developed proteolytic activity had a positive effect on oil-absorption capacity, pasting, and gelation properties. Conversely, the increased α-amylase activity in sprouted samples (≥36 h) altered starch functionality. As regards sorghum-enriched bread, the blends containing 48 h-sprouted sorghum showed high specific volume and low crumb firmness. In addition, enrichment in sprouted sorghum increased both the in vitro protein digestibility and the slowly digestible starch fraction of bread. Overall, this study showed that 48 h-sprouted sorghum enhanced the bread-making performance of wheat-based products.

Keywords: sorghum; germination; flour functionality; rheology; bread; starch digestibility; protein digestibility

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1. Introduction

Although sorghum (*Sorghum bicolor* [L.] Moench) is a staple food for the populations of the sub-Saharan regions, it is becoming an interesting ingredient in those formulations which are typical of the Western countries [1–6]. Sorghum has been defined as the "crop of the future" thanks to its high resistance to semi-arid soils and its low water requirements [7]. In addition to the agronomic traits, from a nutritional standpoint, sorghum is a good source of dietary fiber, vitamins, minerals, and phenolic compounds [8]. Moreover, being a gluten-free cereal, sorghum is also suitable for the diet of people suffering from celiac disease. On the other hand, sorghum is characterized by low protein digestibility, due to the presence of protein bodies formed by kafirins (i.e., storage proteins with high hydrophobicity) stabilized by disulphide bonds [9]. In addition, these structures form a tight starch–protein matrix that leads not only to a decrease in starch and protein digestibility [9,10], but also to a decrease in starch gelatinization properties [11,12]. This is critical from a technological standpoint because starch pasting and gelation properties represent a key aspect in food products by affecting their final characteristics such as viscosity, structure, and texture. For these reasons, the use of sorghum in food production is still limited. As regards wheat-based bread, the presence of sorghum (from 10%) decreases bread volume and increases dry mouthfeel and crumb firmness [13]. For this reason, sorghum should be treated in a way that improves its functionality, to obtain

6.2. Conference's abstracts

6.2.1 Oral communications

1st Telematic Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology
Palermo, September 14th – 15th, 2021

Gaetano Cardone (gaetano.cardone@unimi.it)
Dept. of Food, Environmental and Nutritional Sciences (DeFENS), Università degli Studi di Milano, Milan, Italy
Tutor: Professor Alessandra Marti

Sprouting as a biotechnological process to improve the functional properties of cereal-based products

This PhD project aimed at understanding the effects of controlled sprouting on the technological properties of cereals. Specifically, it focused on the relationship between functional changes in starch, protein, and fiber – induced by sprouting – and their impact on the characteristics of the resulting dough and bread. Results showed that - by monitoring changes in protein and starch functionality - sprouted grains can be used as ingredients in bread-making to produce cereal-based products with improved nutritional and structural features.

La germinazione per il miglioramento delle proprietà funzionali dei prodotti da forno

Lo scopo del presente progetto di dottorato è stato quello di comprendere gli effetti indotti della germinazione in condizioni controllate sulle proprietà tecnologiche dei cereali. Nello specifico, lo studio si è concentrato sulla relazione tra i cambiamenti delle proprietà funzionali di amido, proteine e fibra ed il loro effetto sulle caratteristiche di impasti e pane. I risultati hanno

mostrato che – controllando i cambiamenti a carico di amido e proteine - gli sfarinati da cereali germinati possono essere utilizzati come ingredienti in panificazione per la produzione di prodotti con migliorate caratteristiche nutrizionali e strutturali.

This presentation was selected as a finalist for the " 5th What for Award" promoted by the Italian Network of the PhD Courses in Food Science, Technology and Biotechnology and Federalimentare.

Workshop on the Developments in the Italian PhD Research on Food Systems

Milan, September 15th, 2020

Gaetano Cardone (gaetano.cardone@unimi.it)
Department of Food, Environmental and Nutritional Sciences (DeFENS)
Università degli Studi di Milano, Milan, Italy
Tutor: Prof. Alessandra Marti

Effect of sprouting on the technological properties of grains

The effects of controlled sprouting (48 h; 20° C) on content and functionality of starch (pasting properties), protein (gluten aggregation) and fiber (hydration properties) of both whole grain and refined wheat flours were evaluated. Sprouting did not greatly compromise either pasting or gluten aggregation properties of flours, even if enzymatic activities significantly increased during the process. On the whole, a decrease in gelatinization and gluten weakening was observed in sprouted wheat. As regards the hydration properties of fiber, the sprouting process caused only a slight decrease in its water holding capacity.

Effetto della germinazione sulle proprietà tecnologiche dei semi

Gli effetti della germinazione (48 h; 20° C) sul contenuto e sulle proprietà di amido (*pasting properties*), proteine (aggregazione del glutine) e fibra (proprietà di idratazione) sono stati valutati sulla farina di frumento (sia integrale che raffinata). La germinazione non ha compromesso completamente le proprietà di gelatinizzazione e retrogradazione dell'amido e la capacità di aggregazione del glutine, nonostante l'attività enzimatica sia aumentata significativamente durante il processo. Nel complesso, è stata osservata una diminuzione della capacità di gelatinizzazione ed un indebolimento del reticolo glutinico. Inoltre, la germinazione ha ridotto la capacità di trattenimento dell'acqua da parte della fibra.

18th European Young Cereal Scientists and Technologists Workshop
Camerino, April 15th – 17th 2019

Effects of sprouting process on the bread-making performance of durum wheat

Gaetano Cardone, Anna Scipioni, Silvia Grassi, Alessandra Marti

Università degli Studi di Milano, Department of Food, Environmental and Nutritional Sciences (DeFENS), Via Celoria 2, 20133, Milan, Italy

- *Effects of sprouting on protein and starch properties*
- *Changes in dough rheology as affected by sprouting time*
- *Assessment of bread characteristics when semolina from sprouted wheat is used in bread-making*

This study aimed at evaluating the effects of durum wheat sprouting under controlled conditions on starch and protein characteristics and the relation between chemical/rheological changes and bread-making performance. Durum wheat kernels were sprouted at lab scale (Molino Quaglia S.p.A., Vighizzolo d'Este, Padova, Italy) at 20° C and 90% relative humidity, and sampled after 24, 38, 48, and 62 hours and then milled into semolina flour. Amylase activity was directly (by Ceralpha Method) and indirectly (through the Falling Number) evaluated upon sprouting. In addition, protein (AACCI 46-12.01), total starch (AACCI 76-13.01), damaged starch (AACCI 76-31.01), and simple sugars (Megazyme® enzymatic kit) were measured. Protein and starch features were evaluated in terms of gluten aggregation kinetics (by the Glutopeak®) and pasting properties (by the Rapid Visco Analizer®; RVA), respectively. Finally, dough leavening properties and specific volume of bread were measured.

As regards starch properties, sprouting led to drastic decreases in viscosity values during heating and cooling, due to the increased amylase activity during the sprouting process. In the presence of the enzyme inhibitor ($AgNO_3$), peak and final viscosity greatly increased, indicating that the pasting and gelatinization properties of starch were not compromised by sprouting. Despite the proteolytic activity developed during sprouting, the gluten proteins were still able to aggregate. However, the indices from the GlutoPeak test suggested a weakening of the gluten network. No significant differences were detected between 36 and 48 h, whereas the sample sprouted for 62 h showed the worst aggregation properties, giving rise to a bread with the lowest specific volume (2.69 mL/g). On the contrary, the best results in terms of dough development (180 mL) and bread specific volume (3.1 mL/g) were obtained using semolina from wheat sprouted up to 38 h. The PCA analysis highlighted a particular importance of the chemical indices to distinguish the unsprouted from the sprouted samples, while the changes in gluten were decisive in distinguishing the samples subject to different sprouting hours (24-38 h; 62 h). In conclusion, despite the accumulation of hydrolytic enzymes, sprouting under controlled conditions did not compromise the technological properties of semolina up to 48 h of germination. Furthermore, the germination process led to an improvement in the characteristics of the bread made from semolina obtained from durum wheat sprouted for 38 hours.

Durum wheat is characterized by low bread-making performance, due to its high protein tenacity. The development of a specific enzymatic pattern during the sprouting process may improve the technological performance of durum wheat in bread-making.

This oral presentation was awarded the first prize for best oral presentation.

Advanced School in Protein structure solution, prediction, and validation

Spetses, May 13rd – 17th, 2019

Effects of sprouting under controlled conditions on gluten properties

Gaetano Cardone, Maria Ambrogina Pagani, Alessandra Marti

Università degli Studi di Milano, Department of Food, Environmental and Nutritional Sciences (DeFENS), Milan, Italy

Sprouting is a natural process associated with many nutritional enhancements of grains: i.e., decrease in antinutritional factors increase in bio-availability of vitamins and minerals, and an increase in amino acids and sugars. All these improvements are related to the activity of hydrolytic enzymes (e.g., proteases and amylases) developed during the sprouting process. However, from the technological point of view, a high accumulation of hydrolytic enzymes negatively impacts on flour performance. Usually, a high proteolytic activity causes detrimental effects on gluten aggregation properties, making sprouted wheat unsuitable for baked products. However, recent studies have reported that flours from sprouted wheat under controlled conditions, in terms of time, temperature and relative humidity, could be used to improve the baking performances of flours. To understand why sprouting process under controlled conditions improves the bread-making performance of wheat flours, the purpose of this work was to evaluate the effects of sprouting on gluten properties. Specifically, the study focused on gluten protein formation in different hydration and shear stress conditions (GlutoPeak *vs* Farinograph test), as well as its viscoelastic properties (Glutograph test) and its ability to retain gas during the leavening phase (Rheofermentograph test). Finally, bread characteristics were also measured.

In slurry system, the gluten proteins were still able to aggregate. However, the decrease in maximum torque and in aggregation energy required for gluten aggregation suggested gluten weakening. In dough system, the decrease in dough water absorption, development time and stability during mixing confirmed the worsening of gluten quality due to sprouting time. On the other hand, the sprouting process led to an increase in gluten stretching, suggesting an increase in dough extensibility, giving rise to bread with great capacity to retain gas and high specific volume.

6.2.2 Poster communications

24th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology
Florence, September 11st – 13rd, 2019

Gaetano Cardone (gaetano.cardone@unimi.it)
Department of Food, Environmental and Nutritional Sciences (DeFENS)
Università degli Studi di Milano, Milan, Italy
Tutor: Prof. Alessandra Marti

Effect of sprouting on the technological properties of grains

This PhD project aims at understanding the effects of sprouting - carried out under controlled conditions of time and temperature - on the technological properties of grains (i.e. cereals, pseudocereals, and pulses). The project focuses on the relation between structural/functional changes in starch, protein, and fiber and the characteristics of cereal-based products (i.e., bread and/or pasta). Understanding the relationship between molecular changes and grain functionality will help in re-designing new cereal-based products with improved nutritional, sensory, and textural characteristics.

Effetto della germinazione sulle proprietà tecnologiche dei semi

Questo progetto di dottorato ha come obiettivo la comprensione degli effetti del processo di germinazione - effettuato in condizioni controllate di tempo e temperatura - sulle proprietà tecnologiche di cereali, pseudocereali e legumi. Il progetto ha come focus la relazione tra i cambiamenti strutturali/funzionali delle principali macromolecole dei cereali, ovvero amido, proteine e fibra e le caratteristiche dei prodotti finiti quali pane e pasta. La comprensione della relazione tra modificazioni strutturali e caratteristiche tecnologiche degli sfarinati risulta peculiare nella formulazione di nuovi prodotti a base di cereali con migliori specifiche nutrizionali, sensoriali e di *texture.*