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Molecular characterization of proteins in food matrices: how their structure evolve from raw materials to finished products

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1. ABSTRACT

Proteins have a fundamental importance for their peculiar chemical characteristic, implication in metabolism and structure for all living organisms. In food systems, proteins provide both nutritional and non-nutritional functionalities and, with other macromolecules, contribute in define the characteristics of the food systems. Technological and biotechnological treatments used in food processing are also responsible for the formation of new inter- and intra-molecular interactions, driven by changes in the structure of proteins, often in association with other food components.

In the present PhD thesis, the structural evolution of proteins from raw materials to finished products was studied in different food systems, with the aim of connecting protein molecular features - and the changes they undergo upon processing - with their functional properties. This PhD thesis aims at improving the current understanding of the structure/function relationship of macromolecules in various food systems, and of how the new relationships affect their technological properties. The approaches and results presented are of practical relevance for the food industry, that require to understand how to tailor processes in order to fit the nutritional, sensorial and environmental requests from the consumers.

The introduction (**Chapter 2**) provides a review of the current knowledge and of the approaches used to study the structure of proteins in food systems. The gluten network is used as a model system in this context, because of its high level of complexity and of its importance in defining the properties of cereal-based food systems. The first four **sections (4.1, 4.2, 4.3 and 4.4)** of the Results presents the structural and geometrical events leading to the formation of the gluten-network, and discusses some novel approaches set-up to investigate the three-dimensional rearrangements resulting from a transformation process (such as bread making). These approaches were also applied to clarify the impact of other macromolecules (amylose and amylopectin) in the gluten-network development, that in turn affects the properties of the derived products.

Sections 4.5, 4.6 and **4.7** discusses the impact of technological treatments on proteins in gluten-free flours, using lentils as a model and analyzing protein structural changes as a function of different technological treatments. Biochemical characterization was one prong of a multidisciplinary approach (rheology and calorimetry) that showed how the technological treatments affect the protein structure, improving the transformability of lentis flour in gluten-free pasta.

Pseudo cereals, such as buckwheat (**Section 4.8**), represent a suitable system to show how biotechnological treatments like sprouting can positively affect the nutritional properties,

also thanks to modification of the protein pattern and structure. The overall features of proteins in sprouted grains made them more suitable for the formation of protein network, useful for appropriate texturing of specific foods, like couscous. The nutritional properties were assessed in couscous fortified with the sprouted flour, after a simulation of an *in vivo* digestion.

Finally, proteins can also impact the flavor of the products acting as a source of reactive and/or bioactive components, often originated by breakdown by endogenous or added proteases. In **Sections 4.9, 4.10 and 4.11**, the nature and amount of peptides and amino acids released during fermentation of cocoa was related to the selected inoculum and to the flavor of the final product.

2. INTRODUCTION & LITERATURE REVIEW

2.1 Introduction

Proteins are the most abundant biological macromolecules occurring in all cells in which are virtually responsible and mediate (sometimes together with other biomolecules) every process in the cell, organs and systems, exhibiting an almost endless diversity of functions. Our food is derived from animal and plant tissues, thus by default the proteins of these tissues become food proteins, satisfying our body requirement (human nutrition) as well as the requirements in creating food systems (food functionalities) (Nadathur et al., 2017). Proteins are highly complex polymers, and their functional diversity mainly arises from their tridimensional structure and also by their interaction with other biopolymers and organic or inorganic compounds. In some proteins, some of the amino acid residues are enzymatically modified by the cytoplasmatic enzymes. Example of such modifications include glycosylation (e.g. lectins and vicilin-type proteins of legumes) and phosphorylation (α- and β-caseins, kinases, phosphorylases). In addition, the substituted amide linkage in proteins is a partial double bond increasing the structural complexity of protein polymers. Moreover the interactions between the amino acid combined, combined with the properties of the amide linkage make possible the formations of structural forms to proteins with diverse biological functions (Damodaran, 1997). Beside the functions explicated by proteins in cell, when considered in food proteins provide several useful functional properties. The functional properties therefor play a central role in the definition of all the properties that to make our food desirable and pleasurable to consume. Therefore, we utilize the functionality of proteins to satisfy many non-nutritional needs of eating food (Nadathur et al., 2017). The nutritional value and functional properties go hand-in-hand when making a protein-rich food more desirable to eat. The functional properties are related to the multitude of chemical and physical properties that protein can assume.

Kinsella (1976) firstly defined the functionality of food proteins as “any physico-chemical property which affects the processing and behavior of protein in food systems, as judged by the quality attributes of the final product”, including also the complex interactions of the protein other food components (i.e. carbohydrates, lipids), and the nature of the environment in which these interactions occur (Kinsella 1976). Protein had a fundamental role on the acceptability factor of food like (i) appearance (color, size, shape), (ii) taste and flavor (aroma, smell, taste) and (iii) texture (all perceptions that occurs through tactile, kinesthetic, visual, and hearing senses). All of that factors are firstly affect by the nature of the food matrices, with a great impact of the physico-chemical characteristics (e.g. pH, solvent and solute concentration, presence of other macromolecules such as lipids and carbohydrates). Moreover, the technological transformations affect the characteristics of the food matrices, for

instance by modification the proteins tridimensional structure and aminoacidic side chain residues. During technological processes of food, many proteins modification occur that result in denaturation of proteins. All these events involve modification in the biological properties, in specific domains (e.g. hydrophobic patches) and in residues (e.g. charged residues) on the protein molecule surface that determine the answer of proteins to all the molecular events that occur during the processing of the raw product into food. The manifestation of physicochemical properties of the protein is therefore dictated by the structural attributes of the protein molecule and the interaction with the environment (Nadathur et al., 2017). An in-depth description of the structural and conformational changes of proteins, as well as of the interactions with the other components, as a function of the process variables (including the formulation) is at the basis for elucidating the structure/function relationships. The molecular description of these events potentially has numerous practical implications, which include the fine-tuning of the process, improving the nutritional value, and replacing (undesirable) ingredients. The study of food proteins with the classic methodological approaches is however made difficult due to some intrinsic characteristics of food products: proteins are inserted in complex matrices from which it is difficult to extract them without altering their structure, moreover they are often insoluble. One of the most indicative examples is represented by gluten proteins: insoluble, intrinsically disordered and inserted in complex matrices that influence their molecular properties.

2.2 Studying the structure/function relationship of a protein network: the gluten network

The scientific literature concerning wheat gluten's collects a large number of works by authors from many different fields such as chemistry, biochemistry, food technologists and nutritionists. A first description of wheat's gluten was provided by the Italian chemistry J.B. Beccari in his essay "De Frumento" (On Wheat) published in 1731 (Bock and Seetharaman, 2012). From this early work, by going through over 250 years of study, currently the common definition of gluten is "the rubbery mass that is possible obtain by washing a wheat dough under a stream of running water in order to remove starch and the water-soluble components" (Wieser, 2007). The gluten network originates by the interaction among the insoluble storage proteins, particularly between gliadins and glutenins. From a physiological point of view, the storage proteins family represent the main nitrogen reserve for the germinating embryo (Bietz & Simpson, 1992). This proteins are deposited in the starchy endosperm inside the kernel (Shewry et al., 2002) and, even if starch represent the major constituent of wheat grain (in quantity terms), they are attributable of wheat's unique properties.

Gluten proteins account for 80% of wheat protein and are the largest contributor to wheat quality (D'Ovidio & Masci, 2004). Dough's protein network is formed through interactions between hydrated gluten proteins. The unique viscoelastic properties of dough are at the base

of the technological features of the large number of wheat's derived products (Delcour et al., 2012). The formation of a viscoelastic protein network is crucial for gas retention during dough proofing, and in the final setting into a porous structure in baked products like bread and cakes (Aguilera, 2019).

Based on the assumption that protein-protein interactions are responsible for the formation of the gluten network, and therefore for the quality of derived products, actually a lot of different approach are available to characterize the proteins involved in the formation of the gluten network, focusing also on the evolution of the three-dimensional features of the whole network.

2.2.1 Wheat proteins

Wheat's protein can be fractionated in four family based on their solubility. According to the traditional Osborne's classification (Osborne, 1924) is possible to identify: albumins (soluble in pure water), globulins (soluble in diluted salt solutions), gliadins (wheat prolamins, soluble in alcohol-water solutions, e.g. 60% ethanol), and glutenins (wheat glutelins, soluble in dilute acid or alkali) (Barak et al., 2015; Bonomi et al., 2014). Wheat's protein can be further subdivide in gluten proteins (involved in the formation of the protein network) and non-gluten proteins (that do not take part in the net). The non-gluten proteins generally enclose approximately 15% to 20% of total, and mainly correspond to albumins and globulins (Delcour et al., 2012). Gluten proteins enclose monomeric gliadins and polymeric glutenins, sharing a very low solubility in water or salt solutions. Glutenins, having inter- and intra-chain disulfide bonds (Bonomi et al., 2013), play a key role in the formation of the gluten network via thiols-disulfide exchange reactions that involve also gliadins. Both are fundamental for the rheological properties of the dough: hydrated gliadins contribute mainly to the viscosity and extensivity of the system, while glutenins are mainly responsible to the strength and elasticity of the dough. It can view gluten as a "two-component glue", with gliadins that operate as a "plasticizer" (or "solvent") for glutenins (Wieser, 2007). The main consequence is therefore the gliadins/glutenins ratio is the main factor determining the viscoelastic properties of a dough.

2.2.2 Gluten network organization

Many works have studied the possible correlation between the protein and baking performance of different wheat cultivar (Bean et al., 1998). However, this approach has an intrinsically tricky aspect. Quantification and identification of proteins extracted have an undoubtable importance in every proteomics work. However, to go deeper in the characterization of the gluten network this approach is limited because do not take into account protein/protein interaction, often influenced by other components of the matrix (e.g. lipids, starch, etc.). Moreover, the structure and conformation of proteins must be taken into account, since their evolution during the

transformation process (from the raw materials to the dough), is strictly interconnected with the develop of the gluten network. Interactions between proteins and other matrix constituents are fundamental in the definition of the macroscopic characteristics of the system, in particular for the features of food products.

Isolation of gluten's components and characterization of gluten interactions is one of the most difficult challenge faced by protein chemists. Firstly there is the heterogeneity of wheat's proteins: many polypeptides are enrolled in the formation of gluten network (Bietz & Simpson, 1992) and there are therefore problems related with the identification of the proteins due to the high polymorphism of the involved proteins. Secondly, gluten proteins are low-soluble and intrinsically disordered, thus making it difficult to apply the classical approaches of protein chemistry developed to study ordered proteins in aqueous solutions. Additionally, technological processes affect the three-dimensional structure of each single protein, modifying their chemical reactivity (in term of accessibility of amino acid residues), and so leading the formation of the protein network.

Under a molecular point of view, the description of the gluten requires to pinpoint its high complexity by discriminating different level of complexity that should be summarized as follow:

- the protein pattern, elucidating qualitative differences among wheat lines, responsible for their technological attitude;
- the protein conformation, in term of secondary and tertiary structure, descriptive on one hand of the molecular reorganization that trigger the evolution from flour to dough, and on the other hand of the overall protein reorganization induced (or allowed) by the process;
- the protein network, that is the nature and the extent of protein/protein interaction in gluten.

Moreover, in a complex system such as a dough system, molecular events that lead from the gluten proteins to the gluten network are strictly influenced by other components (starch, lipids, fiber, etc.) with which gluten proteins have to interact directly or indirectly.

2.2.3 Methodological approaches to profile the proteins pattern

Highlight differences in homologous proteins, in different wheat species and varieties, requires the use of a broad range of "classical" analytical techniques: mono- and two-dimension electrophoresis, high performance liquid chromatography (HPLC), and mass spectroscopy (MS) (Bonomi et al., 2013).

2.2.3.1 Electrophoresis

Electrophoresis has been one the first techniques used to characterize the composition of gluten. The peculiar amino acidic composition of the gluten proteins, mainly characterized by few basic or acidic residues, (giving them low charge/mass ratio) made possible to realize their electrophoretic separation with different methods based: (i) both on size and charge influence mobility (by using the two-dimensional electrophoresis, 2-DE); (ii) only on the different size, going to suppress the charge differences using a detergent such as sodium dodecyl sulfate (SDS-PAGE); and (iii) only the isoelectric point (pI), since proteins migrate in a pH gradient to positions where they are electrically neutral (isoelectric focusing, IEF) (Bietz & Simpson, 1992).

The SDS-PAGE (Laemmli, 1970) represent one of the most useful techniques that allow to separate - and also quantify - proteins from a complex mixture. Heating the sample in presence of SDS, an anionic surfactant, proteins are denatured and charge differences are eliminated bounding the SDS. The separations occur totally on the basis of size and the molar mass can be estimated by comparison to standard proteins (Bietz & Simpson, 1992). Adding a reducing agent (such as 2-ME) reduce disulfide bonds so different subunits can migrate independently. An example of the “classical” application is provided by Graybosc and Morris (1990) that described the use of SDS-PAGE in order to obtain an optimal resolution of the low molecular weight glutenin subunit (LMW-GS) from different wheat. This is a clear-cut case of application of this techniques to highlights difference in the protein pattern in two raw materials, but – as the authors themselves explain – is difficult to attribute to a single protein a specific quality parameter. Gao et al. (2010) characterized the high molecular weight glutenin subunit (HMW-GS) from 60 germplasms with a pool of proteomic approach. The main drawback of SDS-PAGE, in which x-type HMW-GS show slower electrophoretic mobility and high molecular weight than y-type, was addressed with its low resolution. Moreover, the quantification of the different subunits is difficult (Shewry et al., 1984; Yan et al., 2004). Other techniques (such as MS and HPLC) provide more reliable high throughput identification and screening. However, SDS-PAGE has the advantage of the technical simplicity and the low equipment requirement, thus it is suitable for large-scale and high-throughput HMW-GS screening for breeding programs, particularly when it is know the glutenin composition of the raw materials (Gao et al., 2010).

2-DE techniques is based on using sequentially a slab IEF gel and a SDS-PAGE, to separate proteins with a very good sensitive (Scheele, 1975; Görg et al., 2000). By using these techniques is possible to obtain a very good separation of the wheat proteins (Bietz & Simpson, 1992), that is usually applied as separative step in proteomic approaches. A first description of this techniques in relation of the separation of cereal proteins was provided by Wrigley (1970). Skylas et al. (2005) published an in-depth proteomic characterization of wheat grain based on 2-DE high-resolution separations. A proteomic study, in which 2-DE was used for

the separation of protein from three durum wheat cultivars different for their technological performances, was provided by Pompa et al. (2013). Poor technological performance was related to low abundance in the LMW 48,000−35,000 region, the absence of the LMW-2 and the down-expression of the LMW Met-typ.

Both SDS-PAGE and 2-DE are suitable to study the molecular determinants at the base of the formation of the gluten network, but for an adequate application it is crucial to take into account the effects of the technological process on the proteins (e.g. mechanical stress such as shearing stress during kneading, heat and pressure during the extrusion), that are at the bases of proteins conformational changes, resulting also in a modification of the solubility and digestibility.

Anderson and Ng (2000) have studied the effects of the twin-screw extrusion processing on the sulfhydryl and disulfide contents, by using SDS-PAGE in reducing and non-reducing condition. Using this approach, the authors have studied how the process affect the ability of the disulfide and sulfhydryl groups to interact to stabilize the protein's structure that are modified during the extrusion process, concluding that there was very little or no thiol-disulfide exchange reaction during extrusion processing under the experimental conditions, and also, that the intramolecular scission of disulfide bonds seemed to be the predominant occurrence during the extrusion process. This is a very good example of the application of the electrophoresis to addressing the evolution of the three-dimensional properties of wheat proteins.

2.2.3.2 Mass spectrometry

Mass spectrometry (MS) is an analytical technique that can provide both qualitative and quantitative information about molecules after their conversion to ions. MS has reached a prominent an outstanding position among the analytical techniques according to its unequalled sensitivity and low detection limits (in order of the pico-, femto- and attomole). The principle at the base of this technique is the production of a gas-phase ions from the compounds under scrutiny; all of these ions, generated during the ionization step, will be separated by the mass spectrometer according to their mass-to-charge ratio (m/z) detecting it in proportion to their abundance (Ho et al., 2003; Hoffmann & Stroobant, 2007).

Electron spray ionization (ESI) MS and matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) MS analysis represent milestones techniques in proteomics (for the description of these techniques please refer to Hoffmann & Stroobant, 2007). MALDI-TOF MS is often used for realize a direct identification of protein mixtures (generally in combination with 2-DE techniques). The crucial point at the base of the success of the us of MALDI matrix, is the desorption and ionization of analytes; another advantage of this techniques is the easy preparation of the sample and the large tolerance to contamination e.g. by salt, buffer or

detergents. MALDI matrix pared with the TOF analyzer has proven to be a sensitive technique to determine the molecular weights of intact HMW-GS (Lagrain et al., 2013). Analysis by MALDI-TOF-MS delivers fast determination of protein molecular mass up to 100kDa, obtaining a fingerprint of gluten proteins, with the identification of gluten/gliadins in finished food and flour sample, achieving the characterizations of wheat gliadin mass pattern in complex matrices (e.g. flours, starches and processed food), both in celiac toxic cereals (such as barley, rye and oats) and celiac non-toxic cereals (mays and rice) (Ferranti et al., 2007). The greatest problem with this technique is related to the loss of accuracy at high mass and with protein mixture with high complexity level: the results is a suppression phenomenon in MALDI that do not allow the identification of the protein subtypes and also a problem in distinction between high and low molecular weight proteins (Mamone et al., 2000).

The ESI-MS is a sensitive, robust and reliable tools for studying at femto-mole quantities in microliter sample volumes, non-volatile and thermally labile biomolecules that are not amenable to analysis by other conventional techniques (Ho et al., 2003); with the production of a range of charged species for each bio-molecule, the precision of mass assignments is increased. A great advantage of ESI-MS is that is a liquid-based method, compatible with typical chromatographic separations of biosamples. The combined use of ESI and liquid chromatography (LC/ESI-MS) is a very useful tool that can provide a lot of information concerning proteins sequences, wheat gluten composition and also gluten structure (Lagrain et al., 2013). A proteomics approach based on combination of HPLC and ESI-MS can been used to rationalize gluten heterogeneity in different wheat varieties, allowing rapid differentiation among varieties; typical application of LC/ESI-MS is the analysis of gliadins in different wheat varieties (Ferranti et al., 2007).

Today there are no standard methods for gluten analysis using LC coupled with MS techniques (LC-MS). LC-MS is considered to be a powerful and highly sensitive proteomics technique that is in high demand for food testing required for gluten-free labelling. There is a definite need to standardize the different data analysis platforms and several researchers advise using multiple platforms to ensure the comparability of results (Haraszi et al., 2020).

2.2.4 Methodological approaches to investigate protein structure

The structure of a proteins represents furthermore a paramount point that must be studied since its changes are essential in the reticulation process. A wide variety of techniques have thus been developed to address three-dimensional change of that proteins.

2.2.4.1 Infrared spectroscopy

Infrared spectroscopy (IR) today is largely applied in support of other more classic techniques (e.g. MS). An important advantages of IR spectroscopy is the no need of protein extraction. Is

thus possible apply IR spectroscopy to asses secondary structure information in complex systems. Furthermore, it is possible to develop that kind of spectroscopy as an alternative strategy for protein fingerprinting (Saeed et al., 2012).

Attenuated total reflectance (ATR)-FTIR spectroscopic techniques was used by several authors to study the gluten secondary structure, showing the presence of β-turn and intermolecular β-sheet (Belton et al., 1995; Li et al., 2006; Pézolet et al., 1992). Moving on the application on processed matrices, the results obtained with ATR-FTIR provide information regarding the evolution of the secondary structures in gluten network as consequence of treatments. In detail it was reported β-sheet structures were the most responsive to pasta-making processing conditions and their increase could be associated with the cooking losses and textural differences (Bock et al., 2015). The cooking of the finished products leads to a complete loss of α-helical structure, accompanied by a decrease of β-turns. All of this modification contributed to a substantial increasing in β-sheets (20–39% higher). These results underline that gluten originated from a strong wheat flour can better preserve an optimal secondary structure distribution, whereas gluten from weak flours was prone to structural collapse of β-turns to β-sheets (Jazaeri et al., 2015). Failure of the secondary structure would be in line with a dominant role for noncovalent interactions in the weaker gluten networks. Also of interest is the significantly lower β-sheet content for whole wheat pasta dough and cooked whole pasta, compared with its refined counterpart. The differences have been attributed at interference from bran, both from a physical and a water distribution (Bock et al. 2013) standpoint.

2.2.4.2 Fluorescence

Spectroscopic techniques are underused in cereals science. They represent a powerful tools to study complex matrices “as such” to obtain information regarding the structures and interactions among the constituents (Belton et al., 1995; Calucci et al., 2003; Chen et al., 2010; Li et al., 2006) and gain insight on chemical characterizations of the system. Fluorescence represent a widely used technique because made possible investigation of the structure of proteins and also the function and reactivity of small molecules, synthetic polymers and other biological molecules (Strasburg & Ludescher, 1995).

The assessment of the conformational evolution of the gluten proteins during hydration and kneading, are made possible by applying a pools of spectroscopic methods. With these techniques is possible to evaluate changes in protein structure by exploiting different luminescent molecules (such as intrinsic and extrinsic fluorophores or using different probes) (Sadat et al., 2019). Fluorescence exploits the emission of “light” by specific fluorophore (Bonomi et al., 2004). This phenomenon is the response in change of the electronic and vibrational state of the fluorophore as consequence of the photoexcitation of “fluorescent

molecules". The returns of electron to its ground state from an excited level is called quantic jump: its result in a loss of excess of energy, accumulated during the excitation step, as photon.

In structural studies, fluorescence represents a powerful and flexible technique. This depend on the sensitivity of the fluorescent molecules to the environment. Tryptophan is the most commonly used intrinsic fluorophore. Fluorescence of tryptophan can be quenched by protonated amino and carboxyl groups, and amide bonds. In a native protein the tryptophan is generally buried in the hydrophobic core of the proteins, where the presence of other residues (such as arginine) have a quenching effect. The result is a blue-shifted fluorescence with a low emission intensity. During the unfolding and moving away from the quencher, the tryptophan become more and more exposed to the solvent (with a progressive increasing of polarity of the environment) resulting in a red-shifted fluorescence and higher emission intensity. A problem that must be taken in account is the general loss of structure related with the solubilization in non-aqueous solvent, or in in denaturing buffer of water-insoluble proteins, such as the glutenins and in generals the prolamins.

Fluorescence techniques represent a solution to this problem because it is applicable on different matrices without the need of solubilize the single components. In cereal-science is largely applied a solid-state spectroscopy know as front-face fluorescence spectroscopy, that need a very limited (and sometimes no one) steps for the preparation of the sample (Mounier, 2017). Front-face fluorescence has been used to study three-dimensional evolution of the insoluble proteins in undisturbed solid and semisolid food system (Bonomi et al., 2004; Sadat et al., 2019). Bonomi et al. (2004) explain how this technique can be used to assess structural features of proteins in common wheat flours and in durum wheat semolina during crucial transformation step such as the (i) protein solvation and (ii) kneading. By using intrinsic front-face fluorescence the authors were able to describe the effect of the addition of water and applying a mechanical stress to the system, responsible and necessary for the exposition of the proteins domain associate with the formation of the gluten network. The exposition to the solvent (in this case water), causes a remarkable red-shift of the emission maximum compared to a residue buried in the protein interior (Bonomi et al., 2004). The change in intrinsic fluorescence (i.e. the structural reorganization of gluten protein) was found to be function of the level of hydration of the raw material. Whereas in wheat flours the maximum structural changes related to tryptophan exposure apparently are completed at water contents close to that optimal for development of the gluten network, in semolina the molecular modifications occur at lower water concentrations with respect to those involved in the formation of network-stabilizing bonds upon kneading.

Structural information can be expanded through the use of extrinsic fluorophores. Proteins are deposited in grain in an almost dry state in compact aggregates. The abundance of glutamine residues, in both gliadins and glutenins, minimizes interactions with water.

(Bonomi et al., 2014). Interaction with water (solvation of charged or hydrophilic groups on amino acid side chains) leads to significant structural changes in the gluten proteins even in the absence of mechanical treatments. When the right amount of water is added (generally at moisture content above the 35%) and a mechanical stress is applied (the kneading phase), gluten proteins are subject to modification of their molecular conformation. Therefore, water operate as plasticizer and through the mixing phase of the hydrated flour/semolina, gluten proteins are made able to interact, resulting in the formation of the gluten network mainly via thiol-disulfide exchange reactions and hydrophobic interactions with a general decrease of the protein extractability (Delcour et al., 2012). In general, extrinsic probes can be used to characterize molecular events in the absence or the extreme presence of a high number of intrinsic probes (Strasburg & Ludescher, 1995). A common used extrinsic probes is 1-anilinonaphtalene-8-sulfonic acid (ANS). ANS is used due to its ability to interact, and so become fluorescent, with the hydrophobic patches that are exposed by the proteins. Results from this approach are used to highlight the effect of technological transformation, in particular during hydration and kneading (that represent a key step for the proteins reticulation). Bonomi et al. (2004) showed that semolina undergo rearrangement of its hydrophobic surfaces upon solvation, even after completion of conformational rearrangements. This suggest the existence of diverse hydrophobic regions in the semolina proteins that may take part of interprotein interaction in gluten network, thus acting in a synergic fashion for establishing the properties of semolina.

Iametti et al., (2006) used ANS front-face fluorescence to determinate the amount of the hydrophobic probe that remain bound to the buffer-insoluble proteins fraction in immature wheat kernels. Results suggest that major compaction of the overall protein structure began when gluten proteins became the most abundant protein component.

2.2.5 Methodological approaches to characterize the protein network

Techniques presented above are useful to characterize single proteins, but present limitations for studying the structure of the whole protein network. Is well know that the secondary structure of proteins is mainly determinate by chemistry and order of the amino acids that made up the proteins. Peculiar amino acidic profile of prolamins is a key aspect for the unique properties of wheat gluten network (Shewry et al., 2002). Addressing the molecular feature of the proteins network (i.e. protein involved, number and nature of intermolecular interaction, compactness of the three-dimensional net, etc.) is therefore a key point to better understand the rheological properties cereal-based products.

2.2.5.1 Chromatographic methods

Chromatographic methods can be used to realize the separation of the proteins based on size (i.e. in the size-exclusion chromatography, SE) or according to the intrinsic characteristics imparted by specific amino acids (i.e. in different types of affinity chromatography, such as ion-exchange, IE, reverse-phase, RP, and hydrophobic interaction, HI). Chromatography represent a technique largely applied to isolate and characterize wheat proteins; in particularly high performance liquid chromatography (HPLC) are a widely used methods in food analysis for analytic and preparative separations, commonly coupled with conventional UV and fluorescence detector (Léonil et al., 2000) or in proteomics approaches for wheat quality characterization (Haraszi et al., 2020).

SE-HPLC was used by Lundh and Macritchie (1989) (in combination with SDS-PAGE), for the molecular characterization of the gluten protein in relation to baking performance. To solubilize and realize the proteins separation in SE-HPLC 0.1 % (w/v) SDS buffer were used. Evaluating also changes in Mixograph peak development time, authors highlight the relation between the ratio of larger to smaller glutenins and bread making quality, underlining the fundamental contribution of HMW-GS to the technological properties of the dough. Interesting, in this work the authors underlined the problems related with the solubility of the gluten proteins, especially related to the highest molecular weight proteins (glutenins) that remain insoluble regardless of the solvent system.

Another application of the SE-HPLC has been described by Singh (2005). In this work the author evaluates molecular changes in protein as consequence of the baking process by SE-HPLC, sonication and reduction. Starting from a baked product, author solubilized the proteins with sonication in 0.5% SDS (w/v), 0.05M sodium phosphate, pH 6.9 buffer. Analyzing the areas of SE-HPLC chromatograms they observed a regular decrease in the total extractable protein with increasing in baking time. That results indicate the formation of insoluble polymers throughout the baking process, underlining a general decrease in solubility related to aggregation and/or cross-linking that occur during the baking. Moreover, the effect was greater in the central section than in the crust or base of the bread, suggesting that the temperature-moisture profile governs cross-linking during baking.

Jazaeri et al. (2015) studied changes in protein extractability, titration of accessible thiols and evaluating the molecular weight distribution of the gluten proteins (by size exclusion separation), highlighting the formation of larger polymeric gluten structures depending on disulfide linkages in hard wheat dough than in soft wheat dough. Jazaeri et al (2005) has moreover assessed the gluten network evolution in terms of changing in hydrophobicity of wheat proteins during mixing using the front-face fluorescence spectroscopy. The totality of evidence form Jazaeri et al. (2005) leads to the conclusion that hydrophobic interactions play the main role in soft wheat gluten network formation. Thus, formation of gluten networks in

different wheat depend on the influence of evolution of the structure of the single proteins, driving the process by hydrophobic interactions or disulfide linkages.

2.2.5.2 Differential solubility

Interprotein disulfides are by far the most abundant – if not the sole – covalent interaction of interest among different proteins prior to baking (at least when specific enzymes, i.e. transglutaminase, are not added). Whether non-covalent interactions significance after the addition of water is debatable, these interactions cannot be assessed on proteins in the dry state (Bonomi et al., 2014).

By studies of differential solubility is possible to obtain information regarding the type of interaction that occur among wheat proteins. Proteins held together by ionic interactions in poorly soluble aggregates are dissociated and brought in solution when raising the ionic strength of an aqueous buffer. With chatropes like urea, guanidine or different detergents, is possible to perturb the hydrophobic interactions that occur between proteins, making soluble those proteins that form homo- and heteropolymeric aggregates, based only on hydrophobic interactions (Bonomi et al., 2013). Finally, is possible to solubilize proteins that are involved in insoluble homo- and heteroprotein complex stabilized by disulfide bonds by using a disulfide-reducing agents like the dithiothreitol (DTT) in addition to the chaotropes.

Differential solubility approach made possible to compare the performance between flour and semolina, from the raw materials and in finished products. Bonomi et al., 2013 observe that the amount of protein soluble in chaotrope decrease significantly in pasta compared to semolina, but that solubility is almost the same in reducing condition, suggestion the formation of cross-linking as result of pastification process.

2.2.5.3 Thiol accessibility

Information about the compactness of the protein network are achieved exploiting change in accessibility of specific residues, such as cysteines. The role of cysteine residues in the generation of the protein network throughout disulfide exchange reaction is well known. The network-forming capacity of proteins involved in the thiols-disulfide exchange reaction is related with various factor such as their relative abundance, the amount and location of reactive thiols and disulfide, and also their availability to exchange events (Iametti et al., 2013). Exposure and burring of the thiols are related to structural modification of the proteins (i.e. during technological transformation), thus thiols represent a specific target to evaluate the three-dimensional changes of proteins structure.

Quantification of cysteine based on using the 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) in widely used. This quantification exploits the reaction of the bis(p-nitrophenyl) disulfide with aliphatic thiols compound to produce a p-nitrothiophenol anion per mole thiol.

The resulting anion is highly colored (ε = 13,600 $M^{-1}cm^{-1}$ at 412 nm) (Ellman, 1959). Accessibility for the relatively large reagent restricts this method to detecting only the sulphydryl groups that are located on the protein surface or in accessible cavities (Visschers & De Jongh, 2005). Measurement of the thiols accessibility in various condition (i.e. presence or absence of dissociating agents like chatropes or detergents) offer information regarding the compactness of the protein network in the system (Bonomi et al., 2013). Combined with solubility studies, DTNB made possible the quantification of the accessible thiol groups (Bonomi et al., 2012; Parizad et al., 2020), that could provide comparative information about raw materials different in their performance trait (Bonomi et. al., 2013) or finished products (Bonomi et. al, 2012).

The fluorescent labelling is a widely used techniques and there are a range of approach available for labelling proteins, with a variety of probe fluorescent probe that can be used (Toseland, 2013). In order to expand the structural information obtained by using the DTNB, in the last years a "thiolomics" approach has been developed. Iametti et al. (2013) labelled protein thiols with a fluorescent probe in absence or presence of a non-polar chaotrope (urea). Combining the fluorescent labeling with SDS-PAGE and 2-DE detection, this approach has the advantage of make possible to study the accessibility and reactivity of proteins involved in the formation of the gluten network. This approach was used to create a map of the accessible thiols. An evolution of this approach to study conformational changes was outlined by Marengo et al. (2019), where the geometrical features of the whole protein network have been studied exploiting the high reactivity of Au^{+} ions on the surface of gold nanoparticles (AuNPs) towards thiols. The results of this work show that is possible to explore the geometrical features of complex protein networks, containing proteins with free thiols, using specific probes, and through the identification of the "fished" proteins using MS techniques.

This kind of approach represent a progression in studying protein network, pointing out the importance of the proteins structure for the finished products. Understanding molecular events are therefore a key to study the macroscopic properties of finished products in order to optimize the transformation at industrial level of the raw materials, and also to evaluate the possibility of innovation in terms of products.

2.2.6 Methodological approaches to describe the role of matrix effects in defining the gluten network

Information presented above are mandatory to understand the effects of interactions between proteins and other wheat components. The interplay between the basic ingredients that characterize the matrix defines most of the physical properties during processing of the materials (Mann et al., 2014). Is therefore of paramount importance to evaluate the interaction between proteins and other macromolecules and polymers that make up the system. In

particular, in the next paragraph are addressed the importance of starch, lipids, and other non-gluten proteins and is possible to evaluate that interactions.

2.2.6.1 Starch

Starch is the most abundant carbohydrate in wheat grains, representing 70-80% of dry weight. Its content seems to be inversely related to proteins, with soft wheat varieties that show in general higher starch contents than hard ones (Bonomi et al., 2014). Starch is composed by two polymers: amylose and amylopectin, both based on units of anhydroglucose. Differences are related in the degree of branching of the D-glycosyl units. Amylose is essentially a linear molecule (in spite of the linear chains can form helical structures) in which the D-glycosyl units are α(1-4) linked (with a degree of polymerization in the range of 500-6000 glucose residues). Amylopectin is also based on α(1-4) D-glucosyl chains, but are presents branches every 20-25 residues due to α(1-6) linkages (and a degree of polymerization in the range of 3×10^5-3×10^6 glucose residues) (Bonomi et al., 2014). Amylose/amylopectin ratio differs among starches, but is in general around the 25-28% and 72-75% respectively (Van Hung et al., 2006). However, the starch of some mutant wheat genotypes, selected via classical breeding and genetics, containing high amount of amylose (high amylose or amylostarch with up to 70% amylose), or are practically amylose free (waxy, with 99-100% amylopectin) (Goesaert et al., 2005; Robert A. Graybosch et al., 2003). In function of the development of the grain structure, wheat starch granules show various size and shapes. Based on size, starch granules are classified in three classes: A- (20-35μm), B- (9-11μm) and C- (2-3μm), with the last two that are usually grouped as small B-types. Regarding the shape, A-types show a lenticular shape meanwhile B-types are spherical (Cao et al., 2015).

Because of the high percentage of starch in wheat flour and semolina, its pasting properties, retrogradation and viscosity characteristics are fundamental for the finished products features. When water is added during kneading, it is adsorbed by the hydrophilic compounds (salt, simple sugar, globular proteins and amino-acids) forming a homogeneous aqueous solution wetting the starch granule (Schiraldi & Fessas, 2003). Resulting from kneading step, the formed gluten acts as a natural hydrocolloid that interacts with water molecules forming dough. The three-dimensional network formed is filled with starch granules of different size and shapes (X. Gao et al., 2020). The main starch-starch and starch-gluten interactions are typically based on van der Waals and hydrogen bond type interactions (Meerts et al., 2017). When gluten is formed, there is a competition with starch for hydration; moreover, gluten proteins is located on the surface of starch granules and acting as diffusion barrier, affecting the diffusion of the water into the starch granules (Jekle et al., 2016). The milling step cause in a small, but significant proportion of starch granules (5-8%), a physical damage. Level

of starch damage varies according with the severity of grinding and wheat hardness. The level of damaged starch affects the rate of water adsorption of dough (Van Der Borght et al., 2005).

In last years is increased the interest for waxy starches due to the positive effects on retarding starch retrogradation resulting in an extension of the products shelf-life. Caramanico et al. (2017) have investigated the relationship between starch and proteins in a waxy wheat with an interdisciplinary approach, comparing two waxy wheat lines with a non-waxy one. The results of Caramanico et al. (2017) suggest that proteins in waxy wheat samples needed more water to complete solvation, likely because in waxy wheat dough, water was tightly bound to starch. Moreover, the low water mobility in waxy wheat resulted in low and retarded gluten hydration and in high stickiness. In samples with the highest stickiness, protein aggregates were stabilized mainly by hydrophobic interactions.

2.2.6.2 Lipids

Lipids are endogenously present in wheat or can be added as ingredients. In bread making lipids carry out important roles that positively affect baking and storage (Pareyt et al., 2011). The medium content of lipids in wheat is about 2-2.5% and are determined not only by genetics and environment but also by the milling and lipid extraction methods (Pareyt et al., 2011; Van Der Borght et al., 2005).

The functions of lipids in bread making are various like “shortening”, lubrication, aeration; moreover, lipids help with heat transfer, extending shelf-life, as well as providing structure and desirable textural properties such as tenderness, richness, and improved mouthfeel (Huschka et al., 2012). The presence of water during the mixing process drive the interaction between flour lipids and gluten proteins via hydrophobic and polar interaction (McCann et al., 2009). In the first steps of baking process, approximately the 70% of total wheat flour lipids become bound or trapped in by the gluten fraction, lining up at the interface of the gas cells giving support in the dough and positively affecting the gas retention (Pareyt et al., 2011). Dough mixing accelerates hydrophobic binding of mainly triglycerides and steryl esters to glutenin and gliadins while polar lipids (chiefly digalactosyl diglyceride and some phospholipids) that mainly interact with glutenin proteins (Chung et al., 1978).

ATR-FTIR approaches, Hesso et al. (2015) studied the impact of ingredients addition on the protein secondary structure in a model batter system. Regarding the addition of fat to flour, the authors report the formation of α-helical structure (increasing from 16% to 35%) and a decrease in β-sheet structure (49% to 22%).

2.2.6.3 Other proteins

Gluten proteins play obviously a key role in the formation of dough and in characterization of the technological properties of wheat. Moreover, non-gluten proteins strongly affect the wheat

technological aspects such as dough forming properties. In particular, the amphiphilic proteins, that are lipid-binding proteins, play a key role in the definition of some important features of the whet. The friabilins is a 15kDa proteins group that can find in on the starch granules surface in larger amount in soft than in hard wheat (Bonomi et al., 2014). The two major polypeptides of the friabilin fraction were found identical to two isoforms of puroindolines (PINs): puroindoline-a (PIN-a) and puroindoline-b (PIN-b) (Bonomi et al., 2014). PINs are basic (pI =11) and cysteine-rich proteins, with an important presence of the amino acid tryptophane that are present in the amphiphilic domain or loop and flanked by two cysteine residues involved in the formation of a disulfide bond (Day et al., 2006). Whilst PINs only representing the 0.1% of the total wheat proteins, they are enrolled in determining the hardness of wheat kernel texture, like the force that is necessary to crush the kernel (Morris, 2002; Quayson et al., 2018). The wheat endosperm hardness is mainly affected by the interaction between PINs, starch granule surface, storage proteins and lipids (Finnie et al., 2010). The interaction between PINs and starch grains surface (and starch surface with polar lipids) are realized thought the tryptophan-rich domain of the proteins, also suggesting the formation of PIN homo- or hetereo-dimers/oligomers via ionic, polar and/or hydrophobic interactions between the residues that are exposed on the loops and helix surface of PINs (Quayson et al., 2018).

Quayson et al. (2018) investigated the possible interaction between PINs and gluten proteins in flour, focusing on the effect of PINs on aggregation of gluten proteins, on protein solvation, and on the exposure of reporter amino acid sidechains in gluten proteins. The technique used by the authors are mainly based on solubility studies, SDS-PAGEs of the samples used, protein solvation and investigating the accessibility of thiols accessibility. The results obtained by the authors highlight the impact of PINs on proteins interaction in flour, enhancing the gluten proteins aggregation resulting in a decreased SDS extractability, less thiols accessibility. Authors suggest that from the interaction between PINs and gluten proteins result an highly compact supra-macromolecular aggregates that are stabilized by local and very tight hydrophobic interactions.

2.3 Remarks on the gluten network

At present, evidences suggest that the network formed using different wheat, e.g. whole wheat and refined, show different properties. Also technological transformation – like the drying process of pasta – affect the protein network (Bock et al., 2015). The effect of mixing time represents, for instance, a major aspects regarding the properties of the gluten because from an optimum mixing time result a protein network with more structurally ordered, homogeneous and elastic characteristics. Also genetic factor plays a key role, with the evidence of different structure function in connection with the wheat varieties was already observed (Kuktaite &

Ravel, 2020; Peter R. Shewry et al., 2000). There are evidence that events like aggregation or pre-aggregation of gluten proteins, that are relevant for the geometrical properties of the network, occur during the earlier stages of transformation process. The main protein-protein interaction (e.g. crosslinking between gluten protein molecules through disulfide/sulfhydryl interchange reactions, hydrophobic interactions and iso-peptide bonding, occurs according to the chosen temperature, additives used or processing method (Kuktaite & Ravel, 2020).

Physical properties of the gluten network have been commonly studied by using a farinographic kneading that provide fundamental information from processing standpoint, but any molecular properties of the gluten network. Spectroscopy techniques can provide new approach in order a molecular evaluation of the events that leading the formation of the gluten network during kneading and their evolution from the raw materials to the end products.

Studying the interaction between gluten and other components of wheat is clear the importance of evaluating the geometrical evolution of the gluten proteins. From the combined use of the classic techniques (such as SDS-PAGES, chromatography and MS) with approach based on solubility is possible to obtain a lot of information about the proteins. Moreover, spectroscopy techniques provide an incredible amount of tools that can shed light on the geometrical evolution of the protein structure, starting from the secondary structure of proteins to evaluating the exposure of specific and a-specific target.

It is clear that the available tools, and their combination, are practically endless, giving to the researcher spoilt for choice. If research concerning the gluten network has a very long stories, today we are far from the conclusion of the investigation about the structural aspects of wheat proteins and the gluten network features.

A strong boost in the investigation of the characteristics of protein network is today driven by consumer demand, and the growing interest in the use of new food matrices, such as legumes, for the production of pasta and other products, traditionally derived from cereals such as wheat. This represent new challenge for all the food industry, that need to improve their knowledge of the relationship between the rheological and molecular aspects at base of the characteristics of the end products.

2.4 References

Aguilera, J. M., 2019. The food matrix: implications in processing, nutrition and health, Crit. Rev. Food Sci. Nutr. 59, 3612-3629.

Ahmad, M. H., Nache, M., Waffenschmidt, S., Hitzmann, B., 2016. Characterization of farinographic kneading process for different types of wheat flours using fluorescence spectroscopy and chemometrics. Food Control 66, 44-52.

Anderson, A. K., Ng, P. K. W., 2000. Changes in disulfide and sulfhydryl contents and electrophoretic patterns and extruded wheat flour proteins. Cereal Chem. 77, 354-359.

Anderson, O. D., Larka, L., Christoffers, M. J., McCue, K. F., Gustafson, J. P., 2002. Comparison of orthologous and paralogous DNA flanking the wheat high molecular weight glutenin genes: Sequence conservation and divergence, transposon distribution, and matrix-attachment regions. Genome. 45, 367-380.

Anklam, E., H.-D. Belitz, W. Grosch, P. Schieberle, 2005. Food Chemistry, Third Edition. Analytical and Bioanalytical Chemistry, 382(1), 10–11.

Barak, S., Mudgil, D., Khatkar, B. S., 2015. Biochemical and Functional Properties of Wheat Gliadins: A Review. Crit. Rev. Food Sci. Nutr. 55, 357-368.

Bartels, D., Altosaar, I., Harberd, N. P., Barker, R. F., Thompson, R. D., 1986. Molecular analysis of γ-gliadin gene families at the complex Gli-1 locus of bread wheat (T. aestivum L.). Theor. Appl. Genet. 72, 845-853.

Bean, S. R., Lyne, R. K., Tilley, K. A., Chung, O. K., & Lookhart, G. L., 1998. A rapid method for quantitation of insoluble polymeric proteins in flour. Cereal Chem. 75, 374-379.

Belton, P. S., Colquhoun, I. J., Grant, A., Wellner, N., Field, J. M., Shewry, P. R., Tatham, A. S., 1995. FTIR and NMR studies on the hydration of a high-Mr subunit of glutenin. Int. J. Biol. Macromol. 17, 74-80.

Bietz, J. A., Simpson, D. G., 1992. Electrophoresis and chromatography of wheat proteins: available methods, and procedures for statistical evaluation of the data. J. Chromatogr. A. 624, 53-80.

Bock, J., Seetharaman, K., 2012. Unfolding gluten: an overview of research on gluten. Cereal Foods World. 57, 209-214.

Bock, J. E., Connelly, R. K., Damodaran, S., 2013. Impact of bran addition on water properties and gluten secondary structure in wheat flour doughs studied by attenuated total reflectance fourier transform infrared spectroscopy. Cereal Chem. 90, 377-386.

Bock, J. E., Damodaran, S., 2013. Bran-induced changes in water structure and gluten conformation in model gluten dough studied by Fourier transform infrared spectroscopy. Food Hydrocolloids. 31, 146-155.

Bock, J. E., West, R., Iametti, S., Bonomi, F., Marengo, M., Seetharaman, K., 2015. Gluten structural evolution during pasta processing of refined and whole wheat pasta from hard white winter wheat: The influence of mixing, drying, and cooking. Cereal Chem. 92, 460-465.

Bonomi, F., D'Egidio, M. G., Iametti, S., Marengo, M., Marti, A., Pagani, M. A., Ragg, E. M., 2012. Structure-quality relationship in commercial pasta: A molecular glimpse. Food Chem. 135, 348-55.

Bonomi, F., Ferranti, P., Mamone, G., 2014. Wheat Flour. In Bakery: Products Science and Technology (eds W. Zhou, Y.H. Hui, I. De Leyn, M.A. Pagani, C.M. Rosell, J.D. Selman and N. Therdthai).

Bonomi, F., Iametti, S., Mamone, G., Ferranti, P., 2013. The performing protein: Beyond wheat proteomics?. Cereal Chem. 90, 358-366.

Bonomi, F., Mora, G., Pagani, M. A., Iametti, S., 2004. Probing structural features of water-insoluble proteins by front-face fluorescence. Anal. Biochem. 329, 104–111.

Calucci, L., Forte, C., Galleschi, L., Geppi, M., Ghiringhelli, S., 2003. 13C and 1H solid state NMR investigation of hydration effects on gluten dynamics. Int. J. Biol. Macromol. 32, 179-189.

Cao, H., Yan, X., Chen, G., Zhou, J., Li, X., Ma, W., Yan, Y., 2015. Comparative proteome analysis of A- and B-type starch granule-associated proteins in bread wheat (Triticum aestivum L.) and Aegilops crassa. J. Proteomics. 112, 95–112.

Caramanico, R., Barbiroli, A., Marengo, M., Fessas, D., Bonomi, F., Lucisano, M., Pagani, M. A., Iametti, S., Marti, A., 2017. Interplay between starch and proteins in waxy wheat. J. Cereal Sci. 75, 198-204.

Chen, X., Zhou, Y., Peng, X., Yoon, J., 2010. Fluorescent and colorimetric probes for detection of thiols. Chem. Soc. Rev. 39, 2120-2135.

Chung, O. K., Pomeranz, Y., Finney, K. F., 1978. Wheat Flour Lipids in Breadmaking. Cereal Chem. 55, 598–618.

D'Ovidio, R., Masci, S., 2004. The low-molecular-weight glutenin subunits of wheat gluten. J. Cereal Sci. 39 (3), p.321-339.

Day, L., Bhandari, D. G., Greenwell, P., Leonard, S. A., Schofield, J. D., 2006. Characterization of wheat puroindoline proteins. FEBS Journal. 273, 5358-5373.

Delcour, J. A., Joye, I. J., Pareyt, B., Wilderjans, E., Brijs, K., Lagrain, B., 2012. Wheat Gluten Functionality as a Quality Determinant in Cereal-Based Food Products. Annu. Rev. Food Sci. Technol. 3, 469-492.

Ellman, G. L., 1959. Tissue sulfhydryl Groups. Arch Biochem Biophys. 70–77.

Ferranti, P., Mamone, G., Picariello, G., Addeo, F., 2007. Mass spectrometry analysis of gliadins in celiac disease. J. Mass Spectrom. 42, 1531-48.

Gao, L., Ma, W., Chen, J., Wang, K., Li, J., Wang, S., Bekes, F., Appels, R., Yan, Y., 2010. Characterization and Comparative Analysis of Wheat High Molecular Weight Glutenin Subunits by SDS-PAGE, RP-HPLC, HPCE, and MALDI-TOF-MS. J. Agric. Food. Chem. 58, 2777-2786.

Gao, X., Tong, J., Guo, L., Yu, L., Li, S., Yang, B., Wang, L., Liu, Y., Li, F., Guo, J., Zhai, S., Liu, C., Rehman, A. ur, Farahnaky, A., Wang, P., Wang, Z., Cao, X., 2020. Influence of gluten and starch granules interactions on dough mixing properties in wheat (Triticum aestivum L.). Food Hydrocolloids. 106, 105885–.

Georget, D. M. R., Belton, P. S., 2006. Effects of temperature and water content on the secondary structure of wheat gluten studied by FTIR spectroscopy. Biomacromolecules. 7, 2, 469–475.

Goesaert, H., Brijs, K., Veraverbeke, W. S., Courtin, C. M., Gebruers, K., Delcour, J. A., 2005. Wheat flour constituents: How they impact bread quality, and how to impact their functionality. Trends Food Sci. Technol. 16, 12-30.

Görg, A., Obermaier, C., Boguth, G., Harder, A., Scheibe, B., Wildgruber, R., Weiss, W., 2000. The current state of two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis. 21, 1037-53.

Graybosch, R. A., Morris, R., 1990. An improved SDS-PAGE method for the analysis of wheat endosperm storage proteins. J. Cereal Sci. 11, 201–212.

Graybosch, Robert A., Souza, E., Berzonsky, W., Baenziger, P. S., & Chung, O., 2003. Functional properties of waxy wheat flours: Genotypic and environmental effects. J. Cereal Sci. 38, 69–76.

Haraszi, R., Ikeda, T.M., Peña, R.J., Branlard, G., 2020. Gluten Analysis. In: Igrejas, G., Ikeda, T., Guzmán, C. (eds) Wheat Quality For Improving Processing And Human Health. Springer, Cham.

Hesso, N., Marti, A., Le-Bail, P., Loisel, C., Chevallier, S., Le-Bail, A., Seetharaman, K., 2015. Conformational changes of polymers in model batter systems. Food Hydrocolloids. 51, 101-107.

Ho, C. S., Lam, C. W. K., Chan, M. H. M., Cheung, R. C. K., Law, L. K., Lit, L. C. W., Ng, K. F., Suen, M. W. M., Tai, H. L., 2003. Electrospray ionisation mass spectrometry: principles and clinical applications. Clin. Biochem. Rev. 24, 3-12.

Hoffmann, E., Stroobant, V., 2007. Mass Spectrometry. Principles and Applications, 3rd Edition, WILEY-VCH Ver- lag GmbH & Co. KGaA, Weinheim,.

Hsia, C. C., Anderson, O. D., 2001. Isolation and characterization of wheat ω-gliadin genes. Theor. Appl. Genet. 103, 37-44.

Huschka, B., Bonomi, F., Marengo, M., Miriani, M., Seetharaman, K., 2012. Comparison of lipid effects on structural features of hard and soft wheat flour proteins assessed by front-face fluorescence. Food Chem. 133, 1011-1016.

Iametti, S., Bonomi, F., Pagani, M. A., Zardi, M., Cecchini, C., D'Egidio, M. G., 2006. Properties of the protein and carbohydrate fractions in immature wheat kernels. J. Agric. Food Chem. 54, 10239-44.

Iametti, S., Marengo, M., Miriani, M., Pagani, M. A., Marti, A., & Bonomi, F., 2013. Integrating the information from proteomic approaches: A "thiolomics" approach to assess the role of thiols in protein-based networks. Food Res. Int. 54, 980–987.

Jazaeri, S., Bock, J. E., Bagagli, M. P., Iametti, S., Bonomi, F., Seetharaman, K., 2015. Structural modifications of gluten proteins in strong and weak wheat dough during mixing. Cereal Chem. 92, 105-113.

Jekle, M., Mühlberger, K., & Becker, T., 2016. Starch e gluten interactions during gelatinization and its functionality in dough like model systems. Food Hydrocolloids. 54, 196-201.

Kikhney, A. G., & Svergun, D. I., 2015. A practical guide to small angle X-ray scattering (SAXS) of flexible and intrinsically disordered proteins. FEBS Letters. 589, 2570–2577. https://doi.org/10.1016/j.febslet.2015.08.027

Kuktaite, R., Ravel, C., 2020. Wheat gluten protein structure and function: Is there anything new under the sun? In: Wheat Quality For Improving Processing And Human Health,

Laemmli, U. K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-5.

Lagrain, B., Brunnbauer, M., Rombouts, I., Koehler, P., 2013. Identification of Intact High Molecular Weight Glutenin Subunits from the Wheat Proteome Using Combined Liquid Chromatography-Electrospray Ionization Mass Spectrometry. PLoS ONE. 8:e58682.

Lavelli, V., Guerrieri, N., Cerletti, P., 1996. Controlled Reduction Study of Modifications Induced by Gradual Heating in Gluten Proteins. J. Agric. Food. Chem. 44, 2549–2555.

Léonil, J., Gagnaire, V., Mollé, D., Pezennec, S., Bouhallab, S., 2000. Application of chromatography and mass spectrometry to the characterization of food proteins and derived peptides. J. Chromatogr A. 881, 1–21.

Li, W., Dobraszczyk, B. J., Dias, A., Gil, A. M., 2006. Polymer conformation structure of wheat proteins and gluten subfractions revealed by ATR-FTIR. Cereal Chem. 83, 407-410.

Lundh, G., Macritchie, F., 1989. Size exclusion HPLC characterization of gluten protein fractions varying in breadmaking potential. J. Cereal Sci. 10, 247-53.

Mamone, G., Ferranti, P., Chianese, L., Scafuri, L., Addeo, F., 2000. Qualitative and quantitative analysis of wheat gluten proteins by liquid chromatography and electrospray mass spectrometry. Rapid Commun. Mass Spectrom. 14, 897-904.

Mann, J., Schiedt, B., Baumann, A., Conde-Petit, B., Vilgis, T. A., 2014. Effect of heat treatment on wheat dough rheology and wheat protein solubility. Food Sci. Technol. Int. 20, 341-351.

Marengo, M., Mamone, G., Ferranti, P., Polito, L., Iametti, S., Bonomi, F., 2019. Topological features of the intermolecular contacts in gluten-forming proteins: Exploring a novel methodological approach based on gold nanoparticles. Food Res. Int. 119, 492–498.

McCann, T. H., Small, D. M., Batey, I. L., Wrigley, C. W., Day, L., 2009. Protein-lipid interactions in gluten elucidated using acetic acid fractionation. Food Chem. 115, 105–112.

Meerts, M., Cardinaels, R., Oosterlinck, F., Courtin, C. M., Moldenaers, P., 2017. The Interplay Between the Main Flour Constituents in the Rheological Behaviour of Wheat Flour Dough. Food and Bioprocess Technol. 4,1357-66.

Morris, C. F., 2002. Puroindolines: The molecular genetic basis of wheat grain hardness. Plant Mol. Biol. 48, 633–647.

Mounier, S., Redon, R., Nicolodelli, G., Milori, D., 2017. Front-face fluorescence spectroscopy of tryptophan and fluorescein using laser induced fluorescence and excitation emission matrix fluorescence. RSC Advances. 7, 56117–56122.

Osborne, T.B., 1924. The vegetable proteins. Longmans, Green and Company. Pareyt, B.,

Finnie, S. M., Putseys, J. A., Delcour, J. A., 2011. Lipids in bread making: Sources, interactions, and impact on bread quality. J. Cereal Sci. 54, 266-279.

Parizad, P. A., Marengo, M., Bonomi, F., Scarafoni, A., Cecchini, C., Pagani, M. A., Marti, A., Iametti, S., 2020. Bio-functional and structural properties of pasta enriched with a debranning fraction from purple wheat. Foods. 9, 163.

Payne, P. I., 1987. Genetics of Wheat Storage Proteins and the Effect of Allelic Variation on Bread-Making Quality. Annu. Rev. Plant Physiol. 38(1):141-53

Pézolet, M., Bonenfant, S., Dousseau, F., Popineau, Y., 1992. Conformation of wheat gluten proteins Comparison between functional and solution states as determined by infrared spectroscopy. FEBS Letters. 299, 247-250.

Pompa, M., Giuliani, M. M., Palermo, C., Agriesti, F., Centonze, D., Flagella, Z., 2013. Comparative analysis of gluten proteins in three durum wheat cultivars by a proteomic approach. J. Agric. Food. Chem. 61, 2606-2617.

Quayson, E. T., Marti, A., Morris, C. F., Marengo, M., Bonomi, F., Seetharaman, K., Iametti, S., 2018. Structural consequences of the interaction of puroindolines with gluten proteins. Food Chem. 253, 255–261.

Rasheed, F., Plivelic, T. S., Kuktaite, R., Hedenqvist, M. S., Johansson, E., 2018. Unraveling the Structural Puzzle of the Giant Glutenin Polymer - An Interplay between Protein

Polymerization, Nanomorphology, and Functional Properties in Bioplastic Films. ACS Omega, 3, 5584–5592.

Robertson, G. H., Gregorski, K. S., Cao, T. K., 2006. Changes in secondary protein structures during mixing development of high absorption (90%) flour and water mixtures. Cereal Chem. 83, 136-42.

Sadat, A., Corradini, M. G., Joye, I. J., 2019. Molecular spectroscopy to assess protein structures within cereal systems. Curr. Opin. Food Sci. 25, 42–51.

Saeed, A., W. Huck, C., Pallua, J., A. Huck-Pezzei, V., Bittner, L., Pezzei, C., Schonbichler, S., M. Qureshi, A., K. Bonn, G., Najam-ul-Haq, M., 2012. Role of Infrared Spectroscopy in Proteomics and Subsequently the Biomarker Analysis. Current Proteomics, 9, 118–131.

Scheele, G. A., 1975. Two-dimensional gel analysis of soluble proteins. Characterization of guinea pig exocrine pancreatic proteins. J. Biol. Chem. 250, 5375-85.

Schiraldi, A., Fessas, D., 2003. Classical and knudsen thermogravimetry to check states and displacements of water in food systems. J. Therm. Anal. Calorim. 71, 225-235.

Shewry, P. R., Halford, N. G., Tatham, A. S., 1992. High molecular weight subunits of wheat glutenin. J. Cereal Sci., 15, 105-120.

Shewry, P. R., Tatham, A. S., 1997. Disulphide bonds in wheat gluten proteins. J. Cereal Sci. 25, 207-227.

Shewry, Peter R., Field, J. M., Faulks, A. J., Parmar, S., Miflin, B. J., Dietler, M. D., Lew, E. J. L., Kasarda, D. D., 1984. The purification and N-terminal amino acid sequence analysis of the high molecular weight gluten polypeptides of wheat. Biochimica et Biophysica Acta (BBA)/Protein Structure and Molecular Enzimology. 788, 23–34.

Shewry, Peter R., Halford, N. G., Belton, P. S., Tatham, A. S., 2002. The structure and properties of gluten: An elastic protein from wheat grain. Philosophical Transactions of the Royal Society B: Biological Sciences. 357, 133–142.

Shewry, Peter R., Popineau, Y., Lafiandra, D., Belton, P., 2000. Wheat glutenin subunits and dough elasticity: Findings of the EUROWHEAT project. Trends in Food Sci. Technol. 11, 433-441.

Shewry, Peter R, Tatham, A. S., Forde, J., Kreis, M., Miflin, B. J., 1986. The classification and nomenclature of wheat gluten proteins: A reassessment. J. Cereal Sci. 4, 97-106.

Singh, H., MacRitchie, F., 2001. Application of polymer science to properties of gluten. J. Cereal Sci. 33, 231–243.

Singh, H., 2005. A study of changes in wheat protein during bread baking using SE-HPLC. Food Chem. 90, 247–250.

Skylas, D. J., Van Dyk, D., Wrigley, C. W., 2005. Proteomics of wheat grain. J. Cereal Sci. 41, 165-179.

Strasburg, G. M., Ludescher, R. D., 1995. Theory and applications of fluorescence spectroscopy in food research. Trends in Food Sci. Technol. 6, 69–75.

Thanhaeuser, S. M., Wieser, H., Koehler, P., 2014. Correlation of quality parameters with the baking performance of wheat flours. Cereal Chem. 91, 333-341.

Toseland, C. P., 2013. Fluorescent labeling and modification of proteins. J. Chem. Biol. 6, 85-95.

Van Der Borght, A., Goesaert, H., Veraverbeke, W. S., Delcour, J. A., 2005. Fractionation of wheat and wheat flour into starch and gluten: Overview of the main processes and the factors involved. J. Cereal Sci. 41, 221–237.

Van Hung, P., Maeda, T., Morita, N., 2006. Waxy and high-amylose wheat starches and flours-characteristics, functionality and application. Trends Food Sci. Technol. 17, 448-456.

Visschers, R. W., De Jongh, H. H. J., 2005. Disulphide bond formation in food protein aggregation and gelation. Biotechnol. Adv. 23, 75-80.

Wellner, N., Mills, E. N. C., Brownsey, G., Wilson, R. H., Brown, N., Freeman, J., Halford, N. G., Shewry, P. R., Belton, P. S., 2005. Changes in protein secondary structure during gluten deformation studied by dynamic fourier transform infrared spectroscopy. Biomacromolecules. 6, 255-261.

Wieser, H. (2007). Chemistry of gluten proteins. Food Microbiol. 24, 115-119.

Wrigley, C. W., 1970. Protein mapping by combined gel electrofocusing and electrophoresis: Application to the study of genotypic variations in wheat gliadins. Biochem Genet. 4, 509-16.

Wrigley, Colin W., 1996. Giant proteins with flour power. Nature. 381, 738-739.

Yan, Y., Jiang, Y., Sun, M., Yu, J., Xiao, Y., Zheng, J., Hu, Y., Cai, M., Li, Y., Hsam, S. L. K., Zeller, F. J., 2004. Rapid identification of HMW glutenin subunits from different hexaploid wheat species by acidic capillary electrophoresis. Cereal Chem. 81, 561-566.

Zhang, Y., Hu, X., Juhasz, A., Islam, S., Yu, Z., Zhao, Y., Li, G., Ding, W., Ma, W., 2020. Characterising avenin-like proteins (ALPs) from albumin/globulin fraction of wheat grains by RP-HPLC, SDS-PAGE, and MS/MS peptides sequencing. BMC Plant Biol. 20, 1-9.

3. AIMS AND OBJECTIVES

The macroscopic properties of food matrices and their characteristics are defined by the molecular events that involve their structure and by interactions among macromolecules in response to technological processes. Studying the transformation process from a molecular point of view therefore allow to understand how the conformational changes of proteins, in respect also the whole matrices under scrutiny, affect the properties of the finished products.

In this PhD thesis, different food matrices - in which proteins have a fundamental role in defining food properties - have been considered:

- cereals, where proteins play a key role in the development of the gluten network thanks to their peculiar structuring functions;
- legumes, where proteins contribute to texture and play a key role in the production of gluten-free products such as pasta;
- pseudo cereals, where protein conformational changes influence the texture and the nutritional properties of final products;
- cocoa, where proteins, through the development of the fermentative microflora, release precursors of aromatic substances contributing to the development of desired sensory properties.

For all these products, suitable methodological approaches have been applied (and developed) to study their protein component, paying particular attention to the molecular characteristics relevant for the functional properties typical of each product.

4. RESULTS AND DISCUSSION

4.1 CEREALS – WHEAT. Thiolomic: set up of the approach

4.1.1 Background

The gluten network originates from the interaction among the monomeric gliadins and the polymeric glutenins. From a physiological point of view these proteins, deposited in the starchy endosperm inside the kernel (Shewry et al., 2002), represent the main nitrogen reserve for the germinating embryo (Bietz & Simpson, 1992). Gluten proteins that constituted the 80% of wheat protein, have a fundamental role in defining the wheat quality (D'Ovidio & Masci, 2004). During the processing (e.g. in pasta or bread making), the gluten proteins undergo - after solvation - structural rearrangements due to the mechanical action in kneading, leading to the formation of the gluten network. In the resulting network, proteins are connected by non-covalent interactions (mostly hydrophobic), and by covalent intermolecular disulfide bonds, originating from thiol-disulfide exchange reactions between free cysteines and pre-existing disulfide bonds (Bonomi, Ferranti and Mamone, 2014). Aside from compositional differences in various types of cereals (e.g. total protein content), structural characteristics of the proteins (such as the amount and location of "native" disulfide bonds and free thiol groups and their accessibility) that control molecular rearrangement achieved during processing, are of paramount relevance to the overall quality of the products.

A first model of the geometrical structure of the gluten network was proposed by Belton (1999): the "loops" and "trains" model attempts to provide an explanation of the viscoelastic properties of the gluten network that are crucial for its peculiar features, like gas retention during dough proofing and the development of a porous structure in baked products (Aguilera, 2019).

Given the central role of thiol-disulfide exchange reactions, assessing their modification during transformation it would add a piece to our understanding of the overall process that leads to gluten formation, and on its impact on the properties of finished products. Although the characteristics of the individual wheat's proteins are well known (Bartels et al., 1986; Shewry et al., 2002), molecular features and geometrical properties of the "gluten network" are still understudied, mainly because of the intrinsic characteristics of the involved proteins (water-insoluble and intrinsically disordered) that make difficult the application of the traditional techniques used to investigate the structural features of proteins and their modification in biotechnological processes. Moreover, the complexity of real food systems is still larger, since the presence of other components (e.g. lipids, other proteins, simple and complex sugars, etc.) may influence at different levels the molecular events that lead to gluten formation.

Starting from the thiolomic approach proposed by Iametti et al. (2013) we have deepened the possibility of studying the structure of wheat proteins, by focusing our attention on the evolution of the accessibility of the thiols group. The general idea was “quite simple” but highly informative: perform a thiol accessibility hierarchy study by labeling thiols in native or destructuring conditions (in order to label thiols redly accessible or buried in hydrophobic grooves), on the raw material and at different stages of the technological process.

4.1.2 Materials and Methods

4.1.2.1 Materials

Commercial flour and semolina were purchased from a local supermarket. Standard gluten sample (Sigma, G5004) was used as reference.

4.1.2.2 Thiols covalent fluorescent labeling

The labeling protocol was developed by improving the method published in Iametti et al. (2013) and is schematically reported in figure 1. The first step of the protocol (called “dealbuminization”) aimed to remove soluble proteins, albumins and globulins. 50 mg of sample were suspended in 1 mL of a 50 mM sodium phosphate, 0.1 M NaCl, pH 7.0, and keep in agitation on a shaker for 1 h, followed by a centrifugation at 10,000 ×*g* for 10 min at 25 °C. The supernatant phase (containing water soluble proteins) was recovered. The precipitate (containing the water insoluble gluten proteins) was resuspended in three different condition: (i) 1 mL of 50 mM sodium phosphate, 0.1 M NaCl, pH 7.0, (ii) 1 mL of 50 mM sodium phosphate, 0.1 M NaCl, pH 7.0, added with 4 M urea, (iii) 1 mL of 50 mM sodium phosphate, 0.1 M NaCl, pH 7.0, added with 2% (w/v) SDS. The supernatant from the dealbuminization step and the three precipitate suspensions were added of 50 µL of 5 mM 5-(iodoacetamido)fluorescein (5-IAF) in dimethylformamide and incubated in the dark under agitation for 2 h to allow the reaction of 5-IAF with free and accessible thiols. The unreacted 5-IAF was blocked by adding 20 µL of 0.25 M dithiothreitol (DTT) and stirring the labelled suspension for 1 h. Finally, the blocked reactions were centrifuged at 5,000 ×*g* for 10 min at 25 °C. For the sample containing the gluten proteins, the supernatant (containing gluten proteins solubilized by the chaotrope or the detergent) and the precipitate (containing proteins insoluble also in destructuring condition, mainly for the presence of aggregates stabilized by disulfide bounds) were collected for future analysis.

4.1.2.3 Electrophoretic protein separation

Sample were prepared by diluting the supernatant, or when indicated by resuspending the precipitate, in denaturing buffer (0.125 M Tris–HCl, pH 6.8, 50% glycerol (w/v), 1.7% SDS (w/v), 0.01% Bromophenol Blue (w/v)) containing 1% (v/v) 2-mercaptoethanol (2-ME) when

indicated, followed by treatment at 100 °C for 10 min. Electrophoretic runs were performed at pH 8.3 (0.025 M Tris-HCl, 0.192 M glycine, 0.1% (w/v) SDS), in a Miniprotein II apparatus (Bio-Rad). At the end of the electrophoretic separation, fluorescent images of the gels were acquired by a VersaDoc^{TM} Image Analysis System (Bio-Rad). Then, the gels were stained by traditional Coomassie Brilliant Blue to obtain a total protein map. The fluorescent image and the Coomassie stained gels were compared and analyzed through the ImageLab software Version 6.0.1 (Bio-Rad).

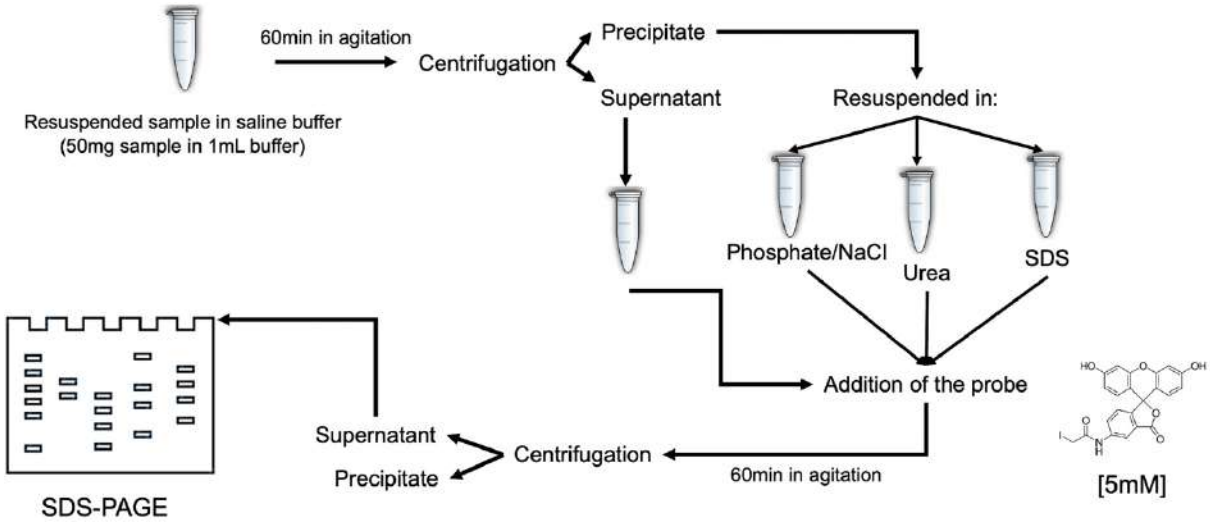

Figure 1. Detail of the thiolomic protocol developed.

4.1.3 Results and discussion

4.1.3.1 Fluorescent labelling of commercial gluten

The protocol for the thiols covalent labelling was set up using standard gluten. Since the standard sample is “pure” gluten, the dealbuminization step was not necessary. The commercial gluten was labelled after resuspension in saline buffer and saline buffer in presence urea and SDS (figure 2). After the labelling, the suspensions were centrifuged and analyzed in SDS-PAGE.

As expected, the standard gluten shows the presence of the three main protein group that are involved in the protein network: polymeric glutenins with High- and Low Molecular Weight subunits (HMW-, LMW-GS) and the monomeric gliadins. The labelling realized in saline buffer show very few proteins solubilized and labelled: this results is due to the water-insoluble nature of the gluten proteins. Both urea (a non-polar chaotrope) and SDS (an anionic surfactant) shows the same ability in solubilizing gluten protein, but different properties as for allowing thiols accessibility, that was found higher in the presence of urea.

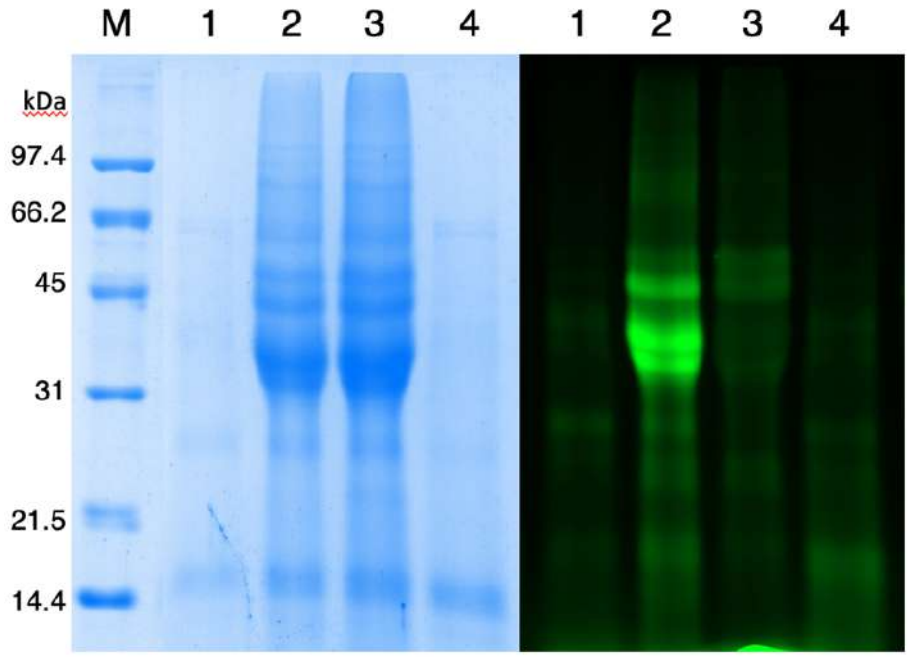

Figure 2. Labelling of commercial gluten in three different condition: 1) in saline buffer; 2) in saline buffer in presence of 4M urea; 3) in saline buffer in presence of 2% (p/v) SDS; 4) soluble protein fraction from the commercial gluten

4.1.3.2 Fluorescent labelling of commercial flour and semolina

The protocol was then applied to a more complex system such as commercial fluor and semolina (figure 3 panel A and B), focusing the attention on the gluten proteins solubilized in the different conditions. Results reported in figure 3 shown that the non-gluten proteins are highly reactive toward the fluorescent probe. Albumins and globulins are only the 15-20% of the total wheat proteins, but are rich in thiols, easily labelled by 5-IAF. As for gluten proteins, urea and SDS were found to be both effective in their solubilization, however, as for the commercial gluten, the accessibility of protein thiols to the fluorescent label was confirmed to be much higher when 4 M urea is used. It is noteworthy that the labelling of the thiols seems to occur independently from protein solubilization, since it is possible to observe a fluorescent signal for both the soluble and insoluble material (figure 3, panel C and D) especially when flour and semolina are resuspended in the saline buffer after the dealbuminization step.

4.1.3.3 Solubilizing capacity of urea and SDS

We investigated the ability of lower concentrations of urea and SDS to solubilize gluten proteins and to “make thiols accessible” (figure 4), starting from the concentration for urea (4 M) and SDS (2% (p/v)) used for the fluorescent labelling of flour and semolina.

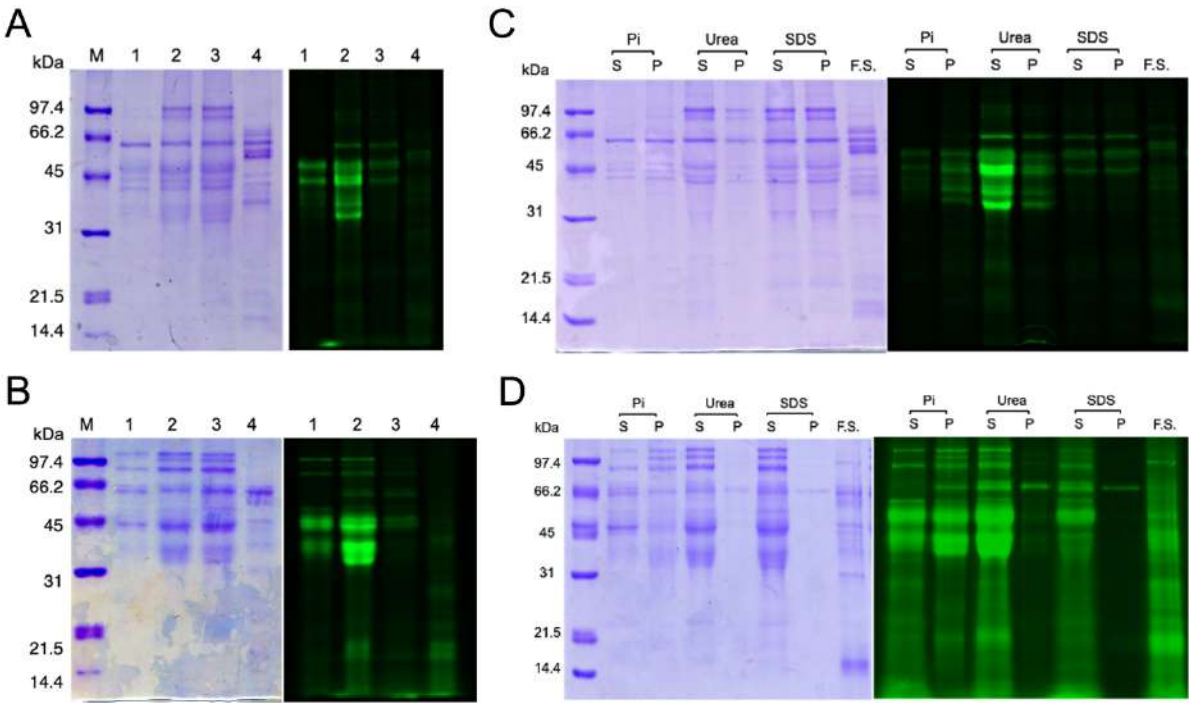

Figure 3. Fluorescent labelling of commercial fluor (panel A) and semolina (panel B); M) Marker, 1) saline buffer, 2) saline buffer in presence of 4 M urea, 3) saline buffer in presence of SDS 2%,4) soluble fraction from delabuminization step; loaded 10µL of sample.
Results from the thiolomic labeling of the soluble and insoluble fraction of semolina (panel C) and flour (panel D). Pi) saline buffer, Urea) saline buffer in presence of 4 M urea, SDS) saline buffer in presence of SDS 2%, F.S.) soluble fraction from delabuminization step, S) soluble fraction, P) insoluble fraction

We found that 2 M urea is the lowest concentration of chaotrope that allows a full accessibility of thiols. In parallel, 2 M urea is also able to solubilize the totality of the gliadins and most of the glutenins, but increasing the concentration up to 3 M improve the solubilization of the HMW-GS.

In the case of SDS, 0.2% w/v (i.e. the critical micellar concentration – CMC – at 20°C) is the threshold to promote protein solubilization, even if 0.5% SDS is needed to solubilize the totally of the gluten protein.

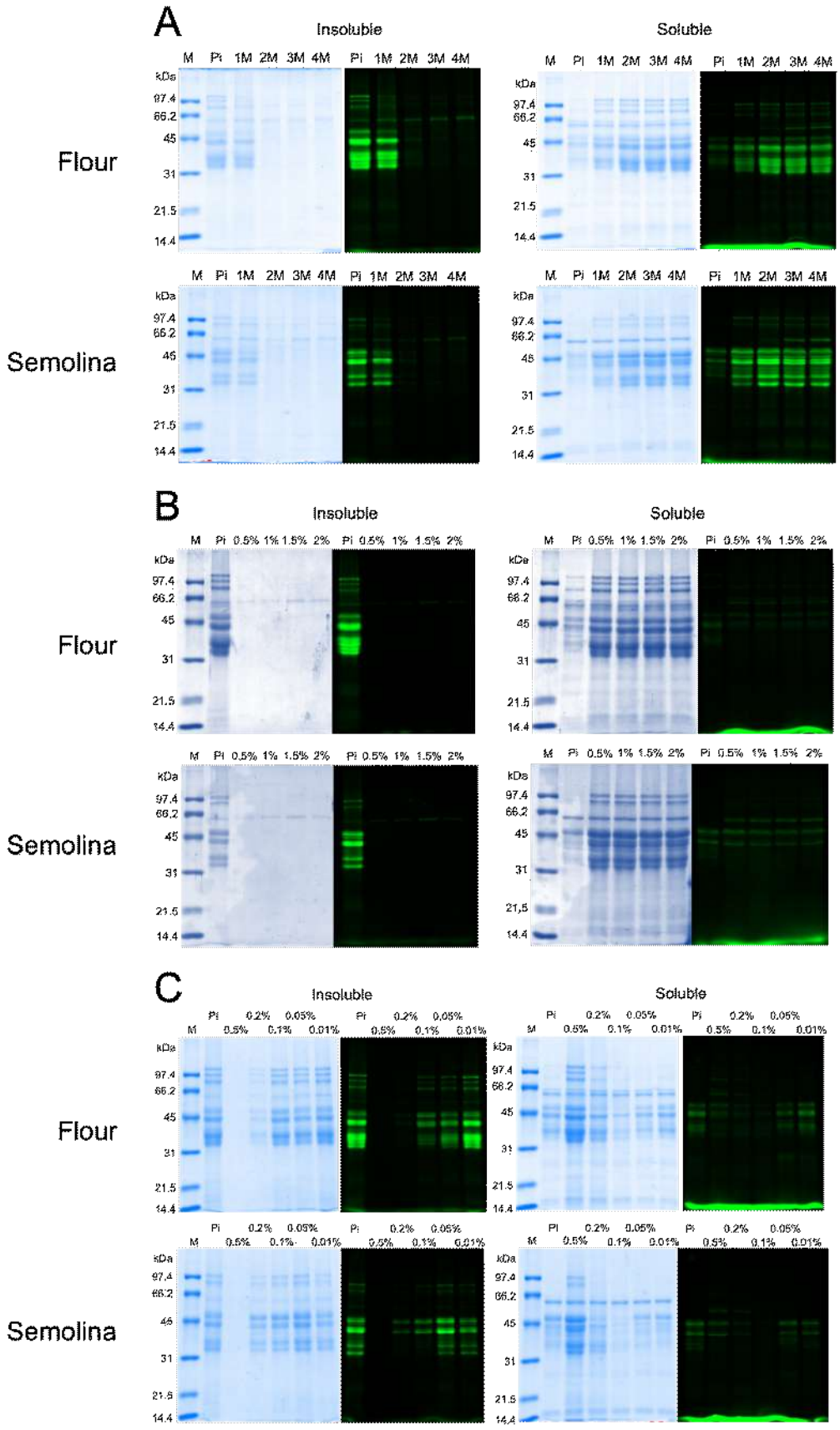

Figure 4. Investigation of the different solubility/destructuring performance of urea (panel A) and SDS (panel B and C).

4.1.3.4 Investigation of the effect of temperature

Temperature is a factor that influences the protein solubility and denaturation. The effect of temperature occurs on the non-covalent interactions (e.g. hydrogen and electrostatic bonds, hydrophobic interactions) that are involved in the stabilization of secondary and tertiary structures (Pelegrine et al., 2005). Therefore, the structural rearrangements of the protein induced by temperature may affect solubility, as well as of thiol accessibility. The effect of the temperature was investigated by performing the labelling in 4 M urea and 2% SDS at 20, 40 and 60°C (figure 5).

The increase of the temperature up to 60°C does not allow an increase of thiols accessibility of flour (figure 5, panel B) and semolina (figure 5, panel C). As for albumin and globulin fraction from the dealbuminization step (figure 5, panel A) it is possible to observe a decrease in protein solubility. This result suggests that the increase of the temperature may promote a thermal coagulation of these proteins as during the baking process.

4.1.4 Conclusions

Performing thiolomic studies can provide numerous information about the protein structure, also because most of these “structural” issues have never been addressed at molecular level. Therefore, matching the structural information from the thiolomic (and most common biochemical approach like differential solubility, thiols titrations with DTNB or fluorescent information from front-face approach) with compositional and rheological data can provide a complete description of the system. The thiolomic approach also is cheap and reliable and can provide information in a (relatively) short time.

However, studying cereal-based matrices, and in particular the gluten proteins, is made particularly tricky by the chemical nature of gliadins and glutenins (intrinsically disordered protein (IDP) with a very low solubility in water, presence of intra- and inter-chain disulfide bounds). Addressing the solubility of that proteins in different condition was therefore of paramount importance to obtain a clear map of the distribution of the thiols in the proteins. The fluorescent results also have to be analyzed always in comparison with the total protein map because the labeled proteins may be present as multiple spots, that made difficult a clear identification.

The developed thiolomic approach and the protocol is graphically represented in figure 1. Here was presented only the development and the set-up of that approach, however it was modified and adapted case by case in function of the investigation and sample under scrutiny.

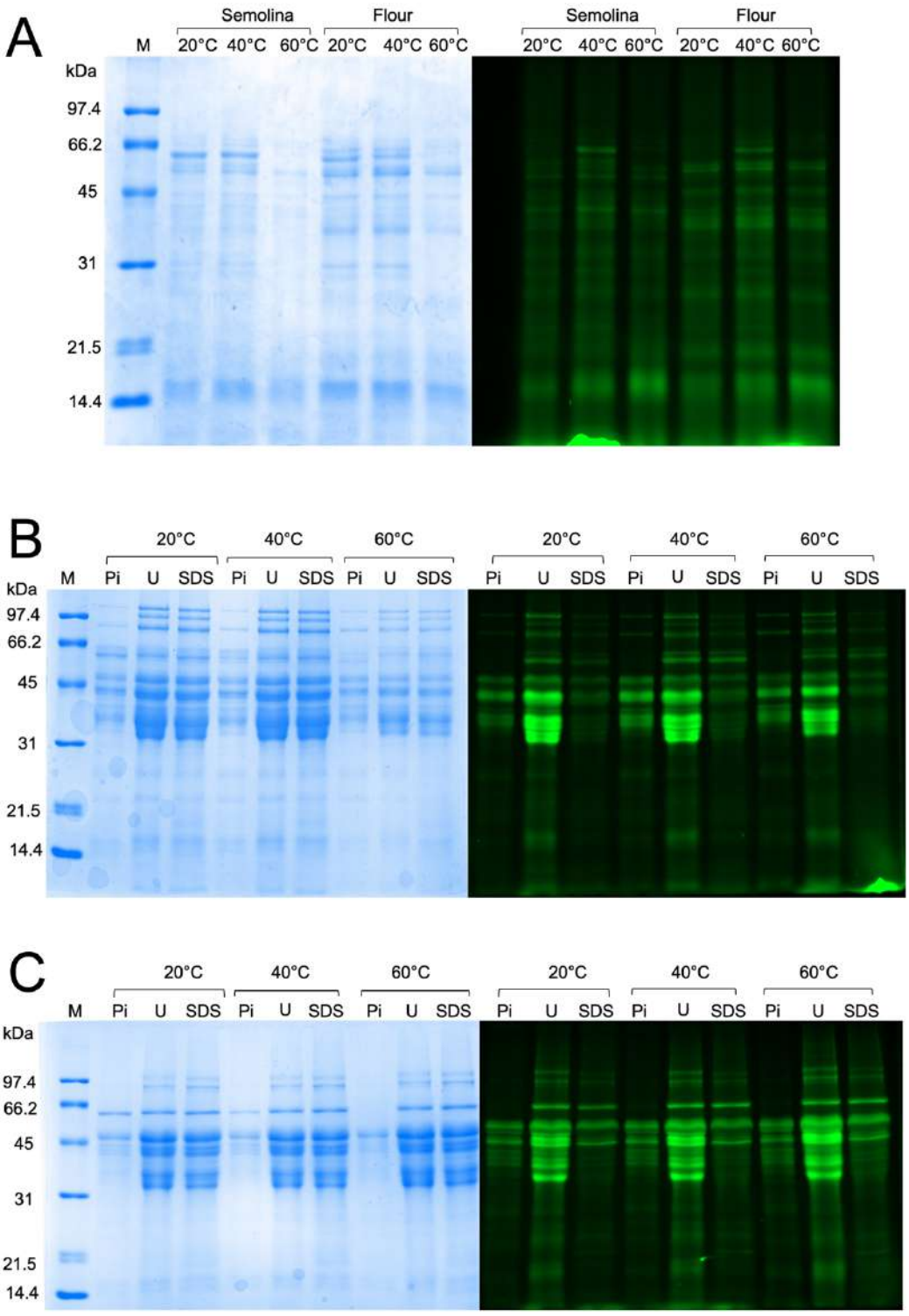

Figure 5. Combined effect of temperature and presence of chaotrope/detergent on solubilizing and destructuring. Panel A) fraction from dealbuminization; Panel B) Flour; Panel C) Semolina.

4.1.5 References

Aguilera, J. M., 2019. The food matrix: implications in processing, nutrition and health. Crit. Rev. Food Sci. Nutr. 59, 3612–3629.

Bartels, D., Altosaar, I., Harberd, N. P., Barker, R. F., Thompson, R. D., 1986. Molecular analysis of γ-gliadin gene families at the complex Gli-1 locus of bread wheat (T. aestivum L.), Theor. Appl. Genet. 72, 845–853.

Belton, P. S., 1999. On the elasticity of wheat gluten. J. Cereal Sci. 29, 103-7.

Bietz, J. A., Simpson, D. G., 1992. Electrophoresis and chromatography of wheat proteins: available methods, and procedures for statistical evaluation of the data. J. Chromatogr. A. 624, 53–80.

Bonomi, F., Ferranti, P., Mamone, G., 2014. Wheat Flour: Chemistry and Biochemistry. In: Bakery Products Science and Technology, Second Edition. John Wiley & Sons, pp. 55–74.

D'Ovidio, R., Masci, S., 2004. The low-molecular-weight glutenin subunits of wheat gluten. J.Cereal Sci. 39, 321-39.

Iametti S, Marengo M, Miriani M, Pagani MA, Marti A, Bonomi F., 2013. Integrating the information from proteomic approaches: A "thiolomics" approach to assess the role of thiols in protein-based networks. Food Res Int. 54, 980-7.

Pelegrine, D. H. G., Gasparetto, C. A., 2005. Whey proteins solubility as function of temperature and pH. WT Food Sci. Technol. 38, 77-80.

Shewry, Peter R., Halford, N. G., Belton, P. S., Tatham, A. S., 2002. The structure and properties of gluten: An elastic protein from wheat grain. Philosophical Transactions of the Royal Society B: Biological Sciences. 357, 133-142.

4.2 CEREALS – WHEAT. Thiolomic: a tool for investigate protein pattern in *Triticum aestivum* flour and a *Triticum durum* semolina

4.2.1 Background

The evolution of the protein structure and formation of inter- and intra-molecular protein network, affects the characteristics of the food products. From this vision comes the necessity to acquire structural information about the protein structure, its evolution and also of the involved proteins. The thiolomic approach can be very useful for ruling out and highlighting differences in apparently similar matrices, such as *Triticum aestivum* flour and a *T. durum* semolina that are epitome of wheat food ingredients. The combination of the thiolomic results and other biochemical parameters (e.g. solubility studies and DTNB titration), can therefore provide detailed information on the whole system. In this paragraph is presented the application of the thiolomic approach to the description of two different wheat matrices such as *Triticum aestivum* flour and a *T. durum* semolina.

4.2.2 Material and methods

4.2.2.1 Materials

Triticum aestivum flour and a *T. durum* semolina was provided by Molino Quaglia, Vighizzolo d'Este, Padova, Italy. Standard gluten (Sigma, G5004) was used as reference.

4.2.2.2 SDS-PAGE

50 mg of sample of flour and semolina and 5 mg of commercial gluten were suspended in 1 mL of 50 mM sodium phosphate, 0.1 M NaCl, pH 7. An aliquot of 50 µL was denatured by diluting 1:1 the sample with a denaturing buffer (0.125 M Tris–HCl, pH 6.8, 50% glycerol (w/v), 1.7% SDS (w/v), 0.01% Bromophenol Blue (w/v)) containing 1% (v/v) 2-ME, when indicated, followed by treatment at 100 °C for 10 min. Electrophoretic runs of the suspension were performed at pH 8.3 (0.025 M Tris–HCl, 0.192 M glycine, 0.1% (w/v) SDS) in a Miniprotein II apparatus (Bio-Rad), and the gels were Coomassie Blue-stained.

4.2.2.3 Thiolomic characterization

The thiolomic characterization was realized as previously described, adapting the protocol in order to include solubility studies and the titration of the accessible thiols. 150 mg of sample were suspended in 3 mL of 50 mM sodium phosphate, 0.1 M NaCl, pH 7.0 and keep in agitation on a shaker for 1 h. The suspension was then centrifuged at 10,000 ×*g* for 10 min at 25 °C in order to separate the supernatant (containing the soluble proteins) and the precipitate. The precipitate phase was resuspended in 3 mL of the same saline buffer, containing when indicated 4 M urea or 2% SDS as reported previously. Once well resuspended, the suspension

was aliquoted in 3 eppendorf tubes with 1 mL. To one of the fraction 50 µL of 5-IAF were added, and leave to stir for 2h in the dark at room temperature. At the end the incubation the excess of probe was blocked by adding 20 µL of 250 mM DTT and leave to stir for another hour in the dark. Finally, the blocked suspension was centrifuged at 5,000 ×*g* for 10 min at 25 °C, collecting the supernatant for the electrophoretic characterizations, as previously described (see the section "4.1.2.2 Thiols covalent fluorescent labeling"). The other two aliquots were used for protein and thiol quantification, as reported below.

4.2.2.4 Protein and thiol quantification

Protein and thiol quantifications were carried out on the same samples used for thiol labelling. Quantification of the solubilized protein in the different condition (i.e. the precipitate from the dealbuminization step resuspended in saline buffer, urea and SDS) was carried out on 1mL of suspension (i.e. one of the three aliquots previously collected), stirred for 2 h (the same time of the fluorescent labeling) and then centrifuged at 10,000 ×*g* for 10 min. The supernatant phase was used for the quantification of the solubilized proteins by using a dye-binding method (Bradford, 1976) using a Perkin-Elmer Lambda 2 UV/VIS Spectrometer (Perkin-Elmer Inc., Waltham, MA, USA). Results are expressed as mean ± standard deviations of three replicates, from two independent measurements.

Accessible thiols were quantified by exploiting the ability of the reagent 5,5-dithio-bis-nitrobenzoate (DTNB) to react with the exposed -SH groups of cysteine, adapting the protocol from Caramanico et al. (2017). To 1 mL of the previous aliquoted suspensions were added 100 µL of 2 mM DTNB (prepared in 50 mM sodium phosphate, 0.1 M NaCl at pH 7.0) and stirred for one hour. At the end the suspension was centrifuged at 13,000 ×*g* for 10 min and the absorbance of the supernatant was read at 412 nm against a DTNB blank using a Perkin-Elmer Lambda 2 UV/VIS Spectrometer (Perkin- Elmer Inc., Waltham, MA, USA). Results are expressed as mean ± standard deviations of three replicates, from two independent measurements.

4.2.3 Results and Discussion

4.2.3.1 Overall protein pattern

The overall protein pattern of the *T. aestivum* flour and the *T. durum* semolina is shown in figure 1**.** The main gluten proteins were identified by comparison with the literature (Dupont at al., 2008; Žilić et al., 2011; Vensel at al., 2014). The glutenin subunits are separated in a molecular weight range from 60 to 100 kDa for the high-molecular weight (HMW-GS) and from 35 to 50 kDa for the low-molecular weight (LMW-GS) (Vensel at al., 2014). Concerning the

gliadin fractions, ω-gliadin are from 45 to 66 kDa, whereas α/β- and γ-glidins from 30 to 45 kDa range (Dupont at al., 2008).

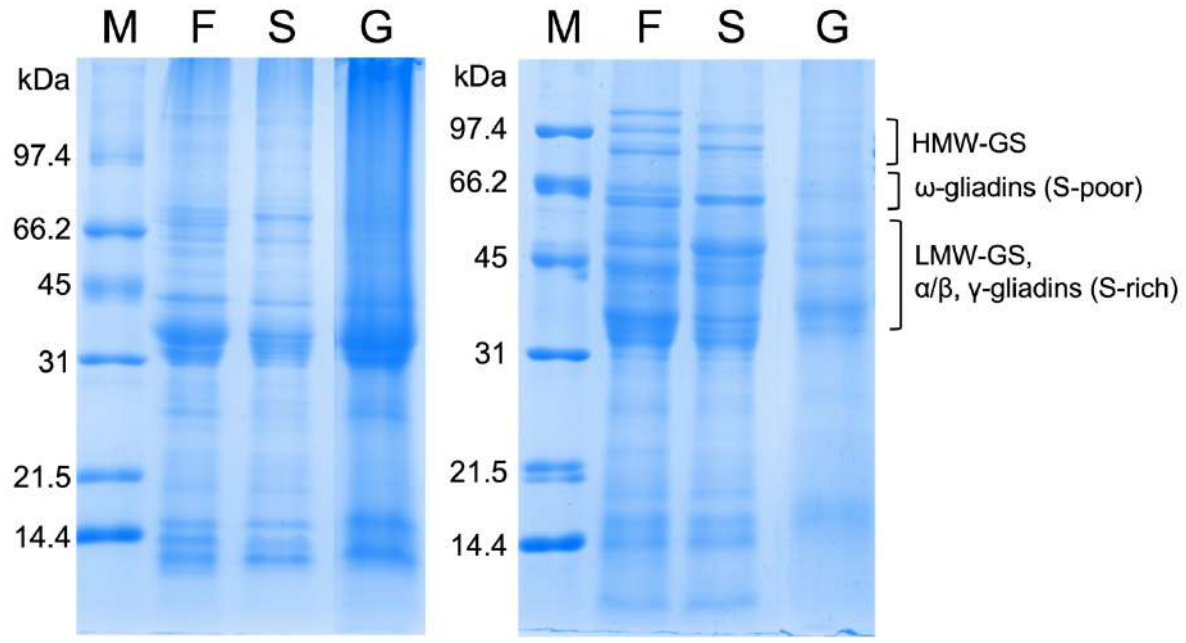

Figure 1. Protein pattern from commercial flour (F) and semolina (S), compared to the Standard gluten (G).

4.2.3.2 Characterization of the proteins by thiolomic approach

The developed thiolomic protocol was applied to a *T. aestivum* flour and the *T. durum* semolina to assess its suitability on these two raw materials (figure 2). Although the differences in the nature of the proteins in these two materials are obvious, the labelling approach shows to be effective to obtain an accurate thiolomic description of the protein fractions (albumins/globulins and gluten).

After the dealbuminization step, when the precipitate is resuspended in the three different condition is possible to observe that for both the matrices the solubilizing performance of urea and SDS are similar (lane 2 and 3), with no major differences observable from the two destructuring conditions. In general is also possible to observe that the *T.aestivum* flour show a less amount of gluten protein compared to *T.durum*, while the amount of albumins and globulins is similar, in agreement with Žilić et al. (2011). Analyzing for first the albumin and globulin fraction (i.e. protein solubilized during the dealbuminization step), is possible to observe a wide molecular weight distributions. As described by Veraverbeke & Delcour (2002) the nongluten proteins are generally monomeric. Their molecular weight is mostly lower than 25 kDa although a significant proportion has a molecular weight between 60 and 70 kDa; the Authors moreover reported that both albumins (mainly β-amylases) and globulins contain

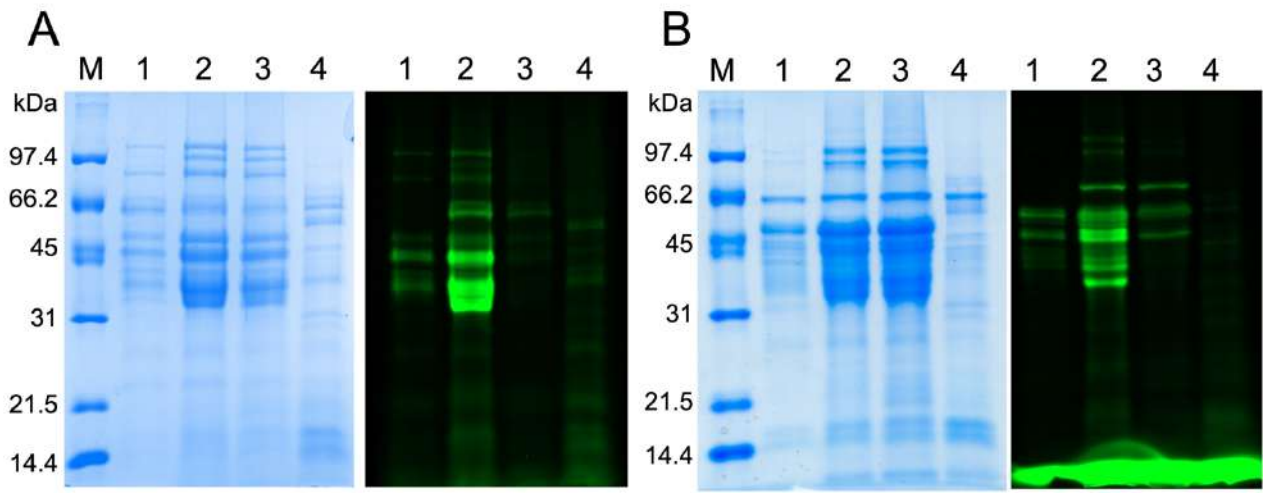

Figure 2. Flour (panel A) and semolina (panel B) proteins labelled with 5-IAF. The protein map is blue-stained on the left, whereas the fluorescent images is green-staines on the right. M) molecular weight markers; 1) “gluten fraction” labelled under non-destructuring conditions; 2) “gluten fraction” labelled in urea 4M; 3) “gluten-fraction” labelled in SDS 2%; 4) “non-gluten fraction” labelled in non-destructuring conditions.

proteins that occur as polymers stabilized by inter-chain disulfide bonds. Although the albumin and globulin fraction mainly represent metabolic (mainly enzymes) or structural proteins, is generally accepted that they are involved with the gluten proteins in the development of the gluten network (Veraverbeke & Delcour, 2002). Concerning the gluten proteins, is possible to observe that by using both 4 M urea and 2% SDS is clearly possible to solubilize the polymeric HMW-GS (molecular weight around 97.4 kDa). In the region of the gliadins (30 – 45 kDa) a clear identification of the solubilized proteins is made particularly tricky by the high amount of solubilized proteins, that results in an overlay of the bands. In non-destructuring condition (lane 1) the amount of solubilized proteins is very low, according to the Osborn’s solubility. Comparing the labeling of flour and semolina, in the region from 97.4 to 66 kDa is possible to observe a very low signal in saline buffer and in SDS, that is higher when urea is used. This structural information is relative to the HMW-GS, and can be explained taking into account the polymeric nature of that proteins. By using urea is possible to modify the tridimensional structure of that proteins, allowing the label of thiols normally inaccessible because buried by the hydrophobic interactions. HMW-GS, that belong to the minor components within the gluten protein family representing around the 10% (Wieser, 2007), play a fundamental role in the determination of the dough properties. For their fundamental contribution to the physical and chemical features of the gluten network, the genes that encode for this fraction have been subject of in-depth studies, representing one of the major focus in wheat bioengineering (O. D. Anderson et al., 2002).

From the thiolomic labeling the gliadins were found to be more reactive toward the 5-IAF than in *T. durum*, likely due to a higher content of free –SH groups. As observed before, urea and SDS show comparable performance in solubilizing the gluten proteins, but the labeling in SDS is lower compared to urea. When 4 M urea is used, is possible to observe a fluorescent signal from all the S-poor and S-rich gluten fraction: this is an evidence of the distribution of -SH (not the disulfide) in the protein structure, that are mainly hidden in the hydrophobic core.

Thanks to the thiolomic approach is possible to compare the different structural organization of the HMW-GS from *T. aestivum* and *T. durum*, with a more intense signal for the first than for the second, indicating a different distribution and accessibility of the thiol residues. Analyzing the region between 66 and 31 kDa, is possible to observe the high amount of thiols from the different proteins. In this region are typically separated the LMW-GS and the gliadins. All of these protein family are usually classified as S-rich (Shewry et al, 1997) and from the fluorescent signal is possible to observe, in each condition an emission of fluorescence.

4.2.3.3 Proteins solubilization and thiols accessibility

To better understand the data from SDS-PAGE, was quantified the amount of solubilized proteins in each condition was quantified, starting from the albumin and globulin, and the amount of proteins solubilized after the resuspension in the different condition (figure 3, panel A). The amount of albumins and globulins solubilized from flour and semolina is comparable, and consistent with the literature (Belderok et al, 2000; Merlino et al, 2009). Urea and SDS are largely used to study the gluten proteins. From our data is possible to observe that 4 M urea show the same solubility performance for both the flour and semolina. When 2% SDS is used however is possible to observe an higher solubility in the case of *T.aestivum* while for *T.durum* the solubility in SDS is not significantly different ($p < 0.05$) to the data obtained in 4 M urea.

Quantification of accessible -SH groups in saline buffer (figure 3, panel B) provided further information on the structural organization and overall compactness of the protein network, as well as on the reactivity of cysteine residues, which are potentially relevant for the formation of intermolecular disulfide bonds (Bresciani et al., 2022). Although their low amount, albumins and globulins (i.e. the soluble fraction) show a relatively high amount of accessible thiols in both the matrices, underlining the importance of removing them to avoid interferences in the labeling of gluten proteins. As regards gluten proteins, the semolina show always an higher content of thiols readily accessible (i.e. in saline buffer) and in destructing conditions (i.e. in urea and SDS). By using SDS interestingly, the amount of thiols accessible in semolina is higher than flour, although a lower solubility.

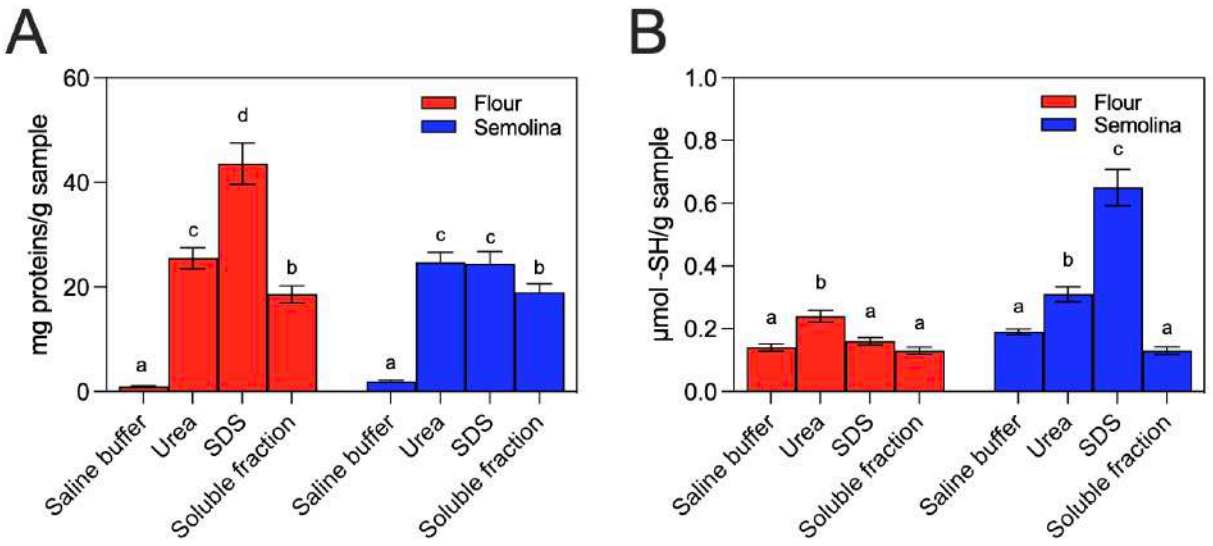

Figure 3. Solubility studies (panel A) and quantification of accessible thiols (panel B) of *T. aestivum* flour and the *T. durum* semolina. Samples with the same superscript letter are not significantly different (Tukey's honestly significant difference test, $p < 0.05$).

4.2.4. Conclusions

The presented results, where a flour and semolina were compared, want to be an example of the potential of the thiolomic approach, combined with more common biochemical approach, to obtain molecular and structural information of proteins from different matrices.

Urea and SDS proven to be very useful in this kind of approach, in particular to realize the obtain structural information about proteins. With our solubility studies we observed a different protein solubility, underlining moreover differences in the amount of thiols in the tested matrices. Combining that results with the thiolomic approach are enhanced information about the protein structure in the different matrices. Though the results from the solubility and thiols titration seem to be in disagreement with the thiolomic results, our hypothesis is that the low fluorescent signal in SDS come from an incompatibility between the anionic detergent and the probe used. The problem could be search in the SDS concentration used: we worked with a concentration 10 times higher to the critical micellar concentration (CMC) of the SDS that is 0.2% at 20°C; by forming SDS micelle the fluorescent labelling is more difficult and the only fluorescent signal is observed in proteins extremely rich in -SH (like the observed gliadins).

One of the great advantage of that combination of approach is the molecular description of matrices that are actually studied mainly from a rheological point of view (e.g. by evaluating the mixing properties). The molecular description of these systems can provide useful information, changing the focus from “the amount” of proteins present in the matrices, to the more complex aspect of the structural properties of these proteins, which are relevant for the subsequent molecular rearrangements that lead to gluten development during transformations and processing.

4.2.5 References

Anderson, O. D., Larka, L., Christoffers, M. J., McCue, K. F., Gustafson, J. P., 2002. Comparison of orthologous and paralogous DNA flanking the wheat high molecular weight glutenin genes: Sequence conservation and divergence, transposon distribution, and matrix-attachment regions. Genome. 45, 367-380.

Belderok B, Mesdag J, Mesdag H, Donner DA., 2000. Bread-making quality of wheat: a century of breeding in Europe. Springer Science & Business Media.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem, 72, 248–254.

Bresciani, A., Emide, D., Saitta, F., Fessas, D., Iametti, S., Barbiroli, A., Marti, A., 2022. Impact of Thermal Treatment on the Starch-Protein Interplay in Red Lentils: Connecting Molecular Features and Rheological Properties. Molecules. 27, 1266.

Caramanico, R., Barbiroli, A., Marengo, M., Fessas, D., Bonomi, F., Lucisano, M., Pagani M. A., Iametti, S., Marti, M., 2017. Interplay between starch and proteins in waxy wheat. J. Cereal Sci. 75, 198-204.

Dupont, F., Samoil, V., Chan, R., 2008. Extraction of up to 95% of Wheat (Triticum aestivum) Flour Protein Using Warm Sodium Dodecyl Sulfate (SDS) without Reduction or Sonication. J. Agric. Food. Chem., 56, 7431-8.

Merlino, M.; Leroy, P.; Chambon, C.; Branlard, G., 2009. Mapping and proteomic analysis of albumin and globulin proteins in hexaploid wheat kernels (Triticum aestivum L.). Theor. Appl. Genet. 18, 1321–1337.

Shewry, P. R., Tatham, A. S., 1997. Disulphide bonds in wheat gluten proteins. J. Cereal Sci. 25, 207–227.

Wieser, H., 2007. Chemistry of gluten proteins. Food Microbiol. 24, 115–119.

Veraverbeke WS, Delcour JA., 2002. Wheat protein composition and properties of wheat glutenin in relation to breadmaking functionality. Crit. Rev. Food Sci. Nutr. 42, 179-208.

Žilić, S., Barac, M., Pešić, M., Dodig, D., Ignjatovic-Micic, D., 2011. Characterization of proteins from grain of different bread and durum wheat genotypes. Int. J. Mol. Sci. 12. 5878-5894.

4.3 CEREALS – WHEAT. Study of the protein structural evolution and protein-protein interactions in a model bread

4.3.1 Background

Bread and baked products, and wheat in general, are worldwide considered as essential in the human nutrition, providing macro- and micro-nutrients, dietary fiber, vitamins, and antioxidants (Kweon, M. et al., 2014). The improvements of the nutritional properties via innovative strategies, approaches, ingredients, and techniques (Cappelli et al., 2020) should meets the expectations of consumers in term of sensory properties and, in general, quality of the products. Studying the evolution of the protein network represents a key point to obtain reliable evidences about the structure/function relationship of proteins and of the whole network, and how it is related to the technological properties of the final products. In this section the thiolomic approach is used to describe the molecular events at the base of the formation of the gluten network and baking process.

4.3.2 Material and Methods

4.3.2.1 Materials

Commercial flour from *Triticum aestivum* was provided by Molino Quaglia, Vighizzolo d'Este, Padova, Italy.

4.3.2.2 Dough production

For the production of dough, 50 g of flour was kneaded in Farinograph-E® (Brabender GmbH & Co. KG, Duisburg, Germany) hydration level (i.e., 60% on dry matter). Sample was taken at different time: before (1 min), at the full development of the gluten network (7 min) and at overmixing (30 min). For each time a new dough was realized. Samples were immediately frozen, lyophilized, and subsequently grinded.

4.3.2.3 Bread making

Bread (figure 4, panel A) was obtained by using a commercial home bread maker machine (SEVERIN, Art. BM3985), following the recipe indicated by the producer, in particular: 500 g flour, 300 g water, 8 g of salt, 8 g of yeast and 3 g of sugar. Bread was cut in portions of around 20 g that were lyophilized and subsequently grinded. An aliquot of dough was collected before the baking step, and lyophilized and grinded prior to perform the analysis.

4.3.2.4 Thiolomic labelling

The thiolomic labelling was realized on 50 mg of samples (flour, dough and bread). In order to perform the dealbuminization, samples were suspended in 1 mL of a 50 mM sodium

phosphate, 0.1 M NaCl, pH 7.0 saline buffer keeping the suspension in the dark in agitation on a shaker for 30 minutes; at the end the suspension was centrifuged at 10,000 ×*g* for 15 min at 25 °C. The precipitate, containing the insoluble proteins, was resuspended in 1 mL of the same saline buffer also in presence of 4 M urea, adding 50 µL of 5 mM 5-(iodoacetamido)fluorescein (5-IAF), keeping the suspension in agitation in the dark for 2 hours. The labelled samples were then centrifuged and the supernatant phase was loaded in SDS-PAGE.

4.3.2.5 Solubility in different media

The solubility of proteins in native and denaturing/reducing conditions was evaluated as reported by Iametti et al. (2006). For these analyses, 150 mg of flour were suspended in 6 mL of saline buffer (50 mM sodium phosphate, 0.1 M NaCl, pH 7.0), containing 4 M urea and 10 mM dithiothreitol (DTT), when required. The suspension was stirred for 90 min at 25 °C and subsequently centrifuged (10,000 ×*g* for 20 min, 25°C). The quantification of proteins in the supernatant was carried out by a dye-binding method (Bradford, 1976) using a Perkin-Elmer Lambda 2 UV/VIS Spectrometer (Perkin-Elmer Inc., Waltham, MA, USA). Results were expressed as mean ± standard deviations of three replicates.

4.3.2.6 Quantification of accessible Thiols

Accessible thiols (-SH) were quantified by exploiting the ability of the reagent 5,5-dithio-bis-nitrobenzoate (DTNB) to react with the exposed -SH groups of cysteine (Ellman, 1959), following a procedure reported in Caramanico et al. (2017). A 50 mg aliquot of flour was suspended in 6 mL of 50 mM sodium phosphate, 0.1 M NaCl, pH 7.0, containing 0.2 mM DTNB, in the presence/absence of 4 M urea. After 1 h at 25 °C, the suspension was centrifuged (13,000 ×*g* for 15 min, 20 °C) to remove solid materials, and the absorbance of the supernatant was read at 412 nm against a DTNB blank, in a Perkin-Elmer Lambda 2 UV/VIS Spectrometer (Perkin-Elmer Inc., Waltham, MA, USA). Results are expressed as mean ± standard deviations of three replicates, from two independent measurements (Bresciani et al., 2022).

4.3.3 Results

4.3.3.1 Structural evolution of proteins: from flour to dough

In figure 1 are reported the overall protein patterns of the samples (panel A) and the farinographic trace (panel B) of the samples. The thiolomic characterization, reported in figure 2, don’t show major differences in the protein separations and in the fluorescent signal, suggesting the absence of differences in the protein “free thiol pattern” among different time of kneading.

Differential solubility (figure 3, panel A) and quantification of accessible thiols (figure 3, panel B), where used to deepen structural information. Differential solubility results (panel A) don't show significant differences ($p < 0.05$) at the different kneading times in all the tested conditions (saline buffer, in presence of 4 M urea and 10 mM DTT). Results reported in figure 3, panel A are in agreement with the expected behavior of the flour: a low solubility in saline buffer (where are soluble albumins and globulins) that increase with the addition of the chaotrope and of the reductant (that allow the solubilization of all the other proteins/aggregates). The results obtained in urea does not change because only albumins and globulins are soluble in phosphate, regardless of whether gluten is formed or not. Moreover, the absence of difference in urea also suggest that disulfide bridges are of little relevance to the network.

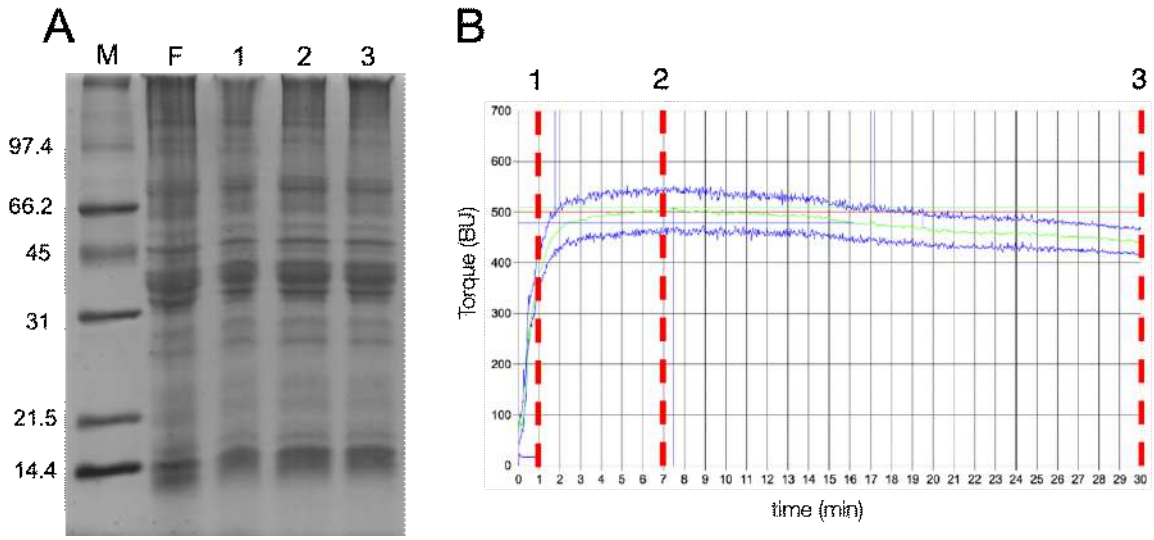

Figure 1. Panel A, SDS-PAGE in reducing condition of flour (F) and dough realized in a farinograph taking sample before (1), at the full development of the gluten network (2) and in overmixing (3). Panel B, farinographic trace of the flour; red lines and numbers represent the kneading time selected for the electrophoretic characterization

The amount of accessible thiols in native condition (saline buffer) (figure 3, panel B) is not significantly different ($p < 0.05$) at each kneading times. In presence of urea however we can observed some differences: starting from the flour, the lower thiols accessibility is observed at the full development of the gluten network (7 min), while before (1 min) and in overmixing (30 min) the accessibility is the higher.

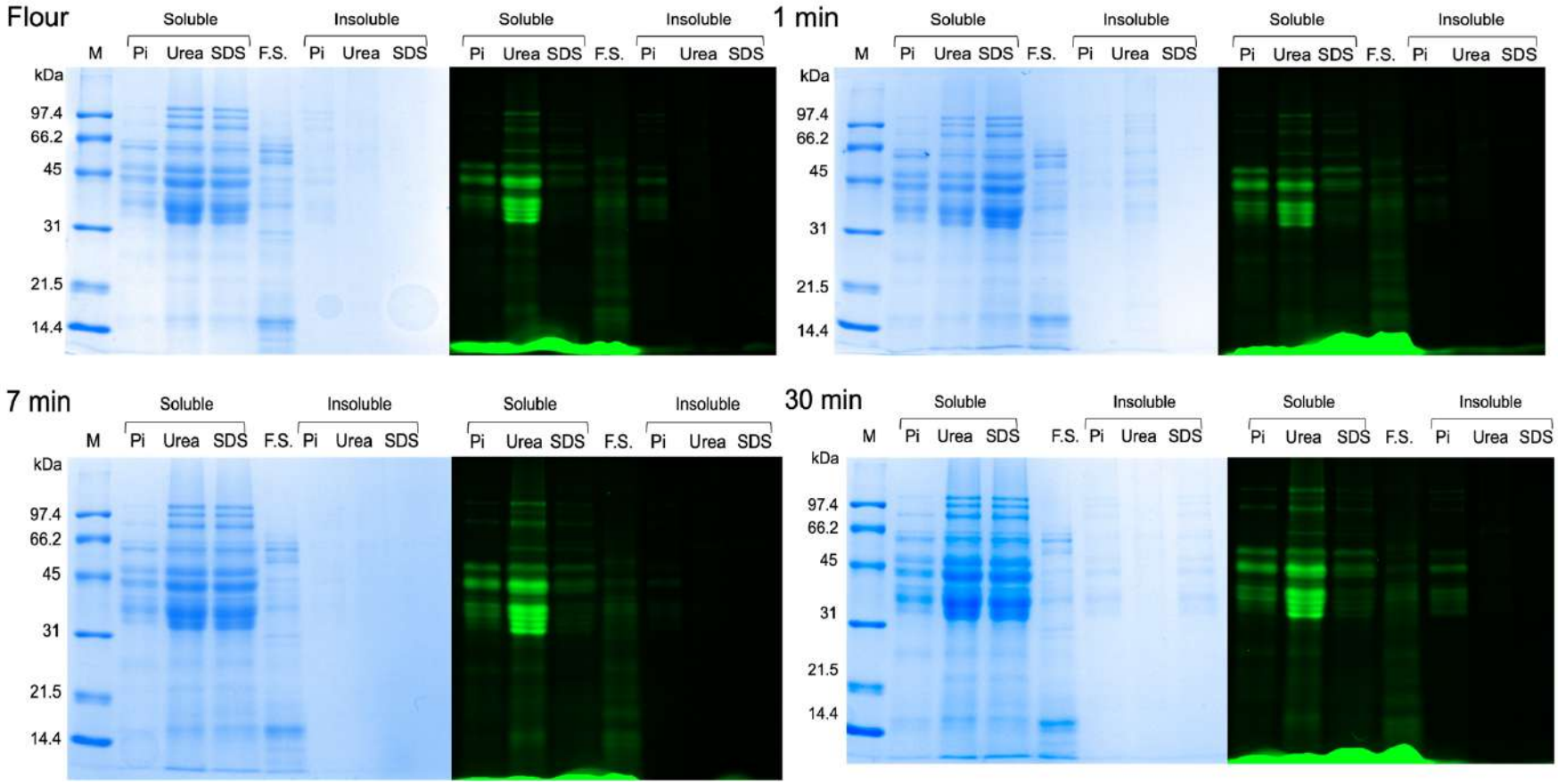

Figure 2. Thiolomic results of flour and sample taken at the different kneading, with results of the labelling of the soluble (Soluble) and insoluble (Insoluble) proteins in reducing conditions; Pi) precipitate after dealbuminization resuspended and labeled in native condition (saline buffer), Urea) precipitate after dealbuminization resuspended in urea, SDS) precipitate after dealbuminization resuspended in SDS, F.S.) soluble fraction obtained from the dealbuminization step.

Taken together, these results suggest that at the full development (i.e., 7 min kneading) gluten has the highest protein compactness and is stabilized mainly via hydrophobic interaction.

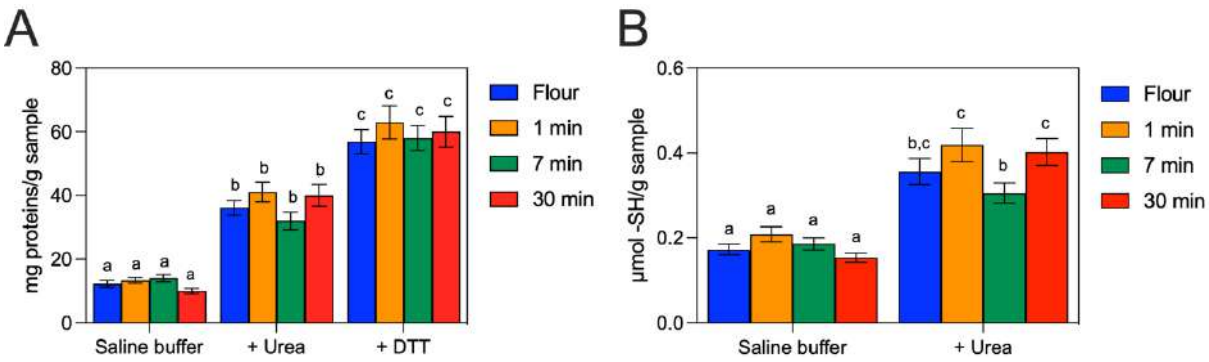

Figure 3. Differential solubility (panel A) and DTNB titration (panel B) of the samples made with the farinograph. Samples with the same superscript letter are not significantly different (Tukey's honestly significant difference test, $p < 0.05$).

4.3.3.2 Structural evolution of proteins: from dough to bread

After the formation of the gluten network with the addition of water and the kneading step, a baking phase represent the crucial step in the production of bread. Bread was prepared by using a home bread maker machine. In literature, the effect of high temperature on proteins are describe as a combination as a combination of drastic changes in the disulfide/sulfhydryl structure of gluten, and in the pattern of non-covalent hydrophobic interactions among gluten proteins (Alamprese et al., 2005). During baking, temperature plays a crucial role in the stabilization of the structure. The thiolomic approach applied on the starting flour, the dough and bred, reported in figure 4 (panel B), shows some differences of thiol accessibility, in particular in the gliadinic fraction (45 to 66.2 kDa). In detail, a band at about 66.2 kDa looks to be less fluorescent in the bread compared to the flour and dough, whereas a bands around 45 kDa always in bread shows higher fluorescent intensity. These results suggest that during the formation of the dough, as observed before, are occurring changes in the protein structure that lead to gluten formation mainly via hydrophobic interaction. The effect of high temperature typical of the baking step stabilizes the new protein organization, triggering the formation of interprotein disulfide bound in the network.

Differential solubility (figure 5, panel A) confirm that electrostatic and hydrophobic interactions mainly occurs in the dough, representing the main protein-protein interactions in that system (also in the flour). The effect of the thermal treatment of baking process however deeply affect the protein-protein interactions of the network, causing structural rearrangements

resulting in a strong influence of the covalent interactions in the stabilization of the aggregates. To support that evidence, when the accessible thiols are titrated with the DTNB (figure 5, panel B), is possible to observe significant differences in the saline buffer, while using 4 M urea the bread show the lowest amount of accessible thiols and the flour and dough are not significantly different ($p < 0.05$).

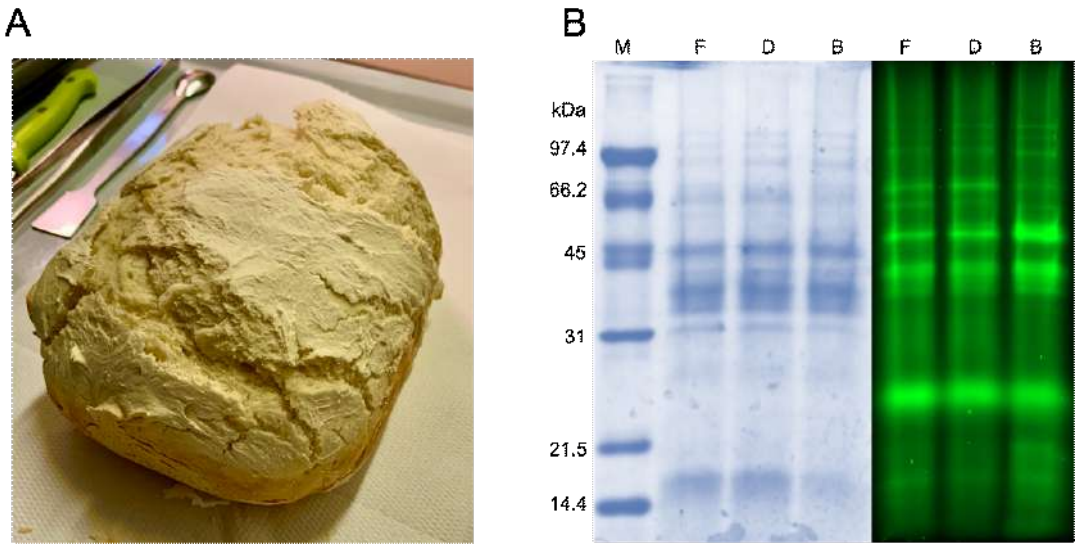

Figure 4. Bread realized (panel A) and electrophoretic characterization (panel B) of the raw materials (Flour, F), the dough (D) and of the bread (B)

Overall, these results show that the cooking process plays a fundamental role in the formation of intermolecular disulfide bridges, determining the characteristics of the protein network of the bread.

4.3.4 Conclusions

Common characterizations based on protein quantification can only provide limited information about the entire gluten system. The evolution of the protein structure and formation of intermolecular interactions drive the change from storage proteins to gluten, and play a key role for the characteristics – and quality – of the products. From this vision comes the necessity of a method suitable to perform three-dimensional investigation about the protein structure in complex systems, in order to investigate and understanding the structure-function relationship in food systems. A central point of the work is the molecular description of the cereal systems: the evolution of the characteristics of the matrix from the dry state of the flour/semolina until the final product (a model bread). The main concept stressed out is that the protein content is not the only important parameter to evaluate the features of protein network. Therefore, for a deep knowledge of the protein network and its impact on the characteristics of finished

products, it is of paramount importance the study at molecular of level the interactions among proteins and the other components of the matrices (e.g. starch, lipids, etc.).

From the presented results we were able to highlight (even if not clearly identify with the presented approach) the proteins that are directly involved in the covalent stabilization of gluten, highlighting the structural evolution that result in changes of the number and accessibility of SH groups. Another evidence of our work is the limited relevance on the disulfide bridges in the formation of the gluten network of the dough: in this system the thiolomic results did not show major differences, while some significant differences can be observed with the DTNB.

With our results we show the entity of the protein structural evolution at the base of the formation and stabilization of the gluten network. Future study will aimed to clarify whether the inter-chain disulfide bridges are formed by disulfide exchange reactions (and therefore the total number of -SH should not change), or if they are newly formed disulfide bridges.

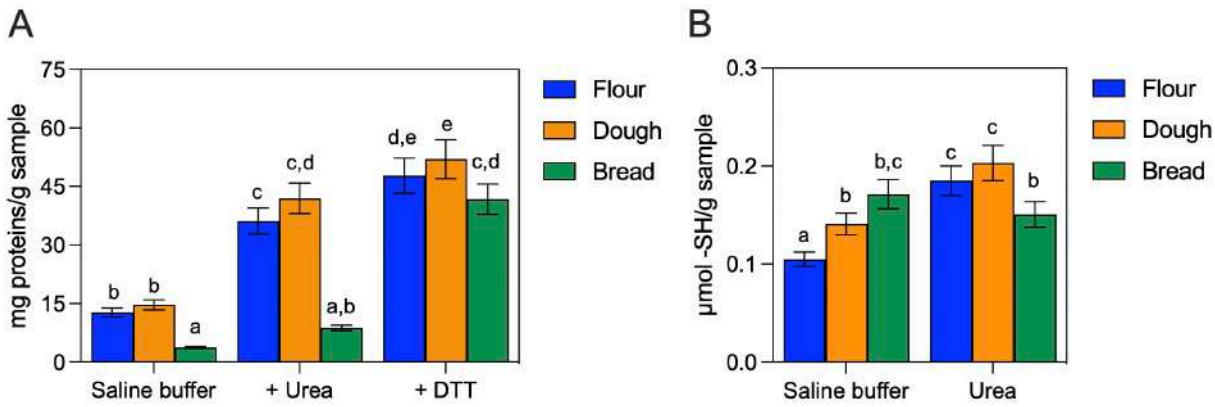

Figure 5. Differential solubility (panel A) and DTNB titration (panel B) of flour, dough and bread. Samples with the same superscript letter are not significantly different (Tukey's honestly significant difference test, $p < 0.05$).

4.3.5 References

Alamprese, C., Iametti, S., Rossi, M., Bergonzi, D., 2005. Role of pasteurisation heat treatments on rheological and protein structural characteristics of fresh egg pasta. Eur. Food Res. Technol. 22, 759-67.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem, 72, 248–254.

Bresciani, A., Emide, D., Saitta, F., Fessas, D., Iametti, S., Barbiroli, A., Marti, A., 2022. Impact of Thermal Treatment on the Starch-Protein Interplay in Red Lentils: Connecting Molecular Features and Rheological Properties. Molecules. 27, 1266.

Cappelli A., Bettaccini L., Cini E., 2020. The kneading process: A systematic review of the effects on dough rheology and resulting bread characteristics, including improvement strategies. Trends Food Sci. Technol. 104, 91-101.

Caramanico, R., Barbiroli, A., Marengo, M., Fessas, D., Bonomi, F., Lucisano, M., Pagani M. A., Iametti, S., Marti, M., 2017. Interplay between starch and proteins in waxy wheat. J Cereal Sci. 75, 198-204.

Ellman, G. L., 1959. Tissue sulfhydryl Groups. Arch Biochem Biophys. 70–77.

Emide D., Bonomi F., Iametti S., Barbiroli A.; From proteins to bread: novel tools for the molecular description of protein-protein interactions (and their evolution) in baked products.

Iametti, S., Bonomi, F., Pagani, M.A., Zardi, M., Cecchini, C., D'Egidio, M.G., 2006. Properties of the protein and carbohydrate fractions in immature wheat kernels. J. Agric. Food. Chem. 54, 10239–10244.

Kweon, M., Slade, L., Levine, H., Gannon, D., 2014. Cookie-versus cracker-baking—what's the difference? Flour functionality requirements explored by src and alveography. Crit. Rev. Food Sci. Nutr. 54, 115-3.

4.4 CEREALS – WHEAT. Molecular insights into the role of amylose/amylopectin ratio on gluten protein organization

The results presented here below are submitted as: ***Emide, D****, Magni, C, Saitta, F, Cardone, C, Botticella, E, Fessas, D., Iametti, S., Lafiandra, D., Sestili, F., Marti, A., Barbiroli, A., Molecular insights into the role of amylose/amylopectin ratio on gluten protein organization.*

4.4.1 Abstract

Waxy (WX) and high-amylose (HA) wheat flours have interesting functional and/or nutritional characteristics, but low technological properties compared to regular wheat. Here a set of three wheat lines, having different amylose content but sharing the same varietal background, were compared to shed light on the role of amylose/amylopectin ratio on the protein conformational changes that lead to gluten formation. Despite the absence of difference in their protein profile, as confirmed by thiolomic approaches, the WX and the HA lines developed weaker gluten than the control sample. The altered amylose/amylopectin ratio exert a matrix effect establishing a competition for water with proteins, leading to a different protein structure and three-dimensional organization of the gluten network. These results add a piece to the understanding of the molecular aspects that oversee matrix effects on gluten formation in wheat, which description can be helpful for a rational optimization of the transformation process.

4.4.2 Introduction

In wheat, the various grain components cooperate to determine the quality and nutritional value of the flour and of derived food products. Protein and starch represent the main constituents, accounting for about 12 and 70% of the total dry weight of the grain, respectively, with lipids, non-starch polysaccharides, minerals and vitamins being minor components.

The protein fraction largely contributes to the rheological and technological quality of wheat dough. In particular, dough strength and the bread-making potential of bread wheat varieties are influenced by both the quality and quantity of gluten, and especially by the composition and abundance of different high molecular weight glutenin subunits (Shewry and Halford, 2002). Extensive research has been devoted to clarifying the contribution of individual glutenin subunits to the gluten network, by investigating the relationships between their structural features and inter/intra subunits interactions (Lucas et al., 2018; He et al., 2013; Wieser, 2007). However, there is increasing evidence that gluten properties also depend on additional intermolecular relationships, that involve other endosperm components, such as starch, lipids, non-gluten proteins (e.g. puroindolines), and non-starch polysaccharides (Quayson et al., 2018; 2016; Pareyt et al., 2011).

Starch, contributing for more than a half to the total dry matter in the endosperm, affects several flour and food product properties, both from a technological/rheological and a nutritional point of view. As for this latter, the rate of starch digestibility control food glycemic index, a measure of blood glucose levels after a meal consumption, with high glycemic index value supposed to be a main factor in the insurgence of serious metabolic disorders and non-communicable diseases (Botticella et al., 2021; Birt et al., 2013). Explicitly, starch digestibility measures its susceptibility to the hydrolysis by amylases and depends on starch chemical structure and on intra- and inter-molecular interactions with other flour components. Starch is a mixture of two glucose polymers: amylose and amylopectin; usually in a 1:3 ratio in common wheat. Amylose and amylopectin contain linear α-1,4 and branching α-1,6 glycosidic bonds, the latter seldom found in amylose but abundant in amylopectin. Modern breeding strategies allow the modulation of amylose/amylopectin ratio in wheat and other cereals providing an amylose gradient from zero to almost 100% in a pool of genotypes (Botticella et al., 2021), thus providing new genetic resources to explore novel functionalities of agrifood interest. Moreover, targeting a few key enzymes catalyzing starch biosynthesis, by silencing the respective genes, allows to produce low-amylose waxy (Granule Bound Starch Synthase I, GBSSI-deficient), intermediate high-amylose (Starch Synthase IIa, SSIIa-deficient), and high-amylose (Starch Branching Enzyme IIa, SBEIIa-deficient) wheat lines in the same varietal backgrounds (Botticella et al., 2018). The creation of high amylose genotypes shed light on a different starch fraction, named “resistant starch”, generally negligible in common wheat but much increased in high-amylose lines. “Resistant starch” is held responsible for lower food glycemic load and promoting beneficial colon fermentation (Botticella et al., 2021). Conversely, low amylose lines correlate with higher starch digestibility, best suited for some food categories (baby foods, baked products) and proven to improve some sensory qualities of such products (Guo et al., 2003; Bhattacharya et al., 2002).

Altered amylose/amylopectin ratio affects granule size and shape as well as the network of interactions in wheat endosperm (Labuschagne et al., 2007). Moreover, wheat lines with altered amylose to amylopectin ratio showed different performances in food processing. Lower amylose contents in waxy lines corresponds to higher peak pasting viscosity, lower peak viscosity temperature and higher resistance to retrogradation than conventional wheat. In contrast, high-amylose flour has significantly higher peak viscosity temperature than waxy and conventional wheats, and also shows a significantly lower peak viscosity (Hung et al., 2006). Doughs made from either waxy and high-amylose wheat flours show higher water absorption than conventional wheat flour, along with lower stability during mixing (Morita et al., 2002) and lower gluten strength (Graybosch et al., 2003). In bread-making, waxy and high amylose flours could give larger and smaller loaf volume - respectively - than bread from conventional wheat, and are not accepted by consumers. On the other hand, incorporation of

waxy starch results in a delay in staling, extending shelf-life of breads (Morita et al., 2002), whereas substitution up to 50% of normal wheat flour with high-amylose wheat flour significantly increases the amount of resistant starch in the final product, thus increasing its nutritional value (Hung et al., 2006).

The potential of wheat genotypes with novel added value in health and nutrition deserves the attention of food technologists called to optimize the process based upon their different properties. A few data on these materials showed that gluten properties are pleiotropically affected by the presence of altered starch composition (De Arcangelis et al 2021), but the reasons behind their poor technological properties are still debated.

Here we present a study where a set of bread wheat genotypes with a gradient of amylose from 0 to 79% (Botticella et al., 2018) are compared to shed light on the role of amylose/amylopectin ratio in the protein conformational changes that govern the protein-protein interactions leading to gluten formation. The investigation has been carried out at different levels: i) the protein pattern of the selected lines was assessed, to rule out possible differences in protein expression; ii) the structural feature and aggregation state of proteins present in the various flours were characterized to highlight possible differences; iii) protein conformational changes occurring during mixing were studied at various hydration levels, addressing their different rheological behaviors in view of a possible competition for water between proteins and starch due to a different amylose/amylopectin ratio among the starch mutants.

4.4.3 Materials and methods

4.4.3.1 Materials

Wheat lines with a different amylose/amylopectin ratio were produced by altering the activity of key starch synthesis enzymes in the common wheat variety Cadenza (Botticella et al., 2018).

The wild type line (Cadenza WT, total starch 57%, amylose 34%), the GBSSI-deficient waxy line (WX, total starch 59%, amylose n.d.), and the SBEIIa deficient high-amylose line (HA, total starch 58%, amylose 79%) were grown in the season 2019-2020 under comparable conditions. In detail the three genotypes were grown in open field at the Experimental Farm “Nello Lupori” (University of Tuscia) located in Viterbo, Italy (lat. 42◦26’ N, long. 12◦04′ E, altitude 310 m a.s.l.). Crop management was performed using standard cultivation methods.

Flour production. Kernels (2 kg) were conditioned (16 h at 20 °C) till reaching 16% moisture, and then milled into flour using a roller mill (RM1300, Erkaya, Ankara, Turkey), equipped with a 250 μm sieve. The protein content of the obtained flours was: WT 10.1%, WX 10.4%, HA 12.0% (AOAC 990.03).

4.4.3.2 Electrophoresis and thiolomics

SDS-PAGE. Ten milligrams of flours were suspended in a mixture made up of 250 µL of 50 mM sodium phosphate, 0.1 M NaCl, pH 7.0, and of 250 µL of denaturing buffer (0.125 M Tris-HCl, pH 6.8, 50% glycerol, 1.7% SDS, 0.01% bromophenol blue (w/v)), containing 1% (v/v) 2-mercaptoethanol (2-ME) when indicated. The suspensions were then heated at 100 °C for 10 min. After centrifugation (10,000 ×*g* for 5 min, 25°C), 20 µL of the clear solutions were loaded into the gel. The electrophoretic run was performed in a Miniprotean II cell (Bio-Rad Laboratories, Hercules, CA, USA), running buffer 0.025 M Tris-HCl, 0.192 M glycine, 0.1% (w/v) SDS. The gels were Coomassie Blue-stained.

Gliadins and glutenins fractionation. Flour proteins (albumins, globulins, gliadins and glutenins) were fractionated by sequential extraction. Albumins and globulins were extracted by suspending 150 mg of sample in 3 mL of 50 mM sodium phosphate, 0.5 M NaCl, pH 7.5. After 1h incubation at 4°C under mild agitation, the suspension was centrifuged at 10,000 ×*g* for 15 min. Albumins and globulins were recovered in the supernatant. The pellet was resuspended in 2.5 mL of 70% ethanol for gliadins extraction. After 1h incubation at 4°C under mild agitation, the alcoholic suspension was centrifuged at 10,000 ×*g* for 15 min. Gliadins solubilized in the supernatant were precipitated over-night by using 4 volumes of cold acetone (-20 °C). Precipitate gliadins, after evaporation of the solvent, were solubilized in 700 µL of 50 mM sodium phosphate, 0.5 M NaCl, pH 7.5, in the presence of 4 M urea. Glutenins, contained in the last pellet, were resuspended in 2 mL of 50 mM sodium phosphate, 0.5 M NaCl, 4M urea, pH 7.5.

Thiols labeling. Fluorescent labelling of protein thiols was adapted from Iametti et al. (2013). Gliadin solutions and glutenin suspensions (500 µL) were mixed with 25 µL of 5 mM 5-(iodoacetamido)fluorescein (5-IAF) and incubated for 2h under shaking in the dark. At the end of the incubation, 3.9 mg of 1,4-dithiothreitol (DTT) (final concentration 50 mM) was directly added to the protein/probe mixture to quench the probe excess. The presence of DTT also allows the solubilization of glutenins. After 1h of incubation in the dark, the mixture was centrifuged at 13,000 ×*g* for 10 min and the clear supernatants (i.e. gliadin and glutenin solutions) were collected for 2D-PAGE characterization.

2D-PAGE. For the 2D-PAGE characterization, samples were prepared by diluting aliquots of the gliadin (45 µL) or of the glutenin (30 µL) solutions to a final volume of 120 µL with rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS (w/v), 65 mM DTT, 2% IPG buffer pH 3–10, and 0.002% Bromophenol Blue (w/v)). The sample was then loaded on 7 cm IPG strips (GE Healthcare) with a linear 3–10 pH gradient. Strips were focused at 11,000 V/h, with a

maximum of 3,000 V, at 20 °C using the Ettan IPGphor II apparatus (GE Healthcare). Prior to the second dimension, strips were incubated in equilibration buffer (0.375 M Tris–HCl, pH 8.8, 6 M urea, 2% SDS, and 20% glycerol) containing 65 mM DTT for 15 min. Then, strips were incubated with the equilibration buffer containing 243 mM iodoacetamide for 10 min. The separation was performed in a 12% polyacrylamide SDS-PAGE gel in a Miniprotean II cell (Bio-Rad Laboratories, Hercules, CA, USA). Fluorescence patterns of 2D-PAGE gels were acquired by using a VersaDoc™ Image Analysis System (Bio-Rad) and analyzed through the ImageLab software Version 6.0.1 (Bio-Rad). Gels analyzed for fluorescent spots were subsequently Coomassie Blue-stained and another round of image analysis was carried out with the same instrument and software.

4.4.3.3 Protein structural organization

The ***differential solubility*** of proteins in native and denaturing/reducing conditions was evaluated as reported by Bonomi et al. (2012). 150 mg of flour were suspended in 6 ml of saline buffer (50 mM sodium phosphate, 0.1 M NaCl, pH 7.0), with sequential addition of 4 M urea and 10 mM dithiothreitol (DTT). The suspension was stirred for 90 min at 25°C and subsequently centrifuged (10,000 ×*g* for 20 min, 25°C). The quantification of proteins in the supernatant was carried out by a dye-binding method (Bradford, 1976). Results were expressed as mean ± standard deviation of three replicates.

Thiols accessibility to the specific reagent 5,5-dithio-bis-nitrobenzoate (DTNB) was used to investigate the compactness of aggregates, essentially as reported in Caramanico et al. (2017). 250 mg of flour were suspended in 5 mL of 50 mM sodium phosphate, 0.1 M NaCl, pH 7.0, containing 0.2 mM DTNB. After 1 h at 25°C, the suspension was centrifuged (13,000 ×*g* for 15 min, 20°C) to remove solids, and the absorbance of the supernatant was read at 412 nm against a DTNB blank. Results are expressed as mean ± standard deviation of three replicates.

4.4.3.4 Spectrofluorimetry

Intrinsic (tryptophan) fluorescence was assessed by front-face (solid state) measurements, as reported by Bonomi et al. (2004). Solvation studies were performed by adding enough water to 1 g of sample to reach final moisture levels between 10% (no addition) and 50%. The mixture was kneaded for 4 min by using a glass stirring rod, and then loaded in the front-face fluorescence cell. Tryptophan fluorescence emission was monitored from 300 to 420 nm (emission slit 2.5 nm), with excitation at 280 nm (excitation slit 2.5 nm), and scan speed 50 nm/min. All the measures were conducted at 25°C in a LS50 B Perkin-Elmer Luminescence Spectrometer (Perkin-Elmer Co., Waltham, MA, USA).

Protein surface hydrophobicity was determined by using 1,8-anilino-naphthalene-sulfonate (ANS) (Bonomi et al., 2004). Enough water was added to 1 g of sample to reach 40% final moisture. Part of the added water was replaced with an ANS stock solution (5 mM in water) to give a final ANS concentration in the resulting dough ranging from 0 to 1 mM. Fluorescence intensity was monitored at 460 nm (emission slit 2.5 nm), with excitation at 390 nm (excitation slit 2.5 nm). All the measurements were carried out at 25°C in a LS50 B Perkin-Elmer Luminescence Spectrometer (Perkin-Elmer Co., Waltham, MA, USA).

4.4.3.5 Thermal properties

Starch gelatinization properties were analyzed through a PerkinElmer DSC6 (PerkinElmer, Waltham, MA, USA) working with stainless steel sealed pans throughout a temperature range from 10°C to 150°C at a scanning rate of 2°C/min. An empty pan was used as reference and calibration was carried out with indium as standard. Samples were prepared by directly adding an adequate amount of water within the pans containing about 15 mg of sample to achieve overall moistures of 50% and 70%.

Data were analyzed with the dedicated software IFESTOS following procedures reported in previous studies (Bresciani et al., 2022a; Bresciani et al., 2022b). In brief, the excess heat capacity $C_P^{exc}(T)$ ($J \cdot K^{-1} \cdot g^{-1}_{dry\ matter}$), *i.e.*, the difference between the apparent heat capacity $C_P(T)$ of the sample and the heat capacity of the pre-transition region, was recorded across the scanned temperature range. Three replicates were performed for each sample and one representative curve for each sample was reported.

4.4.3.6 Thermogravimetric analysis

The water phase separation in doughs prepared from the different flours was analysed by thermogravimetric analysis (TGA). A Setaram TG-DSC111 (Lyon, France) was used to simultaneously record the thermal effect (heat flow versus temperature) and the TG trace (mass loss versus temperature). The typical sample mass was about 30 mg of dough at 40% overall moisture. Scans were performed from 20°C to 200°C at 2° $C \cdot min^{-1}$ scanning rate. Each run was repeated twice. The ratio between the heat flux and the related mass loss rate was found to be equal to the enthalpy of water evaporation. This check confirmed that the mass loss was substantially related to water evaporation only. All the TG traces were normalized to 100 mg of mass sample. Accordingly, the temperature derivative (DTG, Derivative thermogravimetric) traces were expressed as mg of lost mass per K degree (with reference to the scanning rate used).

4.4.3.7 Empiric rheology

Gluten aggregation properties were measured by means of the GlutoPeak (Brabender GmbH and Co KG, Duisburg, Germany) device, by using 9 g of flour and 10 g of water, 2,750 rpm as paddle speed rate, and 35 °C as temperature of water circulating bath (Pagani et al., 2020). Mixing properties were evaluated by using the Farinograph-E (Brabender GmbH and Co KG, Duisburg, Germany) device equipped with a 50 g mixing bowl, according to the ICC standard method 115/1 (ICC, 1992).

4.4.3.8 Statistical Analysis

One-way ANOVA ($p < 0.05$) were performed by using SigmaPlot version 14.0 (Systat Software Inc., San Jose, CA, USA). When a factor resulted significantly different, the difference was determined through the Tukey HSD test.

4.4.4 Results

4.4.4.1 Wild type, waxy and high amylose Cadenza lines share the same protein pattern

Waxy and high amylose Cadenza lines were produced inhibiting the activity of key enzymes of starch biosynthesis (GBSSI and SBEIIa, respectively) (Botticella et al., 2018). Although the genetically modified lines are expected to share the same protein pattern with the wild type variety Cadenza - given their genetic similarity - differences cannot be excluded as a consequence of pleiotropic effects. In order to highlight differences or confirm similarities, the protein pattern of the three lines were characterized by electrophoretic approaches.

The SDS-PAGE, performed on the “whole” sample, showed that the protein profile of the two modified samples were almost identical to control (cv. Cadenza) (figure S1). This similarity is evident regardless the use of reducing conditions (suitable to compare the nature and relative amount of subunits), or of non-reducing conditions (suitable to highlight the presence of disulfide cross-links).

The gliadin and glutenin fractions were isolated and investigated by 2D-PAGE separation coupled with a thiolomic approach based on the labeling of cysteinic thiols with the fluorescent probe 5-IAF. Free thiols (i.e., thiols not involved in disulfide bound) play an essential role on the overall behavior of flours during the formation of the dough (Shewry, 1997). Their accessibility and distribution across glutenins and gliadins are strictly dependent on the structural and supramolecular organization of proteins. Coomassie Blue-stained 2D-PAGE and fluorescent maps of gliadins and glutenins extracted from the three lines are shown in figure 1. As expected, spots corresponding to monomeric gliadins are visible on the gel between 35 and 50 kDa, with isoelectric points (pI) ranging from 6.5 to 8 (figure 1A). Glutenin subunits range from 60 and 100 kDa for the high-molecular weight (HMW-GS) and from 35 to

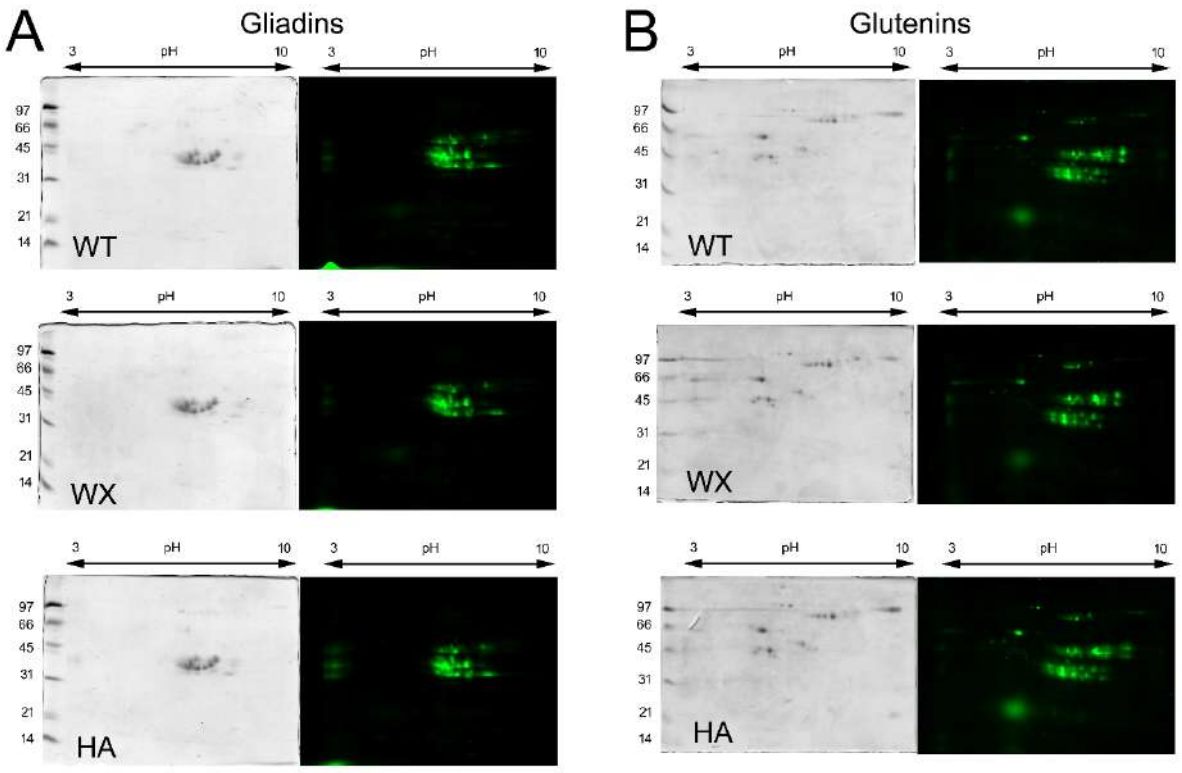

Figure 1. A thiolomic analysis of gliadins (A) and glutenins (B) purified from wild type (WT), waxy (WX) and high amylose (HA) Cadenza lines. For each sample, the 2D-PAGE Coomassie Blue stained maps (dark spots on clear background) are matched with the corresponding fluorescence map (green spots on dark background), originating from labeling of cysteine thiols by 5-IAF.

50 kDa for the low-molecular weight (LMW-GS) subunits respectively, both with a broader range of isoelectric points compared to glutenins (Vensel et al., 2014) (figure 1B). The thiolomic approach confirms that gliadins and LMW-GS are rich in free thiols, as opposed to HMW-GS. In particular, LMW-GS spots show remarkable fluorescent signal in spite of their overall low abundance in the gel. The comparison of the 2D-PAGE images confirms the absence of differences among the samples, for both gliadins and glutenins. Moreover, the almost complete overlap of the fluorescent maps suggest that free thiols have the same distribution across gliadin and glutenin subunits in all the three samples. The image analysis also suggests the absence of quantitative differences in the gliadin/glutenin ratio among the control and the two mutant lines. In fact, both the Coomassie Blue-stained and fluorescent thiol maps of gliadins and glutenins show almost identical intensities regardless of the sample from which they were produced (figure 1).

Taken together, these results showed that the protein patterns of the two mutant lines were identical to those of the control variety. No major differences were found either in the

distribution of protein thiols. This evidence is a prerequisite to address the role of (i) differences in the structural organization and (ii) competition for water played between proteins and starch, in gluten formation.

4.4.4.2 Proteins in wild type, waxy and high amylose Cadenza lines show different accessibility

More insights on the aggregative state of storage proteins can be obtained by differential solubility approaches, that rely on the ability of dissociating and reducing agents to sequentially solubilize the proteins involved in aggregates stabilized by either non-covalent (mainly hydrophobic) or covalent (disulfide bridges) interactions. The protein solubility, regardless of the absence/presence of chaotropes and disulfide reductants, increased in the order: WT<WX<HA (figure 2A).

Considering that the three lines have almost identical protein patterns, the difference in protein solubility could be attributed to differences in protein organization in terms of protein aggregate compactness: the higher the structural compactness, the lower the accessibility to the chaotrope or to the reductant, and consequently the solubility. Differences in the accessibility were confirmed also by the assessment of thiols accessible under non-destructuring conditions (figure 2B). The number of readily accessible thiols was significantly higher in the HA sample, indicating a looser structure of the proteins in HA with respect to proteins in WX and WT**.** The number of readily accessible thiols reflects the same trend observed for protein extractability (figure 2A), suggesting that the behaviors observed in the two experiments are somewhat related and dependent to differences in protein organization induced by the different amylose/amylopectin ratio.

4.4.4.3 Amylose/amylopectin ratio affects protein hydration

Flour is a complex system, where starch and protein coexist in separate and incompatible phases (Schiraldi & Fessas, 2003), competing for available water: the higher the amount of water “adsorbed” by starch, the less water is available for protein hydration.

Protein hydration is essential for proteins to acquire the needed flexibility to undergo conformational modification in response to mechanical stress applied in technological processes (Wrigley et al., 2006). A direct evaluation of protein conformational changes occurring upon mixing was achieved by front-face intrinsic fluorescence, where the position (i.e. the wavelength) of the emission maximum of tryptophan increases as tryptophan side chains move from the hydrophobic protein interior to a more polar (water exposed) environment. The tryptophan emission maximum was plotted *versus* the moisture content in figure 3A. WT and WX show a similar behavior, reaching the maximum exposure of tryptophan at 40% moisture content, whereas HA needed 50% moisture. These results suggest that hydration of proteins requires more water in HA compared to WT and WX.

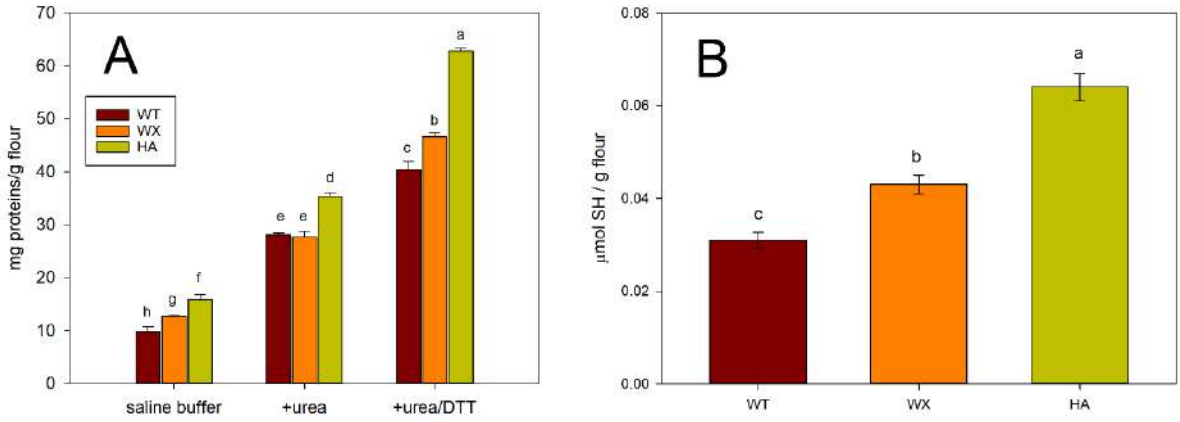

Figure 2. Protein structuring indices. A) Protein differential solubility in saline buffer in the absence or presence of urea 4M and DTT 10 mM for wild type (WT), waxy (WX) and high amylose (HA) Cadenza lines. B) Thiols accessibility in non destructuring condition for wild type (WT), waxy (WX) and high amylose (HA) Cadenza lines. Samples marked with different letters are significantly different ($p < 0.05$).

Structural changes induced by mixing or kneading results also in a different exposition of hydrophobic surface patches, that in turn can be monitored by titration with ANS as a fluorescent hydrophobic probe. The number of hydrophobic surface sites and the overall affinity toward ANS were studied at 40% moisture content, in order to stress the differences observed in front-face intrinsic fluorescence. When flour is mixed in the presence of different concentrations of ANS, the maximum fluorescence (Fmax) reflects the number of exposed hydrophobic bindings. As shown in figure 3B, Fmax is lower in HA compared to WT and WX. Hydrophobic sites in HA also show the highest affinity for ANS (i.e., the lowest dissociation constant, Kd, figure 3B, inset). A low number of surface hydrophobic sites suggests that - at 40% moisture - proteins are not completely stretched during mixing, and some hydrophobic regions remain hidden inside the protein structure. Titration with ANS also highlights differences between WT and WX. Proteins extracted from WX line are characterized by a higher number of binding sites and lower affinity to the probe compared to WT, suggesting a different reorganization into the gluten matrix when kneading. An overall overview of the hydrophobic surface behavior is provided by the Protein Surface Hydrophobicity index (PSH) calculated as Fmax/Kd. The PSH index after mixing at 40% moisture followed the order HA>WT>WX (figure 3B, inset).

4.4.4.4 High amylose and high amylopectin ratio affect competition for water

The gelatinization properties of the different flours were evaluated through Differential Scanning Calorimetry (DSC). Gelatinization profiles (figure 4) were monitored at two water contents (50% and 70%), representing a water-limiting condition and excess water, respectively (Fessas & Schiraldi, 2000).

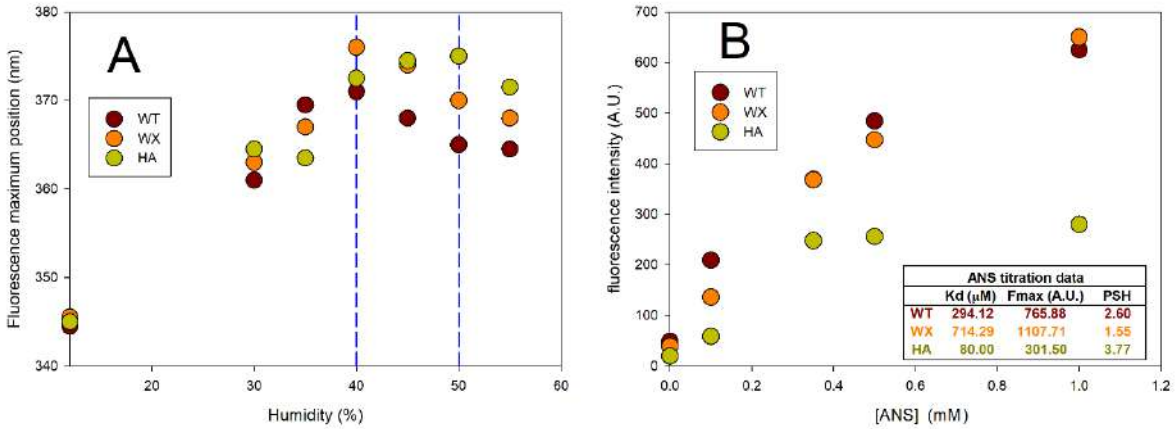

Figure 3. Protein conformational change induced by mixing. A) Wavelength of the maximum of tryptophan emission recorded in front-face fluorescence upon mixing at different water content for wild type (WT), waxy (WX) and high amylose (HA) Cadenza lines. Dashed blue lines indicate the humidity at which the highest value was recorded. B) ANS fluorescent emission recorded in titration experiments carried out at 40% constant humidity for wild type (WT), waxy (WX) and high amylose (HA) Cadenza lines. Inset: Dissociation constant (Kd), maximum fluorescence intensity (Fmax) and protein surface hydrophobicity (PSH) calculated from the titrations.

The thermal profiles exhibited main endothermic events corresponding to individual steps of the starch gelatinization process and to water partition between starch and protein phases (Bresciani et al., 2022a). In brief, the first step of starch gelatinization involves the immediately available water molecules and is followed by a second step at higher temperatures when further water molecules gain enough mobility to complete the process, including the dissociation of amylose-lipid complexes (Fessas & Schiraldi, 2000; Bresciani et al., 2022b).

The gelatinization profile of WT at 70% moisture (figure 4A) shows a main peak that reflects almost the full gelatinization process, indicating that the immediately available water molecules in the sample are enough to gelatinize starch granules to completeness in just one step (Fessas & Schiraldi, 2000). A second peak is mostly attributable to the amylose-lipid complexes dissociation as indicated by the permanence of this signal in a second heating scan

for this sample, confirming that the dissociation of the amylose-lipid complexes is the only reversible phenomenon of the overall gelatinization process. At 50% moisture, the WT sample (figure 4A) shows a lower contribution of the first step of gelatinization to the overall process, followed by an evident second gelatinization step. A shift of the amylose-lipid dissociation towards high temperatures is also evident, in agreement with the literature (Fessas & Schiraldi, 2000).

A one-step overall gelatinization process was observed also for the WX sample at 70% moisture (figure 4B). Although the onset temperature of gelatinization is similar to the WT, the rate of process seems to be decreased in WX, suggesting a different structure and distribution of starch granules. This is evident in the case of WX at 50% moisture, where completion of gelatinization occurs at very high temperature. As expected, the amylose-lipid transition is absent in the WX tracings, because of the very low amylose/amylopectin ratio.

Figure 4C displays the HA thermograms. At 70% moisture the HA thermogram is already biphasic, which could suggest that the immediately available water for the gelatinization is much less than in WT and WX systems at the same moisture level. However, HA thermogram at 50% moisture shows the expected delay in gelatinization, but the differences between the two moisture level are much less evident if compared to WT and WX. This picture suggests that the characteristic gelatinization process of HA at the highest moisture level may be not exclusively ascribable to limitations in the immediately available water, and that differences in the starch granule organization cannot be excluded.

As concerns the overall gelatinization enthalpies among our set of experiments, they were 13 ± 2 $J \cdot g^{-1}_{dry}$, 9 ± 2 $J \cdot g^{-1}_{dry}$, and 9 ± 2 $J \cdot g^{-1}_{dry}$ for WT, WX and HA, respectively. A slight decrease was observed in the case of HA and WX samples with respect to the WT, an effect ascribable to the different starch composition.

TGA showed the water loss from different dough samples as the temperature increase. DTG traces for WT, WX and HA (figure 5) display the rate of water evaporation during heating and represent an indirect index of the strength of water retention in the matrix. More specifically, the broad low-temperature peak reflects the loss of the least retained water molecules (mainly from starch, diffusional water), whereas the sharp high-temperature peak is associated to the release of water molecules strongly bound to gluten. In this regard, the higher the maximum temperature of this latter peak, the stronger the gluten network (Fessas & Schiraldi, 2001). However, it is possible to stress that gluten formation in a dough is a combination of protein characteristics, water amount and mechanical energy through kneading. In order to perform a fair comparison with the protein structural indexes observed in front-face fluorescence, the initial water content of the samples was set at 40%. The release of the diffusional water (mostly from the starch phase) is similar for the three systems indicating that there is no difference in starch-water strength of interaction. On the other hand, as regards

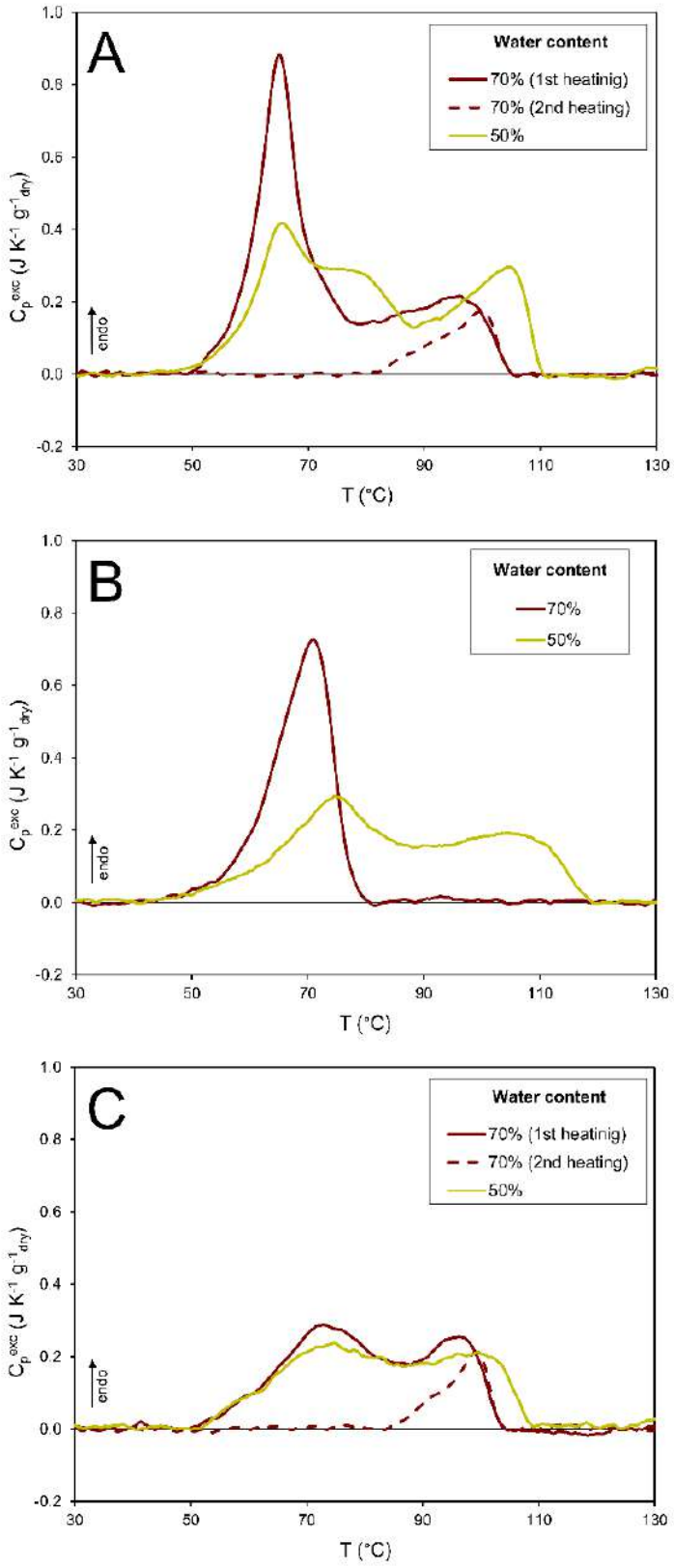

Figure 4. DSC thermograms. DSC traces were obtained for wild type (WT, panel A), waxy (WX, panel B) and high amylose (HA, panel C) Cadenza lines at 70% (red lines) and 50% (yellow line) moisture. Solid line: first heating; dashed line: second heating recorded on samples cooled down after the first heating.

the release of water from the gluten phase, a sharp peak is well distinguishable for WT and WX samples, whereas just a shoulder at about 110°C is exhibited by HA. This picture is in accordance with the results observed in ANS front-face fluorescence, where, at 40% moisture, HA sample shows limited structural changes due to the insufficient hydration of proteins. The downshift of gluten-related peak towards lower temperatures following the order WT-WX-HA revealed a gradual loosening of gluten network in the same direction, in good agreement with GlutoPeak results reported below, in section 4.4.4.5.

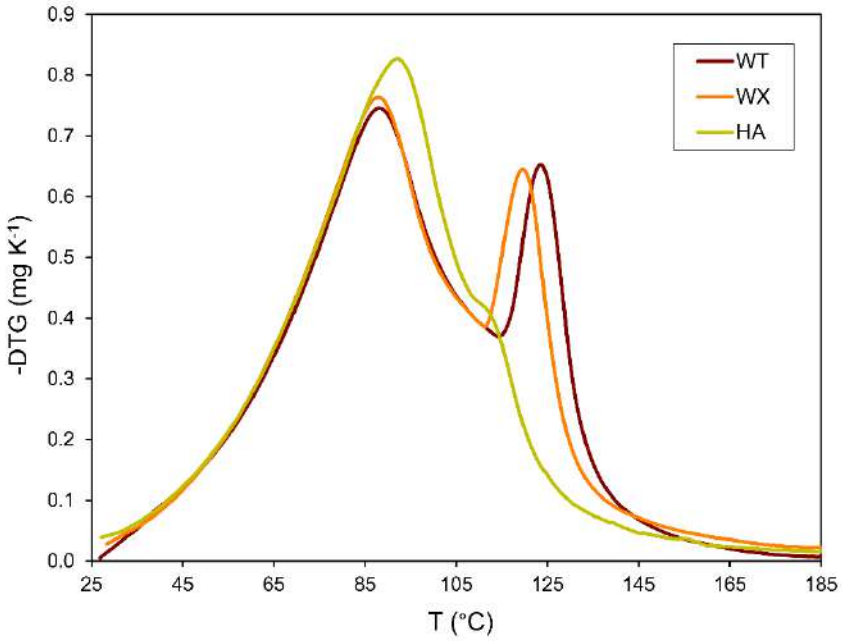

Figure 5. Temperature-derivative of the thermogravimetric traces (DTG) traces obtained for doughs from wild type (WT), waxy (WX) and high amylose (HA) Cadenza lines at 40% moisture.

4.4.4.5 Altered amylose/amylopectin ratio leads to peculiar rheological properties

Rheological properties related to the gluten develop in genetically modified line wheat flours compared to the wild type line were evaluated by the GlutoPeak test. During the test, flour and water are mixed at high speed (2,750 rpm): as the gluten is formed, the device registers an increase in consistency till a maximum value (*i.e.*, maximum consistency at a certain time, called peak maximum time) which corresponds to the maximum gluten aggregation. Weak flours generally show a rapid buildup in dough consistency and a sharply high defined peak followed by a rapid break down, while strong flours will have a much slower build up in dough consistency and a more time to achieve peak consistency (Goldstein et al., 2020). GlutoPeak traces reported in figure 6A showed different aggregation kinetics for the three lines: the time needed for gluten aggregation (also called peak maximum time) followed the order WT >> WX

> HA. The same trend was observed for the energy value (*i.e.*, the area under the curve, till 15 s after the peak): 3,838 GlutoPeak Equivalent (GPE), 2,526 GPE and 2,506 GPE for WT, WX and HA, respectively. Both peak maximum time and energy values suggest the presence of weaker gluten in HA compared to other samples. As for the maximum torque, it followed the order: WX > HA >> WT.

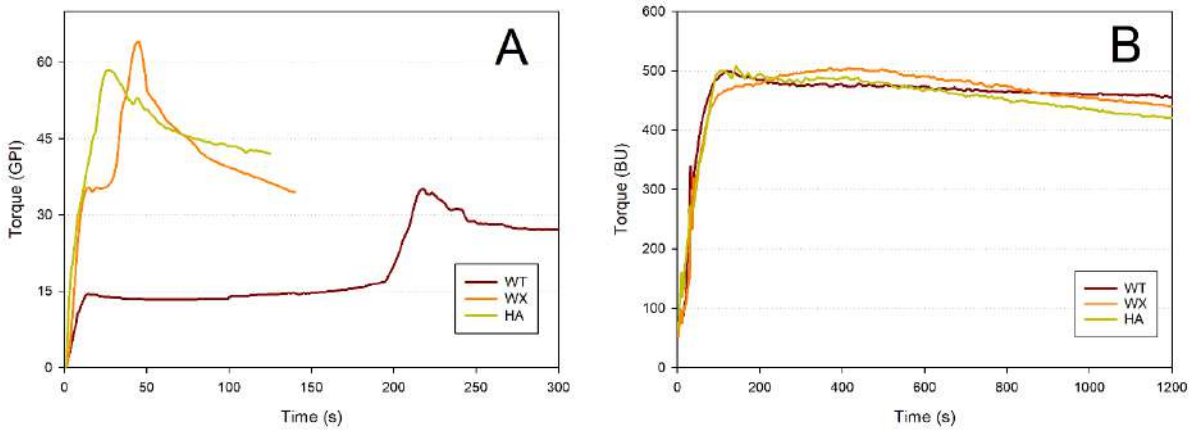

Figure 6. Rheological characterization. GlutoPeak (A) and Farinograph (B) curves of wild type (WT), waxy (WX) and high amylose (HA) Cadenza lines.

Flour quality was also evaluated in a dough system by using the Farinograph (figure 6B). HA samples absorbed the highest amount of water (76 g water/100 g flour) to make a dough with optimal consistency (*i.e.*, 500 Farinograph Units) followed by WX (69.5 g/100g) and WT (55.6 g/100g). This result is consistent with the evidence that the altered amylose/amylopectin ratio interferes with protein solvation making it necessary to add greater quantities of water to allow the formation of gluten. Differences in protein solvation might account also for the longer mixing time required for dough formation observed for WX compared to WT and HA (figure 6B). A similar behavior was observed also in other waxy wheat lines (Caramanico et al., 2018). Finally, dough stability (which is the time by which dough keeps its consistency) followed the order WT (10 min) > WX (8.9 min) > HA (6.7 min), suggesting that the gluten networks from WX and HA were weaker compared to the one in WT, as also reported in figure 6A.

4.4.5 Discussion

The use of wheat with an altered proportion of amylose and amylopectin is widely studied due to the positive technological and nutritional effects. Low amylose wheats - due to their novel viscosity properties - are suitable to enhance the stability and shelf-life of several products

such as bread and frozen foods, whereas high amylose wheat presents benefits associated to the increase in the resistant starch fraction.

Despite their interesting functional and nutritional properties, waxy and high-amylose wheat flours are usually mixed in different proportions with regular wheat flour (Hung et al., 2006). As a general observation, doughs made from waxy and high-amylose wheat flours have higher water absorption than conventional wheat flour (Morita et al., 2002) and decreased gluten strength (Graybosch et al., 2003). A number of hypotheses has been made to explain the lower quality of gluten in waxy and high amylose flour, such as a different protein content, a different protein pattern, and a higher fiber content (Hung et al., 2006).

In this study we took advantage of the availability of a set of three wheat lines, having different amylose content but sharing a unique varietal background. In detail, two mutant genotypes were derived by targeting single genes of the starch pathway in the wild type genotype, in order to inhibit amylose or amylopectin accumulation, respectively (Botticella et al., 2018). Results shown here indicate that the protein profile of the two mutant lines is almost identical to that of wild type (figure. 1). Therefore, differences in the technological properties can be attributed to the matrix effect exerted by starch in view to the distinct amylose/amylopectin ratio. Doughs produced by using flours from the three lines franked in the HA>WX>WT order for water adsorption, and exactly in the reverse order for dough stability (figure 6B). Rheological assessment carried out at fixed amount of water (i.e.Glutopeak) (figure 6A) suggest that proteins in HA and in WX have impaired gluten development compared to the WT. Although differences among GlutoPeak traces are usually related to differences in flours, such as the protein content (Rakita et al., 2018) and the amount of gliadins (Marti et al., 2015), the three lines under investigation share the same protein pattern profile and have a very similar protein content, ruling out any influences coming from the “protein quality”. Overall, the rheological assessment suggest that water availability plays a primary role in gluten development, as well as on dough properties.

Under a molecular point of view, gluten development follows a series of steps that concur to the quality of the end product. The gluten network is formed when wheat flour and water are mixed, and mechanical energy is applied. Water undergoes redistribution during kneading, thus altering the hydration state of both starch and gluten (Parenti et al., 2021), and due to thermodynamic incompatibility between polysaccharides and proteins, water is partitioned between coexisting phases. Mechanical energy (tension, compression, and/or shear) induces structural modifications in the water-swollen proteins, triggering the exposition of hydrophobic regions and thiols groups, originally buried inside the proteins. The exposition of reactive regions in turns starts the reactions of thiol-disulfide exchange as well as the formation of intermolecular hydrophobic interactions, that are at the basis of gluten development (Wieser, H., 2007). Structural modification of gluten protein during mixing is

limited by the “hydration level” of proteins, which is essential to provide proteins with the necessary flexibility to undergo conformational changes (Bonomi et al., 2004). HA flour required a larger amount of water (50%, figure 3A) compared to WX and WT flours (40%, figure 3A) to allow for the same level of structural changes in proteins. Indeed, when HA flour is mixed with an amount of water non optimal for protein solvation (40%), proteins exposed fewer hydrophobic region than WX and WT flour (figure 3B). On the other side, Caramanico et al. (2017) observed that protein conformational changes in waxy flour follow a sigmoidal behavior as a function of added water, with a first step in which small effects were observed at low amount of added water, and major conformational changes are observed only above a given hydration threshold. All these results suggest that the water distribution in the systems with altered amylose/amylopectin ratio impairs protein solvation, thus limiting the conformational changes required for gluten development. Although water availability and distribution among phases may involve other components (i.e. non starch polysaccharides, and soluble proteins (Fessas and Schiraldi, 2001)), the marked differences observed among WT, WX and HA lines cannot stem from differences in their composition. Indeed, only a slight variation in the total soluble carbohydrate content were observed, with an accumulation of soluble alpha-glucans in WX line (Botticella et al., 2018). The low relevance of these components on the water availability is also demonstrated by the fact that WX flour, which has the highest content of soluble alpha-glucans, absorbs less water than HA flour.

An investigation on the distribution of water within wheat flour dough phases can be achieved by TGA (figure 5). Indeed, TGA allows to discriminate the water bound to the starch and gluten phases and, also reveals the degree of development of the gluten itself during kneading (Fessas & Schiraldi, 2001). On dough prepared at 40% humidity, WX showed some differences in the water bound to the gluten compared to the WT. Stronger differences were observed for HA, where the water bound to the gluten phase was barely detected, suggesting both a limited amount of available water and a limited development of gluten network.

All the information provided by different approaches indicated that competition for water seems to be one of the main factors influencing gluten formation in flour with an altered amylose/amylopectin ratio. In HA wheat, where the absorption of water is the highest, protein hydration is impaired, preventing structural changes essential to trigger all the molecular events responsible for gluten formation. In waxy wheat the absorption of water by the starch phase fall in the middle compared to the “normal” and the HA wheat. In turns, also effects on protein structure, and ability to undergo structural changes, was found intermediate, as suggested by ANS titration and TGA measurement. In both WX and HA flours, remarkable effects on rheological properties were observed.

4.4.6 Conclusions

In this work, three wheat lines having different amylose content but sharing a common varietal background were characterized in order to shed light on the role of the amylose/amylopectin ratio on the gluten development and, in general, on the rheological properties of doughs. The three lines were found to have the same protein profile, with overlapping patterns of free thiols on individual protein subunits. However, proteins appear to have different intermolecular relationships in the various materials, as indicated by their overall solubility, and by the accessibility of their thiol groups. These differences suggest that amylose/amylopectin ratio could somehow influences the process of deposition of storage proteins, since the same proteins in the three lines form deposits characterized by a different compactness.

The altered amylose/amylopectin ratio also exerts a matrix effect which influences the hydration of proteins, as made evident by evaluating the conformational changes induced by the mechanical stresses applied during mixing. Gluten proteins in HA sample need more water than WT and WX proteins to acquire the structural flexibility required for gluten development. Since the proteins in the three samples are the same, this result suggests that the amylose-rich sample absorbs more water than the other two systems. This hypothesis is confirmed by the calorimetric data, where the behavior of starch in gelatinization has a shape typical of experiments recorded in limiting water conditions even when “large” percentages of water are used. A remarkable negative effect on the rheological properties were observed for both WX and HA, suggesting that *ad hoc* protocols for the production of end-products from these raw material need to be developed.

Waxy and high amylose wheats have unique functional and nutritional benefits, for this reason it is very important to study their molecular properties to optimize the transformation process based on their specific technological properties. In this frame, the potential of wheat genotypes with an altered content of amylose and amylopectin sharing their overall protein composition allows to clarify the molecular aspect at the basis of the poor technological properties of these samples. Future efforts will be dedicated to extend these approaches on doughs obtained under various operating conditions, and on the respective final products, in order to improve the current view of the matrix effects at the basis of the molecular events responsible for gluten develop, and on their effects on the overall quality of end products.

4.4.7 Rererences

Bhattacharya, M., Erazo-Castrejón, S. V., Doehlert, D. C., & McMullen, M. S. (2002). Staling of bread as affected by waxy wheat flour blends. *Cereal Chemistry, 79(2)*, 178-182. https://doi.org/10.1094/CCHEM.2002.79.2.178

Birt, D. F., Boylston, T., Hendrich, S., Jane, J. L., Hollis, J., Li, L., Mc Clelland, J., Moore, S., Phillips, G. J., Rowling, M., Schalinske, K., Scott, M. P., & Whitley, E. M. (2013). Resistant starch: promise for improving human health. *Advances in Nutrition, 4(6)*, 587-601. https://doi.org/ 10.3945/an.113.00432

Bonomi, F., Mora, G., Pagani, M. A., & Iametti, S. (2004). Probing structural features of water-insoluble proteins by front-face fluorescence. *Analytical Biochemistry, 329(1)*, 104–111. https://doi.org/10.1016/j.ab.2004.02.016

Bonomi, F., D'Egidio, M. G., Iametti, S., Marengo, M., Marti, A., Pagani, M. A., & Ragg, E. M. (2012). Structure-quality relationship in commercial pasta: A molecular glimpse. *Food Chemistry, 135(2),* 348-355. https://doi.org/10.1016/j.foodchem.2012.05.026

Botticella, E., Sestili, F., Sparla, F., Moscatello, S., Marri, L., Cuesta-Seijo, J. A., Falini, G., Battistelli, A., Trost, P., & Lafiandra D. (2018). Combining mutations at genes encoding key enzymes involved in starch synthesis affects the amylose content, carbohydrate allocation and hardness in the wheat grain. *Plant Biotechnology Journal, 16(10)*, 1723–1734. https://doi.org/10.1111/pbi.12908

Botticella, E., Savatin, D. V., & Sestili, F. (2021). The Triple Jags of Dietary Fibers in Cereals: How Biotechnology Is Longing for High Fiber Grains. *Frontiers in Plant Science*, 12:745579. https://doi.org/10.3389/fpls.2021.745579

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem, 72*, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Bresciani, A., Emide, D., Saitta, F., Fessas, D., Iametti, S., Barbiroli, A., & Marti, A. (2022a). Impact of thermal treatment on the starch-protein interplay in red lentils: Connecting molecular features and rheological properties. *Molecules, 27(4)*, 1266. https://doi.org/10.3390/molecules27041266

Bresciani, A., Vaglia, V., Saitta, F., Fessas, D., Casiraghi, M. C., Erba, D., Pagani, M. A., Lee, J. Y., Kang, J. W., Ko, J. M., Bocchi, S., Cho J. H. & Marti, A. (2022b). High-amylose and Tongil type Korean rice varieties: physical properties, cooking behaviour and starch digestibility. *Food Science and Biotechnology, 31(6)*, 681-690. https://doi.org/10.1007/s10068-022-01075-2

Caramanico, R., Barbiroli, A., Marengo, M., Fessas, D., Bonomi, F., Lucisano, M., Pagani M. A., Iametti, S., & Marti, M. (2017). Interplay between starch and proteins in waxy wheat. *Journal of Cereal Science*, *75*, 198–204. https://doi.org/10.1016/j.jcs.2017.04.008

Caramanico, R., Marti, A., Vaccino, P., Bottega, G., Cappa, C., Lucisano, M., & Pagani, M. A. (2018). Rheological properties and baking performance of new waxy lines: Strengths and weaknesses. *Lwt, 88*, 159-164. https://doi.org/10.1016/j.lwt.2017.09.035

D'Ovidio, R. & Masci, S. (2004). The low-molecular-weight glutenin subunits of wheat gluten. *Journal of Cereal Science, 39 (3),* 321-339. https://doi.org/10.1016/j.jcs.2003.12.002

De Arcangelis, E., Trivisonno, M. C., Angelicola, M., Quiquero, M., Di Nardo, V., Falasca, L., Sestili, F., Messia, M. C., & Marconi, E. (2021). Milling and rheological properties of high amylose wheat. *Journal of Cereal Science, 102*, 103335. https://doi.org/10.1016/j.jcs.2021.103335

Fessas, D., & Schiraldi, A. (2000). Starch Gelatinization Kinetics in Bread Dough. DSC investigations on 'simulated' baking processes. *Journal of Thermal Analysis and Calorimetry*, *61*(2), 411-423. https://doi.org/10.1023/A:1010161216120

Fessas, D., & Schiraldi, A. (2001). Water properties in wheat flour dough I: classical thermogravimetry approach. *Food Chemistry*, *72*(2), 237-244. https://doi.org/10.1016/S0308-8146(00)00220-X

Goldstein, A., Ashrafi, L., & Seetharaman, K. (2010). Effects of cellulosic fibre on physical and rheological properties of starch, gluten and wheat flour. *International journal of food science & technology, 45(8)*, 1641-1646. https://doi.org/10.1111/j.1365-2621.2010.02323.x

Graybosch, R. A., Souza, E., Berzonsky, W., Baenziger, P. S., & Chung, O. (2003). Functional properties of waxy wheat flours: Genotypic and environmental effects. *Journal of Cereal Science, 38*, 69–76. https://doi.org/10.1016/S0733-5210(02)00139-X

Guo, G., Jackson, D. S., Graybosch, R. A., & Parkhurst, A. M. (2003). Asian salted noodle quality: Impact of amylose content adjustments using waxy wheat flour. *Cereal Chemistry, 80(4)*, 437-445. https://doi.org/10.1094/CCHEM.2003.80.4.437

He, J., Penson, S., Powers, S. J., Hawes, C., Shewry, P. R., & Tosi, P. (2013). Spatial patterns of gluten protein and polymer distribution in wheat grain. *Journal of agricultural and food chemistry, 61(26)*, 6207-6215. https://doi.org/10.1021/jf401623d

Hung P.V., Maeda T., & Morita N. (2006) Waxy and high-amylose wheat starches and flours—characteristics, functionality and application. *Trends in Food Science & Technology, 17(8)*, 448-456. https://doi.org/10.1016/j.tifs.2005.12.006

Iametti, S., Marengo, M., Miriani, M., Pagani, M. A., Marti, A, & Bonomi F. (2013). Integrating the information from proteomic approaches: A “thiolomics” approach to assess the role of thiols in protein-based networks. *Food Research International*, *54(1)*, 980–987. https://doi.org/ 10.1016/j.foodres.2012.12.054

ICC (International Association for Cereal Science and Technology). (1992). ICC Standard No. 115/1, Method for using the Brabender Farinograph, Vienna, Austria.

Labuschagne, M. T., Geleta, N., & Osthoff, G. (2007). The influence of environment on starch content and amylose to amylopectin ratio in wheat. *Starch-Stärke, 59(5)*, 234-238. https://doi.org/10.1002/star.200600542

Lucas, I., Becker, T., & Jekle, M. (2018). Gluten polymer networks—A microstructural classification in complex systems. *Polymers, 10(6)*, 617. https://doi.org/10.3390/polym10060617

Marti, A., Augst, E., Cox, S., & Koehler, P. (2015). Correlations between gluten aggregation properties defined by the GlutoPeak test and content of quality-related protein fractions of winter wheat flour. J*ournal of Cereal Science, 66*, 89-95. https://doi.org/10.1016/j.jcs.2015.10.010

Morita, N., Maeda, T., Miyazaki, M., Yamamori, M., Miura, H., & Ohtsuka, I. (2002) Dough and baking properties of high amylose and waxy wheat flours. *Cereal Chemistry, 79*, 491–495. https://doi.org/10.1094/CCHEM.2002.79.4.491

Pagani, M. A., Giordano, D., Cardone, G., Pasqualone, A., Casiraghi, M. C., Erba, D., Blandino, M., & Marti, A. (2020). Nutritional features and bread-making performance of wholewheat: Does the milling system matter? *Foods, 9(8)*, 1035. https://doi.org/10.3390/foods9081035

Pareyt, B., Finnie, S. M., Putseys, J. A., & Delcour J. A. (2011) Lipids in bread making: Sources, interactions, and impact on bread quality. *Journal of Cereal Science, 54(3)*, 266–279. https://doi.org/10.1016/j.jcs.2011.08.011

Parenti, O., Guerrini, L., Zanoni, B., Marchini, M., Tuccio, M. G., & Carini E. (2021) Use of the 1H NMR technique to describe the kneading step of wholewheat dough: The effect of

kneading time and total water content. *Food Chemistry, 338*, 128120. https://doi.org/10.1016/j.foodchem.2020.128120

Quayson, E. T., Marti, A., Morris, C. F., Marengo, M., Bonomi, F., Seetharaman, K., & Iametti, S. (2018) Structural consequences of the interaction of puroindolines with gluten proteins. *Food Chemistry, 253*, 255-261, https://doi.org/10.1016/j.foodchem.2018.01.146

Rakita, S., Dokić, L., Dapčević Hadnađev, T., Hadnađev, M., & Torbica, A. (2018). Predicting rheological behavior and baking quality of wheat flour using a GlutoPeak test. *Journal of texture studies, 49(3)*, 339-347. https://doi.org/10.1111/jtxs.12308

Schiraldi, A., & Fessas, D. (2003). Classical and Knudsen thermogravimetry to check states and displacements of water in food systems. *Journal of thermal analysis and calorimetry*, *71*(1), 225-235. https://doi.org/10.1023/A:1022290922801.

Shewry, P. R., & Tatham, A. S. (1997) Disulphide bonds in wheat gluten proteins. *Journal of Cereal Science, 25(3)*, 207–227. https://doi.org/10.1006/jcrs.1996.0100

Shewry, P. R., & Halford, N. G. (2002). Cereal seed storage proteins: structures, properties and role in grain utilization. *Journal of Experimental botany, 53(370)*, 947-958. https://doi.org/10.1093/jexbot/53.370.947

Vensel, W. H., Tanaka, C. K. and Altenbach, S. B. (2014). Protein composition of wheat gluten polymer fractions determined by quantitative two-dimensional gel electrophoresis and tandem mass spectrometry. *Proteome Science, 12, 8.* https://doi.org/10.1186/1477-5956-12-8

Wang, X., Appels, R., Zhang, X., Diepeveen, D., Torok, K., Tomoskozi, S., Bekese, F., Mab, W., Sharpf, P., & Islam, S. (2017). Protein interactions during flour mixing using wheat flour with altered starch. *Food chemistry, 231,* 247-257. https://doi.org/10.1016/j.foodchem.2017.03.115

Wieser, H. (2007). Chemistry of gluten proteins. *Food microbiology, 24(2)*, 115-119. https://doi.org/10.1016/j.fm.2006.07.004

Wrigley, C.W., Békés, F., & Bushuk, W. (2006). Gluten: A Balance of Gliadin and Glutenin. In C. Wrigley, F. Békés, & W. Bushuk (Eds.), *Gliadin and Glutenin: The Unique Balance of Wheat Quality* (pp. 3-32). AACC International, Inc.

4.5 PULSES – LENTILS. Proteins molecular features of pulses

4.5.1 Background

Pulses represent a key food in the Mediterranean diet pattern (Tucci et al, 2021). The interest of the food industry for legumes is increased in the last years for the several health benefits associated with their consumption and in response to sustainability and food security issues (Bresciani et al., 2022).

In the following paragraph we describe the proteins structural characterization of pulses flour and after its transformation in pasta. This study would like to provide information for optimizing the processing parameter in order to improve the technological properties of the raw matrices as well as the characteristics of the final products.

4.5.2 References

Bresciani, A., Emide, D., Saitta, F., Fessas, D., Iametti, S., Barbiroli, A., Marti, A., 2022. Impact of Thermal Treatment on the Starch-Protein Interplay in Red Lentils: Connecting Molecular Features and Rheological Properties. Molecules. 27, 1266.

Tucci, M., Martini, D., Del Bo, C., Marino, M., Battezzati, A., Bertoli, S., Porrini, M., Riso, P., 2021. An Italian-Mediterranean dietary pattern developed based on the EAT-Lancet reference diet (EAT-IT): A nutritional evaluation. Foods. 10, 558.

4.6 PULSES – LENTILS. Impact of Thermal Treatment on the Starch-Protein Interplay in Red Lentils: Connecting Molecular Features and Rheological Properties

The results presented here below are published in: Bresciani, A.; ***Emide, D.;*** *Saitta, F.; Fessas, D.; Iametti, S.; Barbiroli, A.; Marti, A. Impact of Thermal Treatment on the Starch-Protein Interplay in Red Lentils: Connecting Molecular Features and Rheological Properties. Molecules. 2022, 27,1266.*

4.6.1 Abstract

Thermal treatments are widely applied to gluten-free (GF) flours to change their functionality. Despite the interest in using pulses in GF formulations, the effects of thermal treatment at the molecular level and their relationship with dough rheology have not been fully addressed. Raw and heat-treated red lentils were tested for starch and protein features. Interactions with water were assessed by thermogravimetric analysis and water-holding capacity. Finally, mixing properties were investigated. The thermal treatment of red lentils induced a structural modification of both starch and proteins. In the case of starch, such changes consequently affected the kinetics of gelatinization. Flour treatment increased the temperature required for gelatinization, and led to an increased viscosity during both gelatinization and retrogradation. Regarding proteins, heat treatment promoted the formation of aggregates, mainly stabilized by hydrophobic interactions between (partially) unfolded proteins. Overall, the structural modifications of starch and proteins enhanced the hydration properties of the dough, resulting in increased consistency during mixing.

4.6.2 Introduction

Pulses are key to the Mediterranean diet pattern [1] due to several health benefits associated with their consumption [2], such as a reduction in the risk of cardiovascular diseases and type 2 diabetes. In addition, pulse consumption is expanding in response to sustainability and food security issues [3]. Despite some differences among countries, most dietary guidelines recommend an adherence to a plant-based diet [4]. Among plant species, pulses are a good source of proteins, in terms of both protein content (17–30%) and amino acid composition, in particular lysine [5]. When combined with cereal proteins, pulses sustainably provide balanced nutrition [6].

Pulses are mainly consumed as grains, but this entails long preparation and cooking times. Moreover, pulses may contain antinutrients such as phytic acid, trypsin inhibitors, and some non-digestible oligosaccharides, which are related, among others, to some digestive disorders [7].

One approach to increase the consumption of pulses is to use them in the form of flour, which can be added to formulations of cereal-based products [8,9]. Specifically, pulses (or their fractions) enhance the nutritional value of gluten-free products due to their high protein content (21–25%) and dietary fiber (12–20%) [10].

The production of gluten-free products, such as bread, cookies, and pasta, requires the use of flours that have been subjected to a thermal treatment able to modify starch properties. Heat-treated flours exhibited a high water-binding capacity at room temperature, thus impacting on the rheological properties of the dough [11–13]. Several studies describing the effect of thermal treatments on cereals (mainly rice and corn) are reported in the literature [12]. Since starch properties (including swelling and water-binding capacity) can vary according to processing conditions and starch type [12], there might be a change in the competition for the available water between starch gelatinization and protein denaturation processes during the heat-treatment of pulses. In the case of pulses, the modification of starch and proteins upon processing have seldom been evaluated at the molecular level. As a matter of fact, countless studies underscore that understanding individual molecular events in terms of their impact on each class of macromolecules - and of specific components within each class - may be of great value, not only from the standpoint of science at large but also for when treatment parameters need to be adjusted according to a specific raw material or a specific food.

In current practice, pulses are thermally treated at industrial level on an empirical basis, most often duplicating the same processes and technologies used for cereals. Heat-treated pulses are expected to have increased nutritional properties, with a lower content of anti-nutritional factors [14], and improved technological properties [12]. Nevertheless, to the best of our knowledge, there is no evidence regarding the effect of the above-mentioned process on the functional properties of pulse flour and their relationship with the structural organization of starch and proteins or their interactions.

For this reason, this study primarily aims to investigate the effects of a thermal treatment carried out on an industrial scale, and thus representative of common industrial practice, on the molecular characteristics of starch and proteins in pulses. Red lentils (Lens culinaris) have been chosen as the raw material because they are the second-most consumed pulse after chickpeas and because their use in cereal-based products is growing [15,16]. To this aim, one thermal-treated and one non-treated sample from the same commercial batch of red lentils, produced by an Italian mill as an ingredient for food production, were compared. Investigating the relationship between the molecular changes and rheological properties of red lentil dough is the second objective of the present study. Since no studies are available on the effect of thermal treatments on starch and protein features in red lentils, addressing this topic could help to identify ideal raw materials for specific applications and/or to design specific process conditions.

4.6.3 Results and Discussion

4.6.3.1 Impact of Heat Treatment on the Color of Red Lentil Flour

Color plays a fundamental role in consumer choice and product acceptability. The heat treatment led to a significant ($p < 0.001$) increase in the degree of brightness (91.1 versus 81.1) and redness (10.5 versus 9.3) of the red lentil flour. These differences were not attributable to differences in flour particle size distribution, which was similar in both samples (data not shown). However, the modest change in overall color (ΔE less than 4) suggests that the overall differences in color might not be perceivable to the human eye [17].

4.6.3.2 Starch Properties

4.6.3.2.1 Heat Treatment Affects Starch Susceptibility to α-Amylase Hydrolysis

An evaluation of the amount of starch susceptible to rapid hydrolysis (i.e., 10 min) by α-amylase was used to provide information on process-related changes to starch: the higher the value of readily hydrolyzable starch, the higher the degree of gelatinization [18]. The thermal treatment applied to red lentils led to a modest but significant ($p < 0.01$) increase in susceptibility to rapid α-amylase hydrolysis. Heat-treated flour showed a slightly higher (+8%) amount of starch that was quickly accessible to hydrolysis compared to the untreated sample (5.4 ± 0.1 versus 5.0 ± 0.1 g/100 g of total starch).

However, it is important to note that the effect of heat treatment is reportedly much greater in cereals, such as rice or corn, than in pulses. For example, heat-treated rice shows a more than 50% increase in the amount of starch susceptible to enzymatic hydrolysis [18]. Pulse starch is characterized by a high amount of amylose (30%) [19,20] that requires high energy for gelatinization [21], a feature that may limit the effects of the thermal treatment of red lentils. Moreover, the low starch content in pulses, together with their high content in protein and fiber, may further contribute to limiting starch gelatinization during heat treatment (likely due to the competition for water between starch and proteins) and, therefore, the susceptibility of starch to rapid α-amylase hydrolysis even after the thermal treatment.

4.6.3.2.2 Thermal Analysis Highlights the Modification of Both Starch and Proteins

The thermal behavior of raw and heat-treated flours from red lentils was assessed through differential scanning calorimetry (DSC) (Figure 1A). A complex multiphasic endothermic signal was observed in untreated red lentil flour, where the signal mainly reflected the starch gelatinization process. In particular, the main peak (at about 70 °C) corresponds to the starch gelatinization that relies on water immediately available in the system (the higher the water content, the higher the percentage of gelatinized starch in this first step), whereas the second shoulder in the thermograms in Figure 1A (above 80 °C) corresponds to the completion of the process [22]. On the other hand, an initial shoulder is observed in the DSC trace (visible below

60 °C). Such a shoulder is not common for starch gelatinization occurring in DSC experiments carried out at a high moisture level, which instead is generally observed to begin with a very steep increase in Cp^{exc} [22]. This small additional thermal contribution might be ascribed to proteins. Indeed, lentils contain proteins with a distribution of different thermal stabilities [23]. Moreover, the thermal stability of globular proteins in complex systems is moisture-dependent and, in our DSC conditions (i.e., 60% moisture, 2 ◦C/min), a low-temperature onset of protein denaturation is plausible [24]. The overall enthalpy of these changes corresponds to ΔH_{raw} = 10.5 ± 0.5 J/g_{dry}.

The DSC thermogram of the flour from treated red lentils reveals substantial differences in the system's thermal behavior. Specifically, the protein denaturation contribution is no longer detectable, indicating that the treatment led to a sensible protein denaturation. The starch gelatinization onset appears shifted towards higher temperatures: from about 60 °C in untreated flour to 70 °C in the flour from treated materials. Since the gelatinization onset temperature relies heavily on starch composition and structure [22], the observed upshift suggests that the treatment had an impact on the starch granule structure as to make the first penetration of water more difficult. In the case of the treated samples, the overall enthalpy remained the same when measured in the raw flour ($\Delta H_{treated}$ = 10.5 ± 0.5 J/g_{dry}).

The overall picture indicates that the heat treatment of red lentils affected the starch granule structure and its gelatinization properties in terms of onset and possibly its kinetics, but was not able to induce starch gelatinization, as suggested by the similarity of the enthalpy values measured on the treated and untreated samples. Evidence for the absence of significant gelatinization is observed in Figure S1 in the Supplementary Material, showing the absence of starch retrogradation in a treated flour sample kept in the presence of excess water at 4 °C for 48 h, a condition that should allow starch retrogradation [25].

4.6.3.2.3 Structural Modifications Lead to Different Pasting Properties

The effect of the thermal treatment on starch pasting and retrogradation properties was assessed by measuring the changes in viscosity upon heating and cooling steps under controlled conditions. As the temperature increased, the viscosity of the system increased because starch (in the presence of excess water) undergoes swelling, followed by the rupture of its granules and loss of original organization (i.e., gelatinization), leading to a decrease in viscosity. As the temperature decreases, the viscosity increases due to amylose reorganization in a more organized structure (i.e., retrogradation).

The pasting profiles of individual samples are reported in Figure 1B. Raw flour showed a significantly lower ($p < 0.05$) pasting temperature than the heat-treated sample (70.6 and 72.9 °C), likely depending on the change in the onset of gelatinization observed in DSC thermograms (Figure 1A). This confirms that the industrial process applied in this study might

have resulted in starch and/or starch granule structure reorganization. Indeed, starch in heat-treated flour required a higher temperature to start the gelatinization [26]. Moreover, the heat-treated flour showed a significantly higher ($p < 0.05$) peak viscosity (202.5 ± 7.8 BU) than the raw flour (164.5 ± 7.9 BU). This suggests that the thermal treatment, even if modifications were only minor, made the starch granules more capable of absorbing water during the test, resulting in a higher viscosity due to the higher gelatinization capacity. During cooling, a significant ($p < 0.05$) higher final viscosity was observed in the treated flour (394 ± 12 BU versus 320 ± 21 BU), indicating that the treated material underwent a greater reorganization as a consequence of a greater gelatinization, with no impact on the retrogradation capacity, since no differences in setback values were observed (data not shown).

Our findings are consistent with those reported for yellow pea [25]. In the previous study, a high viscosity was associated also with changes occurring to the protein fraction, that could form a network capable of retaining water and increasing the viscosity of the system. Jiang et al. [26] attributed the higher viscosity of thermally treated pulses to the denaturation of water-soluble protein. Proteins can have a significant impact on starch pasting properties by forming aggregates through protein-protein interactions [27], by changing the leaching behavior of starch components [28], and by increasing water retention [29].

4.6.3.3 Protein Features

4.6.3.3.1 Heat Treatment Affects Protein Structure and Aggregation

The compactness of the protein structure was studied by investigating their susceptibility to the actions of proteolytic enzymes. In most cases, a low accessibility to proteases is associated with a native and compact structure, whereas increased accessibility is associated with the flexible structure typical of partially unfolded proteins [32]. Proteins in the heat-treated flour showed a significant ($p < 0.001$) increase in susceptibility to tryptic hydrolysis compared to those in the raw sample, releasing a higher amount of TCA-soluble peptides (absorbance at 280 nm of the TCA supernatant was 0.286 ± 0.027 and 0.115 ± 0.025, respectively).

The protein pattern of the raw and heat-treated red lentil flour was characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The comparison of the protein patterns obtained in the presence/absence of a disulfide-reducing agent is shown in Figure 2A. No main differences were observed between raw and heat-treated flour in non-reducing conditions (Figure 2A, left), suggesting that the thermal treatment did not induce the formation of covalent aggregates stabilized by disulfide bridges. In this condition, the two main storage protein families, namely the 11S/legumin-like (60 kDa subunit), and the 7S/vicilin-like (50 kDa subunits), are grouped in the upper part of the electrophoretic separation [33]. This observation is confirmed by the addition of 2-ME as a disulfide reductant (Figure 2A, right) that

breaks the disulfide bridge inside the 11S/legumin-like subunit and releases its 20 and 40 kDa polypeptides [33].

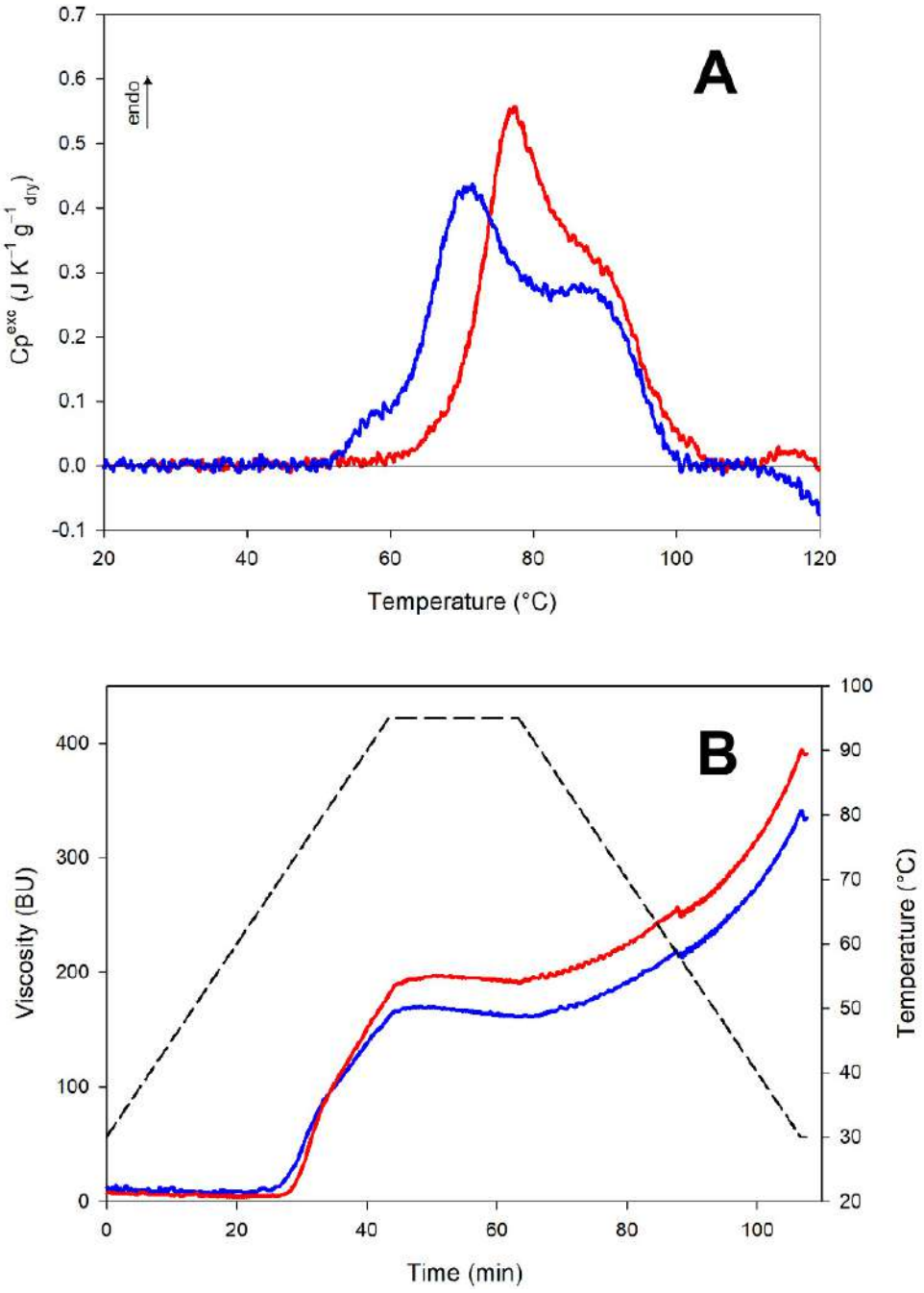

Figure 1. Starch properties. Raw flour (blue line) and heat-treated flour (red line) from red lentils. Panel A: Differential Scanning Calorimetry (DSC) traces recorded at 60% moisture content. Panel B: Micro Visco-Amylo-Graph traces.

More insights on the formation and properties of protein aggregates can be obtained by differential solubility approaches [34,35], which rely on the ability of dissociating and

reducing agents to sequentially solubilize the proteins in aggregates stabilized by either non-covalent (mainly hydrophobic) or covalent (disulfide bridges) interactions. The number of proteins solubilized in saline buffer (i.e., when the soluble proteins are not involved in aggregates) was lower in heat-treated than in raw flour (Figure 2B), suggesting that the heat treatment promoted the formation of aggregates between somehow unfolded proteins. In the presence of a chaotrope (4 M urea) and a reductant (4 M urea + 10 mM DTT) (i.e., a condition where soluble proteins are involved in non-covalent or covalent aggregates, respectively) the number of solubilized proteins increased, but no significant differences were observed. This observation suggests that aggregates formed during the thermal treatment are mainly stabilized by hydrophobic interactions (and not by covalent interactions). This is consistent with both the SDS-PAGE separation (Figure 2A, where non-covalent interactions cannot be detected, since the test is carried out in denaturing conditions) and the DSC measurements (Figure 1A, where the endothermic contribution related to protein denaturation was no longer visible in the treated flour), suggesting protein denaturation and possible aggregation during the treatment.

The quantification of the accessible -SH groups in saline buffer provided further information on the structural organization and overall compactness of the protein network, as well as on the reactivity of cysteine residues, which are potentially relevant for the formation of intermolecular disulfide bonds in further processing steps. Thermal treatment resulted in a significant ($p < 0.05$) decrease in the accessibility of thiols (1.10 ± 0.16 and 1.62 ± 0.13 μmol -SH/g fluor in heat-treated compared to raw flour, respectively). The thermal treatment influenced the structure of proteins by increasing their compactness, resulting in a lower accessibility of the cysteine thiols that became buried in the protein network. The formation of aggregates and the reduction in thiol accessibility as a consequence of thermal treatments is also a general trait in other gluten-free raw materials, such as rice [36], no matter whether thermal treatments are applied before or during the pasta-making process.

4.6.3.3.2 Water Availability in Drive Protein Modifications during Kneading

Tryptophan (intrinsic) front-face fluorescence was used to study structural modifications of proteins induced by kneading, as a function of the water content of the system. Reportedly, solvation allows proteins to achieve the structural flexibility needed to undergo the modifications induced by physical, chemical, or enzymatic treatments [37]. By increasing the water content of the matrix (from 10% up to 40%), a progressive red-shift (toward higher wavelength) of the maximum tryptophan emission is observed (Figure 2C). The red-shift occurs when the chemical environment of the tryptophan side chains becomes more polar, which happens when hydrophobic regions are exposed to water during protein unfolding. Tryptophan exposure was achieved at 30% moisture content, although the red shift in the heat-

treated sample was more pronounced than in the raw flour sample (Figure 2C). Increasing the water content by up to 40% resulted in a further evolution of the systems, observable as a slight decrease in the red-shift. This trend reversal was likely due to increased hydrophobic interactions among the regions exposed at a lower water content so that tryptophan side chains were again buried in these “new” hydrophobic surroundings. Overall, the heat-treated sample showed a greater exposure of tryptophan, suggesting greater modification.

4.6.3.4 Hydration Properties

4.6.3.4.1 Thermogravimetric Analysis Shows Effects on Starch and Protein Phase Separation

A thermogravimetric analysis (TGA) was carried out to investigate the water distribution in the matrices. Figure 3 reports the derivative thermogravimetric (DTG) traces for raw and treated flours. These traces reflect the rate of water evaporation during heating and represent an indirect index of the strength of water retention in the matrix. The biphasic traces for both the raw and treated materials indicate a non-homogenous water distribution among the starch and protein phases [40]. This phase separation appears more evident in the case of the treated flour. These measurements by themselves do not allow for the assignment of the low and high water retention contributions to starch and/or protein phases. Such a distinction is reported for starch/gluten matrices [40], but no information is available for starch/globular protein systems. A deeper investigation on such a topic is beyond the scope of this phenomenological analysis. It seems safe to say that the treatment produced a variation in water distribution that is coherent with both the modifications of starch granules observed by DSC (section 4.6.3.2.2) and the presence of protein aggregates evidenced by differential solubility experiments (section 4.6.3.3.1) and by surface hydrophobicity measurements since protein aggregates are characterized by a decrease in the water-exposed hydrophobic surface (see the ANS binding data in 4.6.3.3.2).

4.6.3.4.2 Thermal treatment affects Water Holding Capacity

The hydration capacity of both raw and heat-treated flours was mostly imputable to their high content of dietary fiber and to the presence of hydrophilic groups in pulse proteins [39]. However, the heat-treated flour showed a slight but significant ($p < 0.001$) increase in the ability to absorb and hold water. In detail, the water holding capacity was 1.12 ± 0.02 and 0.99 ± 0.02 (g water)/(g flour) for heat-treated and raw flour, respectively.

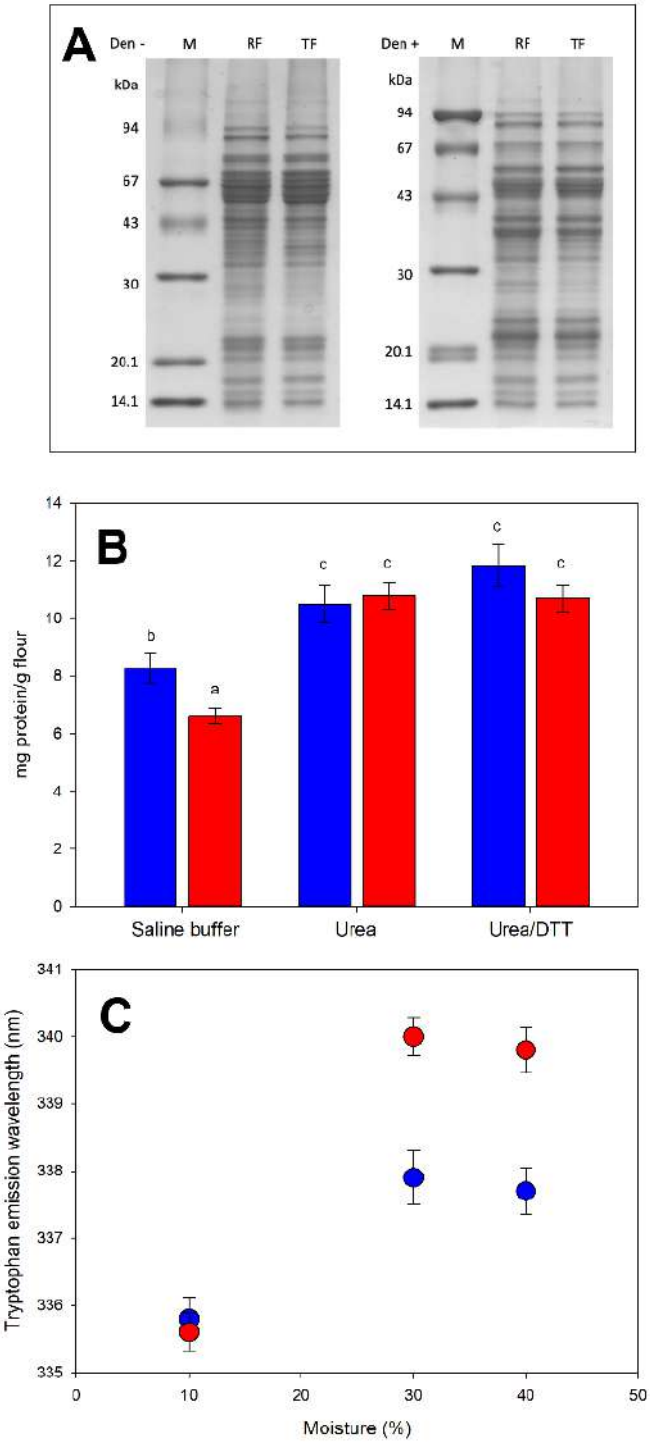

Figure 2. Protein properties. Panel A: SDS-PAGE in non-reducing (Den-, left) and reducing (Den+, right) conditions; M: markers; RF: raw flour; TF: heat-treated flour. Panel B: Protein differential solubility in non-dissociating (saline buffer), dissociating (urea), and reducing (urea/DTT) buffers for raw (blue) and heat-treated (red) flour. Samples marked with the same letter are not significantly different ($p < 0.05$). Panel C: Tryptophan emission maximum recorded in front-face fluorescence upon kneading raw (blue dots) or heat-treated (red dots) flour with different amounts of water. DTT, dithiothreitol.

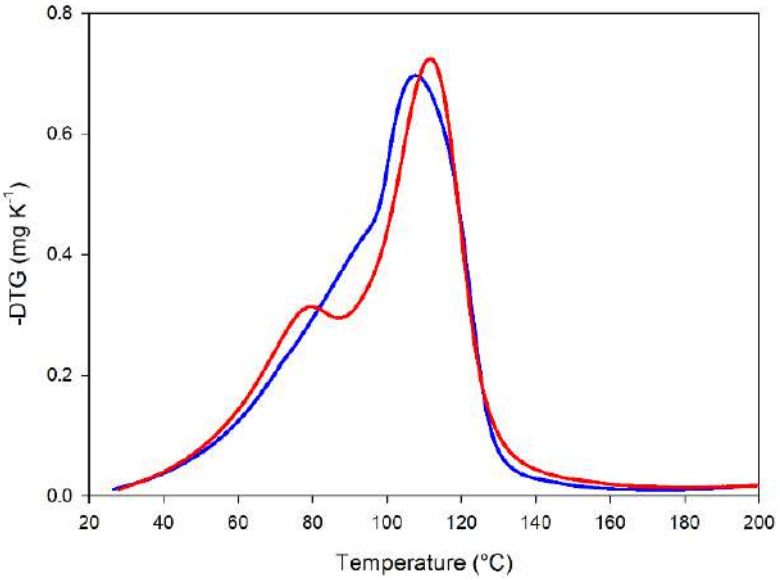

Figure 3. Derivative thermogravimetric (DTG) traces of dough from of raw (blue line) and heat-treated (red line) flours from red lentils at 60% moisture content.

The greater hydration capacity of the heat-treated flour could be related to the more open structure of starch, as also shown by the higher susceptibility to enzymatic hydrolysis. This could favour the hydration of starch granules and lead to a better capacity in the absorption and subsequent retention of water. This behaviour was also observed when lentil flour was suspended in water and then heated under control conditions (Figure 1B). The higher viscosity observed in the heat-treated was probably due to the greater ability of starch to absorb and bind water. Nevertheless, changes in WHC can also be related to protein denaturation [25,40], as proteins, upon unfolding, expose previously hidden peptide bonds and polar side chains, that contribute to their water holding ability [41].

4.6.3.4 Mixing properties

The effect of the heat-treatment on the ability of red lentil flour to form a dough was measured at 30°C and constant hydration level [42]. Results are reported in Figure 4 The heat-treated samples showed a better ability to form the dough visible from the higher torque values. This result could be due to the better capacity of absorbing water (see section 4.6.3.4.2) which therefore allows the formation of a dough with higher consistency/torque compared to the raw sample. Similar results were observed for heat treated cereals like corn and rice and other types of heat treated pules (e.g., chickpea) [42].

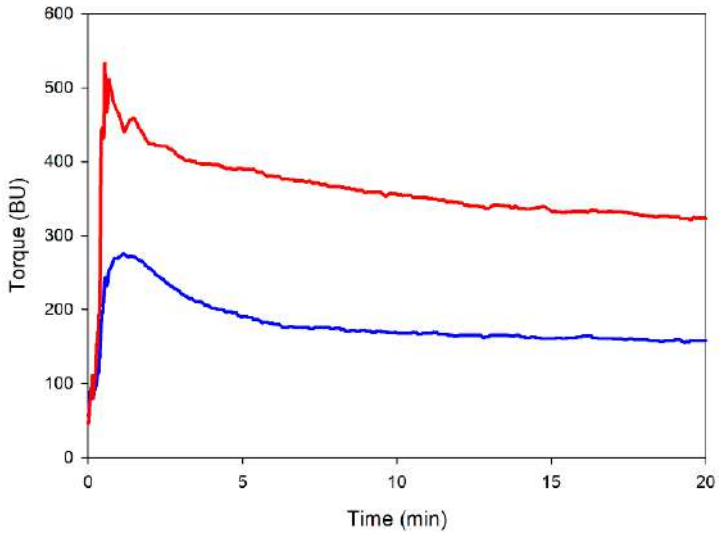

Figure 4. Mixing properties of raw (blue line) and heat-treated (red line) flours from red lentils at 50% hydration level. BU, Brabender Units.

4.6.4 Materials and Methods

4.6.4.1 Samples

Flours from raw and heat-treated red lentils available on the Italian market were tested. Both samples belonged to the same batch of red lentils.

4.6.4.2 Color

The color of the flours was measured using a reflectance color meter (CR 210, Minolta Co., Osaka, Japan) to measure the lightness and saturation of the color intensity. Results were expressed in the CIE L* a* b* color space. Color test was carried out on fifteen replicates. Color difference (ΔE) was calculated as:

$$\Delta E = \sqrt{(L2 - L1)^2 + (a2 - a1)^2 + (b2 - b1)^2} \qquad (1)$$

4.6.4.3 Starch properties

4.6.4.3.1 Starch susceptibility to α-amylase hydrolysis

This index was assessed as damaged starch (AACC 76-31.01; [43]). Results were expressed on the total starch content, determined by the standard method AACC 76-13.01 [43]. Results were expressed as mean ± standard deviation of three replicates.

4.6.4.3.2 Thermal properties

The starch gelatinization properties and the thermal behavior of the protein fraction in flours were analyzed through a PerkinElmer DSC6 (PerkinElmer, Waltham, MA, USA) working with

stainless steel sealed pans and runs from 10°C to 150°C at 2°C/min. An empty pan was used as reference and calibration was carried out with indium as standard. Samples were prepared by directly adding an adequate amount of water within the pans containing about 18 mg of sample to achieve 60% moisture content. Specifically, for the heat-treated flour, additional DSC measurements were performed for samples that were highly hydrated (72% moisture) and stored at 4°C for 48 hours before the measurement in order to highlight possible starch retrogradation. The real final water content for each sample was assessed after the DSC analysis, on the cold pans, by piercing the pans and desiccating their contents at 105°C.

Data were analyzed with the dedicated software IFESTOS following procedures reported in previous studies [44]. In brief, the excess heat capacity C_P^{exc}(T) (J K-1 g-1dry matter), i.e., the difference between the apparent heat capacity C_P(T) of the sample and the heat capacity of the pre-transition region, was recorded across the scanned temperature range. Three replicates were performed for each sample and one representative curve for each sample was reported.

4.6.4.4 Pasting properties

Pasting properties of pulse flours were evaluated using a Micro Visco-Amylo-Graph, MVAG (Brabender GmbH., Duisburg, Germany) as reported by Bresciani et al. [24]. Briefly, 12 g of flour were dispersed in 100 ml of distilled water, scaling both sample and water weight on a 14% flour moisture basis. The moisture content of flours was evaluated at 130°C until the sample weight did not change by 1 mg for 60 s in a moisture analyzer (MA 210.R, Radwag; Wagi Elektroniczne, Chorzòw, Poland). The suspensions were subjected to the following temperature profile: heating from 30 up to 95°C, holding at 95°C for 20 minutes and cooling from 95 to 30°C with a heat/cooling rate of 1.5°C/min. The analysis was carried out in duplicate and one representative curve for each sample was reported.

4.6.4.4 Protein properties

4.6.4.4.1 Protein susceptibility to tryptic hydrolysis

Susceptibility of protein samples to tryptic hydrolysis was studied as described by Iametti et al. [30]. In this study, flour (75 mg) was suspended in 6 ml of 50 mM sodium phosphate, 0.1 M NaCl, pH 7.0, and added of 150 µl of a trypsin solution (2 mg/ml in 20 mM sodium acetate pH 4.5) to give an enzyme/protein ratio around 1:50. After incubation at 37°C for 40 min, the proteolytic reaction was stopped by adding an equal volume of 20% (w/v) trichloroacetic acid (TCA). The precipitated undigested proteins were removed by centrifugation (13,000 ×*g* for 10 min), and soluble peptides were quantified spectrophotometrically by reading the absorbance of the supernatant at 280 nm versus a blank containing only phosphate buffer and TCA. Results were expressed as mean ± standard deviation of three replicates.

4.6.4.4.2 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

For SDS-PAGE, the samples were prepared by suspending 2 mg of flour in 100 µl of water and 100 µl of denaturing buffer (0.125 M Tris-HCl, pH 6.8, 50% glycerol, 1.7% SDS, 0.01% bromophenol blue (w/v)), containing 1% (v/v) 2-mercaptoethanol (2-ME) when indicated. The suspension was then heated at 100°C for 10 min. The electrophoretic run was performed at pH 8.8 (0.025 M Tris-HCl, 0.192 M glycine, 0.1% (w/v) SDS), in an Miniprotean II cell (Bio-Rad), and the gels were Coomassie Blue-stained.

4.6.4.4.3 Protein differential solubility

The solubility of proteins in native and denaturing/reducing conditions was evaluated by differential solubility studies, conducted as reported by Iametti et al. [45]. For these analyzes 150 mg of flour was suspended in 6 ml of saline buffer (50 mM sodium phosphate, 0.1 M NaCl, pH 7.0) containing 4 M urea and 10 mM dithiothreitol (DTT) when required. The suspension was stirred for 90 min at 25°C and subsequently centrifuged (10,000 × g for 20 min, 25°C). The quantification of proteins in the supernatant was carried out by a dye-binding method [46]. Results were expressed as mean ± standard deviation of three replicates.

4.6.4.4.4 Quantification of accessible thiols

Accessible thiols (-SH) were quantified exploiting the ability of the reagent 5,5-dithio-bis-nitrobenzoate (DTNB) to react with the exposed -SH groups of cysteine [47], following a procedure reported elsewhere [48]. A 50 mg aliquot of flour was suspended in 6 ml of 50 mM sodium phosphate, 0.1 M NaCl, pH 7.0, containing 0.2 mM DTNB, in the presence/absence of 4 M urea. After 1 h at 25°C, the suspension was centrifuged (13,000 × g for 15 min, 20°C) to remove solid materials, and the absorbance of the supernatant was read at 412 nm against a DTNB blank. Results are expressed as mean ± standard deviation of three replicates, from two independent measurements.

4.6.4.4.5 Front-face fluorescence

Intrinsic (tryptophan) fluorescence was assessed by front-face (solid state) measurements, as reported by Bonomi et al. [35]. Solvation studies were performed by adding to 1 g of sample enough water to reach a final moisture between 10% (i.e., the moisture content of flour) and 40%. The mixture was kneaded for 2 min by using a glass stirring rod, and then loaded in the front-face fluorescence cell. Tryptophan fluorescence emission was monitored from 300 to 420 nm (emission slit 2.5 nm), by exciting samples at 280 nm (excitation slit 2.5 nm), with scan speed of 50 nm/min. All the measures were conducted at 25°C in a LS50 B Perkin-Elmer Luminescence Spectrometer (Perkin-Elmer Co., Waltham, MA, USA). Results were expressed as mean ± standard deviation of three replicates. Protein surface hydrophobicity was

determined by using 1,8-anilino-naphthalene-sulfonate (ANS) as the hydrophobic fluorescent probe [35]. Samples were prepared as reported above for the intrinsic fluorescence except that part of the added water was replaced with an ANS stock solution (5 mM in water) to give a final concentration of 0.5 mM probe in the resulting dough. Fluorescence intensity was monitored at 460 nm (emission slit 2.5 nm), by exciting samples at 390 nm (excitation slit 2.5 nm). Results are expressed as mean ± standard deviation of three replicates.

4.6.4.5 Hydration properties

4.6.4.5.1 Thermalgravimetric analysis (TGA)

The water phase separation in the different lentil flours was analysed by TGA. A Setaram TG-DSC111 (Lyon, France) allowed the simultaneous monitoring of thermal effects (heat flow versus temperature, and mass loss versus temperature). The typical sample mass was 30 mg with 60% of overall water content (as in DSC experiments) and runs were carried out from 20°C to 200°C at 2°C/min. Each run was repeated twice. One representative profile for each sample is reported. The ratio between the heat flux and the related mass loss rate was found equal to the enthalpy of water evaporation temperature range. This check confirmed that the mass loss was substantially related to water evaporation only. All the TG traces were normalized to 100 mg of sample mass. DTG traces, i.e., the temperature derivative of TG traces, were obtained and expressed as mg of lost mass per temperature unit.

4.6.4.5.2 Water holding capacity (WHC)

WHC was determined by calculating the amount of water absorbed and retained by the flours. Briefly, 1.0 ± 0.1 g flour was mixed with 10 mL distilled water, vortexed for 30 s, then left for 30 min at 25°C. Mixtures were centrifuged at 2,500 × g for 20 min, and the supernatant decanted. WHC was calculated as the ratio between grams of water retained per gram of solid. Results were expressed as mean ± standard deviation of three replicates.

4.6.4.6 Mixing properties

Mixing properties were measured by using a Farinograph-E® (Brabender GmbH & Co. KG, Duisburg, Germany) equipped with a 50 g mixing bowl and operating at a constant hydration level (i.e., 50% on dry matter) as reported by Bresciani et al [42]. The analysis was carried out in duplicate and one representative curve for each sample was reported.

4.6.4.7 Statistics

The data was subjected to t-Test (two-tailed distribution) using Statgraphics Plus 5.1 (Statpoint Inc., Warrenton, VA, USA). Differences at $p < 0.05$ (*); $p < 0.01$ (**) and $p < 0.001$ (***) were considered significant. Data from differential solubility were subjected to analysis

of variance (one-way ANOVA; $p < 0.05$) by using SigmaPlot version 14.0 (Systat Software Inc., San Jose, CA, USA). When a factor resulted significantly different, the difference was determined through the Tukey HSD test.

4.6.5 Conclusions

The thermal treatment considered in this study promoted structural changes on both starch and proteins in red lentils. The treatment was not able to promote extensive starch gelatinization, since the enthalpy values of raw and treated flour were closely comparable. At the same time, some molecular change happens, since the kinetics of gelatinization observed in DSC, as well as the gelatinization, pasting temperatures, and hot viscosity in pasting properties (Figure 1) were affected. Overall, our results suggest that the treatment likely affected the external regions of the starch granules, i.e., those regions that resulted slightly (but significantly) more susceptible to α-amylase hydrolysis.

The effects of the heat treatment on proteins appears to be generally more pronounced, since evidence on their denaturation in the thermally treated samples comes from the lack of the thermal peak at 60°C in DSC studies, as well as from the increased susceptibility to trypsin and the red-shift observed in tryptophan front-face fluorescence. As a consequence of denaturation, the exposure of hydrophobic regions leads to the formation of protein aggregates stabilized mainly through hydrophobic interactions. Disulfide bonds, that are one of the main actors in many thermal coagulation events [49,50], as well as in the formation of the gluten network [51] do not appear to play a significant role in the case of red lentil proteins, at least in these conditions. However, thiol accessibility decreases significantly in the treated red lentils, suggesting that residual thiols may be hidden within compact regions in the protein network formed upon temperature-induced aggregation.

Changes in both starch and protein organization affected the hydration and mixing properties of the heat-treated flour. Specifically, the heat-treated flour absorbed more water but showed a lower retention capacity (Figure 3). The higher water holding capacity, together with the greater protein aggregation tendency/properties, resulted in a dough with higher consistency during mixing (Figure 4).

This study indicates that red lentils subjected to a heat treatment at industrial level show a peculiar behaviour. Starch apparently undergoes only minimal structural changes, but these minor changes are able to influence its rheological properties. Storage proteins, that in legumes are well-structured globulins, unfold and aggregate contributing to the modification of the rheological properties of the flour and to the water partition between the two classes of biopolymers.

The practical relevance of these observations is manifold. For instance, the intensity and duration of the thermal process could be increased to promote starch gelatinization above

what reported here, so to reach an extent that could be desirable for specific gluten-free products and formulations. Another factor that may come into play – as suggested by this study – is the amount of water in the system at the onset of the thermal treatment. Subtle differences may mean a lot here, as water availability relates to the rate (and fate) of both protein unfolding and starch gelatinization. A thorough study of these parameters was outside the scope of what reported here, as this study was aimed at characterizing materials of common industrial use. In spite of these limitations, this study provides a methodological basis for improving our current understanding of how any modification of the main macromolecules present in a given system may affect its technologically relevant properties, and how processes may be tailored to fulfil specific requirements from the food industry.

4.6.6 References

1. Tucci, M.; Martini, D.; Del Bo, C.; Marino, M.; Battezzati, A.; Bertoli, S.; Porrini, M.; Riso, P. An Italian-Mediterranean dietary pattern developed based on the EAT-Lancet reference diet (EAT-IT): a nutritional evaluation. *Foods* **2021**, *10*, 558; DOI:10.3390/foods10030558.

2. Martini, D.; Godos, J.; Marventano, S.; Tieri, M.; Ghelfi, F.; Titta, L.; Lafranconi, A.; Trigueiro, H.; Gambera, A.; Alonzo, E.; Sciacca, S.; Buscemi, S.; Ray, S.; Galvano, F.; Del Rio, D.; Grosso, G. Nut and legume consumption and human health: an umbrella review of observational studies. *Int. J. Food. Sci. Nutr.* **2021**, *72*, 871-878; DOI:10.1080/09637486.2021.1880554.

3. Sozer, N.; Holopainen-Mantila, U.; Poutanen, K. Traditional and new food uses of pulses. *Cereal Chem.* **2017**, *94*, 66-73; DOI:10.1094/CCHEM-04-16-0082-FI.

4. Martini, D.; Tucci, M.; Bradfield, J.; Di Giorgio, A.; Marino, M.; Del Bo, C.; Porrini, M.; Riso, P. Principles of sustainable healthy diets in worldwide dietary guidelines: efforts so far and future perspectives. *Nutrients* **2021**, *13*, 1827; DOI:10.3390/nu13061827.

5. Day, L. Proteins from land plants – Potential resources for human nutrition and food security. *Trends Food Sci. Technol.* **2013**, *32*, 25-42; DOI:10.1016/j.tifs.2013.05.005.

6. Boye, J.; Zare, F.; Pletch, A. Pulse proteins: Processing, characterization, functional properties and applications in food and feed. *Food Research International.* **2010**, *43*, 414-431; DOI:10.1016/j.foodres.2009.09.003.

7. Hall, C.; Hillen, C.; Garden Robinson, J. Composition, nutritional value, and health benefits of pulses. *Cereal Chem.* **2017**, *94*, 11-31; DOI:10.1094/CCHEM-03-16-0069-FI.

8. Boukid, F.; Zannini, E.; Carini, E.; Vittadini, E. Pulses for bread fortification: a necessity or a choice? *Trends Food Sci. Technol.* **2019**, *88*, 416-428; DOI:10.1016/J.TIFS.2019.04.007.

9. Bresciani, A.; Marti, A. Using pulses in baked products: lights, shadows, and potential solutions. *Foods* **2019**, *8*, 451; DOI:10.3390/foods8100451.

10. Foschia, M.; Horstmann, S.W.; Arendt, E.K.; Zannini, E. Legumes as functional ingredients in gluten-free bakery and pasta products. *Annu. Rev. Food.* **2017**, *8*, 75-96; DOI:10.1146/annurev-food-030216-030045.

11. Witczak, M.; Ziobro, R.; Juszczak, L.; Korus, J. Starch and starch derivatives in gluten-free systems – A review. *J. Cereal Sci.* **2016**, *67*, 46-57; DOI:10.1016/j.jcs.2015.07.007.

12. Gómez, M.; Martínez, M.M. Changing flour functionality through physical treatments for the production of gluten-free baking goods. *J. Cereal Sci.* **2016**, *67*, 68-74; DOI:10.1016/j.jcs.2015.07.009.

13. Marti, A.; Pagani, M.A. What can play the role of gluten in gluten free pasta? *Trends Food Sci. Technol.* **2013**, *31*, 63-71; DOI:10.1016/j.tifs.2013.03.001.

14. Patterson, C.A.; Curran, J.; Der, T. Effect of processing on antinutrient compounds in pulses. *Cereal Chem.* **2017**, *94*, 2-10; DOI:10.1094/CCHEM-05-16-0144-FI.

15. Marchini, M.; Carini, E.; Cataldi, N.; Boukid, F.; Blandino, M.; Ganino, T.; Vittadini, E.; Pellegrini, N. The use of red lentil flour in bakery products: how do particle size and substitution level affect rheological properties of wheat bread dough? *LWT* **2021**, *136*, 110299; DOI:10.1016/j.lwt.2020.110299.

16. Bresciani, A.; Iametti, S.; Emide, D.; Marti, A.; Barbiroli, A. Molecular features and cooking behavior of pasta from pulses. *Cereal Chem.* **2021**, *00*, 1– 5; DOI:10.1002/cche.10490.

17. Fois, S.; Campus, M.; Piu, P.P.; Siliani, S.; Sanna, M.; Roggio, T.; Catzeddu, P. Fresh pasta manufactured with fermented whole wheat semolina: physicochemical, sensorial, and nutritional properties. *Foods* **2019**, *8*, 422; DOI:10.3390/foods8090422.

18. Marti, A.; Caramanico, R.; Bottega, G.; Pagani, M.A. Cooking behavior of rice pasta: effect of thermal treatments and extrusion conditions. *LWT - Food Science and Technology* **2013**, *54*, 229-235; DOI:10.1016/j.lwt.2013.05.008.

19. Asif, M.; Rooney, L.W.; Ali, R.; Riaz, M.N. Application and opportunities of pulses in food system: a review. *Crit. Rev. Food. Sci. Nutr.* **2013**, *53*, 1168-1179; DOI:10.1080/10408398.2011.574804.

20. Hoover, R.; Hughes, T.; Chung, H.J.; Liu, Q. Composition, molecular structure, properties, and modification of pulse starches: a review. *Food Res. Int.* **2010**, *43*, 399-413; DOI:10.1016/j.foodres.2009.09.001.

21. Bresciani, A.; Giordano, D.; Vanara, F.; Blandino, M.; Marti, A. The effect of the amylose content and milling fractions on the physico-chemical features of co-extruded snacks from corn. *Food Chem.* **2021**, *343*, 128503; DOI:10.1016/j.foodchem.2020.128503.

22. Fessas D.; Schiraldi A. Starch gelatinization kinetics in bread dough. DSC investigations on 'simulated' baking processes. *J. Therm. Anal. Calorim.* **2000**, *61*,411-423; DOI:10.1023/A:1010161216120.

23. Riva, M.; Fessas, D.; Schiraldi, A. Starch retrogradation in cooked pasta and rice. *Cereal Chem*. **2000,** *77*, 433-438; DOI:10.1094/CCHEM.2000.77.4.433.

24. Bresciani, A.; Giuberti, G.; Cervini, M.; Marti, A. Pasta from yellow lentils: how process affects starch features and pasta quality. *Food Chem.* **2021**, *364*, 130387; DOI:10.1016/j.foodchem.2021.130387.

25. Sopiwnyk, E.; Bourré, L.; Young, G.; Borsuk, Y.; Lagassé S.; Boyd, L.; Sarkar, A.; Jones, S.; Dyck, A.; Malcolmson, L. Flour and bread making properties of whole and split yellow peas treated with dry and steam heat used as premilling treatment. *Cereal Chem.* **2020**, *97*, 1290-1302; DOI:10.1002/cche.10357.

26. Jiang, Z.Q.; Pulkkinen, M.; Wang, Y.J.; Lampi, A.M.; Stoddard, F.L.; Salovaara, H.; Piironen, V.; Sontag-Strohm, T. Faba bean flavour and technological property improvement by thermal pre-treatments. *LWT - Food Science and Technology*, **2016**, *68*, 295-305; DOI:10.1016/j.lwt.2015.12.015.

27. Xie, L.; Chen, N.; Duan, B.; Zhu, Z.; Liao, X. Impact of proteins on pasting and cooking properties of waxy and non-waxy rice. *Journal of Cereal Science*. **2008**, *47*, 372-379; DOI:10.1016/j.jcs.2007.05.018.

28. Zhou, Z.; Robards, K.; Helliwell, S.; Blanchard, C.; Baxterb, G. Rice ageing. I. Effect of changes in protein on starch behaviour. *Starch/Stärke*. **2003**, *55*, 162-169; DOI:10.1002/star.200390030.

29. Ribotta, P.D.; Colombo, A.; León, A.E.; Añón, M.C. Effects of soy protein on physical and rheological properties of wheat starch. *Starch/Stärke*. **2007**, *59*, 614-623; DOI:10.1002/star.200700650.

30. Iametti, S.; Donnizzelli, E.; Pittia, P.; Rovere, P.P.; Squarcina, N.; Bonomi, F. Characterization of high-pressure-treated egg albumen. *J. Agric. Food. Chem.* **1999**, *47*, 3611–3616; DOI:10.1021/jf9808630.

31. Derbyshire, E.; Wright, D.J.; Boulter D. Legumin and vicilin, storage proteins of legume seeds. *Phytochemistry* **1976,** *15*, 3-24; DOI:10.1016/S0031-9422(00)89046-9.

32. Marti, A.; Barbiroli, A.; Marengo, M.; Fongaro, L.; Iametti, S.; Pagani, M.A. Structuring and texturing gluten-free pasta: egg albumen or whey proteins? *European Food Research Technology* **2014**, *238*, 217-224; DOI:10.1007/s00217-013-2097-4.

33. Bonomi, F.; D'Egidio, M.G.; Iametti, S.; Marengo, M.; Marti, A.; Pagani, M.A.; Ragg, E.M. Structure-quality relationship in commercial pasta: a molecular glimpse. *Food Chem.* **2012**, *135*, 348-55; DOI:10.1016/j.foodchem.2012.05.026.

34. Barbiroli, A.; Bonomi, F.; Casiraghi, M.C.; Iametti, S.; Pagani, M.A.; Marti, A. Process conditions affect starch structure and its interactions with proteins in rice pasta. *Carbohydr. Polym.* **2013**, *92*, 1865-1872; DOI:10.1016/j.carbpol.2012.11.047.

35. Bonomi, F.; Mora, G.; Pagani, M.A.; Iametti, S. Probing structural features of water-insoluble proteins by front-face fluorescence. *Anal. Biochem.* **2004,** *329*, 104–111; DOI: 10.1016/j.ab.2004.02.016.

36. Mariotti, M.; Lucisano, M.; Pagani, M.A.; Iametti. S. Macromolecular interactions and rheological properties of buckwheat-based dough obtained from differently processed grains. *J. Agric. Food Chem.* **2008**, *56*, 4258–4267; DOI:10.1021/jf800009e.

37. Eynard, L.; Iametti, S.; Relkin, P.; Bonomi, F. Surface hydrophobicity changes and heat-induced modifications of alpha-lactalbumin. *J. Agric. Food Chem.* **1992**, *40*, 1731-1736; DOI:10.1021/jf00022a002.

38. Fessas D.; Schiraldi A. Water properties in wheat flour dough I: Classical thermogravimetry approach. *Food Chem.* **2001**, *72*, 237-244; DOI:10.1016/S0308-8146(00)00220-X.

39. Pasqualone, A.; Costantini, M.; Labarbuta, R.; Summo, C. Production of extruded-cooked lentil flours at industrial level: effect of processing conditions on starch gelatinization, dough rheological properties and techno-functional parameters. *LWT - Food Science and Technology.* **2021**, *147*, 111580; DOI:10.1016/j.lwt.2021.111580.

40. Aguilera, Y.; Esteban, R.M.; Benitez, V.; Molla, E.; Martin-Cabrejas, M.A. Starch, functional properties, and microstructural characteristics in chickpea and lentil as affected by thermal processing. *J. Agric. Food Chem.* **2009**, *57*, 10682-10688; DOI:10.1021/jf902042r.

41. Singh, U. Functional properties of grain legume flours. *Journal of Food Science and Technology-mysore* **2001**, 38, 191-199.

42. Bresciani, A.; Annor, G.A.; Gardella, M.; Marti, A. Use of the farinograph for gluten-free grains. In *The Farinograph Handbook: Advances in Technology, Science and Applications*, 4th ed.; Bock, J.E., Don., C., Eds.; Woodhead Publ. & Cereals & Grains Assoc. Bookstore: Saint Paul, MN, USA, 2021, *in press*.

43. AACC International. Approved Methods of Analysis, 11th ed.; Cereals & Grains Association, St. Paul, MN, USA, 2011.

44. Marengo, M.; Barbiroli, A.; Bonomi, F.; Casiraghi, M.C.; Marti, A.; Pagani, M.A.; Manful, J.; Graham-Acquaah, S.; Ragg, E.; Fessas, D.; Hogenboom, J.A.; Iametti, S. Macromolecular traits in the African rice Oryza glaberrima and in glaberrima/sativa crosses, and their relevance to processing. *J. Food Sci.* **2017**, *82*, 2298-2305; DOI:10.1111/1750-3841.13853.

45. Iametti, S.; Bonomi, F.; Pagani, M.A.; Zardi, M.; Cecchini, C.; D'Egidio, M.G. Properties of the protein and carbohydrate fractions in immature wheat kernels. *J. Agric. Food. Chem.* **2006**, *54*, 10239-10244; DOI:10.1021/jf062269t.

46. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248-254; DOI:10.1016/0003-2697(76)90527-3.

47. Ellman, G.L. Tissue sulfhydryl groups. *Archives of Biochemistry and Biophysics* **1959**, *82*, 70–77; DOI:10.1016/0003-9861(59)90090-6.

48. Caramanico, R.; Barbiroli, A.; Marengo, M.; Fessas, D.; Bonomi, F.; Lucisano, M.; Pagani, M.A.; Iametti, S.; Marti, A. Interplay between starch and proteins in waxy wheat. *J. Cereal Sci.* **2017**, *75*, 198–204; DOI:10.1016/j.jcs.2017.04.008.

49. Monahan, F.J.; German, J.B.; Kinsella, J.E. Effect of pH and temperature on protein unfolding and thiol/disulfide interchange reactions during heat-induced gelation of whey proteins. *J. Agric. Food Chem.* **1995**, *43*, 46–52; DOI:10.1021/jf00049a010.

50. Mine, Y.; Noutomi, T.; Haga, N. Thermally induced changes in egg white proteins. *J. Agric. Food Chem.* **1990**, *38*, 2122–2125; DOI:10.1021/jf00102a004.

51. Shewry, P.R.; Tatham, A.S. Disulphide bonds in wheat gluten proteins. *J. Cereal Sci.* **1997**, *25*, 207-227; DOI:10.1006/jcrs.1996.0100.

4.7 PULSES – LENTILS. Molecular features and cooking behavior of pasta from pulses

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4.7.1 Abstract

Background and objectives: Pulse pasta is one of the latest responses of the food industry to meet the consumers request for healthy and sustainable foods. Among pulses, red lentils and chickpeas are the preferred raw materials for making 100% pulse pasta. This study aimed at addressing starch and protein features in commercial pulse pasta to provide an insight on how their molecular organization may affect cooking behavior.

Findings: Differences in starch pasting profile and in protein overall organization were found among commercial pasta samples. Considering the same pulse, the best performing pasta showed a protein network characterized by a more compact structure. Regardless of the producer, lentils gave pasta with the best cooking behavior (low cooking loss and high firmness).

Conclusions: Cooking quality of pulse pasta depends on both the type of pulse (chickpeas or red lentils) and pasta-making process. Significance and novelty: This study lays some molecular groundwork as for elucidating the role of individual pulses and of the pasta-making process in determining the quality of pulse pasta.

4.7.2 Introduction

The production of 100% pulse pasta represents the most recent innovation in the pasta industry in response to the consumer request for healthy and sustainable food products (Lascialfari et al., 2019; Tucci et al., 2021). Pasta from 100% lentils, beans, green peas, and chick- peas is currently available on the market; and differences in nutritional traits and cooking behavior have been reported for a few commercial products (Turco et al., 2019). Such differences are related to differences in raw materials and in processing conditions, as both are known to drive the molecular rearrangements affecting the quality of pasta from both wheat (Bock et al., 2015; Bonomi et al., 2012) and gluten-free cereals (Barbiroli et al., 2013; Marti et al., 2010). However, no information is available on the relation between starch and protein features and cooking behavior in pulse pasta. In this study, commercial pasta from 100% chickpea and 100% red lentil flours (each from two different brands) was characterized from several standpoints to address the nature and role of starch and protein features that may impact on cooking behavior.

4.7.3 Materials and Methods

4.7.3.1 Pasta samples

Pasta produced by two companies (brand A and brand B) was considered. For each brand, two production batches of pasta from 100% chickpeas and 100% red lentils were analyzed. Chemical composition as reported in the label is shown in Table S1. Pasta samples were used as such for assessing cooking quality. For furosine, starch, and protein analysis, pasta samples were ground to <250 μm using a laboratory mill (IKA Universalmühle M20; IKA Laborteknic), with a water-cooling system to avoid over- heating. Furosine was determined according to Resmini et al. (1990).

4.7.3.2 Cooking behavior

Pasta cooking quality (i.e., water absorption, cooking loss, and firmness) was assessed at the optimum cooking time (7 min) and upon overcooking (9 min), as reported by Bresciani et al. (2021).

4.7.3.3 Pasting properties

Pasting properties were evaluated by using a Micro Visco-Amylo-Graph (Brabender GmbH), using a heat/ cooling rate of 1.5°C/min (Bresciani et al., 2021).

4.7.3.4 Protein properties

The nature of protein aggregates was assessed by the differential solubility approach followed by SDS-PAGE (Barbiroli et al., 2013; Bonomi et al., 2012) with modifications. In particular, ground pasta (0.15 g) was extracted in 5 ml of saline buffer (50 mM phosphate buffer, 0.1 M NaCl, and pH 7), and the concentration of urea - when present - was kept at 4 M instead of 8 M. Readily accessible and total thiols were assessed by adapting the original protocol (Bonomi et al., 2012), by using 0.05 g sample in 6 ml buffer and 4 M urea.

4.7.3.5 Statistical analysis

Three independent trials were carried out on each sample for water absorption, cooking loss, and texture analysis. Furosine, pasting properties, protein aggregation state, and accessibility of protein thiols were measured in tripli- cate. All results represent the average of the data from two sample batches. Statgraphics Plus 5.1 (StatPoint Inc) was used for data analysis.

Table 1. Cooking behavior of chickpea and red lentil pasta from different brands

		Chickpea pasta		Red lentil pasta	
		Sample A	**Sample B**	**Sample A**	**Sample B**
Heat damage, uncooked pasta	Furosine (mg/100 g)	96***	926	91***	699
Cooking quality at 7 min	Water absorption (g/100 g)	109	113	104	107
	Cooking loss (g/100 $g_{d.m.}$)	11.4***	8.0	8.1***	7.5
	Firmness (*N*)	282***	411	398*	437
Cooking quality at 9 min	Water absorption (g/100 g)	113*	119	123	124
	Cooking loss (g/100 $g_{d.m.}$)	13.1***	8.9	9.1**	8.0
	Firmness (*N*)	270***	407	369**	439

Asterisks indicate significant differences (*t* test; *$p < .05$; **$p < .01$; ***$p < .001$). Separate *t* test was carried out for chickpea and red lentil pasta. Abbreviation: d.m., dry matter.

4.7.4 Results and Discussion

Data will be discussed focusing on the comparison between pasta samples prepared by two companies (A and B), rather than on the comparison between pasta samples made from different pulses (chickpeas or red lentils). Regardless of the producer, differences between pasta prepared from different raw materials (chickpeas and red lentils) are expected, due to differences in the protein nature, abundance, and organization and in starch structure between the two types of pulses (Boye et al., 2010; Keskin et al., 2021; Wani et al., 2016). On the other hand, for the same pulse, differences between brands A and B might be accounted by pasta-making process since their chemical composition—as reported on the label—was similar (Table S1).

Regardless of the type of pulse, sample A showed a higher cooking loss and lower firmness than sample B, either at optimal cooking time or upon overcooking (Table 1). Brand-related differences in cooking behavior were greater in chickpea pasta than in red lentil pasta. The heat damage parameters in Table 1 indicate that pasta from brand B was likely produced using a different heat treatment than A, regardless of the pulse used as the in- gredient. Furosine (ε-N-furoylmethyl-l-lysine) is the most widely used molecular marker of Maillard reaction in pasta (Resmini & Pellegrino, 1994). In wholegrain pasta, furosine levels higher than

300 mg/100 g protein are indicative of a mild heat damage and medium temperature drying cycle (Marti et al., 2017).

When comparing chickpea pasta, pasta A exhibited a significant lower maximum viscosity ($p < .01$) and set- back ($p < .001$) values, indicating lower swelling capacity and retrogradation tendency, respectively (Figure 1, upper panel). No significant differences in starch swelling were measured in red lentil pasta (Figure 1, lower panel). On the contrary, sample A exhibited a slight (but significant) higher retrogradation tendency than B, contrarily to what found for chickpea pasta. Data suggested that the pasta- making process adopted by the two brands might differently impact the starch properties of chickpeas and red lentils. Differences in starch organization in the different pulses might account for the obtained results.

Differential solubiity is a way of discriminating among the different intermolecular bonds in protein aggregates, by quantifying protein soluble in saline buffer (ionic interactions), in high urea (hydrophobic interactions), and in urea/dithiothreitol (disulfide bonds). No major differences between samples belonging to the same pulse type and no qualitative difference among protein solubilized in different conditions was evident from SDS-PAGE (Figure S1). However, the solubility data suggested some difference in the nature of the polymeric network of various pasta samples. As for the pasta from different pulses, the data shown in Table 2 indicated that more proteins are soluble in chickpea pasta than in red lentil pasta, suggesting that the pasta processes had a different impact on proteins from individual pulses.

By comparing the solubility figures in Table 2, it is evident that about 50%–60% of the total solubilized proteins were bound by ionic interactions in chickpea pasta, with respect to about 40% in red lentil pasta, suggesting a lower overall level of denaturation/aggregation in chickpea pasta. As for red lentils, pasta A was the only sample in which protein solubility did not in- crease further when a disulfide reductant was added to the urea/buffered saline extractant, suggesting that low heat treatment (see Table 1) may impair or prevent the formation of red lentil protein aggregates stabilized by covalent disulfide bonds. On the contrary, in pasta B, we observed an increase in the amount of proteins solubilized by buffered saline in the presence of urea and of a disulfide reductant, suggesting that the treatment used for the production of pasta promotes the formation of protein aggregates stabilized by covalent disulfide bonds. As for the chickpea pasta, the solubility data suggested a different protein polymerization between samples A and B. However, the effect of thermal treatments on the interprotein interactions is much less evident in chickpea pasta.

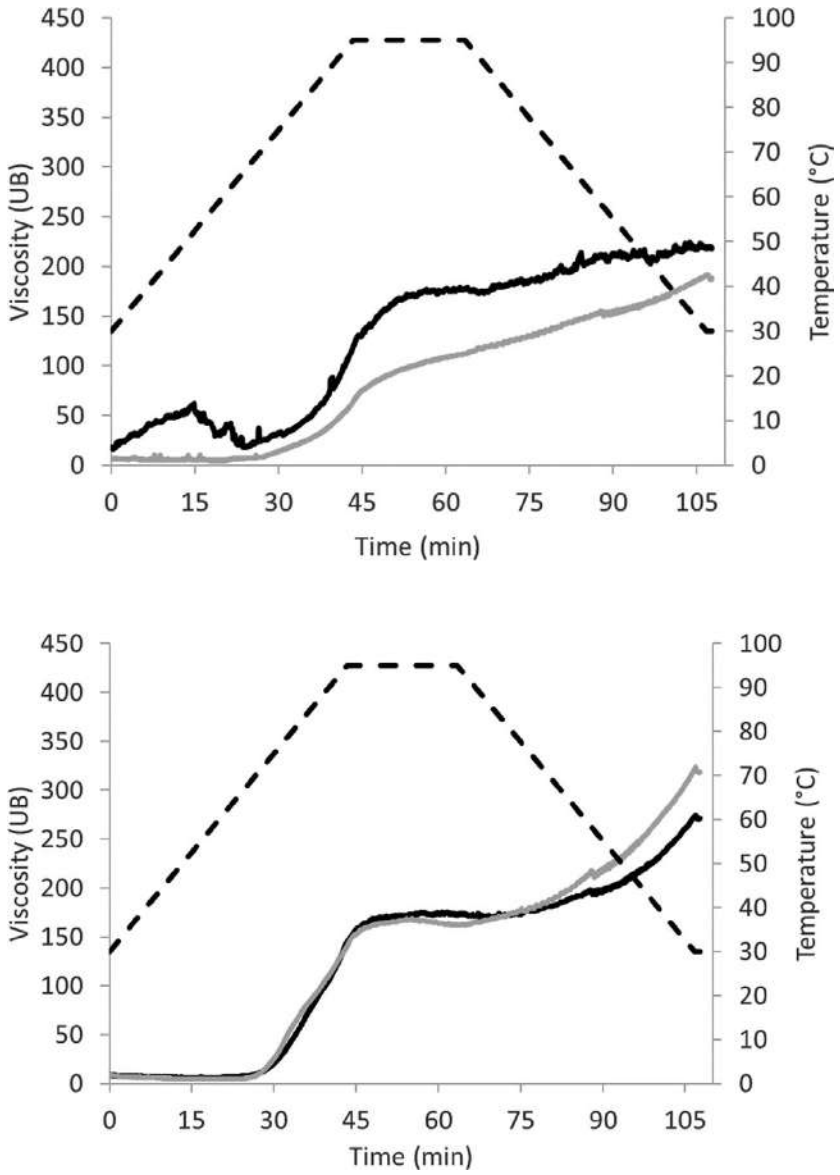

Figure 1. Pasting properties of chickpea (upper panel) and red lentil (lower panel) pasta from brands A (gray solid line) and B (black solid line). One representative curve for each sample was reported. Black dotted line: temperature profile

Regardless of the type of pulse, high-temperature-treated pasta B had a lower content of readily accessible thiol residues, and thiol accessibility in pasta B was markedly increased upon addition of urea. Thus, thiol residues in pasta B are somehow hidden within protein aggregates stabilized by hydrophobic interactions, similar to what is observed for high-temperature dried semolina pasta (Bock et al., 2015; Bonomi et al., 2012) or in pasta nonpulse from gluten-free materials (Barbiroli et al., 2013; Marengo et al., 2015).

All the pasta samples exhibited a good cooking quality (Table 1), as indicated by the following observations: (1) cooking losses were similar - or even lower - than those reported for commercial pasta from either pulses (Turco et al., 2019) as well as from gluten-free

cereals/pseudo cereals (Morreale et al., 2019); (2) instrumental firmness was similar to semolina pasta (data not shown); and (3) cooking loss and firmness did not dramatically change upon overcooking.

The stiffer protein network formed by some high- temperature treatment in the production of sample B resulted in a better cooking behavior than sample A, especially in the case of chickpea pasta. Differences among the samples cannot be accounted simply by their protein content, since it was similar (21%) in samples A and B (see Table S1). In semolina pasta, high-temperature drying promotes the formation of a continuous network that traps the swollen starch granules upon cooking, ensuring a firm texture and low leaching (De Noni & Pagani, 2010). Low-temperature drying leads to semolina pasta with high pasting viscosity (Bonomi et al., 2012; Marti et al., 2013), but an opposite trend was evident in chickpea pasta, whereas no difference was apparent for red lentil pasta, suggesting that factors other than drying temperature might intervene.

Table 2. Differential solubility of chickpea and red lentil pasta protein from different brands

		Chickpea pasta		Red lentil pasta	
		Sample A	**Sample B**	**Sample A**	**Sample B**
Protein solubility (mg protein/g pasta)	Buffered saline	160^{a}	221^{b}	112^{A}	117^{A}
	Buffered saline + 4 M urea	271^{c}	305^{cd}	291^{D}	231^{B}
	Buffered saline + 4 M urea + 10 mM DTT	322^{d}	375^{e}	300^{D}	270^{C}
Accessibility of cysteine thiols (μmol SH/g pasta)	Buffered saline	1.54^{b}	0.49^{a}	1.17^{C}	0.26^{A}
	Buffered saline + 4 M urea	2.04^{c}	1.37^{b}	1.50^{D}	0.86^{B}

Samples with the same superscript letter are not significantly different (Tukey's honestly significant difference test, $p < .05$). Separate one-way analysis of variance (ANOVA) was carried out for chickpea (lowercase letters) and red lentil (uppercase letters) pasta. Abbreviation: DTT, dithiothreitol.

4.7.5 Conclusions

SDS-PAGE profiling indicated that the same proteins were present in the samples from the same pulse in closely comparable amounts. However, it is possible that their structure in the starting materials was not similar, so that sensitivity to heat treatment or to shear forces in the pasta-making process was different. Of course, as commented above, also the process conditions used by each individual producer were different. In this frame, characterization of the pulse preparation used by individual producers and/or availability of samples taken at various processing steps would have provided valuable information on process-related changes, including those recently reported for starch in extrusion-cooked yellow lentil products (Bresciani et al., 2021).

4.7.6 References

Barbiroli, A., Bonomi, F., Casiraghi, M. C., Iametti, S., Pagani, M. A., & Marti, A. (2013). Process conditions affect starch structure and its interactions with proteins in rice pasta. Carbohydrate Polymers, 92, 1865–1872. https://doi.org/10.1016/j.carbp ol.2012.11.047

Bock, J. E., West, R., Iametti, S., Bonomi, F., Marengo, M., & Seetharaman, K. (2015). Gluten structural evolution during pasta processing of refined and whole wheat pasta from hard white winter wheat: The influence of mixing, drying, and cooking. Cereal Chemistry, 92, 460–465. https://doi.org/10.1094/ CCHEM-07-14-0152-R

Bonomi, F., D'Egidio, M. G., Iametti, S., Marengo, M., Marti, A., Pagani, M. A., & Ragg, E. M. (2012). Structure-quality relationship in commercial pasta: A molecular glimpse. Food Chemistry, 135, 348–355. https://doi.org/10.1016/j.foodc hem.2012.05.026

Boye, J., Zare, F., & Pletch, A. (2010). Pulse proteins: Processing, characterization, functional properties and applications in food and feed. Food Research International, 43, 414–431. https://doi. org/10.1016/j.foodres.2009.09.003

Bresciani, A., Giuberti, G., Cervini, M., & Marti, A. (2021). Pasta from yellow lentils: How process affects starch features and pasta quality. Food Chemistry, 364, 130387. https://doi.org/10.1016/j. foodchem.2021.130387

De Noni, I., & Pagani, M. A. (2010). Cooking properties and heat damage of dried pasta as influenced by raw material character- istics and processing conditions. Critical Reviews in Food Science and Nutrition, 50, 465–472. https://doi.org/10.1080/10408 390802437154

Keskin, S. O., Ali, T. M., Ahmed, J., Shaikh, M., Siddiq, M., & Uebersax, M. A. (2021). Physico-chemical and functional prop- erties of legume protein, starch, and dietary fiber—A review. Legume Science, e117. https://doi.org/10.1002/leg3.117

Lascialfari, M., Magrini, M. B., & Triboulet, P. (2019). The drivers of product innovations in pulse-based foods: Insights from case studies in France, Italy and USA. Journal of Innovation Economics, 1, 111–143. https://doi.org/10.3917/jie.028.0111

Marengo, M., Bonomi, F., Marti, A., Pagani, M. A., Abd Elmoneim, O. E., & Iametti, S. (2015). Molecular features of fermented and sprouted sorghum flours relate to their suitability as components of enriched gluten-free pasta. LWT - Food Science and Technology, 63, 511–518. https://doi.org/10.1016/j. lwt.2015.03.070

Marti, A., Cattaneo, S., Benedetti, S., Buratti, S., Abbasi Parizad, P., Masotti, F., Iametti, S., & Pagani, M. A. (2017). Characterization of whole grain pasta: Integrating phys- ical, chemical, molecular, and instrumental sensory approaches. Journal of Food Science, 82, 2583–2590. https://doi. org/10.1111/1750-3841.13938

Marti, A., Seetharaman, K., & Pagani, M. A. (2010). Rice-based pasta: A comparison between conventional pasta-making and extrusion-cooking. Journal of Cereal Science, 52, 404–409. https://doi.org/10.1016/j.jcs.2010.07.002

Marti, A., Seetharaman, K., & Pagani, M. A. (2013). Rheological approaches suitable for investigating starch and protein proper- ties related to cooking quality of durum wheat pasta. Journal of Food Quality, 36, 133–138. https://doi.org/10.1111/jfq.12015

Morreale, F., Boukid, F., Carini, E., Federici, E., Vittadini, E., & Pellegrini, N. (2019). An overview of the Italian market for 2015: Cooking quality and nutritional value of gluten-free pasta. International Journal of Food Science & Technology, 54, 780–786. https://doi.org/10.1111/ijfs.13995

Resmini, P., & Pellegrino, L. (1994). Occurrence of protein-bound ly- sylpirrolaldehyde in dried pasta. Cereal Chemistry, 71, 254–262. Resmini, P., Pellegrino, L., & Battelli, G. (1990). Accurate quantifi- cation of furosine in milk and dairy products by a direct HPLC method. Italian Journal of Food Science, 2, 173–183.

Tucci, M., Martini, D., Del Bo, C., Marino, M., Battezzati, A., Bertoli, S., Porrini, M., & Riso, P. (2021). An Italian-Mediterranean di- etary pattern developed based on the EAT-Lancet reference diet (EAT-IT): A nutritional evaluation. Foods, 10, 558. https://doi.org/10.3390/foods10030558

Turco, I., Bacchetti, T., Morresi, C., Padalino, L., & Ferretti, G. (2019).Polyphenols and the glycaemic index of legume pasta. Food and Function, 10, 5931–5938. https://doi.org/10.1039/C9FO00696F Wani, I. A., Sogi, D. S., Hamdani, A. M., Gani, A., Bhat, N. A., & Shah, A. (2016). Isolation, composition, and physicochemical properties of starch from legumes: A review. Starch-Stärke, 68, 834–845. https://doi.org/10.1002/star.201600007

4.8 PSEUDOCEREALS - BUCKWHEAT. Proteins characterization in sprouted buckwheat

4.8.1 Background

Buckwheat (*Fagopyrum esculentum*) is one of the ancient domesticated crops of Asia, Central, and Eastern Europe that has been mainly used as a staple food especially in arid regions of the world (Farooq et al.,2016). Buckwheat is a pseudocereal particularly rich in functional and nutraceutical components (Mariotti et al., 2008; Wijngaard & Arendt, 2006) and represents a protein source with high biological value. Its seed contains antioxidants, such as rutin, tocopherols and phenolic acids, and can thus be stored for a long time without apparent chemical changes (Dietrych-Szostak & Oleszek, 1999). In particular the rutin – a flavonol glycoside plant metabolite - content of buckwheat is higher than most plants, making that pseudocereal interesting for the anti-oxidative, -inflammatory and -carcinogenic effects associated with rutin, and can also reduce the fragility of blood vessels related to hemorrhagic disease and hypertension in humans (Oomah & Mazza, 1996). Moreover the interest for buckwheat is increased by the fact that it does not contain coeliac-toxic sequences and therefore is suitable for the production of gluten-free foods.

Environmental problems like drought and global warming are the most prominent abiotic stresses limiting crop production and productivity. Buckwheat represent a sustainable crop as it does not require particular agronomic practices (Farooq et al.,2016). In this scenario the interest in buckwheat has been renewed as an alternative crop and health food (Biacs et al., 2002). It is also an excellent candidate for the development of new processes/products, offering new possibilities to the market and consumers (Zanoletti et al., 2017). In spite of the high protein and fiber content, the use of buckwheat in human nutrition may be limited by the presence of several anti-nutritional factors (e.g. the phytic acid) that limit nutrient bioavailability. Sprouting and fermentation are green, cost friendly and easy to scale up food processing employed for improving e the bioavailability of nutrients and bioactive compounds a and for obtaining foods with enhanced nutrition quality from sustainable species (Barbiroli et al., 2022). During sprouting, high-molecular-weight reserve substances present in the seeds (proteins, carbohydrates and fats) are broken down into simple compounds that are easily assimilated by the human body. Furthermore, enzymes taking part in the breakdown of these substances facilitate the digestion of foods (Zych-Wężyk et al, 2012).

In this study we investigate the overall protein features and antioxidant properties of the sprouted buckwheat, as such and after its transformation in couscous, in comparison with the non-sprouted buckwheat.

4.8.2 Materials and Methods

4.8.2.1 Materials

Buckwheat grains, commercial flour and couscous were provided by Molino Filippini (Teglio, SO, Italy). Sprouted grains were produced in lab scale sprouted apparatus (Memmert GmbH Co. KG, Schwabach, Germany). After 16h of soaking (27°C, with a grains:water ratio of 1:3), the grains were treated at 27°C, 90% of relative humidity for 48 and 72h, respectively and dried was at 50°C for 8h. The sprouted grains were grinded using a M20 Universal Mill (IKA, Werke Staufen, Germany; particle size < 500μm).

Couscous were produced without and with addition of25 and 50% of sprouted flours obtained by treatment for Couscous were produced with non-sprouted flour, added when indicated of 25 and 50% sprouted flour for 48 and 72 hours in lab scale plant. The couscous sample was milled in a bead beater with two cycle at 30 s^{-1} for 30 s with one minute of stop between the cycles. The details of the samples (flours and couscous) are reported in table 1.

Table 1: List of the samples prepared and used in this work

Sample	Sample identification	Sprouting time (h)	Level of substitution with sprouted flour (%)
Buckwheat flour	Standard flour	n.s.	0
Sprouted flour 48h	48h flour	48	0
Sprouted flour 72h	72h flour	72	0
Couscous 100% STD	Standard couscous	n.s.	0
Couscous 25% 48h + 75%	48h couscous (25%)	48	25
Couscous 50% 48h + 50%	48h couscous (50%)	48	50
Couscous 25% 72h + 75%	72h couscous (25%)	72	25
Couscous 50% 72h + 50%	72h couscous (50%)	72	50

Samples with n.s. was not sprouted.

4.8.2.2 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

For SDS-PAGE, the samples were prepared by suspending 5 mg of flour and milled couscous in 100 μL of 0.5 M Tris-HCl pH 6.8 buffer and 100 μL of denaturing buffer (0.125 M Tris-HCl, pH 6.8, 50% glycerol, 1.7% SDS, 0.01% bromophenol blue (w/v)), containing 1% (v/v) 2-mercaptoethanol (2-ME), when indicated. The suspension was then heated at 100 ◦C for 10 min. The electrophoretic run was performed at pH 8.8 (0.025 M Tris-HCl, 0.192 M glycine, 0.1% (w/v) SDS), in a Miniprotean II cell (Bio-Rad), and the gels were Coomassie Blue-stained.

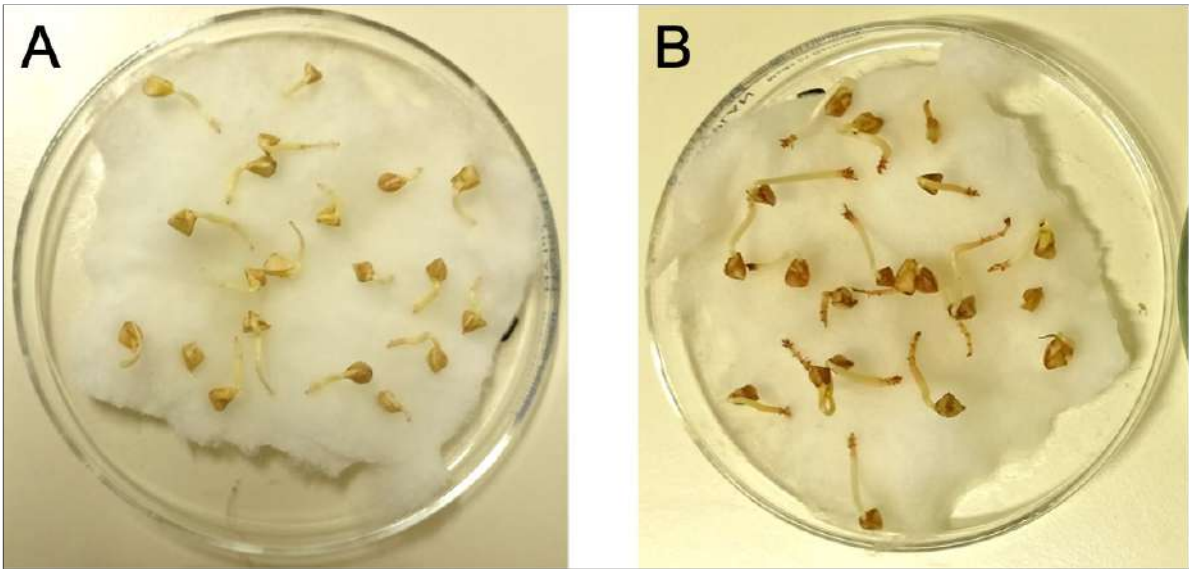

Figure 1. Grains at the end of sprouting for 48 (panel A) and 72H (panel B).

4.8.2.3 Proteolytic activity

Evaluation of proteolytic activity was adapted from Cardone et al. (2020) by using azocasiein as substrate. 500 mg of flour were suspended in 5 mL of 50 mM Tris-HCl buffer in presence of 0.1 M NaCl at pH 7.5, incubating suspension for 2 h. The extracts was centrifuged (1,500 ×*g*) for 15 min and 500 µL of supernatant was incubated with 500 µL of azocasein (1% (w/v), prepared in 0.5 M Tris-HCl, pH 8) for 2 h at 37° C. At the end, 1mL of trichloro acetic acid (TCA, 10% final concentration) was added and incubate in ice for 10 min, followed by centrifugation at 13,000 ×*g* for 10 min. To develop the color to 1 mL of supernatant was added to 80 µL of 0.5 M NaOH. The absorbance was read at 440 nm using a Perkin-Elmer Lambda 2 UV/VIS Spectrometer (Perkin- Elmer Inc., Waltham, MA, USA). The proteolytic activity was expressed as mg of azocasein hydrolyzed in 1 h by 1 g of flour sample, and reported as mean ± standard deviation of three replicates.

4.8.2.4 Chromatographic characterization

Reverse Phase High Performance Liquid Chromatography (RP-HPLC) separations were performed on an extract obtained by suspending 150 mg of sample in 5.0 mL of 0.1% trifluoroacetic acid (TFA) for 60 min under mild agitation and followed by centrifugation at 10,000 ×*g* for 10 min. The supernatant was then precipitated by adding 5% TFA (final concentration) and centrifuged (13,000 ×*g* for 10 min). The clear supernatant was used for the chromatographic characterizations.

Sample extracts were injected in a SIMMETRY300 C18 (5 µm) (4.6 × 250 mm) column (Waters) fitted on a chromatographic apparatus composed by a Waters 600E multisolvent

delivery system and a Waters 2487 Dual λ Absorbance Detector (Waters, Sesto San Giovanni, Italy). Separations were run at 0.8 mL/min, mixing solution A (0.1% TFA in water) and solution B (0.1% TFA in acetonitrile (ACN)) as follows: 5 min isocratic 100% solution A, 45 min linear gradient to 50% solution A and 50% solution B.

4.8.2.5 Phytic acid quantification

Quantification of phytic acid in flour samples were carried-out by using the PHYTIC ACID (PHYTATE)/TOTAL PHOSPHORUS kit (K-PHYT) from Megazyme using a Perkin-Elmer Lambda 2 UV/VIS Spectrometer (Perkin- Elmer Inc., Waltham, MA, USA). Data were expressed as mean ± standard deviation of g of phytic acid on 100 g of flour from three replicates.

4.8.2.6 Proteins solubility

The solubility of proteins in native and denaturing/reducing conditions was evaluated according to Iametti et al. (2006). 150 mg of flour were suspended in 6 mL of saline buffer (50 mM sodium phosphate, 0.1 M NaCl, pH 7.0), containing 4 M urea and 10 mM dithiothreitol (DTT), when indicated. The suspension was stirred for 90 min at 25 °C and subsequently centrifuged (10,000× g for 20 min, 25 °C). The quantification of proteins in the supernatant was carried out by a dye-binding method (Bradford, 1976) using a Perkin-Elmer Lambda 2 UV/VIS Spectrometer (Perkin-Elmer Inc., Waltham, MA, USA). Results were expressed as mean ± standard deviations of three replicates.

4.8.2.7 *In vitro* digestion

Couscous samples were coocked according to the producer indication, and digested under *in vitro* oral gastrointestinal human digestion conditions (Hollebeeck et al., 2013). Briefly, all three stages, salivary (pH 6.9, final volume 10.433 mL of MiliQ water, 5 min, 3.9 U α-amylase/mL, aerobic), gastric (pH 2, final volume 13.6 mL, 90 min, 71.2 U pepsin/mL, aerobic) and abiotic duodenal step (pH 7, final volume 16 mL, 150 min, 9.2 mg pancreatin/mL, aerobic, in presence of 55.2 mg/mL of porcine bile extract) were performed in the same centrifuge tube at 37 °C, shaking all the mixture at 350 rpm in a ThermoMixer® C (Eppendorf). At the end of the three steps, the reaction was stopped by freezing the digested overnight at -20°C. To remove the bile salt, the defrosted digested was centrifugated at 5,000 ×*g* at 4°C for 20 minutes. To the supernatant phase, the cholestyramine (Sigma) activated resin was added (final concentration 10% (m/v)) shaking the mix for 1 h at 350 rpm in ThermoMixer® C (Eppendorf). Resin was removed by centrifugation at 5,000 ×*g* for 20 min and the supernatant was gravimetrically filtered. The filtered digest was frozen at -20 °C until further analysis.

The activation of the cholestyramine resin was activated with PBS buffer in a ratio 1:20 and shaking for 15 min, followed by gravimetric filtration and drying overnight at 70°C. The activated activated resin was stored under dry conditions in a desiccator until use.

4.8.2.8 Preparation of ABTS working solution

Stock solution of 2,2'-azinobis(3- ethylbenzothiazoline-6-sulfonic acid) (ABTS) was prepared by mixing a ABTS aqueous solution with potassium persulfate (7 mM and 2.40 mM final concentrations, respectively) and kept in dark at room temperature for 12–16 h to obtain a stable ABTS cationic radical ($ABTS^{\bullet+}$). Working solution of ABTS was prepared by mixing the $ABTS^{\bullet+}$ cationic radical solution in a ratio of 1:75 with 10 mM PBS buffer at pH 7.4, until an absorbance at 734 nm of 0.7 ± 0.02 was achieved (adjusted by adding more ABTS or PBS) (Re et al, 1999).

4.8.2.9 Evaluation of the Total Antioxidant Capacity (TAC)

4.8.2.9.1 Direct measurement of the TAC with the ABTS-QUENCHER method (QUENCHER)

Direct QUENCHER (QUick, Easy, New, CHEap and Reproducible) method was described by Gökmen et al. (2009). The essay was carried out for buckwheat flour and couscous adapting the assay from Serpen et al. (2008). Solid sample particles were diluted 20-fold with powdered cellulose to fit into the linearity range of the method. Dilution with cellulose allowed weighing 10 mg per sample, thus ensuring good reproducibility, also for materials with high antioxidant capacity (Serpen et al., 2007). 10 mg of the solid-diluted samples were transferred to a centrifuge tube. The reaction was started by adding 6 mL of ABTS working solution and, after mixing the suspension with a vortex. The sample was incubated with constant stirring in a ThermoMixer® C (Eppendorf) at 600 rpm,37°C for 25 minutes. At the end of the incubation time the samplewas centrifuged at 9,000× *g* for 5 min at 25°C. Three thunder µL of sample were used for the quantification of the TAC, reading the absorbance at 734 nm in an Epoch 2 microplate spectrophotometer (BioTek). The antioxidant capacity was expressed as mmol of Trolox equivalent antioxidant capacity per kg of sample by means of a calibration curve (Serpen et al. 2012). The calibration curve was realized by performing the assay described above on 10 mg of cellulose mixed with 100 µL of diluted Trolox (starting from a stock solution of 4 mM prepared in 50% methanol) and 5.9 mL of the ABTS working solution. Data were then read against a blank containing 10 mg of cellulose added with 100 µL of PBS and 5.9 mL of ABTS working solution.

4.8.2.9.2 Indirect measurement of the TAC on the extract and digested samples

The indirect TAC quantification was performed on a saline and alcoholic extract, and after the *in vitro* digestion according to Martinez-Saez et al. (2014).

Saline and alcoholic extract were prepared by suspending 500 mg of sample in 3.75 mL of 50 mM sodium phosphate at pH 7.0, containing 0.1 M NaCl or in 65 volumes ethanol 96% and 35 volumes of 0.3 M HCl (Abasi et al., 2020). Extractions were performed in a ThermoMixer® C (Eppendorf) by shaking it at 350 rpm for 4 hours at 37°C in the dark. At the end the samples were centrifuged at 10,000 × g for 15 min at 25°C and the supernatant phase was recovered and stored at -20°C.

The indirect TAC measurement was performed by mixing 30 µL of extract with 270 µL of ABTS working solution in a microplate. The absorbance was measured at 734 nm for one hour at 25°C in an Epoch 2 microplate spectrophotometer (BioTek) with measurements every 5 min. Sample blank and reagent blank were also analysed in each set of samples. Results were expressed as mmol of Trolox equivalent (TE) and rutin equivalent (RE) antioxidant capacity per kg (Serpen et al. 2012).

4.8.2.10 Quantification of the Total Phenolic Content (TPC)

The TPC was performed on the extracts and digested samples by using the Folin-Chiocolteau reagents (Singleton et al.,1999). The extracts used for the TPC were obtained as described for the TAC measurement. TPC protocol was adapted from Contini et al. (2008). The reaction was started by mixing 10 µL of sample with 150 µL of Folin–Ciocalteu solution. After incubation at room temperature for 3 min, 50 µL of 0.28 M sodium carbonate solution were added and the absorbance was read at 735 nm after 120 minutes of incubation at 37°C. Sample blank and reagent blank were also analyzed in each set of samples. Results were expressed as mmol of gallic acid equivalent (GAE) and rutin equivalent (RE) per kg of sample. All measurements were performed in triplicate.

4.8.2.11 Statistical analysis

Analysis of variance (ANOVA) and Tuckey's test were applied to determine differences between means. Differences were considered to be significant at $p < 0.05$.

4.8.3 Results

4.8.3.1 Effect of sprouting and processing on the buckwheat protein pattern

The effect of sprouting and transformation process on proteins are reported in figure 2. A first approach to evaluate the action of endogenous seed proteases after short-time sprouting (48-72h) was represented by electrophoresis. The comparison of the protein profiles in both non-reducing (panel A) and reducing (panel B) conditions showed that a modification of the

protein pattern is achieved. Analyzing the protein pattern under non-reducing condition (panel A), bands of buckwheat proteins were found at 41kDa (proposed to be albumin) and 56–69kDa (proposed to be legumin-type globulins), and minor bands were found at 57–58 kDa (proposed to be vicilin-type proteins), 26–36 kDa and 14–23 kDa (Radovic et al., 1996; Ma and Xiong 2009). In reducing condition (panel B) the 56–69 kDa bands dissociated into low-molecular weight subunits whereas the 41kDa and minor bands remained as such (Radovic et al., 1996; Guo and Yao, 2006). Thus, the major globulin fraction was proposed to be composed of acidic and basic subunits that are connected through disulfide linkages (Radovic et al. 1996).

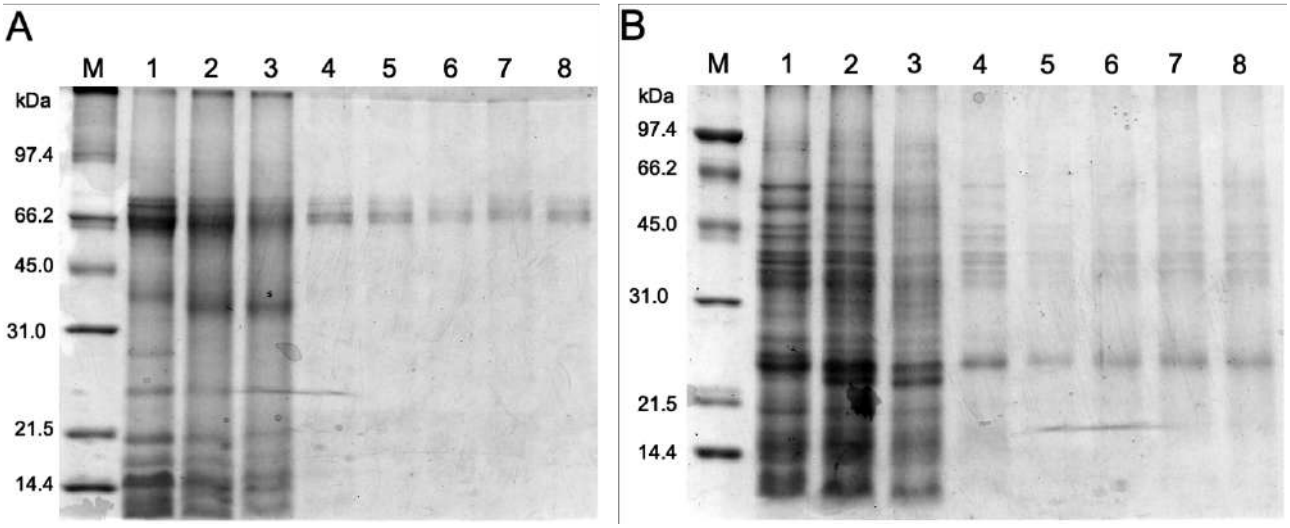

Figure 2. SDS-PAGE in non-reducing (panel A) and reducing (panel B) conditions; M) Marker, 1) Standard flour, 2) Standard flour, 3) 48h flour, 4) 72h flour, 5) Standard couscous, 6) 48h couscous (25%), 7) 48h couscous (50%) 8) 72h couscous (25%), 9) 72h couscous (50%).

As the sprouting time proceed the main modification that can be observed are imputable to the 13S globuline, that mainly consist of about eight different forms of subunits within a molecular weight range from 68 to 43 kDa (Radović et al., 1996). From the SDS-PAGE is possible to observe a wide range of modification at 66 kDa, with an increment of species with molecular weight under 45kDa. The protein pattern evolution, in both the condition, is consistent with Dunaesky et al., (1989) who reported that main modification that can be observed in 13S globulin regards subunits with molecular weights of 57.5, 47.6 and 44.7 kDa with strongly modification during the seed sprouting, in particular with longer sprouting time. The sprouted sample moreover shows a high proteolytic activity, in particular the 72h sample (0.30 ± 0.04 mg azocasein $\times h^{-1} \times$ g flour dwb^{-1}) is significantly different ($p < 0.05$) from the 48h (0.13 ± 0.02 mg azocasein $\times h^{-1} \times$ g flour dwb^{-1}), that in turn is not significantly different from the non-sprouted (0.077 ± 0.004 mg azocasein $\times h^{-1} \times$ g flour dwb^{-1}).

To better understand the protein pattern evolution observed in SDS-PAGE reported in figure 2, RP-HPLC was realized. From those results (figure 3) can be suggested that the activity of the endogenous seed proteases broke down the protein structure releasing peptides. The chromatograms of thesamples obtained at different treatment times (48 and 72 h) showed differences at 25 min and 28-29 min, retention times. The sample obtained at 72h a showed peak with higher areas.

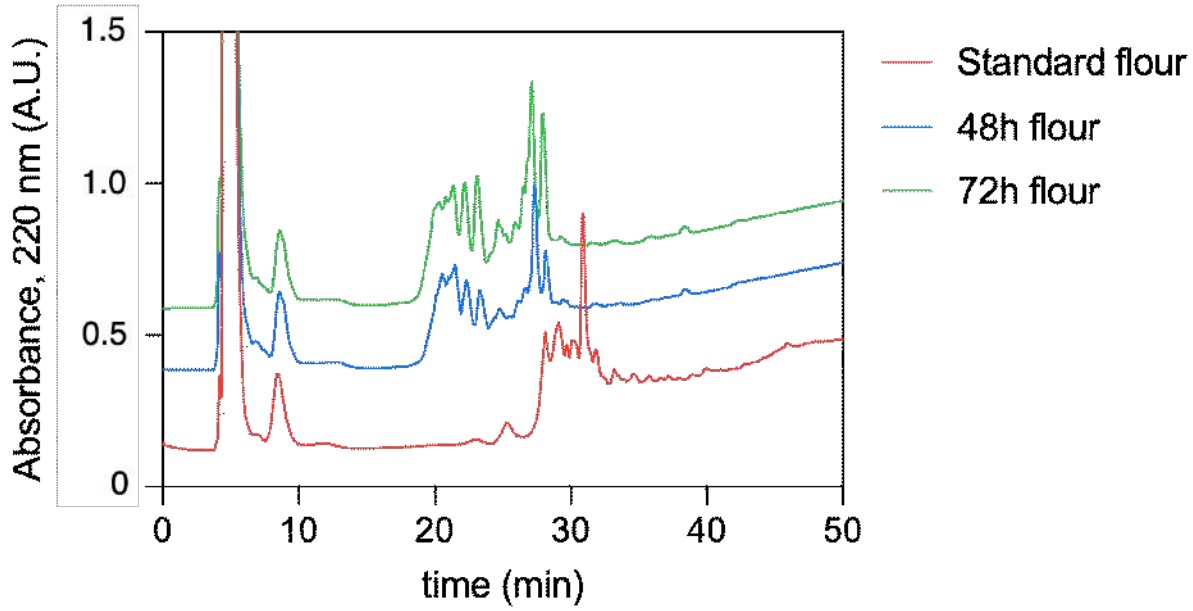

Figure 3. Chromatogram from RP-HPLC of buckwheat flour samples and after 48h and 72h of sprouting.

Couscous proteins (figure 2, lanes 4 to 8) appear to be very little extractable in SDS-PAGE. Only a band with a molecular weight around 66 kDa can be observed under non-reducing conditions (figure 2, panel A) whereas under reducing conditions (fig.2, panel B) a wider distribution of bands at different molecular weights can be observed. In both non-reducing and reducing conditions, couscous proteins have a very low intensity compared to the buckwheat flour. Therefore, the transformation process seems to greatly influence the extractability of the proteins.

The nature of the protein-protein interaction was assessed by differential solubility studies (figure 4) was carried out on the Standard and 72h flours, as well as on Standard and the 72h couscous (50%) (i.e., the couscous with the highest percentage of replacement). The high amount of proteins solubilized in saline buffer is consistent with the nature of proteins in buckwheat. Winjingaard and Arendt (2006) report that common buckwheat seeds consist of 64.5% globulin,12.5% albumin, 8.0% glutelin, and a small percentage of prolamins (2.9%).

The sprouting of 72h deeply affect the total protein pattern, as observed in SDS-PAGE, with a lower amount of proteins that are solubilized. Adding urea the protein extractability seem

increased, suggesting that some proteins are poorly soluble by their nature, or because they are aggregated. The addition of DTT does not seem to further increase the extractability, so the disulfide bridges looks to be less relevant. The transformation into couscous further decreases the protein solubility, probably because of the increase of overall compactness of the system increases. Interestingly, the solubility in urea/DTT in couscous is slightly higher than in urea alone, suggesting that disulfide bridges may play a role in stabilizing the aggregates. Is interesting to underline that the sprouting process affect the protein structure with changes in the hydrophobic patches that, in the standard flour results to be prominent, while in the sprouted and in the two analyzed couscous look lower and (in the case of the couscous) non-significantly different. The same trend is shown for the covalent interactions, that are higher in the standard flour, but are substantially the same in all the other samples.

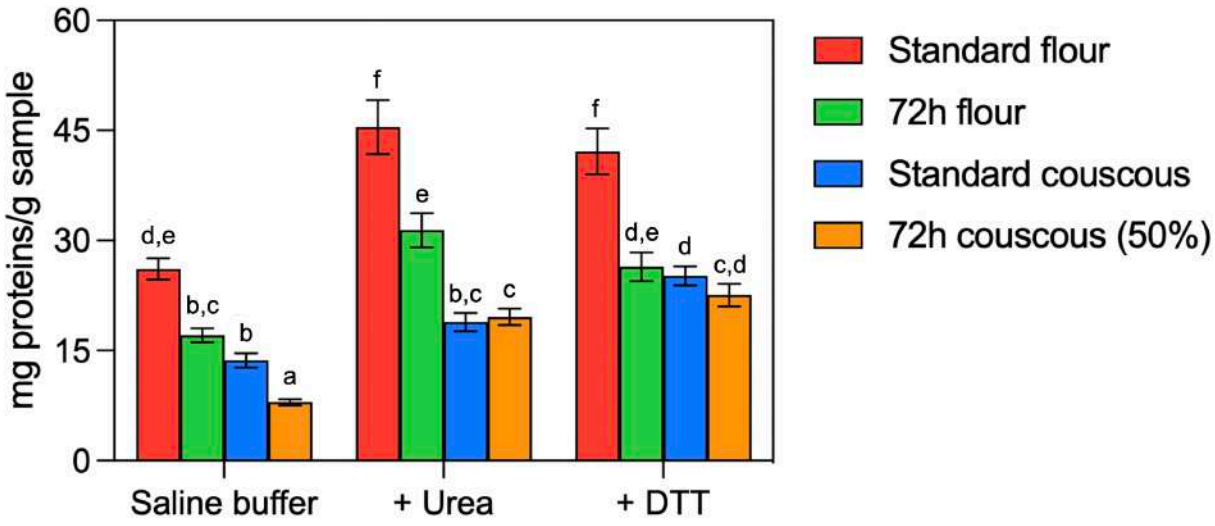

Figure 4. Differential solubility of samples; data were compared grouped for the different condition. Samples with the same superscript letter are not significantly different (Tukey's honestly significant difference test, $p < 0.05$).

From all of the presented data is clear that the sprouting treatment of buckwheat for 48 and 72 hours is responsible for modification in the protein pattern. This is the consequence of activation of various enzymes resulting in modifications of the nutritional and structural properties of the macromolecules. The modification of the protein pattern in sprouted samples is so characterized by the formation of peptides and a less compact structures. Comprehending the effect of sprouting on protein is important since these components are responsible for the technological properties and digestibility of final product.

4.8.3.2 Changes in the nutritional properties

4.8.3.2.1 Quantification of phytic acid

Buckwheat seeds are a good source of many essential minerals that must be obtained through the diet, mostly from plants. Phytic acid is a strong chelator of various metal cations, especially potassium, magnesium and calcium, forming phytate. Phytic acid and associated minerals are deposited in protein bodies of the embryo (cotyledons and axis tissues) and aleurone layer of seeds. While phytic acid provides storage of many minerals in plant tissues, as a dietary component it has been labelled an “anti-nutrient” because of its potential to reduce mineral absorption (Steadman et al., 2001). After 72h of sprouting the phytic acid content show a significant ($p < 0.05$) decrement compared to the non-sprouted flour (1.14 ± 0.03 and 1.33 ± 0.03 g phytic acid × 100 g of flour $^{-1}$, respectively).

4.8.3.2.2 Evaluation of the Total Antioxidant Capacity (TAC)

Buckwheat has a high antioxidant activity and is rich in rutin, catechins, and other polyphenols, which have significant dietary value (Ren and Sun, 2014). The measure of TAC represents a useful tool to give the health potential of antioxidant-rich products in a glance (Gökmen et al., 2009), with a growing interest as a tool for exploring the putative role of that products in the prevention of degenerative diseases (Serafini et al., 2002) and for the selection of varieties with potentially positive health benefits (Frusciante et al., 2007). However, the TAC determination is quite complicated because food contained a mixture of hydrophilic and lipophilic compounds, which can be in free, form as well as bound to other macromolecules. In cereals moreover most antioxidants are covalently bound to cell wall (Perez-Jimenez and Saura-Calixto, 2005), resulting in a more difficult evaluation of the TAC. Therefore, there is not a unique solvent or mixture of solvents that can solubilize all antioxidant compounds embedded in the food matrices. For these regions, we evaluated the TAC with different approach (table 2), starting with the QUENCHER (QUick, Easy, New, CHEap and Reproducible) methods, followed by the determination of the TAC on saline and alcoholic extract. In general, the procedure followed for the TAC measurement is based on the direct measurement on solid (in the case of QUENCHER) or liquid sample by mixing them with the free radicals (ABTS) followed by a subsequent spectrophotometric measurement.

Results reported in table 2 show the effect of the sprouting and processing of the TAC. Flour was the one that showed always the higher TAC value, in particular when was evaluated on the saline extract. When TAC was evaluated with the QUENCHER the value were higher (for the flour and the couscous) than with the extract; this evidence suggest that a large part of the antioxidant species were not extractable with solvent. Interestingly TAC value of non-sprouted and the 72h sprouted flour are not significantly different ($p < 0.05$) when is directly evaluated on the flour sample with the QUENCHER and on the alcoholic extract. Only in the

saline buffer is possible to observe difference. This trend can be analyzed taking into account the data of the differential solubility that show high amount of proteins solubilized in the saline buffer. Rutin, was used as standard because of its large presence in buckwheat seed. Comparing the results, the value are lower than the Trolox.

The processing seemed to play an important role on TAC of the samples. When the flour was processed the product the later showed lower antioxidant capacity than the raw material. However, the couscous made with the sprouted flour showed always the higher antioxidant capacity than that produced employing the untreated flour. After digestion mimicking physiological conditions, the results showed TAC values of the two couscous were not significantly different ($p < 0.05$) showing TAC values similar to that found for the saline extract. When using rutin as a standard, is observed the same trend of the extract (lower value) and the difference in TAC compared of the two digested is not significant ($p < 0.05$).

4.8.3.2.3 Quantification of the Total Polyphenol Content (TPC)

The total polyphenol content (TPC) represent a measurement of the amount of extractable polyphenols (table 3). TPC value of the saline extract of sprouted flour showed higher value than the non-sprouted, both evaluating it as using the gallic acid equivalent (GAE) and as rutin equivalent (RE). When the TPC was evaluated in the alcoholic extract, the values were higher compared to the saline buffer. As observed also for the TAC values, when flour is processed into couscous the values of TPC are lower. When the TPC was evaluated on the digested samples, the differences between the two couscous are not significant ($p < 0.05$).

This data shown that the sprouting positively affects the TPC of the flour, but during the processing into couscous the content of polyphenols apparently decrease. This decrement could be due to the molecular events that occurs during the processing (i.e. starch swelling, formation of protein-protein interactions and protein structural changes, etc…), inducing “matrix effects” that limit the extractability of polyphenols. Polyphenols and other antioxidants are susceptible to degradation during digestion due to the effects of pH and enzymes (Szawara-Nowak et al., 2016).

Table 2: Total Antioxidant Capacity (TAC) of the selected samples

	Standard flour	72h flour	Standard couscous	72h couscous (50%)
			mmol TE/kg sample	
QUENCHER	170.07 ± 10.46^{c}	169.93 ± 10.65^{c}	53.39 ± 3.22^{a}	88.06 ± 5.73^{b}
Saline buffer	61.23 ± 0.09^{f}	67.90 ± 1.7^{g}	26.42 ± 0.34^{b}	39.76 ± 2.38^{d}
Alcoholic extract	48.82 ± 1.03^{e}	53.28 ± 3.39^{e}	3.26 ± 0.19^{a}	11.77 ± 1.13^{c}
***In vitro* digestion**			28.58 ± 0.38	30.56 ± 1.65
			mmol RE/kg sample	
Saline buffer	21.06 ± 0.03^{f}	23.49 ± 0.62^{g}	8.42 ± 0.12^{c}	13.27 ± 0.86^{d}
Alcoholic extract	16.58 ± 0.37^{e}	18.18 ± 1.22^{e}	0.23 ± 0.07^{a}	3.29 ± 0.41^{b}
***In vitro* digestion**			7.93 ± 0.10	8.44 ± 0.43

TAC data obtained with the direct QUENCHER method, the saline buffer and alcoholic extraction, and from the *in vitro* digestion. The mean values designated by the different letters in the columns are significantly different (Tukey's honestly significant difference test, $p < 0.05$).

4.8.3.2.3 Quantification of the Total Polyphenol Content (TPC)

The total polyphenol content (TPC) represent a measurement of the amount of extractable polyphenols (table 3). TPC value of the saline extract of sprouted flour show higher value than the non-sprouted, both evaluating it by using the gallic acid equivalent (GAE) and rutin equivalent (RE). When the TPC are evaluated in the alcoholic extract the value are higher compared with the saline buffer. As observed also for the TAC value, when flour are processed into couscous the value of TPC are lower. When the TPC are evaluated on the digested samples, the differences between the two couscous are not significant ($p < 0.05$).

This data shown that the sprouting positively affect the TPC of the flour, but during the processing into couscous the content of polyphenols decrease: it could be due to a matrix effect caused by the molecular events that occurs during the processing (i.e. starch swelling, formation of protein-protein interaction and protein structural changes), with matrix effects that limit the extractability of that molecules. During the digestion, however the molecular event related with the biochemistry of the process. Polyphenols and other antioxidants are susceptible to degradation during digestion due to the effects of pH and enzymes (Szawara-Nowak et al., 2016).

Table 3: Total polyphenol content (TPC) of the selected samples

	Standard flour	72h flour	Standard couscous	72h couscous (50%)
			mmol GAE/kg sample	
Saline buffer	10.1 ±1.66^{a,c}	21.21± 0.03^{b}	4.95 ± 0.46^{a}	8.46 ± 0.67^{a,c}
Alcoholic extract	59.69 ± 3.0^{d}	62.17±5.51^{d}	5.89±0.15^{a}	14.02±0.77^{c}
***In vitro* digestion**			10.75 ± 0.12	10.22 ± 0.06
			mmol RE/kg sample	
Saline buffer	10.07 ± 1.17^{a,c}	24.98 ± 2.66^{b}	4.63 ± 0.00^{a}	8.35 ± 0.82^{a,c}
Alcoholic extract	69.02 ± 3.49^{d}	85.24 ± 2.99^{d}	5.21 ± 0.14^{a}	14.78 ± 0.9^{c}
***In vitro* digestion**			6.17 ± 0.32	5.52 ± 0.69

TPC data of the selected samples obtained from the saline buffer and alcoholic extraction, and from the *in vitro* digestion. The mean values designated by the different letters in the columns are significantly different (Tukey's honestly significant difference test, $p < 0.05$).

4.8.4 Conclusions

It is known from the publisheddata that in plant extract the phenols are the main antioxidant components, and that the total polyphenols content is directly proportional to their antioxidant capacity (Ke-Xue Zhu et al., 201). In our work we observed that during the sprouting of buckwheat some nutritional properties of buckwheat and couscous are enhanced compared to the non-sprouted samples. We observed a decrement of the content of phytic acid and an increment of the TAC and TPC values in the sprouted samples. These canges could be related also to the structural modification of proteins, that in turn lead to an evolution of the interaction with the antioxidant and polyphenols species. The biochemical changes ensuing from the action of endogenous seed proteases after short-time sprouting (like 72h) of buckwheat grains included a less compact protein structure and a high release of peptides (Barbiroli et al., 2022), that can affect also the overall antioxidant capacity of the sprouted flour. The synergism among the antioxidants in the mixture made the antioxidant activity, not only dependent on the concentration of antioxidant, but also on the structure and interaction among the antioxidants. That is why samples with similar concentrations of total phenolics may vary remarkably in their antioxidant activity (Sun, T. and Ho, C. T., 2005). The overall features of proteins in the sprouted grains made them more suitable for formation of the inter-protein network fundamental for appropriate texturing of specific foods. Sprouting-associated enzymes also decreased the levels of antinutritional factors such as enzymatic inhibitors, phytates and chelating compounds, increasing the bioavailability of metals, fatty acids (data not shown), and free and extractable polyphenols, as observed from the TPC data.

When the flour is processed into a finished product like the couscous, the antioxidant capacity and the polyphenols contents showed a decrease. This behavior suggest a matrix

effect exerted by the structural changes that occurs on the macromolecules in buckwheat - like starch and proteins – affecting the bioavailability of polyphenols and other compounds.

From our evidence results can be suggested that thee sprouting positively affected the nutritional properties of buckwheat, but during the processing into couscous, molecular events took place reducing the level of antioxidants. However, the bioaccesibility of antioxidants, measurable with the methods hereby used, after digestion process seemed to be not affected by the processing. Future work for clarify the modification in bioaccessibility of the molecules after the digestion have to be performed.

4.8.5 Acknowledgment

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4.8.6 References

Abbasi Parizad, P., Marengo, M., Bonomi, F., Scarafoni, A., Cecchini, C., Pagani, M. A., Marti, A., Iametti S., 2020. Bio-functional and structural properties of pasta enriched with a debranning fraction from purple wheat. Foods. 9, 163.

Barbiroli A., Di Nunzio M., Marti A., Gardella M. and Iametti S., 2022. Molecular aspects of nutritionally improved sorghum and buckwheat, two sustainable crops; IUBMB-FEBS-PABMB 2022 Congress. 9 - 14 July 2022, Lisboa.

Biacs, P., Aubrecht, E., Léder, I., and Lajos, J., 2002. Buckwheat. In: Pseudocereals and Less Common Cereals. P. Belton and J. Taylor, eds. Springer Verlag: Berlin. pp. 123-151

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

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.

Contini, M., Baccelloni, S., Massantini, R., Anelli, G., 2008. Extraction of natural antioxidants from hazelnut (Corylus avellana L.) shell and skin wastes by long maceration at room temperature. Food Chem. 110, 659–669.

Dietrych-Szostak, D., Oleszek, W., 1999. Effect of processing on the flavonoid content in buckwheat (Fagopyrum esculentum Moench) grain. J Agric. Food. Chem. 47, 4384-4387.

Dunaevsky, Y. E., Belozersky, M. A., 1989. Proteolysis of the main storage protein of buckwheat seeds at the early stage of germination, Physiol Plant. 75, 424–428.

Farooq, S., Rehman, R. U., Pirzadah, T. B., Malik B., Dar, F. A., Tahir, I., 2016. Cultivation, agronomic practices, and growth performance of buckwheat. In: Molecular breeding and nutritional aspects of buckwheat, Academic Press, pp. 299-319.

Frusciante, L., Carli, P., Ercolano, M.R., Pernice, R., Di Matteo, A., Fogliano, V., Pellegrini, N., 2007. Antioxidant nutritional quality of tomato. Mol. Nutr. Food Res. 51, 609–617.

Gökmen, V., Serpen, A. and Fogliano, V., 2009. Direct measurement of the total antioxidant capacity of foods: the “QUENCHER” approach’. Trends Food Sci. Technol. 20, 278-288.

Guo, X., Yao, H., 2006. Fractionation and characterization of tartary buckwheat flour proteins. Food Chem. 98, 90-4.

Hollebeeck, S., Borlon, F., Schneider, Y. J., Larondelle, Y., Rogez, H., 2013. Development of a standardised human in vitro digestion protocol based on macro- nutrient digestion using response surface methodology. Food Chem. 138, 1936-44.

Iametti, S., Bonomi, F., Pagani, M.A., Zardi, M., Cecchini, C., D’Egidio, M.G., 2006. Properties of the protein and carbohydrate fractions in immature wheat kernels. J. Agric. Food. Chem. 54, 10239–10244.

Ma, Y., Xiong, Y. L., 2009. Antioxidant and bile acid binding activity of buckwheat protein in vitro digests. J. Agric. Food. Chem. 57, 4372-80.

Mariotti, M., Lucisano, M., Pagani, M. A., Iametti, S., 2008. Macromolecular interactions and rheological properties of buckwheat-based dough obtained from differently processed grains. J. Agric. Food. Chem. 56, 4258–4267.

Martinez-Saez, N., Ullate, M., Martin-Cabrejas, M. A., Martorell., P., Genovés, S., Ramon, D., del Castillo, M. D, 2014. A novel antioxidant beverage for body weight control based on coffee silverskin. Food Chem. 150, 227-34.

Oomah, B. D., Mazza, G., 1996. Flavonoids and antioxidative activities in buckwheat. J. Agric.l Food Chem. 44, 1746–1750.

Perez-Jimenez, J., Saura-Calixto, F., 2005. Literature data may underestimate the actual antioxidant capacity of cereals. J. Agric. Food Chem. 53, 5036–5040.

Radović, S. R., Maksimović, V. R. and Varkonji-Gašić, E. I., 1996. Characterization of buckwheat seed storage proteins. J. Agric. Food Chem. 44, 9721-4.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evants, C., 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biol. Med. 26, 1231-1237.

Ren, S. C., Sun, J. T., 2014. Changes in phenolic content, phenylalanine ammonia-lyase (PAL) activity, and antioxidant capacity of two buckwheat sprouts in relation to germination. J. Funct Foods. 7, 298-304.

Serafini, M., Bellocco, R., Wolk, A., Ekstrom, A.M., 2002. Total antioxidant potential of fruit and vegetables and risk of gastric cancer. Gastroenterology. 123, 985–991.

Serpen, A., Capuano, E., Fogliano, V., Go¨kmen, V., 2007. A new procedure to measure the antioxidant activity of insoluble food components. J. Agric. Food Chem. 55, 7676–7681.

Serpen, A., Gökmen, V., Pellegrini, N., Fogliano, V., 2008. Direct measurement of the total antioxidant capacity of cereal products', J. Cereal Sci. 48, 816–820.

Serpen, A., Gökmen, V. and Fogliano, V., 2012. Total antioxidant capacities of raw and cooked meats. Meat Sci. 90, 60–65.

Singleton, V. L., Orthofer, R., Lamuela-Raventós, R. M., 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. Methods Enzym. 299, 152-178.

Steadman, K. J., Burgoon, M. S., Lewis, B. A., Edwardson, S. E., Obendorf, R. L., 2001. Minerals, phytic acid, tannin and rutin in buckwheat seed milling fractions. J. Sci. Food Agric. 81, 1094-100.

Sun, T., Ho, C. T., 2005. Antioxidant activities of buckwheat extracts. Food Chem. 90, 743-749.

Szawara-Nowak, D., Bączek, N., Zieliński, H., 2016. Antioxidant capacity and bioaccessibility of buckwheat-enhanced wheat bread phenolics, J. Food Sci. Technol. 53, 621-630.

Wijngaard, H. H., Arendt, E. K., 2006. Buckwheat. Cereal Chem. 83, 391-401.

Zanoletti, M., Marti, A., Marengo, M., Iametti, S., Pagani, M. A., Renzetti, S., 2017. Understanding the influence of buckwheat bran on wheat dough baking performance:

Mechanistic insights from molecular and material science approaches. Food Res. Int. 102, 728-737.

Zhu, K. X., Lian, C. X., Guo, X. N., Peng, W., Zhou, H. M., 2011. Antioxidant activities and total phenolic contents of various extracts from defatted wheat germ. Food Chem. 126(3):1122-6

Zych-Wężyk, I., Krzepiłko, A., 2012. Determination of Total Phenolic Compound Content and Antioxidant Properties of Edible Buckwheat Sprouts. Ecol. Chem. Eng. 19, 441-449.

4.9 COCOA. Characterization of protein in fermented food: the cacao case

4.9.1 Background

The development of a full range of cacao aroma and taste precursors is likely to occur during fermentation since unfermented beans do not provide cacao aroma upon roasting (Rohan, 1964). Formation of the final cacao product is achieved during the manufacturing processes involving drying and roasting. The evolution of the precursors of this flavors, occurring during fermentation, is mainly due to enzymatic reactions (Lerceteau et al., 1999). Proteins in cocoa represent the second most abundant constituent accounting for 10-15% of the dry weight (the first is the cocoa fat) (Marseglia et al., 2014). The volatile compounds of the cocoa aroma and chocolate are formed during the roasting of the bean, transforming the molecules known as aroma precursors, which are generated during fermentation by proteolysis of the proteins stored inside this bean (Janek et al., 2016). Proteolytic events therefor modify the protein pattern with the formation of oligopeptide and free amino acid that lead the bioactive potential and sensory properties of cocoa and cocoa products.

Typically the fermentation process at industrial level is a spontaneous process, realized with different methodologies (e.g. in heaps, boxes or trays, with banana or plantain leaves used to contain the cocoa) (Korcari et al., 2022).

In the presented work we investigated the possibility of using two minority lactic acid bacteria (LAB) species (*Lactiplantibacillus fabifermentans* and *Furfurilactobacillus rossiae*) as starter for a controlled fermentation process and their impact on the fermentation parameters, chemical composition and sensory profile of fine cocoa and chocolate.

4.9.2 References

Janek, K., Niewienda, A., Wöstemeyer, J., Voigt, J., 2016. The cleavage specificity of the aspartic protease of cocoa beans involved in the generation of the cocoa-specific aroma precursors. Food Chem. 211, 320-328.

Korcari, D., Ricci, G., Fanton, A., Emide, D., Barbiroli, A., Fortina, M. G., 2022. Exploration of *Lactiplantibacillus fabifermentans* and *Furfurilactobacillus rossiae* as potential cocoa fermentation starters. J. Appl. Microbiol. 1-12.

Lerceteau, E., Rogers, J., Pétiard, V. and Crouzillat, D., 1999. Evolution of cacao bean proteins during fermentation: a study by two-dimensional electrophoresis. J. Sci Food Agric. 79, 619-625.

Marseglia, A., Sforza, S., Faccini, A., Bencivenni, M., Palla, G. and Caligiani, A., 2014. Extraction, identification and semi-quantification of oligopeptides in cocoa beans. Food Res. Int. 63, 382-389.

Rohan, T.A., 1964. The precursors of chocolate aroma: a comparative study of fermented and unfermented cocoa beans. J. Food Sci. 29, 456-459.

4.10 COCOA. Exploration of *Lactiplantibacillus fabifermentans* and *Furfurilactobacillus*

*The results presented here below are published in: Korcari, D., Ricci, G., Fanton, A., **Emide, D**., Barbiroli, A., Fortina, M. G., 2022. Exploration of Lactiplantibacillus fabifermentans and Furfurilactobacillus rossiae as potential cocoa fermentation starters. J. Appl. Microbiol. 1-12.*

4.10.1 Abstract

Aims: To investigate the characteristics of two minority autochthonous LAB species, with particular regard to those properties that could be exploited in an improved cocoa fermentation process from a quality and safety point of view.

Methods and Results: Bacterial, yeast and mould strains characteristic of spontaneously fermented Dominican cocoa beans were isolated and identified by 16S or 26S rRNA gene sequencing. The potential of two autochthonous strains of LAB belonging to the species *Lactiplantibacillus fabifermentans* and *Furfurilactibacillus rossiae* were investigated. The two selected LAB strains were able to utilize glucose and fructose, produced mainly D-L lactic acid and had a good ability to resist to cocoa-related stress conditions such as low pH, high temperature and high osmotic pressure, as well as to grow in sterile cocoa pulp. The strains did not inhibit the growth of yeasts and acetic acid bacteria, that are essential to the cocoa fermentation process, and possessed a complex pool of peptidases especially active on hydrophobic amino acids. The strains also showed antifungal activity against mould species that can be found at the final stages of cocoa fermentation, as *Aspergillus tamarii, A. nidulans, Lichtheimia ornata* and *Rhizomucor pusillus*.

Conclusions: The tested strains are good candidates for the design of starter cultures for a controlled cocoa fermentation process.

Significance and Impact of the Study: This research showcases the potential of two alternative LAB species to the dominating *Lactiplantibacillus plantarum* and *Limosilactibacillus fermentum* as cocoa fermentation starters, with an interesting activity in improving the safety and quality of the process.

4.10.2 Introduction

Cocoa bean fermentation is a crucial step in the production process of chocolate and its quality. This step is fundamental in determining the flavour profile and composition of chocolate, and in reducing the excessive bitterness and astringency of unfermented cocoa. At industrial level cocoa fermentation is typically a spontaneous process carried out with different methodologies such as in heaps, boxes or trays, with banana or plantain leaves sometimes used to contain the cocoa.

The variable nature of fermented cocoa is to this day a major problem for the cocoa and chocolate producing industries. With all other factors remaining unchanged, the outcome of the spontaneous fermentation is still difficult to control, and this is a drawback both for chocolate makers that need to use cocoa blends to maintain the uniformity of their recipes, and for cocoa producing companies for which the variable quality is often translated in an economic loss. Although the understanding of the cocoa fermentation process and the setup of controlled fermentations have been an important step toward a more controlled process, a wider understanding of the impact of the different bacterial species on the fermentation is needed to design starter cultures that have a positive role in the flavour and quality of cocoa (Calvo et al., 2021).

Cocoa beans inside the cocoa pod are sterile, and the microbial species that conduct the fermentation derive from environmental contaminations such as the surface of the pods, from the hands and machetes used to open them, from the sacks and baskets used for transportation, from fermentation boxes and plantain leaves (De Vuyst & Weckx, 2016).

The fermentation is carried out by yeasts and lactic acid bacteria (LAB) in the initial anaerobic stage. Glucose, fructose and citric acid are fermented into organic acids and ethanol by both LAB and yeasts. Yeasts are also able to convert the pulp pectin in simple sugars thanks to their pectinolytic activity. After 1–2days the fermenting mass is mixed, a process that introduces oxygen and causes a shift in the microbial population: whereas the presence of yeasts and LAB decreases, aerobic acetic acid bacteria (AAB) begin to develop and convert ethanol and residual sugars into acetic acid. This phase is characterized by an increase in temperature that in synergy with the presence of acetic acid kill the embryo, activating its hydrolytic enzymes and inhibiting the germination. At the end of the fermentation spore forming *Bacillus* species and mould may grow.

Independently of the region of cultivation, the dominating LAB species in cocoa fermentation are facultatively heterofermentative *Lactiplantibacillus plantarum* and strictly heterofermentative *Limosilactibacillus fermentum* (Schwendimann et al., 2015). Other species such as *Fructobacillus* spp. and *Leuconostoc* spp. have been reported to play an important role at the beginning of the fermentation, however, previous starter culture design experiments for cocoa fermentation have focused on the former two species (De Vuyst & Weckx, 2016).

The goal of this work was to evaluate the potential of two autochthonous strains of LAB belonging to the species *Lactiplantibacillus fabifermentans* and *Furfurilactibacillus rossiae*, isolated from fermented cocoa, to be used as adjunct cultures for an improved cocoa bean fermentation process.

These species are not considered to be dominant in cocoa fermentations. Indeed, the facultatively heterofermentative *L. fabifermentans* first described by De Bruyne et al. (2009) from Ghanaian cocoa fermentations, was reported as a minority species at the first stages of

Ecuadorian cocoa fermentation (Papalexandratou et al., 2011), and represented only 1.23% of the LAB isolated from fermented cocoa in the Côte d'Ivoire (Adiko et al., 2018), although, because of the high similarity with *L. plantarum*, this species may have been underestimated in previous cocoa fermentation microbiota research. *L. fabifermentans* has one of the biggest genomes of LAB which shows a great genomic versatility, with a wide range of carbohydrate utilization (Campanaro et al., 2014). The reported preference for fructose, rather than glucose, can reduce the competition with yeast, making this species a good candidate for a mixed starter culture (Lefeber et al., 2011).

F. rossiae, on the other hand, has never been described in cocoa fermentations before, to the authors' knowledge. This obligately-heterofermentative LAB has been isolated from a wide range of environmental niches, such as sourdoughs, fruit, fermented meat, and animal and human gut. This species can metabolize a substantial number of carbohydrates, and genomic research has shown its potential for polysaccharide degradation, as revealed by an in- silico analysis (De Angelis et al., 2014).

This research aims to investigate some characteristics of these minority, scarcely studied species, with particular regard to those properties that could be exploited in an improved cocoa fermentation process from a quality and safety point of view.

4.10.3 Material and Methods

4.10.3.1 Strain isolation and maintenance

Microbial population was isolated from a sample of cocoa of the Criollo variety provided by Rizek Cacao S.A.S., at different fermentation times. The LAB isolation was performed by a culture-dependent method, plating adequate dilutions in Man Rogosa Sharpe (MRS-Difco Lab) agar plates, incubated at 30°C for 48 h. For each fermentation day, approximately 10 colonies were isolated. Pure cultures of each strain were routinely subcultured in MRS broth at 30°C for 24 h.

Similarly, yeast strains were isolated by plating adequate dilutions in Yeast extract Glucose Chloramphenicol (YGC) agar plates (MilliporeSigma) incubated at 28°C for 72 h. Isolated strains were maintained in Yeast extract Peptone Dextrose (YPD) broth incubated at 30°C for 24 h. The composition of the YPD medium is (g L^{-1}): yeast ex- tract 10, glucose 20, peptone 20.

Four mould isolates were obtained from a contaminated batch of cocoa at the end of the fermentation period. For isolation YGC plates were used. Plates were incubated at 28°C for 5–7days. Moulds were isolated and transferred to Malt Extract Agar (MEA) plates (Thermo Fisher Scientific) for their maintenance and identification. In this study we also used other mould strains previously isolated from fermented food and identified in our laboratory, as *Aspergillus flavus*, *Aspergillus niger*, *Mucor circinelloides* and *Fusarium verticillioides*. Spores

were collected by pouring sterile distilled water containing 9 g L^{-1} NaCl on the plates after complete sporification, slowly agitating and storing in sterile tubes.

All isolates were deposited in the culture Collection of the Department of Food, Environmental and Nutritional Sciences, University of Milan, Italy, at −80°C in their maintenance medium, with the addition of 150 ml L^{-1} glycerol.

L. fabifermentans SAF13, *F. rossiae* SAF51, *L. plantarum* B7 and *Saccharomyces cerevisiae* TB2.3 were selected for further studies.

Acetobacter pasteurianus DSM 3509 was used for coculture experiments. The strain was maintained in Glucose Yeast extract Calcium Carbonate (GYC) agar plates incubated at 28°C for 48 h. The composition of the GYC agar medium is as follows (g L^{-1}): glucose 50, yeast extract 10, calcium carbonate 30, agar 15 (MilliporeSigma).

4.10.3.2 Molecular identification of the isolates and q-PCR experiments

The identification of yeast isolates was performed by 26S rRNA gene sequencing or ITS amplification and restriction with restriction enzymes *Hind*III and *Hinf*I, as previously reported (Decimo et al., 2017). Mould identification was carried out by 26S rRNA gene sequencing (White et al., 1990).

LAB isolates were identified by 16S rRNA gene sequencing. For the subsequent detection of *F. rossiae* and *L. fabifermentans* both in single culture and in co-cultures, species-specific probes were used or designed. For the detection of *F. rossiae*, primers designed by Riedl et al. (2017) were used. For the detection of *L. fabifermentans*, primers were designed in this study using the 16S rRNA gene deposited in the GeneBank database and aligning to the 16S rRNA genes of the affine *L. plantarum* species to check for specificity. Furthermore, the melting temperature (T_m) was optimized to avoid mismatches. The primers and thermal cycles are reported in Table 1. For qPCR experiments, calibration curves were designed by inoculating decimal dilutions of an overnight grown culture of each *F. rossiae* and *L. fabifermentans* strain in a range from 3–8 log cycles in MRS broth. Cell lysis of the bacterial pellet recovered from MRS and DNA extraction was performed as reported by Mora et al. (2000).

The PCR reaction was carried out in a total volume of 15µl, containing 7.5 µl of qPCR mix (SSO Fast Supermix, BioRad, Hercules, USA), 0.36 µl of each primer (0.3 µmol L^{-1}), 1.78µl of PCR grade water and 5 µl of DNA. The efficiency of the standard curves was in the range 90%–110% and the R2 was >0.99.

4.10.3.3 Carbon source utilization and organic acids production

The growth of the strains in presence of glucose, fructose and sucrose was tested in MRS broth containing 10 g L^{-1} of each sugar. Overnight grown cultures of each strain were twice

washed with sterile saline water and inoculated in 200 µl of medium at a concentration of 10^6 CFU ml^{-1} in 96 well plates. The growth was measured using an automated microplate reader (EonTM Microplate Spectrophotometer, BioTek) at 600nm. The lag time was calculated by the instrument.

The production of acetic acid and lactic acid after 24 h incubation at 30°C was measured using commercial assay kits according to the manufacturer's instructions (R-Biopharm AG).

Table 1. Primers and thermal cycles for species-specific PCR analyses

Species	Primer sequences	Thermal cycle	Reference
L. fabifermentans	F: CTGGTATTGATTGGTACTTGT R: ACCTCACCATCTAGCTAATG	95 °C × 10 s 59 °C × 20 s 72 °C × 20 s	This study
F. rossiae	F: GGCGTGCCTAATACATGCAA R: TGTCTCGTCAATCTGGTGCAA	95 °C × 10 s 60 °C × 20 s 72 °C × 20 s	Riedl *et al*. 2017

4.10.3.4 Tolerance to stress conditions

The ability of *L. fabifermentans* SAF13 and *F. rossiae* SAF51 to resist to different stress conditions were tested in MRS broth at different temperatures (from 25 to 50°C) and pH values (from 3.0 to 6.5). The tolerance to high osmolarity was evaluated in MRS broth containing 150 g L^{-1} and 300 g L^{-1} glucose or fructose.

The growth was evaluated by measuring the Optical Density at 600nm ($OD600_{nm}$), after 48h of incubation. The autochthonous *L. plantarum* strain B7 was used as comparison.

4.10.3.4 Autolytic activity in buffer system

The rate of autolysis was determined according to the method described by Ayad et al. (2004) and Ma et al. (2011). The cell pellets were resuspended in citrate buffer (0.1 mol L^{-1}pH 4.0) containing 0.5 mol L^{-1} NaCl and then diluted to OD 650 nm = 1.0. The rate of autolysis was determined as absorbance decrease at 650 nm after incubation at 30°C for 48h, using the formula $\%autolysis = (1 - OD_{48h} / OD_{t0}) * 100$.

4.10.3.5 Proteolytic activity

The proteolytic (aminopeptidase, dipeptidyl peptidase and endopeptidase) activity of *L. fabifermentans* SAF13 and *F. rossiae* SAF51 was characterized in comparison to *L. plantarum* B7.

A cellular pellet of the three strains was recovered from an overnight grown culture in MRS medium by centrifugation at 8,000 × g for 10 min, 4°C. The pellet was washed twice, resuspended in physiological solution (9 g L^{-1} NaCl), and then the cells lysed in a TissueLyzer

(Qiagen, Hilden, Germany) at 30 s^{-1} for 3 min. Cellular debris were removed by centrifugation at 13,000 × g for 15 min, 4°C, and the clear supernatant (cell lysate) was stored at −20°C until use. The protein content of cell lysates was quantified by a dye-binding method (Bradford, 1976), whereas the protein pattern was characterized by SDS-PAGE (Laemmli, 1970).

Proteolytic activities were determined by using the following synthetic p-nitroanilide (pNA) sub- strates: Leu-pNA, Met-pNA, Ala-pNA, Pro-pNA, Lys- pNA for aminopeptidase activities; Gly-Pro-pNA for prolylpeptidases activity; N-Benzoyl-Arg-pNA (BAPA) and N-Suc-Ala-Ala-Pro-Phe-pNA (SUNA) for trypsin- and chymotrypsin-like, respectively, endoprotease ac- tivities (Merck Life Science S.r.l., Milano, Italy). Activity assays were adapted from Iametti et al. (2002). The pNA substrate solutions were prepared at the concentration of 0.5 mmol L^{-1} in sodium acetate buffer (50 mol L^{-1}, pH 4.5). The reactions were started by adding 0.2 mL cell lysate (diluted at about 0.4 mg mL^{-1} protein concentration) to 0.8 mL substrate solution. The pNA released in 60 min at 37°C was determined spectrophotometrically at 405 nm. One unit of specific activity was defined as a 0.001 increment in absorbance per hour per amount of protein (mg), under the assay condition.

4.10.3.6 Antifungal activity assay

Antifungal activity against *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus tamarii*, *Aspergillus nidulans, Lichtheimia ornata*, *Rhizomucor pusillus*. *Mucor circinelloides* and *Fusarium verticillioides* was tested using the overlay method (Axel et al., 2015). Each LAB strain tested was streak plated in MRS agar and grown at 30°C for 72 h. Afterwards a layer of MEA soft agar (7 g mL^{-1} agar) containing 104 mL^{-1} spores suspension was slowly added to the plates that were incubated at 30°C for 3 days. Antifungal activity was evaluated as clear zones of inhibition around the bacterial smears.

4.10.3.7 Evaluation of growth in cocoa

The bacterial growth ability was evaluated in cocoa pulp. For this purpose, cocoa pods were harvested, and the surface was cleaned with alcohol. The pulp was collected in sterile conditions and was pasteurized (65°C for 30 min), to ensure the absence of contamination. The sterility was confirmed by plating in MRS agar, YPD agar and PCA and incubating at 30°C for 48 h. Approximately 10^5 CFU mL^{-1} of each LAB strain tested were inoculated in the cocoa pulp and the growth was evaluated by dilution and plating in MRS agar after 16 and 40 h of incubation at 30°C.

4.10.3.8 Growth in co-culture

To investigate the possibility of *F. rossiae* SAF51 and *L. fabifermentans* SAF13 strains to be used in conjunction with one another, co-culture assays were set up. The growth was

performed in MRS broth by inoculating $10^5 – 10^6$ CFU mL^{-1} of each strain and incubating at 30°C for 16 and 40 h. The growth of each strain was evaluated by qPCR.

The two LAB strains were also grown in conjunction with the autochthonous strain *S. cerevisiae* TB2.3. The growth of the yeast was evaluated with the plate count method in YGC agar and the growth of bacterial strains was evaluated with a qPCR assay as previously described.

Co-cultures of LAB strains and *A. pasteurianus* DSM 3509 were carried out by inoculating 10^6 CFU mL^{-1} of cells in MRS broth in agitation at 150 rpm and 28°C. The growth of LAB was evaluated by plate count in MRS agar incubated in anaerobic conditions, the growth of *A. pasteurianus* DSM 3509 was evaluated in basal MRS agar medium without glucose and with 40 ml L^{-1} ethanol as sole carbon source.

4.10.3.9 Statistical analysis

All results are expressed as mean ± SD of three independent replicates of each experiment. Analysis of variance (one-way ANOVA; $p < 0.05$) was performed by using SigmaPlot version 14.0 (Systat Software Inc.,). Differences were considered statistically significant for $p < 0.05$.

4.10.4 Results

4.10.4.1 Microbial population composition

The LAB and yeast populations isolated from the Dominican cocoa fermentation are reported in table 2. The LAB population had a larger diversity in the first 48 h of fermentation. After 72h, in correspondence with the mixing of the cocoa mass, the biodiversity of LAB was significantly reduced, and the population was comprised of mainly *Lactiplantibacillus plantarum*, that was present throughout all days of fermentation. One *L. plantarum* strain, strain B7 was chosen as representative of the dominant species already adapted to cocoa fermentation habitat and used in these comparative studies. Apart from other species commonly associated with fermented cocoa, such as *Lacticaseibacillus paracasei* and *Levilactobacillus brevis*, minority species such as *Lacticaseibacillus rhamnosus*, *Furfurilactibacillus rossiae*, *Lactiplantibacillus fabifermentans* and *Liquorilactobacillus satsumensis* were also isolated. We focused the attention toward the minority *F. rossiae*, and *L. fabifermentans* species, since their presence in fermented cocoa and/or in fermented food has been demonstrated but poorly studied. The two strains *L. fabifermentans* SAF13 and *F. rossiae* SAF51 were selected for their evaluation.

The dominating yeast species was *S. cerevisiae*, which is also typical of cocoa fermentations. This species represented 65% of all isolates identified. Other identified yeasts, such as *Hanseniaspora opuntiae*, *Torulaspora delbrueckii* and *Pichia* spp., are also typical of fermented cocoa. For co-culture experiments with LAB strains, one strain of the more

representative yeast of the cocoa fermentation, *Saccharomyces cerevisiae* TB2.3, was chosen.

The four mould strains isolated from a batch of mouldy cocoa belonged to the species *Aspergillus tamarii*, *Aspergillus nidulans*, *Lichtheimia ornata* and *Rhizomucor pusillus*.

4.10.4.2 Carbon source utilization, lactic and acetic acid production and tolerance to stress conditions

L. fabifermentans SAF13 and *L. plantarum* B7 utilized glucose, fructose and sucrose as carbon sources, whereas *F. rossiae* SAF51 could only use glucose and fructose. Moreover, *F. rossiae* SAF51 had a significantly higher lag time (14.32 ± 0.01 h) compared to *L. plantarum* B7 (4.64 ± 0.01 h) and to *L. fabifermentans* SAF13 (3.75 ± 0.01 h), when grown either in glucose or in fructose.

Table 2. LAB and yeast species isolated at different fermentation times

	Fermentation time (h)						
	24	*48*	*72*	*96*	*120*		
Microbial species	Number of isolates					Total isolates	Percentage
LAB species							
L. plantarum	0	9	2	23	10	44	73.3
L. paracasei	0	2	0	2	0	4	6.7
L. brevis	0	1	2	0	0	3	5.0
L. rhamnosus	0	1	0	0	2	3	5.0
L. fabifermentans	0	0	3	0	0	3	5.0
F. rossiae	0	2	0	0	0	2	3.3
L. satsumensis	0	0	0	1	0	1	1.7
Yeast species							
Saccharomyces cerevisiae	7	14	16	9	7	53	65.4
Torulaspora delbrueckii	3	0	0	4	0	7	8.6
Schizosaccharomyces pombe	0	0	0	0	6	6	7.4
Hanseniaspora opuntiae	3	2	0	0	0	5	6.2
Wickherhamomyces pijperi	3	0	0	0	0	3	3.7
Starmerella bacillaris	1	2	0	0	0	3	3.7
Pichia kudriavzevii	2	0	0	0	0	2	2.5
Pichia manshurica	0	2	0	0	0	2	2.5

After 24 h of growth, *L. fabifermentans* SAF13 and *L. plantarum* B7 produced mainly D-L lactic acid: its final concentration (about 11 g L^{-1} L-lactic acid and 7.5 g L^{-1} of D-lactic acid for *L. fabifermentans*, 11 g L^{-1} L-lactic acid and 6.3 g L^{-1} of D-lactic acid for *L. plantarum*) was 53 times higher than the concentration of acetic acid for both strains (0.33–0.34 g L^{-1}*). F.*

rossiae SAF51 also produced lactic acid as the main product of its fermentation, but the proportion of acetic acid was closer to 1:2 (3.7 g L^{-1} D-lactic acid, 4.9 g L^{-1} L-lactic acid and 3.5 g L^{-1} acetic acid).

All tested strains were able to grow in cocoa-related stress conditions such as low pH, high temperature and high osmotic pressure (Figure 1). *L. fabifermentans* SAF13 and *L. plantarum* B7 had an optimal growth at pH 6, but a moderate level of growth was also observed at pH 3; *F. rossiae* SAF51 was more sensitive to low pH values, showing very limited growth at pH 3. *L. fabifermentans* SAF13 was the most resistant strain to high temperatures, growing at temperatures up to 42°C. No growth was registered at 50°C.

4.10.4.3 Autolysis

The autolytic phenotype of the LAB species under investigation was studied at pH 4 and 0.5 mol l^{-1} NaCl to simulate some of the conditions of cocoa pulp. The results indicate that *F. rossiae* SAF51 and *L. plantarum* B7 have a moderate autolysis rate, as the results obtained were 28% and 23.9%, respectively. *L. fabifermentans* SAF13 on the other hand had a very limited autolytic ability, as the OD was reduced by only 5.4%.

4.10.4.4 Antifungal activity

The ability of the two selected strains to inhibit the fungal growth was evaluated in comparison with the strain *L. plantarum* B7.

As shown in figure 2, *L. fabifermentans* SAF13 and *L. plantarum* B7 showed the highest inhibitory activity towards the mould species that can be found at the final stages of cocoa fermentation. Particularly, *L. fabifermentans* SAF13 was the most effective strain in inhibiting *L. ornata* and *A. tamarii*. *F. rossiae* SAF51 showed low level inhibition towards *R. pusillus* and no inhibition towards *L. ornata* and *A. tamarii*, whereas it could efficiently inhibit the growth of *A. nidulans*. Moreover, the three LAB tested showed interesting antifungal activity toward *A. flavus* and *F. verticillioides*, while no inhibition was evaluable for *A. niger* and *M. circinelloides.*

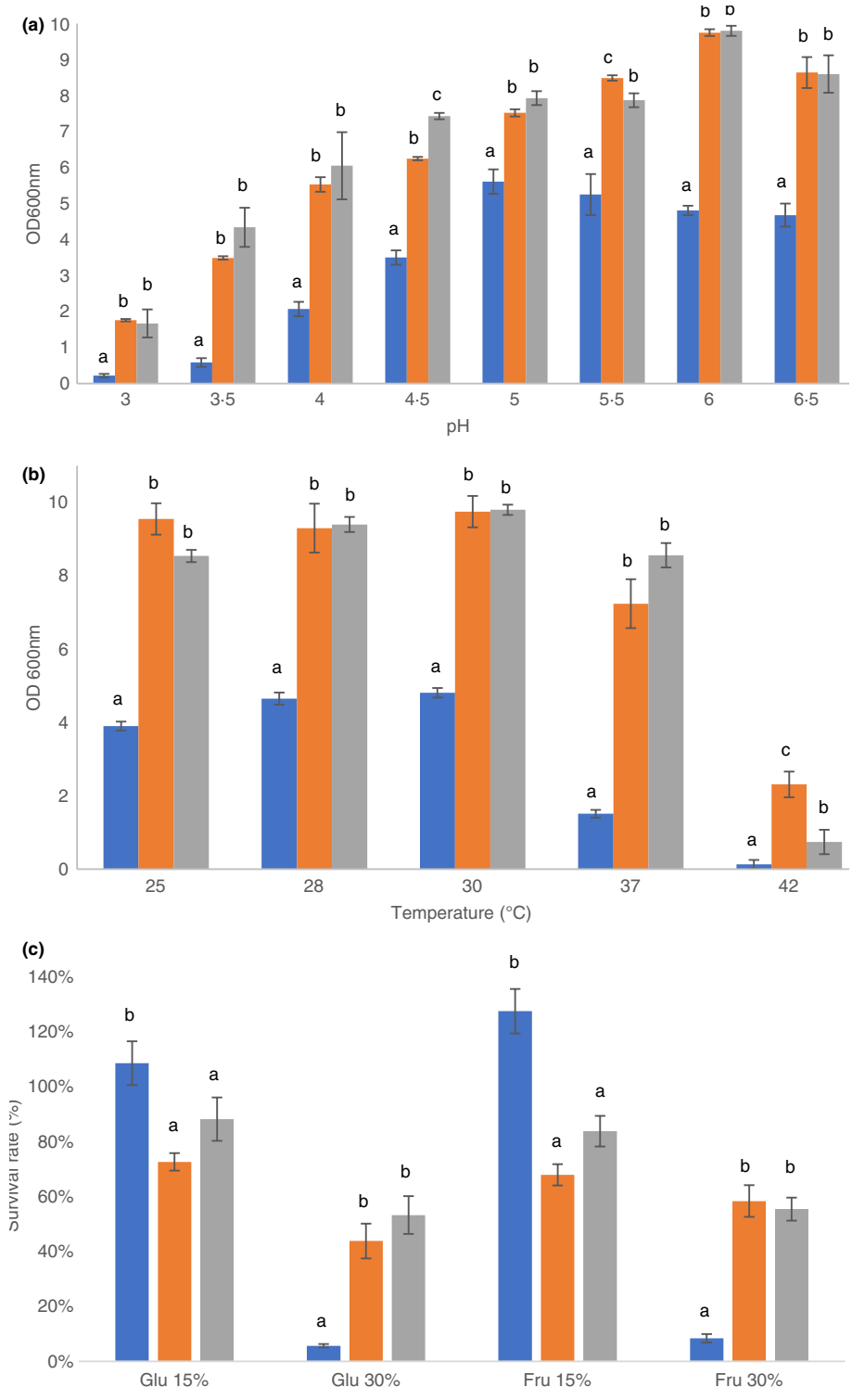


Figure 1 Growth at different pH (A) and temperatures (B) and survival rate relative to standard MRS medium (C) of the strains *F. rossiae* SAF51 (■), *L. fabifermentans* SAF13 (■) and *L. plantarum* B7 (■). Different letters indicate significant differences ($p<0.05$).

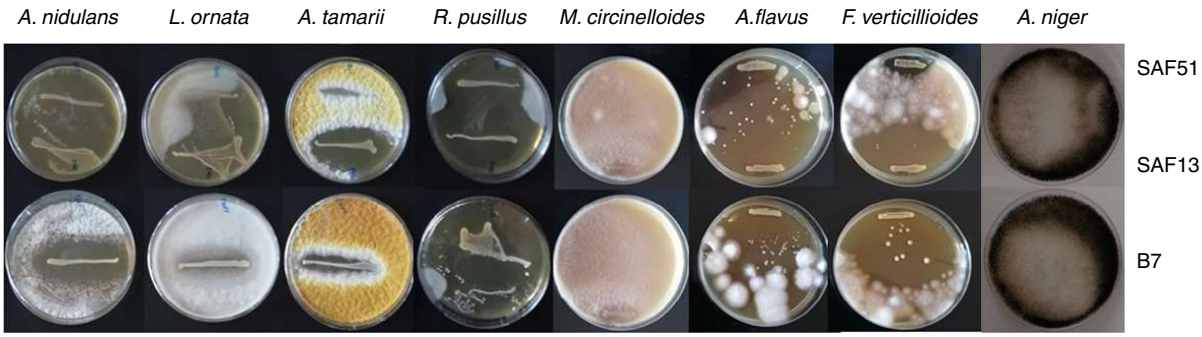


Figure 2. Antifungal activity overlay assay of strains *F. rossiae* SAF51, *L. fabifermentans* SAF13 and *L. plantarum* B7. Antifungal activity is represented by the clear zone surrounding the bacterial smear.

4.10.4.5 Proteolytic activity

In order to evaluate the potential proteolytic activity released upon lysis, cell lysates of *L. fabifermentans* SAF13, *F. rossiae* SAF51 and *L. plantarum* B7 was produced in a bead mill. *L. fabifermentans* SAF13 cell lysate generally had a protein content three time lower than the other two strains. This evidence suggests a greater resistance of *L. fabifermentans* SAF13 also toward mechanical stress compared to *F. rossiae* SAF51 and *L. plantarum* B7, in apparent agreement with autolysis data. The three cell lysates showed peculiar protein patterns (Figure 3a), with the greatest differences observed in *L. fabifermentans* SAF13.

Proteolytic activities of the strains *L. fabifermentans* SAF13, *F. rossiae* SAF51 and *L. plantarum* B7 are shown in Figure 3b. All strains possessed a complex pool of peptidases. The highest aminopeptidase activity was observed toward the hydrophobic amino acids leucine, but significant activity was also found on alanine, methionine and lysine. In addition, iminopeptidase activity on Pro-pNA, prolylpeptidase activity on Gly-Pro-pNA and endoproteolytic activity on BAPA and SUNA were also present in all the three strains. *L. fabifermentans* SAF13 had a significantly higher activity on all tested substrates except for Leu-pNA and Met-pNA, whereas *F. rossiae* SAF51 in general was the strain that possessed the lowest activity.

4.10.4.6 Proteolytic activity

The growth of *L. fabifermentans* SAF13 and *F. rossiae* SAF51 strains in cocoa pulp is represented in Figure 4. Compared to the growth in liquid MRS broth, the growth in cocoa pulp is slower, but after 40 h the final cell concentration in the two conditions is similar.

Mixed culture experiments were set up in MRS broth (Figure 5). Firstly, the ability of each LAB strain to grow in association with the cocoa related yeast strain *S. cerevisiae* TB2.3

was investigated. After 16 or 40 h of incubation, the yeast growth was slightly limited by the presence of the LAB strains.

In the three-strain combination, whereas the growth of *L. fabifermentans* SAF13 and *S. cerevisiae* TB2.3 closely resembled the growth in two-strain co-cultures, *F. rossiae* SAF51 was strongly inhibited. Under these conditions *L. fabifermentans* SAF13 takes over *F. rossiae* SAF51.

When grown in association with the strain *A. pasteurianus* DSM 3509, *F. rossiae* SAF51 increased of about 4 log cycles its growth and had no effect on the growth of the *A. pasteurianus* strain. The same results were observed when *L. fabifermentans* SAF13 was grown in association with the *A. pasteurianus* strain. In this case the growth of *A. pasteurianus* incremented of about 1 log cycle, in relation to the presence of the strain *L. fabifermentans* SAF13.

4.10.5 Discussion

In this research we characterized the microbial population of the Dominican cocoa fermentation, with particular regards to LAB strains, from which we selected the minority strains *L. fabifermentans* SAF13 and *F. rossiae* SAF51 whose potential role in fermentation was investigated. In order to add to the body of knowledge on these poorly characterized species, we studied phenotypical attributes of interest, such as the ability of these strains to grow at low pH, high temperatures and high osmotic pressure, as the most important stressors of fermenting cocoa. In comparison to a cocoa related strain of the most representative species, *L. plantarum* B7, *L. fabifermentans* SAF13 behaved in a similar fashion in these conditions, demonstrating a good ability to survive and grow at low pH and high temperature, up to 42°C. *F. rossiae* SAF51, on the other hand, grew less efficiently in all conditions except in 150 g L^{-1} of sugar added medium, the approximate sugar content of cocoa pulp (Schwan and Wheals, 2004), in which this strain not only performed better than the other two strains, but grew more efficiently than in standard MRS medium (20 g L^{-1} glucose). Furthermore, the optimal pH for the growth of this strain was 5, lower than the strains of *L. plantarum* B7 and *L. fabifermentans* SAF13 that grew better at pH 6. This demonstrates the good adaptation that the strain of *F. rossiae* SAF51 has for conditions like those of fermenting cocoa: indeed, in cocoa pulp its growth after 40 h was comparable to *L. fabifermentans* SAF13 and to the growth in standard MRS medium.

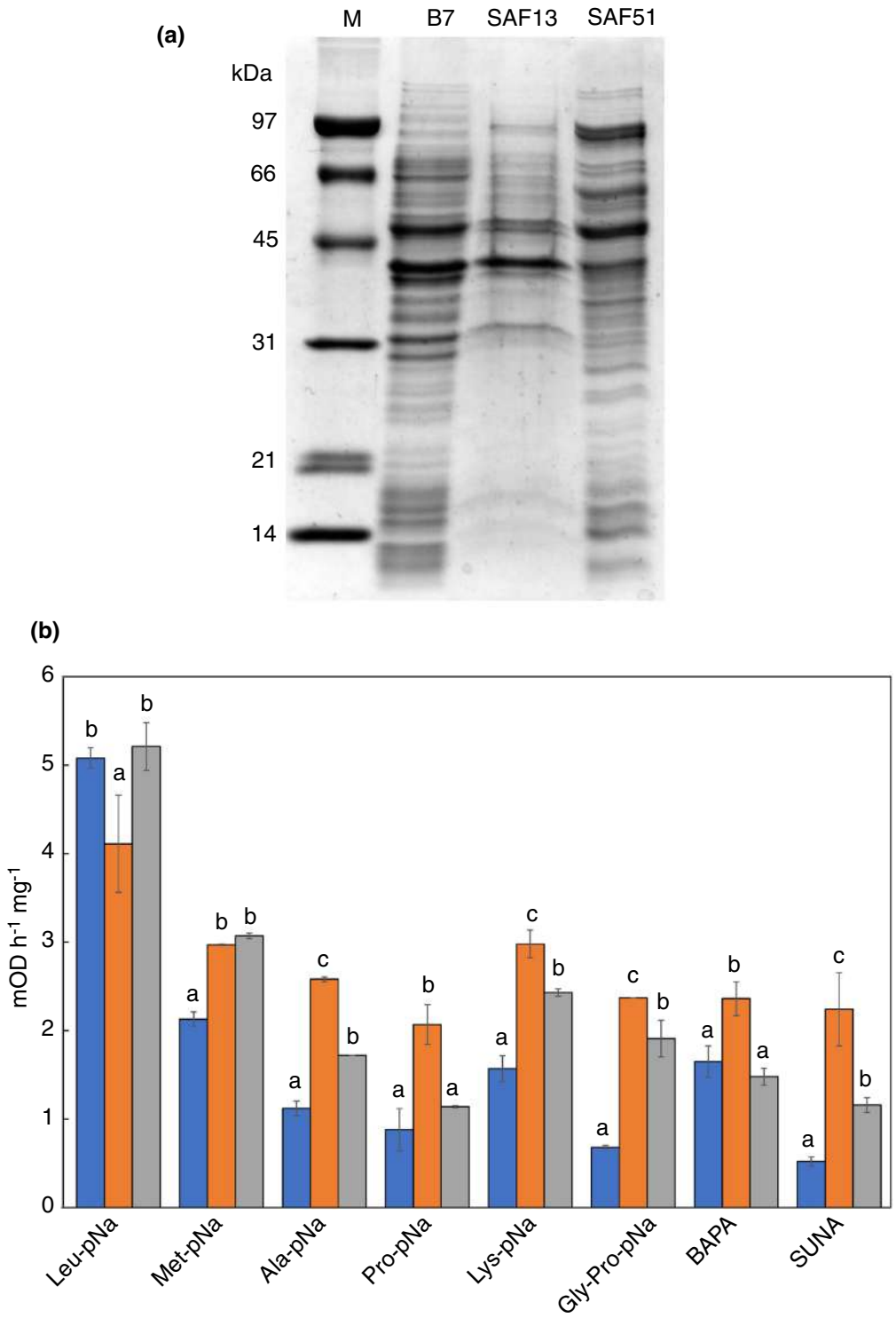


Figure 3. Proteasic activity of cell lysates. A: SDS-PAGE of the cell lysate of *L. plantarum* B7, *L. fabifermentans* SAF13 and *F. rossiae* SAF51; for each sample 5µg of protein were loaded. B: Proteolitic activity of strains *F. rossiae* SAF51 (■), *L. fabifermentans* SAF13 (■) and *L. plantarum* B7 (■) expressed in mOD h-1 mg-1. Different letters indicate significant differences ($p<0.05$).

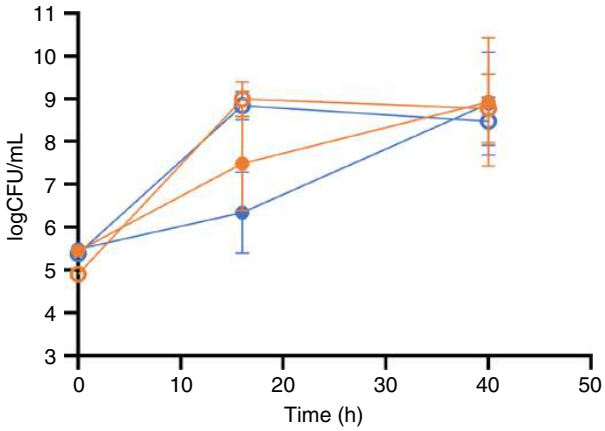


Figure 4. Growth in MRS (empty shapes) and cocoa pulp (full shapes) of the LAB strains *F. rossiae* SAF51 (■), *L. fabifermentans* SAF13 (■).

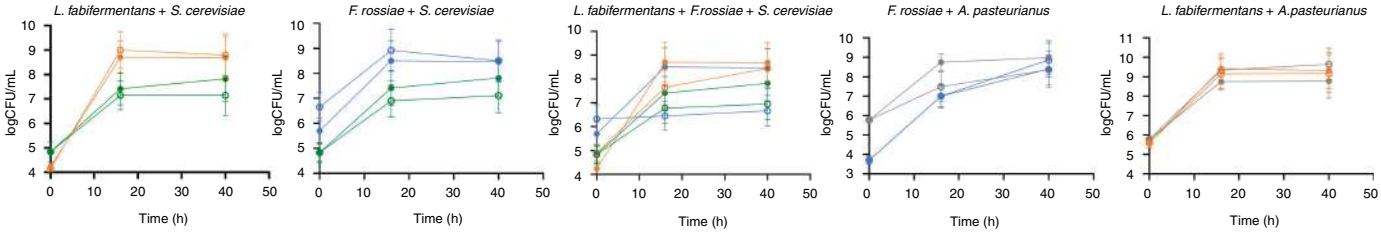


Figure 5. Viability of strains grown in monoculture (full symbols), or co-culture (empty symbols) grown in MRS broth and analysed at 16 and 40h. The two LAB strains in the three-strain mixed culture were discriminated by qPCR. ■ *L. fabifermentans* SAF13 ● *S. cerevisiae* TB 2.3 ▲ *F. rossiae* SAF51.

The two strains behaved differently during the fermentation of glucose, fructose and sucrose, the main fermentable sugars present in cocoa pulp (Afoakwa *et al.* 2013). *L. fabifermentans* SAF13, similarly to *L. plantarum* B7, grew efficiently using all three substrates, whereas *F. rossiae* SAF51 had significantly longer lag times when growing in glucose and fructose, and, as reported by Corsetti *et al.* (2005), the species cannot ferment sucrose. The inability to ferment sucrose makes this species adequate for co-culture growth as it reduces the competition for the carbon source. The production of lactic and acetic acid when fermenting glucose reflected their metabolic nature, as facultatively heterofermentative *L. plantarum* and *L. fabifermentans* mainly produced lactic acid, whereas strictly heterofermentative *F. rossiae* produced a significantly higher amount of acetic acid. Strictly heterofermentative LAB are known to dominate the later stages of cocoa fermentation, mainly due to their higher resistance to stress factors and higher efficiency in ATP production (Gänzle, 2015).

Despite some beneficial activities have been proposed, generally the growth of mould in cocoa is considered non beneficial for the quality of the chocolate. Their growth during the pre-processing stages of chocolate production, mainly during drying and storage, poses a

significant risk due to the production of mycotoxins, mainly ochratoxin A and aflatoxin (Copetti *et al.* 2013). Given the stable nature of these compounds, prevention becomes key to avoiding their presence in the production chain. The ability of the strains of *F. rossiae* SAF51, and especially *L. fabifermentans* SAF13 to inhibit the growth of aflatoxinogenic species *A. flavus*, as well as other toxin-producing *Aspergillus* species isolated from cocoa makes these strains good candidates for the biocontrol of aflatoxin-producing mould species. From the literature, strains belonging to *A. tamarii* have been indicated to being producers of aflatoxins (Klich *et al.* 2000), whereas *A. nidulans* produces sterigmatocystin, a toxic precursor of aflatoxins (Keller and Adams, 1995).

The potential ability of *L. fabifermentans* SAF13 and *F. rossiae* SAF51 to produce cocoa-specific aroma precursors by adding to the enzymatic pool of the fermentation process and increasing the amount of free amino acids that can be further metabolized (De Vuyst and Leroy, 2020) has been studied by evaluating their proteolytic activity at pH 4.5. Although proteolytic activity of LAB is generally studied at neutral pH (El Soda and Desmazeaud, 1982) we decided to stay closed to the pH of the cocoa beans during fermentation, in order to obtain results having a straightforward correlation with the real system. Moreover, the activity was studied on the cellular lysate. *L. fabifermentans* SAF13 shows an overall proteolytic activity slightly higher than *L. plantarum*, including the peptidase activity essential to release free amino acids. On the contrary, *F. rossiae* SAF51 has the lower specific activity on most of the substrate, but shows good endoprotease trypsin-like activity and high peptidase activity on the hydrophobic amino acids Leucine. It is worth noting that at this acidic pH, all the three LAB show interesting endoprotease activity, that could boost the peptidase activity by producing new amino-termini. The autolytic phenotype of *F. rossiae* SAF51 is an added advantage to this activity as it can carry on after the initial stages of fermentation when LAB are dominant.
In conclusion, the two strains *L. fabifermentans* SAF13 and *F. rossiae* SAF51 showed a high level of adaptation to the cocoa matrix, had a good resistance to stress factors related to the fermentation and possessed interesting activities that can be beneficial in a controlled cocoa fermentation process.

Further studies are in progress to evaluate the impact of adding these strains in a guided fermentation of cocoa on fermentation parameters and flavour profile of fine cocoa and chocolate.

4.10.6 References

Adiko, E., Ouattara, H., Doué, G. and Niamké, S. (2018) Assessment of the diversity of lactic acid bacteria involved in cocoa fermentation of six main cocoa producing regions of Côte d'Ivoire. *Ann Res Rev Biol* **27**, 1–16. https://doi.org/10.9734/arrb/2018/42194

Afoakwa, E.O., Kongor, J. E., Takrama, J.F. and Budu, A.S. (2013) Changes in acidification, sugars and mineral composition of cocoa pulp during fermentation of pulp pre-conditioned cocoa (*Theobroma cacao*) beans. *Int Food Res J* **20**(3), 1215-1222.

Axel, C., Brosnan, B., Zannini, E., Peyer, L.C., Furey, A., Coffey, A. and Arendt, E.K. (2015) Antifungal activities of three different lactobacillus species and their production of antifungal carboxylic acids in wheat sourdough. *Appl Microbiol Biotechnol* **100**(4), 1701–1711. https://doi.org/10.1007/s00253-015-7051-x

Ayad, E.H.E., Nashat, S., El-Sadek, N., Metwaly, H. and El-Soda, M. (2004) Selection of wild lactic acid bacteria isolated from traditional Egyptian dairy products according to production and technological criteria. *Food Microbiol* **21**(6), 715–725. https://doi.org/10.1016/j.fm.2004.02.009

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**(1-2), 248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Campanaro, S., Treu, L., Vendramin, V., Bovo, B., Giacomini, A. and Corich, V. (2014) Metagenomic analysis of the microbial community in fermented grape marc reveals that *Lactobacillus fabifermentans* is one of the dominant species: insights into its genome structure. *Appl Microbiol Biotechnol* **98,** 6015–6037. https://doi.org/10.1007/s00253-014-5795-3

Copetti, M.V., Iamanak, B.T., Pitt, J.I. and Taniwaki, M.H. (2014) Fungi and mycotoxins in cocoa: from farm to chocolate. *Int J Food Microbiol* **178,** 13-20. https://doi.org/10.1016/j.ijfoodmicro.2014.02.023

Copetti, M.V., Iamanaka, B.T., Nester, M.A., Efraim, P. and Taniwaki, M. H. (2013) Occurrence of ochratoxin A in cocoa by-products and determination of its reduction during chocolate manufacture. *Food Chem* **136**(1), 100–104. https://doi.org/10.1016/j.foodchem.2012.07.093

Corsetti, A., Settanni, L., van Sinderen, D., Felis, G.E., Dellaglio, F. and Gobbetti, M. (2005) *Lactobacillus rossii* sp. nov., isolated from wheat sourdough. *Int J Syst Evol Microbiol* **55**(1), 35–40. https://doi.org/10.1099/ijs.0.63075-0

De Angelis, M., Bottacini, F., Fosso, B., Kelleher, P., Calasso, M., Di Cagno, R., Ventura, M., Picardi, E., van Sinderen, D. and Gobbetti, M. (2014) *Lactobacillus rossiae*, a vitamin

B12 producer, represents a metabolically versatile species within the genus *Lactobacillus. PLoS ONE,* **9,** e107232. https://doi.org/10.1371/journal.pone.0107232

De Bruyne, K., Camu, N., De Vuyst, L. and Vandamme, P. (2009) *Lactobacillus fabifermentans* sp. nov. and *Lactobacillus cacaonum* sp. nov., isolated from Ghanaian cocoa fermentations. *Int J Syst Evol Microbiol* **59,** 7–12. https://doi.org/10.1099/ijs.0.001172-0

De Vuyst, L. and Leroy, F. (2020) Functional role of yeasts, lactic acid bacteria and acetic acid bacteria in cocoa fermentation processes. *FEMS Microbiol Rev* **44**(4), 432–453. https://doi.org/10.1093/femsre/fuaa014

De Vuyst, L. and Weckx, S. (2016) The cocoa bean fermentation process: from ecosystem analysis to starter culture development. *J Appl Microbiol* **121,** 5–17. https://doi.org/10.1111/jam.13045

Decimo, M., Quattrini, M., Ricci, G., Fortina, M.G., Brasca, M., Silvetti, T., Manini, F., Erba, D., Criscuoli, F. and Casiraghi, M.C. (2017). Evaluation of microbial consortia and chemical changes in spontaneous maize bran fermentation. *AMB Express*, **7**(1). https://doi.org/10.1186/s13568-017-0506-y

Delgado-Ospina, J., Molina-Hernández, J.B., Chaves-López, C., Romanazzi, G. and Paparella A. (2021) The role of fungi in the cocoa production chain and the challenge of climate change. *J Fungi,* **7,** 202. https://doi.org/10.3390/jof7030202

El Soda, M.E. and Desmazeaud, M.J. (1982) Les peptide-hydrolases des lactobacilles du groupe thermobacterium. i. Mise en évidence de ces activités chez *Lactobacillus helveticus*, *L. acidophilus, L. lactis* et *L. bulgaricus*. *Can J Microbiol* **28**(10), 1181–1188. https://doi.org/10.1139/m82-174

Gänzle, M. G. (2015) Lactic metabolism revisited: metabolism of lactic acid bacteria in food fermentations and food spoilage. *Curr Opin Food Sci* **2,** 106–117. https://doi.org/10.1016/j.cofs.2015.03.001

Iametti, S., Rasmussen, P., Frøkiaer, H., Ferranti, P., Addeo, F. and Bonomi, F. (2002). Proteolysis of bovine β-lactoglobulin during thermal treatment in subdenaturing conditions highlights some structural features of the temperature-modified protein and yields fragments with low immunoreactivity. *Eur J Biochem* **269**(5), 1362–1372. https://doi.org/10.1046/j.1432-1033.2002.02769.x

Keller, N.P. and Adams, T.H. (1995) Analysis of a mycotoxin gene cluster in *Aspergillus nidulans*. *SAAS Bulletin Biochem Biotechnol* **8,** 14-21. PMID: 7546572.

Klich, M.A., Mullaney, E.J., Daly, C.B. and Cary, J.W. (2000) Molecular and physiological aspects of aflatoxin and sterigmatocystin biosynthesis by *Aspergillus tamarii* and *A. ochraceoroseus*. *Appl Microbiol Biotechnol* **53**(5), 605–609. https://doi.org/10.1007/s002530051664

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**(5259)**,** 680–685.

Lefeber, T., Janssens, M., Moens, F., Gobert, W. and De Vuyst, L. (2011) Interesting starter culture strains for controlled cocoa bean fermentation revealed by simulated cocoa pulp fermentations of cocoa-specific lactic acid bacteria. *Appl Environ Microbiol* **77,** 6694–6698. https://doi.org/10.1128/aem.00594-11

Ma, C.L., Zhang, L.W., Yi, H.X., Du, M., Han, X., Zhang, L.L., Feng, Z., Zhang, Y.C. and Li, Q.(2011) Technological characterization of lactococci isolated from traditional Chinese fermented milks. *J Dairy Sci* **94**(4) 1691-1696. https://doi.org/10.3168/jds.2010-3738

Mora, D., Parini, C., Fortina, M.G. and Manachini, P.L. (2000) Development of molecular RAPD marker for the identification of *Pediococcus acidilactici* strains. *Syst Appl Microbiol* **23**(3), 400–408. https://doi.org/10.1016/s0723-2020(00)80071-5

Papalexandratou, Z., Falony, G., Romanens, E., Jimenez, J. C., Amores, F., Daniel, H.-M. and De Vuyst, L. (2011) Species diversity, community dynamics, and metabolite kinetics of the microbiota associated with traditional Ecuadorian spontaneous cocoa bean fermentations. *Appl Environ Microbiol* **77,** 7698–7714. https://doi.org/10.1128/aem.05523-11

Riedl, R., Goderbauer, P., Brandl, A., Jacob, F. and Hutzler, M. (2017) Bavarian wheat beer, an example of a special microbe habitat–cultivation, detection, biofilm formation, characterization of selected lactic acid bacteria hygiene indicators and spoilers. *Brew Sci* **70,** 39–50. doi:10.23763/BrSc17-04riedl

Schwan, R. F. and Wheals, A. E. (2004). The microbiology of cocoa fermentation and its role in chocolate quality. *Crit Rev Food Sci Nutr* **44**(4), 205–221. https://doi.org/10.1080/10408690490464104

4.11 COCOA. Fine cocoa fermentation with selected Lactic Acid Bacteria: fermentation performance and impact on chocolate composition and sensory properties

The results presented here below are submitted as: Dea Korcari, Alberto Fanton, Giovanni Ricci, Noemi Sofia Rabitti, Monica Laureati, Johannes Hogenboom, Luisa Pellegrino, ***Davide Emide****, Alberto Barbiroli, Maria Grazia Fortina. Fine cocoa fermentation with selected Lactic Acid Bacteria: fermentation performance and impact on chocolate composition and sensory properties.*

4.11.1 Abstract

In this research we performed controlled fermentations of a fine cocoa variety, to evaluate the impact of adjunct cultures of selected Lactic Acid Bacteria (LAB) on fermentation parameters, chemical composition and sensory profile of fine cocoa and chocolate. Improved fermentation processes employing two strains of LAB, previously isolated from cocoa, and belonging to *Lactiplantibacillus fabifermentans* and *Furfurilactibacillus rossiae* species, were carried out at the Centre for the Integral Transformation of Cacao (CETICO) in Dominican Republic. Fermentation parameters, protein, peptide and free amino acid profiles of the fermented cocoa and volatile molecules were determined. Sensory analysis of the derived chocolate was also carried out. The results obtained indicated that the addition of the adjunct cultures significantly influences the extent and the kinetics of the proteolytic processes and the free amino acid profile. Finally, the adjunct cultures seemed to increase the complexity of the flavour profile of the chocolate as they received a higher score for descriptors commonly used for fine chocolate such as honey and red fruits. The results obtained showed that the selected strains can be an added value to the development of specific flavours that are desirable at an industrial level.

4.11.2 Introduction

Cocoa (*Theobroma cacao L.*) is the main ingredient in the production of chocolate, one of the most important luxury foods with a rapidly expanding market. Before being suitable for chocolate production, cocoa seeds extracted from cocoa pods need to undergo a series of transformations, such as fermentation, drying and roasting. Each of these steps play an important role in the development of the typical chocolate flavour and in the reduction of undesirable notes such as bitterness and astringency, even though the fermentation process is regarded as the most important post-harvest factor influencing the flavour potential of cocoa (De Vuyst & Weckx, 2016). The spontaneous fermentation is characterised by a consortium of naturally occurring yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAB) that causes a series of chemical changes within the cocoa bean that are crucial to the development of the complex, typical chocolate flavour (De Vuyst & Leroy, 2020).

From a quality standpoint, the cocoa beans are classified into “fine” or “flavour” cocoa and “bulk” cocoa. According to the International Cocoa Agreement (United Nations, 2010), fine cocoa can be defined as “cocoa that is recognised for its unique flavour and colour”. Generally, fine cocoa is produced from Criollo, Trinitario and Nacional cocoa varieties, whereas bulk cocoa is obtained from Forastero varieties. Although fine cocoa makes up a small percentage of the cocoa market, estimated around 12%, the demand for this product has increased rapidly in recent years. Only a restricted number of countries can export fine cocoa, and Ecuador, the Dominican Republic and Peru are the main exporters of this product (ICCO, 2021).

In order to be classified as fine cocoa, the product has to meet a number of criteria from a qualitative point of view, but the main factor is the flavour. Specifically, fine cocoa has a lower intensity of undesirable sensory properties such as acidity, bitterness and off-flavours, and it is characterised by flavours such as fruity, flowery, nutty, herbal or caramel. Obtaining products with a standardised sensory profile can be problematic at industrial level, because cocoa fermentation is still carried out using a spontaneous, in-farm process that generally results in end-products of variable quality (Kadow et al., 2013). For this reason, the use of specific starters or adjunct cultures has been and is still investigated (Crafack et al., 2013; de C. Lima et al., 2021; Lefeber et al., 2012; Magalhães da Veiga Moreira et al., 2017; Pereira et al., 2012) with the purpose of standardising the fermentation process.

The aim of this work is to evaluate the impact of adjunct cultures of selected Lactic Acid Bacteria (LAB) on fermentation parameters, flavour and sensory profile of fine cocoa and chocolate. The fermentation processes were carried out at the Centre for the Integral Transformation of Cacao (CETICO) in Dominican Republic. Two strains of LAB, *Lactiplantibacillus fabifermentans* SAF13 and *Furfurilactibacillus rossiae* SAF51, previously isolated from spontaneously fermented Dominican cocoa and investigated for their potential as starter or adjunct cultures, were used as inoculum in improved fermentation processes, in comparison with spontaneous cocoa fermentation. Sensory analyses of the chocolate from the fermented cocoa were carried out for testing the ability of selected strains to modify the flavour and sensory profile of a fine, Criollo cocoa variety.

4.11.3 Materials and Methods

4.11.3.1 Strain growth and maintenance

Two LAB strains, *L. fabifermentans* SAF13 and *F. rossiae* SAF51 previously isolated from spontaneously fermented Dominican cocoa were cultivated in Man Rogosa Sharpe (MRS) broth (Difco Lab., Augsburg, Germany) at 30 °C for 24h. The strains were maintained in MRS with 15% glycerol at -80 °C and were deposited at the Culture Collection of the Department of Food, Environmental and Nutritional Sciences, University of Milan, Italy.

Four mould strains, previously isolated from a contaminated batch of cocoa at the end of the fermentation period and deposited at the culture Collection of the Department of Food, Environmental and Nutritional Sciences, University of Milan, Italy, were used for the biocontrol assay. The mould strains were cultured in Malt Extract Agar (MEA) plates (Thermo Fisher Scientific, Massachusetts, US). Spores were collected by pouring sterile distilled water containing 0.9% NaCl on the plates after complete sporification, slowly agitating and storing in sterile tubes.

4.11.3.2 Fermentation step

For the inoculum, LAB cultures, grown as reported above, were centrifuged at 7,000 ×*g* for 20 min at 4 °C and the cellular pellet was resuspended in a 5% sucrose solution to a final cell concentration between 10^8 and 10^9 CFU/mL. The vitality of the suspension was evaluated by plate counting in MRS agar incubated at 30 °C for 48h. The cell suspension obtained was used to inoculate 60 kg of freshly harvested cocoa beans of the Criollo variety, provided by Rizek Cacao S.A.S., San Francisco de Macorìs, Dominican Republic. The final cell concentration for each strain, ranging between 10^6 and 10^7 CFU/g, is shown in Tab. 1. The fermentation was carried out in closed plastic containers, that were perforated to allow an adequate pulp drainage. The fermenting mass was mixed every 48h for a total of 6 days of fermentation, and then sun dried to a final humidity lower than 8%.

Two different inoculation setups were performed: in the first setup, the inoculation was carried out at the beginning of the fermentation, whereas in the second setup the inoculation was performed both at the beginning and after 48h. A mixed culture fermentation comprised of both strains was also set up to explore the synergies between the strains. A control fermentation without inoculum was performed, for comparison (Tab. 1).

4.11.3.3 Fermentation parameters measurement

For each trial, total LAB and yeast counts were performed in MRS agar with 0.1% cycloheximide (Merck, NJ, US) and Sabouraud Dextrose Agar with 0.1% Tetracycline (Merck, NJ, US), respectively. The temperature was recorded daily. The pH of the pulp was evaluated by resuspending 10g of cocoa in 90 mL of distilled water. Similarly, the cotyledon pH was measured by resuspending 10g of pulp-free cocoa beans in 90 mL of distilled water.

Table 1. Inoculation protocols and microbial counts for each inoculum.

Sample	Fermentation protocol	Inoculum (CFU/g of cocoa)
Control	Spontaneous fermentation	-
A	Inoculum with strain *L. fabifermentans* SAF13 at t_0	$6.25x10^6$
B	Inoculum with strain *L. fabifermentans* SAF13 at t_0 and t_{48}	$6.25x10^6$ at t_0 $1.6x10^7$ at t_{48}
C	Inoculum with strain *F. rossiae* SAF51 at t_0	$1.4x10^7$
D	Inoculum with strain *F. rossiae* SAF51 at t_0 and t_{48}	$1.4x10^7$ at t_0 $1.9\ x10^7$ at t_{48}
E	Inoculum with strains *L. fabifermentans* SAF13 and *F. rossiae* SAF51 at t_0 and t_{48}	SAF13: $6.25x10^6$ at t_0 +$1.6x10^7$ at t_{48} SAF51: $1.4x10^7$ at t_0 + $1.9\ x10^7$ at t_{48}

4.11.3.4 Mould growth inhibition

To assess the ability of the inoculated strains to prevent mould growth during fermentation, 20 g cocoa samples were retrieved at the end of the 6-day fermentation period and were inserted in sterile 50 mL tubes. A 0.5 mL suspension of approximately 10^4 fungal spores of four mould species isolated from a contaminated batch of cocoa at the end of the fermentation period, belonging to *Aspergillus tamarii*, *Aspergillus nidulans*, *Rhizomucor pusillus* and *Lichteimia ornata* was added to each tube, that were incubated at 30°C for 3 days. A non-inoculated sample was also incubated as a control. The growth of mould was evaluated visually.

4.11.3.5 Protein extraction and electrophoretic characterisation

The electrophoretic characterisation of cocoa bean's proteins was adapted from Kumari et al. (2016). Before the extraction, the seed coats of beans were removed by peeling them off with a scalpel, and beans were then finely grinded. Protein's extraction was done by suspending 250 mg of finely grinded cocoa beans in 1 mL of 100 mM Tris-HCl pH 8.1, in presence of 1% DTT and 1% SDS. The suspension was heated in boiling water for 10 min and then stirred for 90 min. The suspension was centrifuged at 13,000 ×*g* for 20 min at 4 °C. Lipids were removed and the liquid phase, containing the solubilised proteins, was collected.

Electrophoretic protein pattern of cocoa beans was characterised through a NuPAGE® electrophoresis system (Invitrogen by Thermo Fisher Scientific, Monza, Italy) by using NuPAGE® 4–12% Bis-Tris Gel and NuPAGE® MES SDS Running Buffer. Samples were prepared in denaturing buffer (0.125 M Tris-HCl, pH 6.8, 50% glycerol, 1.7% SDS, 0.01% bromophenol blue (w/v)), containing 1% (v/v) 2-mercaptoethanol (2-ME), and run according to manufacturer's instructions for reduced condition (Invitrogen by Thermo Fisher Scientific,

Monza, Italy). At the end of the run, proteins were fixed by soaking the gel in 5% glutaraldehyde and 50% methanol for 60 min, whereupon the gel was stained in Coomassie Blue.

4.11.3.6 Chromatographic characterisations

Peptide profiles were studied by chromatographic techniques. Samples were prepared by suspending 250 mg of finely grinded cocoa beans in 5 mL of PBS. Suspensions were stirred at 4°C for 60 min and then centrifuged at 10,000 ×*g* for 40 min at 4°C. The liquid supernatant phase was collected, added of 2% trifluoroacetic acid (TFA) and centrifuged at 13,000 ×*g* for 10 min at 4 °C. The clear supernatant was used for the chromatographic characterisations.

Size Exclusion High Performance Liquid Chromatography (SE-HPLC) separations were performed in a Superdex^{TM} Peptide 10/300 GL column (Cytiva Europe GmbH, Milano, Italy) fitted on a chromatographic apparatus composed by a Waters 600E multisolvent delivery system and a Waters 2487 Dual λ Absorbance Detector (Waters, Sesto San Giovanni, Italy). Mobile phase was PBS, at 0.5 mL/min. Before the use, the pH of the sample was corrected at around 7 by using 5 M NaOH.

Reverse Phase High Performance Liquid Chromatography (RP-HPLC) separations were performed in a SIMMETRY300 C18 (5 µm) (4.6 × 250 mm) column (Waters) fitted on a chromatographic apparatus composed by two Waters 510 HPLC pumps, a Waters 717plus Autosampler and a Waters 996 Photodiode Array Detector (Waters). Separations were run at 0.8 mL/min, mixing solution A (0.1% TFA in water) and solution B (0.1% TFA in acetonitrile (ACN)) as follows: 5 min isocratic 100% solution A, 50 min linear gradient to 70% solution A and 30% solution B.

4.11.3.7 Free amino acids profile

For the extraction of free amino acids, 4.5 g of finely grinded dry cocoa beans were dispersed under magnetic stirring in 40 mL of sodium citrate buffer (0.2 N, pH 2.2) for 40 min. The solution obtained was sonicated for 5 min and filtered using a Whatman No. 41 filters (Whatman plc, Maidstone, UK). 10 mL of the filtrate were deproteinated with 10 mL solution of 7.5% (p/v) sulfosalicylic acid at pH 1.75, under magnetic stirring, for 5 min. After the addition of 250 µL of Nor-Leucine as internal standard the solution was brought to the final volume of 25 mL and filtered using a Whatman No. 42 filter (Whatman) and subsequently it was filtered using a 0.2 µm size pore size syringe filter.

100 µL of the suspension obtained were used for quantification by Ion Exchange Chromatography (IEC) using a Biochrom 30+ chromatograph equipped with an automatic sampler, as described by Hogenboom et al. (2017). An unfermented dry cocoa sample was used to evaluate the changes in the concentration of free amino acids due to the fermentation process.

4.11.3.8 Cocoa liquor and chocolate preparation

Cocoa liquor and chocolate samples for further evaluations were prepared at the KahKow Experience, Santo Domingo, Dominican Republic. Dried cocoa beans were roasted at 100°C for 20 min. Roasted beans were then cracked and dehulled using an automatic winnower. The cocoa nibs were ground to obtain cocoa liquor.

The formulation used to produce the chocolate samples was: cocoa liquor 58%, deodorised cocoa butter 10%, powdered white sugar 32%. After refining through a roller press, the mixture was conched (50 °C, 24h) and tempered by heating the mass at 50 °C and cooling at 31 °C. Finally, the chocolate was moulded into 7g pieces that were individually wrapped and stored at 20 °C until the sensory evaluation.

4.11.3.9 Volatile molecules assay

To analyse the aromatic compounds of the cocoa liquor, 100 mg of sample were inserted in 20 mL headspace vials. Each sample was concentrated in a Gerstel MPS 2 with Dynamic Headspace DHS instrument (GERSTEL GmbH & Co., GERSTEL Inc. USA) at 70 °C under 750 rpm agitation. The volatile fraction was trapped with helium (10 min, 20 °C), and analysed in GC-MS (Agilent GC 6890N, Agilent Technologies Inc. USA) equipped with an Agilent DB 624 column (60 m x 0,32 mm x 1,80 µm) after thermodesorption (heat rate 30-260 °C). The carrier gas was helium at a flow rate of 2 mL/min. The oven temperature was set at a heat rate of 40-255 °C. The mass detector was an Agilent MSD 5975 with an electronic ionisation at 70 eV. The data was analysed using the Agilent ChemStation software.

4.11.3.10 Sensory analysis

In order to describe the chocolate samples sensory properties, the sensory profile method was applied (ISO 13299, 2010).

Ten subjects (7 women and 3 men aged between 20 and 33 years) were selected from a panellist pool consisting of University of Milan students and employees. To participate in the study, subjects had to like and consume dark chocolate. The method consisted of a preliminary training phase to acquire familiarity with the product and the methodology, followed by a second phase focused on the sample evaluation. Subjects were trained over a period of one month (nine sessions of approximately 1 h each). During this phase, several commercial dark chocolates (including samples from Criollo variety) were selected and presented to the assessors in order to provide a wide range of sensory variability for each attribute and thus stimulate the generation of descriptors. As training progressed, descriptive terms and relevant reference standards were defined through panel discussion. Fourteen sensory descriptors covering appearance (brown colour), odour (cocoa, red fruits, toasted, honey), taste (sweet, bitter, sour), flavour (cocoa, red fruits, toasted, honey) and mouthfeel sensations (hardness,

astringency) were defined (see Table S1 supplementary materials). Once the vocabulary was set up, the assessors performed three preliminary sessions to acquire familiarity with the scale.

After the training phase, judges evaluated the six chocolate samples, named Control, A, B, C, D, E (according to Table 1) in three replicates (of which two were retained for data analysis). The three replicates were performed in different days at the sensory laboratory of the Department of Food, Environmental and Nutritional Sciences (University of Milan) designed according to ISO guidelines (ISO 8589, 2007). Assessors were asked not to smoke, eat or drink anything, except water, for 1 hour before the tasting sessions.

Assessors received one 7 g piece of each sample, for a total of six chocolates in each tasting session and rated the intensity of each sensory attribute using a 9-point scale ("1" = "absence/minimum intensity of the descriptor"; "9" = "highest intensity of the descriptor").

Chocolate samples were served at room temperature in plastic plates that were coded with 3-digit numbers. The order in which the samples were presented was systematically varied over assessors and replicates in order to balance the effects of serving order and carryover (Macfie, Bratchell, Greenhoff, & Vallis, 1989). Chocolate samples were evaluated in individual booths under white light. The assessors were provided with unsalted crackers and water to clean their mouths during the evaluation. The data were collected using Fizz software v2.47 (Biosystemes, Couternon, France).

4.11.3.11 Statistical analysis

Experiments reported were performed in triplicate, and results are shown as mean ± standard deviation. T-tests were performed using GraphPad Prism 8 (v. 8.4.3, GraphPad Software Inc., California, USA) and significance levels are indicated as n.s. for non-significant differences, one asterisk (*) for $p<0.05$, two asterisks (**) for $p<0.01$, three asterisks (***) for $p<0.001$.

Sensory data were first analysed by means of 3-way ANOVA considering Samples (6), Judges (10), Replicates (2) and their relevant second order interactions as factors and sensory attributes as dependent variables. When the ANOVA showed a significant effect ($p<0.05$), the least significant difference (LSD) was applied as a multiple comparison test using the statistical software program STATGRAPHICS PLUS version 5.0 (Manugest KS Inc., Rockville, USA).

In order to examine the results from a multidimensional point of view, Principal Component Analysis (PCA) was then performed on sensory data averaged across judges and replicates (matrix Samples × Descriptors) using XLSTAT version 2019.2.2 (Addinsoft, Boston, MA, USA).

4.11.4 Results

4.11.4.1 Fermentation parameters

To study the progress of the fermentation, the temperature and pH of the cocoa samples were recorded daily. Counts of LAB and yeasts were also performed during the initial 72h of fermentation; after this period the LAB and yeast counts were <10 CFU/g of cocoa. The results for each fermentation setup are reported in figure 1. The inoculation of the selected strains did not have a significant impact on the temperature or pH of the pulp and cotyledon. Whereas the strain *L. fabifermentans* SAF13 did not significantly impact the total LAB or yeast counts, the inoculum of the strain *F. rossiae* SAF51 led to a significant increase in the total LAB count after 24h of fermentation, but this difference was not observed in the subsequent days. The inoculum of this strain after 48h of fermentation led to a significant decrease in total yeast count at the 72h mark. Similarly, the inoculum of the consortia of the two strains did not have a significant impact on the fermentation parameters considered.

The pH of the pulp did not significantly change during the first 24h of fermentation, despite the organic acid production by the LAB. This aspect is related to the consumption of citric acid by the LAB, that is known to raise the pH of the cocoa pulp at the beginning of the fermentation (Ouattara et al., 2016). The pH was lowest at 48h, then it increased up to 4-4.5. The pH of the cotyledon, on the other hand, steadily decreased from the initial pH of 5-5.5 to about 4.5, matching the pH of the pulp. The temperature profile depended on the mixing of the fermenting mass, as it rose significantly after 48h when the first mixing was performed, to reach a temperature of 45-50°C that was maintained throughout therest of the fermentation period.

The LAB appeared to have their maximum growth at 24-48h from the beginning of the fermentation, whereas the growth of yeasts appeared to decrease earlier compared to LAB, that may be due to the higher sensitivity of yeasts to the stress factors such as the low pH and high temperature that start to take place after 48h of fermentation.

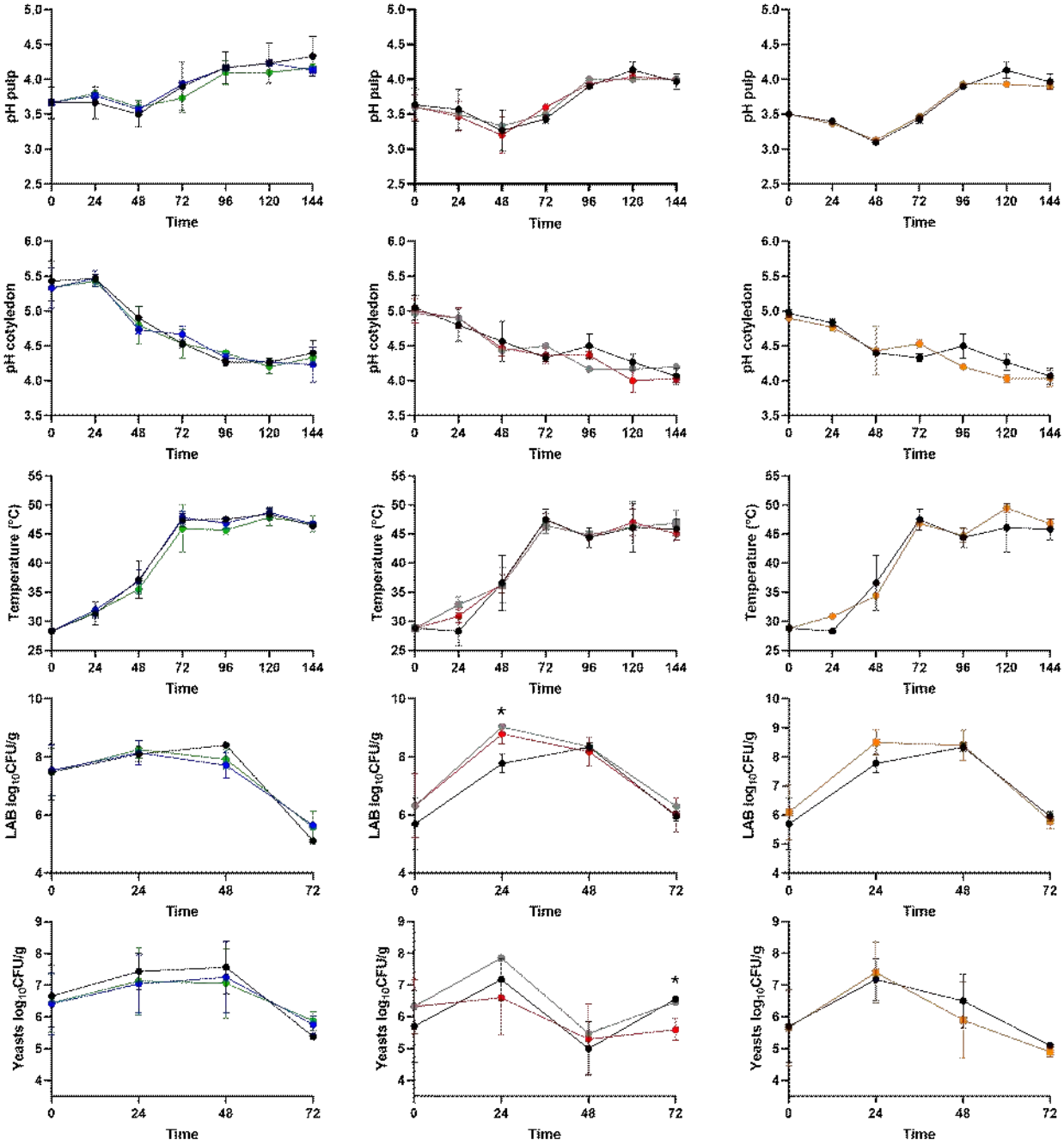


Figure 1. Temperature, pulp and cotyledon pH and LAB and yeast counts for each inoculated species and different inoculation protocol, in comparison with spontaneous fermentation (black): 1) *L. fabifermentans* at t_0 (blue) and t_0+t_{48} (green), 2) *F. rossiae* at t_0 (grey) and t_0+t_{48} (red), 3) co-culture at t_0+t_{48} (orange)

4.11.4.2 Mould growth inhibition

The results of the in-situ antifungal activity towards mould strains isolated from contaminated cocoa are represented in figure 2. Both strains showed some level of inhibition but, while the

strain *F. rossiae* SAF51 only delayed the growth of the four mould strains, these were completely inhibited by *L. fabifermentans* SAF13 as no growth was observed after three days of incubation. On the other hand, all four mould species had a good ability to grow in the spontaneously fermented control.

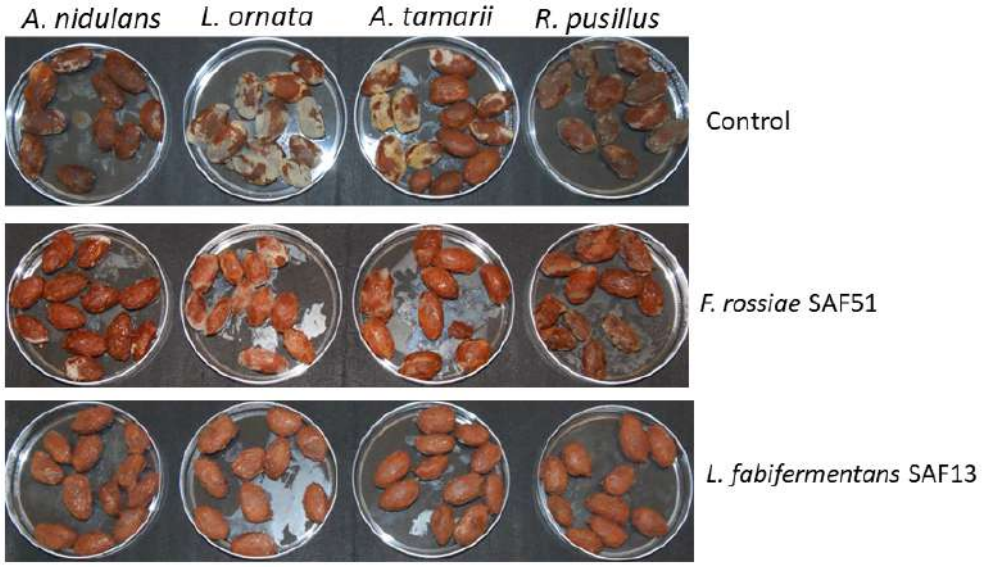


Figure 2. Antifungal activity of strains *L. fabifermentans* SAF13 and *F. rossiae* SAF51 against four mould strains isolated from cocoa. Spontaneously fermented cocoa was used as control.

4.11.4.3 Protein and peptide characterisation

The unfermented sample showed the peculiar protein profile of *Theobroma cacao* seeds. In the protein pattern (figure. S1, panel A) it is possible to identify the Vicilin-type storage proteins (23% of the soluble seed proteins, corresponding to the bands at 47, 31 and 14.5 kDa), and the Kunits-type trypsin inhibitor (14% of the soluble seed proteins, 21 kDa) (Lerceteau et al., 1999). During the fermentation, most of the proteins are hydrolysed: vicilin-type proteins showed a complete degradation, whereas trypsin inhibitor was still visible indicating that proteolysis of this protein was only partial (Kumari et al, 2016). An accumulation of relatively large peptides was observed around 5-7 kDa, together with a light band at 3.5 kDa. No smaller peptides were observed in the gel (figure S1, panel A).

Low molecular weight peptides (< 5 kDa) have been extracted from fermented and unfermented cocoa beans and characterised by chromatographic approaches. SE-HPLC showed a modest accumulation of peptides at dimensions just higher than 500 Da (figure. S1, panel B). According to their molecular weights, these peptides could correspond to the band visible at 3.5 kDa in the electrophoretic separation.

A more efficient separation of peptides was achieved by RP-HPLC. Although this technique does not give information about peptide sizes, the chromatograms showed the presence of three main peaks in the unfermented sample, eluting at 21.5 (peak 1), 23 (peak 2) and 24 (peak 3) minutes (figure. 3, panel A; figure. S1, panel C). The area of these peaks decreased in the fermented samples, suggesting that they refer to three peptides already present in the unfermented sample, digested by proteases during fermentation. Interestingly, the area of these peaks can be correlated to the inoculum and fermentation protocol applied. The largest area reduction was observed with the spontaneous fermentation (figure. 3, panel B), while, in the presence of the double inoculum (SAF13+SAF51 at t_0 and t_{48}) the reduction was close, but not equal. Noteworthy, the inoculum of *L. fabifermentans* SAF13 or *F. rossiae* SAF51 separately, resulted in an intermediated reduction of the areas, with the inoculum at t_0 + t_{48} more active than the t_0 alone.

4.11.4.4 Free amino acids profile

The results obtained are represented in table 2, expressed as g/kg of total protein. The chromatographic profiles of the unfermented control and the control are shown in figure 4.

The total amount of free amino acids in the fermented samples was almost twice that of the unfermented sample. The inoculation of the strain *F. rossiae* SAF51 appears to promote the liberation of free amino acids when compared to the samples inoculated with the strain *L. fabifermentans* SAF13.

The fermentation protocol influenced the final amino acid concentration as well the total amount of amino acids slightly decreased in samples inoculated both at t_0 and at t_{48}. Whereas the amount of some amino acids decreased with the fermentation (Asn, Gln, Gaba, His, Arg), some remained unchanged (Asp, Glu, Pro), the amount of some amino acids increased significantly (Thr, Met, Leu Phe, Lys).

The amino acid profile of the samples inoculated with the strain *L. fabifermentans* SAF13 was similar to the one inoculated with the strain *F. rossiae* SAF51 but, when considering the relative proportion of the single amino acids, the samples inoculated with the strain SAF13 had a consistent abundance of Asn, Glu and Ala, whereas the sample inoculated with SAF51 contained a higher relative amount of Ile, Tyr, Orn and Lys. The sample inoculated with the consortia *L. fabifermentans* SAF13 and *F. rossiae* SAF51 had a profile that closely resembled that obtained with *F. rossiae* SAF51 alone.

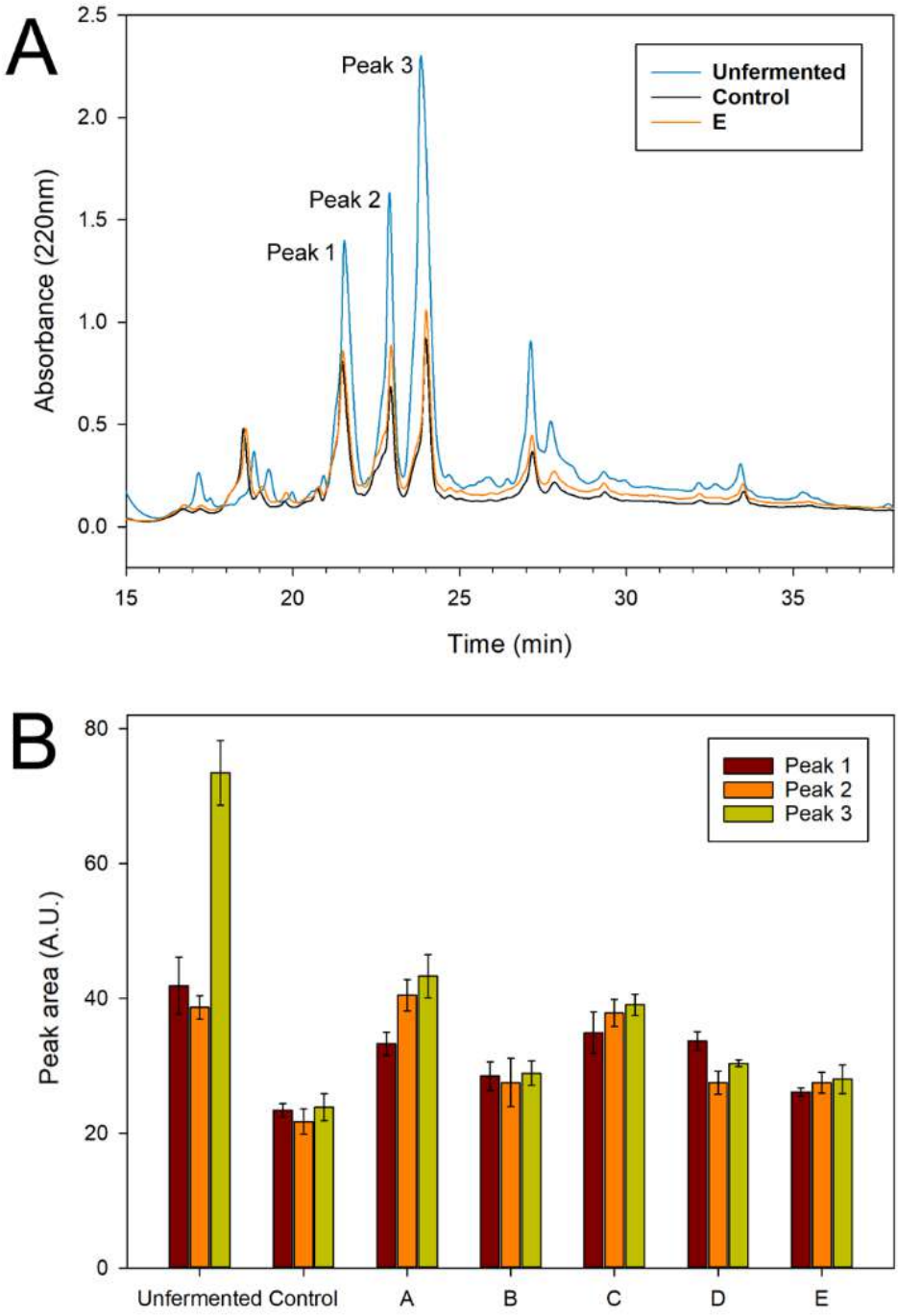


Figure 3. Peptide profile of unfermented and fermented samples. Panel A: RP-HPLC chromatograms of peptide extracted from cocoa beans. For the sake of comparison, only chromatograms from unfermented, control and co-culture at t_0+t_{48} have been reported. Chromatograms of all the samples are available in fig.S1. Panel B: Areas of the three main peaks observed in RP-HPLC chromatograms; Unfermented: unfermented cocoa beans; Control: spontaneous fermentation; A: inoculated with *L. fabifermentans* at t_0; B: inoculated with *L. fabifermentans* at t_0 and t_{48}; C: inoculated with *F. rossiae* at t_0; D: inoculated with *F. rossiae* at t_0 and t_{48}; E: inoculated with both strains at t_0 and t_{48}.

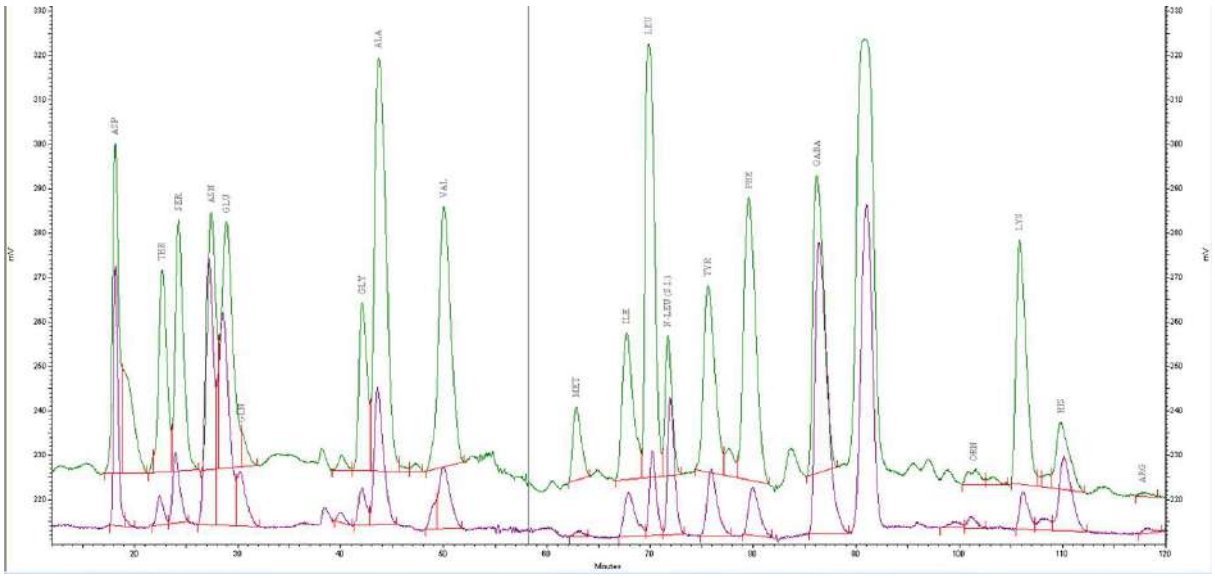


Figure 4. Chromatographic profiles of the unfermented sample (purple) and fermented control (green).

4.11.4.5 Volatile molecules of cocoa mass

The results obtained are reported in figure 5. The flavour descriptors for each molecule are reported according to The Good Scents Company. The amount in ppm of each volatile molecule detected is reported in table S2.

In general, the samples inoculated with the strain *L. fabifermentans* SAF13 presented a higher total amount of volatiles when compared to the control, whereas the amount was lower in samples inoculated with the strain *F. rossiae* SAF51.

In the samples inoculated with *L. fabifermentans* SAF13, the compounds that increased the most, with concentrations more than twice that of the control, were 2,3-butanedion, hexanal, 2-heptanone and 2-nonanone. Furthermore, in the sample inoculated at t_0 only, an increase of approximately 50% was recorded for 2-methyl butyric acid and 3-methyl butyric acid. On the other hand, a reduction was recorded for benzyl acetaldehyde, tetramethyl pyrazine and phenethyl acetate.

Considering the samples inoculated with the strain *F. rossiae* SAF51, a reduction in the majority of compounds was noted, that was more pronounced in the sample inoculated both at t_0 and at t_{48}. 2-methyl butyric acid and 3-methyl butyric acid were the only compounds that increased in both samples.

The sample inoculated with both strains shows an increase in the concentration of hexanal and 2-nonanone, probably due to the high production of these compounds in presence of *L. fabifermentans* SAF13 as previously mentioned.

Significant differences were also observed for the acetic acid amount in the different samples. The fermentations performed with *F. rossiae* SAF51, as well as the one performed by the consortia of the two strains, had lower acetic acid amounts when compared to the control; the samples inoculated with the strain *L. fabifermentans* SAF13, on the other hand, contained higher amounts of acetic acid.

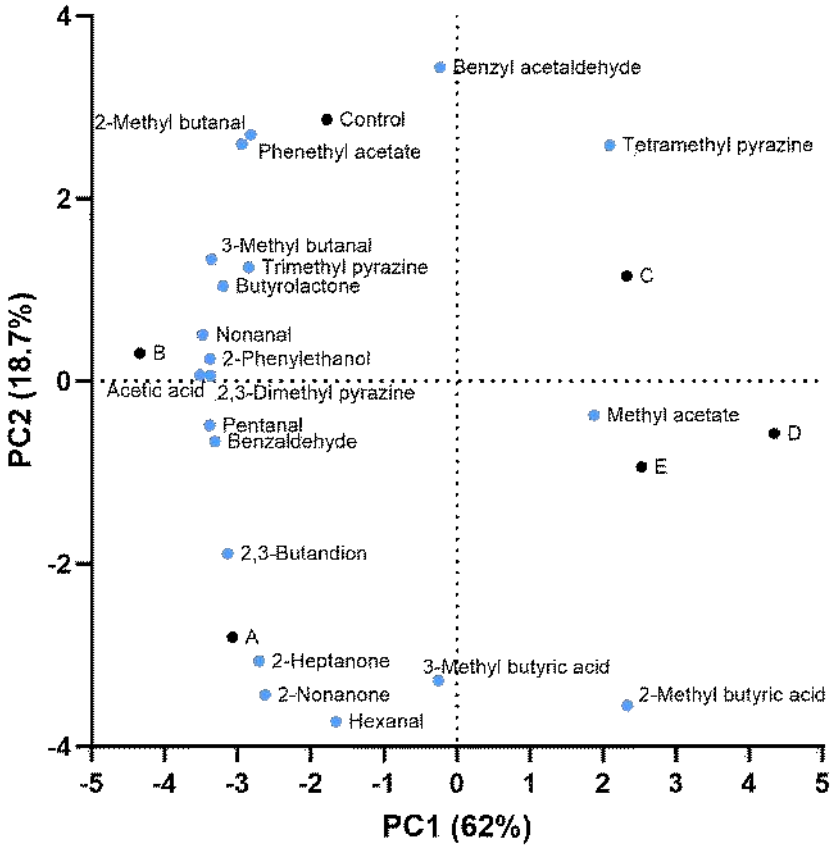


Figure 5. Volatile compounds analysis of cocoa liquor. Control: spontaneous fermentation; A: inoculated with *L. fabifermentans* at t_0; B: inoculated with *L. fabifermentans* at t_0 and t_{48}; C: inoculated with *F. rossiae* at t_0; D: inoculated with *F. rossiae* at t_0 and t_{48}; E: inoculated with both strains at t_0 and t_{48}.

4.11.4.6 Sensory analysis

Three-way ANOVA results indicated that the panel of assessors was reliable (see table S3) and that all the sensory descriptors significantly ($p<0.05$) discriminated the samples.

Since ANOVA results indicated that the mean scores for each sample given by the panel for each attribute could be assumed satisfactory estimates of the sensory profile of samples (table S3), sensory data were averaged across assessors and replicates and submitted to PCA. According to correlation loadings plot, variables with less than 50% explained variance were left out from the analysis (i.e., red fruits odour, astringency and bitter). The biplot based

on samples and the remaining variables is shown in figure 6. The variance explained by the first two principal components was 80.80%. Moving from left to right along the first component (explained variance 64.56%) of figure 6, the control sample and samples A and E were separated from the rest of the samples. The second component (explained variance 16.24%) distinguished samples C from E. The control sample (positioned in the upper left pane, figure 6) was mainly described by brown colour, cocoa odour and flavour, sourness, and hardness and only partially by roasted odour and flavour. Samples A and E were mainly perceived with high intensity of roasted odour and flavour as well as red fruits flavour. These samples (A, E, control) had also the lowest intensity of sensory properties (honey odour and flavour and sweetness) located in the positive part of PC1 of figure 6 which mainly characterise samples D and B. Sample C (located in the upper right pane, figure 6) was mainly sour.

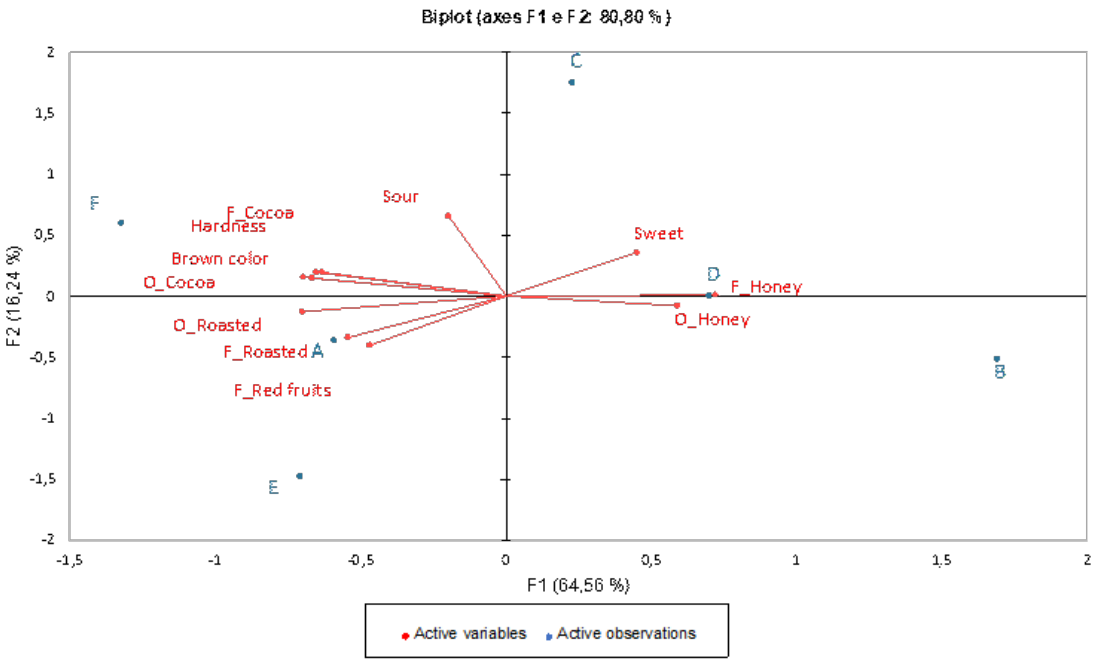


Figure 6. Biplot obtained by the PCA model of chocolate sensory data. A: inoculated with *L. fabifermentans* at t0; B: inoculated with *L. fabifermentans* at t0 and t48; C: inoculated with *F. rossiae* at t0; D: inoculated with *F. rossiae* at t0 and t48; E: inoculated with both strains at t0 and t48. O indicates odour, F indicates flavour.

4.11.5 Discussion

Controlled cocoa fermentations have received a strong interest from chocolate manufacturers and cocoa producers as a natural way to obtain specific flavour profiles without the need of using additives or flavourings. Although a lot of effort from the scientific community has been put into the study of different yeast species, the research is still lacking when it comes to LAB, especially in the study of minor species that do not dominate the fermentation but that may

play a role in the flavour profile of the final product. Furthermore, there is a gap in knowledge about the optimal fermentation parameters to apply in a controlled fermentation and their role in the quality of the final product.

In this research we performed controlled fermentations of a fine flavour cocoa variety using two selected LAB strains, *F. rossiae* SAF51 and *L. fabifermentans* SAF13 as adjunct cultures. Two different inoculation setups were performed: the fermenting cocoa was inoculated either at t_0 only, or both at t_0 and at t_{48}; the second inoculation was performed because from the available scientific literature LAB are indicated to develop and perform at their optimum after the first stage dominated by yeasts (De Vuyst & Weckx, 2016).

The results obtained show that, whereas the two strains did not significantly impact the fermentation parameters such as the temperature and the pH, they gave different profiles when considering the final product. The fact that the fermentation parameters are similar to those of a typical well-fermented cocoa is a positive attribute, as they are crucial for the outcome of the fermentation process. Furthermore, there is no excessive acidity that has been perceived as the main drawback of the presence of LAB in cocoa fermentations (De Vuyst et al., 2010). Indeed, the sensory profile showed that the spontaneous control was the sample with the highest perceived acidity (Table S4). This can be partially due to the heterofermentative phenotype of the LAB used, that produce different metabolites depending on the carbon source used, as opposed to homofermentative LAB that produce lactic acid exclusively. Furthermore, the GC-MS analysis of the cocoa liquor showed a different concentration of acetic acid in the fermentation with each strain, so an interaction with the microbiota as a whole can also be hypothesised, given that acetic acid bacteria are the main producers of acetic acid.

Free amino acids, di- and tripeptides are some of the most important precursors of flavour formation in chocolate. Not only they participate in the Maillard reaction during roasting, giving the typical chocolate flavour, but they also go through degradation to produce aldehydes, ketones and other volatile molecules that have a significant impact in the flavour profile (Hinneh et al., 2018). Vicilin-type proteins were found to be selectively degraded by endogenous proteases under fermentation conditions where optimal levels of aroma precursors were obtained (Voigt et al., 1993). Our results showed that cocoa proteins are digested almost completely whether the fermentation occurs spontaneously or if it is driven by the inoculum of *F. rossiae* SAF51 and/or *L. fabifermentans* SAF13. On the other hand, the inoculum influences the extent and the kinetics of the proteolytic processes occurring on some protein fractions: competition seems to take place between inoculated LAB and spontaneous microflora, since the digestion of some peptides has apparently been reduced compared to the spontaneous fermentation. These results are in agreement with our recent evidence (unpublished data) that *F. rossiae* SAF51 and *L. fabifermentans* SAF13 strains have a different

peptidasic and proteolytic set, even compared to *L. plantarum*, the predominant LAB in cocoa fermentation.

The addition of the adjunct cultures affected the free amino acid profile, either due to the peptidases from the LAB that may be released after the cell death or from the creation of conditions closer to the optimum for the endogenous aspartic proteases and carboxypeptidases that have been suggested as the main enzymes that cleave and release amino acids in cocoa. Hydrophobic amino acids such as alanine, tyrosine and phenylalanine have been indicated as the main precursors of cocoa flavour formation (Afoakwa et al. 2008, Rawel et al., 2019). Their concentration increased with cocoa fermentation, and the inoculation of the strain *F. rossiae* SAF51 appeared to promote their accumulation. The amino acid liberation kinetic observed during cocoa fermentation has been reported previously by Kirchhoff et al. (1989a), that has also noted the elevated proportion between hydrophobic and acidic amino acids (Kirchhoff et al. 1989b), that in our samples is approximately 70:15.

Because the cocoa liquor undergoes significant transformations to obtain chocolate, especially during conching, no direct correlation can be drawn between the volatile compounds detected in cocoa liquor and the flavour profile of the final product. Despite this, the analysis of the volatile compounds is a useful method to highlight the potential activity of selected strains in a controlled fermentation. The adjunct cultures seemed to increase the complexity of the flavour profile of the chocolate as they received a higher score for descriptors commonly used for fine chocolate such as honey and red fruits (Tab. S4). The fermentation protocol also played an important role in all aspects considered, so it is important for future starter culture development to take this factor into consideration.

Finally, the cultures had a protective role against mould contamination, especially the strains *L. fabifermentans* SAF13 that inhibited the growth of the tested moulds completely. These data are of interested as the strains tested, particularly *L. fabifermentans* SAF13, can be considered a good biocontrol agent of aflatoxin-producing mould species during cocoa fermentation. The possibility of using selected LAB to prevent mould contamination in cocoa has been previously investigated with good outcomes (Romanens et al., 2019; Essia Ngang et al., 2014; Ruggirello et al., 2019), so this aspect is an added value to the use of LAB in starter cultures for cocoa fermentation.

4.11.6 Conclusions

In conclusion, this work showed that LAB play a significant role to the flavour and quality of chocolate, and there are significant differences both between different strains and between different inoculation timepoints. Although their presence may not be essential to obtain a product with an acceptable taste, they can be an added value to the development of specific flavours that are desirable at an industrial level.

4.11.7 References

Afoakwa, E.O., Paterson, A., Fowler, M. & Ryan, A. (2008). Flavor formation and character in cocoa and chocolate: a critical review. *Critical Reviews in Food Science and Nutrition*, *48*, 840-857. https://doi.org/10.1080/10408390701719272

Crafack, M., Mikkelsen, M. B., Saerens, S., Knudsen, M., Blennow, A., Lowor, S., Takrama, J., Swiegers, J. H., Petersen, G. B., Heimdal, H., & Nielsen, D. S. (2013). Influencing cocoa flavour using *Pichia Kluyveri* and *Kluyveromyces marxianus* in a defined mixed starter culture for cocoa fermentation. *International Journal of Food Microbiology*, *167*(1), 103–116. https://doi.org/10.1016/j.ijfoodmicro.2013.06.024

de C. Lima, C. O., Vaz, A. B. M., De Castro, G. M., Lobo, F., Solar, R., Rodrigues, C., Martins Pinto, L. R., Vandenberghe, L., Pereira, G., Miúra da Costa, A., Benevides, R. G., Azevedo, V., Trovatti Uetanabaro, A. P., Soccol, C. R., & Góes-Neto, A. (2021). Integrating microbial metagenomics and physicochemical parameters and a new perspective on starter culture for fine cocoa fermentation. *Food Microbiology*, *93*, 103608. https://doi.org/10.1016/j.fm.2020.103608

De Vuyst, L., & Weckx, S. (2016). The cocoa bean fermentation process: from ecosystem analysis to starter culture development. *Journal of Applied Microbiology*, *121*(1), 5–17. https://doi.org/10.1111/jam.13045

De Vuyst, L., Lefeber, T., Papalexandratou, Z., & Camu, N. (2010). The functional role of lactic acid bacteria in cocoa bean fermentation. In: F. Mozzi, R.R. Raya & G.M. Vignolo (Eds.), *Biotechnology of Lactic Acid Bacteria*: *Novel Applications* (pp. 301–326). Wiley-Blackwell, Ames, USA,. https://doi.org/10.1002/9780813820866.ch17

De Vuyst,L., & Frédéric Leroy, F. (2020) Functional role of yeasts, lactic acid bacteria and acetic acid bacteria in cocoa fermentation processes. *FEMS Microbiology Reviews*, *44* (4), 432–453. https://doi.org/10.1093/femsre/fuaa014

Essia Ngang, J.-J., Yadang, G., Sado Kamdem, S. L., Kouebou, C. P., Youte Fanche, S. A., Tsochi Kougan, D. L., Tsoungui, A., & Etoa, F.-X. (2014). Antifungal properties of selected lactic acid bacteria and application in the biological control of ochratoxin A producing fungi during cocoa fermentation. *Biocontrol Science and Technology*, *25*(3), 245–259. https://doi.org/10.1080/09583157.2014.969195

Hinneh, M., Semanhyia, E., Van de Walle, D., De Winne, A., Tzompa-Sosa, D. A., Scalone, G. L., De Meulenaer, B., Messens, K., Van Durme, J., Afoakwa, E. O., De Cooman, L.,

& Dewettinck, K. (2018). Assessing the influence of pod storage on sugar and free amino acid profiles and the implications on some Maillard reaction related flavor volatiles in Forastero cocoa beans. *Food Research International*, *111*, 607–620. https://doi.org/10.1016/j.foodres.2018.05.064

Hogenboom, J. A., D'Incecco, P., Fuselli, F., & Pellegrino, L. (2017). Ion-exchange chromatographic method for the determination of the free amino acid composition of cheese and other dairy products: An inter-laboratory validation study. *Food Analytical Methods*, *10*(9), 3137–3148. https://doi.org/10.1007/s12161-017-0876-4

ICCO (2021, November 9). Fine or flavor cocoa. Retrieved December 11, 2021, from https://www.icco.org/fine-or-flavor-cocoa/.

ISO 13299 (2010). Sensory analysis—Methodology—General guidance to establish a sensory profile. Geneva, Switzerland: International Organization for Standardization.

ISO 8589 (2007). Sensory analysis — General guidance for the design of test rooms. Geneva, Switzerland: International Organization for Standardization.

Kadow, D., Bohlmann, J., Phillips, W., Lieberei, R. (2013) Identification of main fine flavour components in two genotypes of the cocoa tree (*Theobroma cacao* L.). *Journal of Applied Botany and Food Quality, 86(1)*, 90–98. https://doi.org/10.5073/JABFQ.2013.086.013

Kirchhoff, P.-M., Biehl, B., & Crone, G. (1989b). Peculiarity of the accumulation of free amino acids during cocoa fermentation. *Food Chemistry*, *31*(4), 295–311. https://doi.org/10.1016/0308-8146(89)90071-x

Kirchhoff, P.-M., Biehl, B., Ziegeler-Berghausen, H., Hammoor, M., & Lieberei, R. (1989a). Kinetics of the formation of free amino acids in cocoa seeds during fermentation. *Food Chemistry*, *34*(3), 161–179. https://doi.org/10.1016/0308-8146(89)90137-4

Kumari, N., Kofi, K.J., Grimbs, S., D'Souza, R.N., Kuhnert, N., Vrancken, G.& Ullrich, M.S. (2016). Biochemical fate of vicilin storage protein during fermentation and drying of cocoa beans. *Food Research International, 90*, 53–65. doi: 10.1016/j.foodres.2016.10.033.

Lefeber, T., Papalexandratou, Z., Gobert, W., Camu, N., & De Vuyst, L. (2012). On-farm implementation of a starter culture for improved cocoa bean fermentation and its

influence on the flavour of chocolates produced thereof. *Food Microbiology*, *30*(2), 379–392. https://doi.org/10.1016/j.fm.2011.12.021

Lerceteau, E., Rogers, J., Pétiard, V., & Crouzillat, D. (1999). Evolution of cacao bean proteins during fermentation: A study by two-dimensional electrophoresis. Journal of the Science of Food and Agriculture, 79(4). https://doi.org/10.1002/(SICI)1097-0010(19990315)79:4<619::AID-JSFA230>3.0.CO;2-O

Macfie, H. J. H., Bratchell, N., Greenhoff, K., & Vallis, L. V. (1989). Designs to balance the effect of order of presentation and first-order carry-over effects in hall tests. *Journal of Sensory Studies, 4(2),* 129–148.

Magalhães da Veiga Moreira, I., de Figueiredo Vilela, L., da Cruz Pedroso Miguel, M., Santos, C., Lima, N., & Freitas Schwan, R. (2017). Impact of a microbial cocktail used as a starter culture on cocoa fermentation and chocolate flavor. *Molecules*, *22*(5), 766. https://doi.org/10.3390/molecules22050766

Ouattara, H. D., Ouattara, H. G., Droux, M., Reverchon, S., Nasser, W., & Niamke, S. L. (2017). Lactic acid bacteria involved in cocoa beans fermentation from Ivory Coast: species diversity and citrate lyase production. *International Journal of Food Microbiology*, *256*, 11–19. https://doi.org/10.1016/j.ijfoodmicro.2017.05.008

Pereira, G. V., Miguel, M. G., Ramos, C. L., & Schwan, R. F. (2012). Microbiological and physicochemical characterization of small-scale cocoa fermentations and screening of yeast and bacterial strains to develop a defined starter culture. *Applied and Environmental Microbiology*, *78*(15), 5395–5405. https://doi.org/10.1128/aem.01144-12

Rawel, H., Huschek, G., Sagu, S., & Homann, T. (2019). Cocoa bean proteins: characterization, changes and modifications due to ripening and post-harvest processing. *Nutrients*, *11*(2), 428. https://doi.org/10.3390/nu11020428

Romanens, E., Freimüller Leischtfeld, S., Volland, A., Stevens, M. J. A., Krähenmann, U., Isele, D., Fischer, B., Meile, L., & Miescher Schwenninger, S. (2019). Screening of lactic acid bacteria and yeast strains to select adapted anti-fungal co-cultures for cocoa bean fermentation. *International Journal of Food Microbiology*, *290*, 262–272. https://doi.org/10.1016/j.ijfoodmicro.2018.10.001

Ruggirello, M., Nucera, D., Cannoni, M., Peraino, A., Rosso, F., Fontana, M., Cocolin, L., & Dolci, P. (2019). Antifungal activity of yeasts and lactic acid bacteria isolated from

cocoa bean fermentations. *Food Research International*, *115*, 519–525. https://doi.org/10.1016/j.foodres.2018.10.002

United Nations. (2010). International Cocoa Agreement. *Treaty Series*, *2871*, 3. TD/COCOA.10/3

Voigt, J., Biehl, B., & Wazir, S. K. S. (1993). The major seed proteins of *Theobroma cacao* L. Food Chemistry, 47(2). https://doi.org/10.1016/0308-8146(93)90236-9

5. GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

In food proteins contribute to nutritional properties, but also to food quality, texture, aroma, flavor, feeling of satiety, and ease of processing. Moreover, the interaction between proteins and the other macromolecules play a crucial role in the definition of the technological properties of food products. Is clear that the high level of complexity of food matrices, in which a lot of different phase coexist and cooperate to determine peculiar feature appreciated by the food industry and the final consumers, adds a degree of difficulty to the study of food proteins. In this context, the main goal of this PhD thesis was to define novel methodological approaches suitable to study the protein structure/association in food matrices in relation to the processes they have undergone and the function they perform.

The structural properties of proteins play a key role in cereal systems. In this frame, the molecular and “geometrical” description of the gluten network, as well as its evolution from the dry state in the flour/semolina until the final product (a model bread) represent a great challenge. We developed a novel thiolomic approach based on the labeling with a fluorescent probe of accessible free thiols, suitable to study the structural evolution of the proteins involved in the formation of the gluten network. Gluten proteins has a fundamental role in the definition of the properties and quality of wheat-based bakery products. Despite their importance, studies regarding gluten protein structure and their interactions with other dough components are made particularly difficulty due to the intrinsic physical and chemical properties of the storage wheat proteins (insoluble, intrinsically disordered and inserted in a complex matrix). Actually, common characterizations of cereal-based products are based on rheological approaches that provide information about the technological attitude of the matrices (i.e. mixing properties by using the farinograph). A molecular description can therefore clarify and better explain differences in the technological attitude of similar matrices. However, assessing interactions and structural features of gluten network is challenging since most methods require the extraction or isolation of the proteins before analysis (Sadat et al., 2019), resulting in the formation of artefacts. The thiolomic approach had proven to be a very powerful tool in the study of the molecular events at the base of the development of the gluten network, understanding also the role of the technological transformations. By applying the thiolomic approach and other “more conventional” characterization, we was able to describe the molecular events occurring during the transformations from the flour to the final product (a bread) (Emide 2022) . Our results showed that in the raw dough the glutinic network is mainly stabilized by hydrophobic interaction, whereas baking triggers the formation of intermolecular disulfide bound via thiol-disulfide exchange reaction. The approach has also been extended to the study of the role of the amylose/amylopectin ratio in affecting the development of the gluten network, and consequently the features of the finished products, in sample sharing the same

protein pattern. We found that altered amylose/amylopectin ratio strongly influences the molecular rearrangement occurring in gluten development by competing for the water available for protein (and starch) hydration. As a future perspective, the thiolomic approach based on the fluorescent labelling will be enhanced by exploiting the reactivity of gold nanoparticles (AuNPs) with thiols. The use of AuNPs of different size (from 5 to 200 nm) should allow to gain information on the “geometrical” accessibility of thiols in proteins that are involved in the formation of the network. Proteins “fished” by AuNPs have been identified - in a proof-of-concept study - by mass spectrometry (Marengo et al., 2019). The overall results will provide an in-depth characterization of the protein network under the molecular and geometrical point of view. The molecular description will be related to the rheological properties in order to increase the level of knowledge of the structure/function relationships of these systems.

The impact of a heat treatments was evaluated also in lentils used in the production of gluten-free products (pasta). The production of gluten-free products, such as bread, cookies, and pasta, take some advantage from the use of flours that have been subjected to a thermal treatment able to modify starch and protein properties (Bresciani et al., 2022). When applied to lentils, the considered thermal treatment proven to lead structural changes in both starch and proteins, without promoting an extensive starch gelatinization. The greatest effect was observed on proteins, leading to the formation of aggregates mainly stabilized through hydrophobic interactions. Even if disulfide bonds are one of the main actors in many thermal coagulation events (such as for milk and egg proteins), they don’t play a significant role in the case of lentils, probably due to their low content of sulfur amino acids. Structural modifications of starch and proteins affect many technological properties relevant for pasta production. Flour from heat treated lentils showed a higher water adsorption and a lower retention capacity. The higher water-holding capacity, together with the greater protein aggregation tendency/properties, resulted in a dough with higher consistency during mixing. When the heat-treated flour is processed in gluten-free pasta, the modification induced by the treatments results in improved behaviors during cooking (Bresciani et al., 2021).

Change in structure/function relationship play a key role not only in the definition of the technological properties of matrices, but also in the nutritional characteristics of the finished products. Sprouting deeply affects the characteristics and composition of the raw materials, and consequently the characteristics of the finished products. The sprouting process could be a useful approach to improve the technological and nutritional properties of buckwheat, 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. We evaluated the impact of sprouting on buckwheat (as a raw material) and on a couscous enriched in sprouted buckwheat (as a final product). In details, both protein structure and nutritional properties were evaluated. The overall features of proteins in the sprouted grains

made them more suitable for formation of the inter-protein network fundamental for appropriate texturing of specific foods. Sprouting-associated events also decreased the levels of antinutritional factors such as enzymatic inhibitors, phytates and chelating compounds, increasing the bioaccessibility of metals, fatty acids, and free polyphenols. Moreover, sprouting increased the antioxidant capacity and the polyphenols content. When the sprouted buckwheat is processed into couscous, the technological treatments negatively affect the total antioxidant capacity and the polyphenols contents. However, during simulated *in vivo* digestion, couscous enriched in sprouted sample showed better nutritional properties than the couscous made with the non-sprouted flour.

Fermentation, that is a fundamental step for the production of many food products, has proved effective in increasing the nutritional and/or the sensory properties. In the case of cocoa, fermentation represent the first step for the production of chocolate. Lactic acid bacteria, molds and yeasts are the main actors in the fermentation process of cocoa where proteins represent the nitrogen source for their metabolisms. During the fermentation, specific proteolytic events help the development of the peculiar sensory profile of the finished chocolate through the release of free amino acids precursor of aromatic compound. Controlled cocoa fermentations received a strong interest from chocolate manufacturers and cocoa producers as a natural way to obtain specific flavor profiles without the need of using additives or flavorings. The obtained results showed that inoculum of selected LAB strains gave different peptide profiles, influencing sensory properties of the final product. Vicilin-type proteins were found to be selectively degraded by endogenous proteases under fermentation conditions where optimal levels of aroma precursors were obtained. Differences in the proteolytic (peptidase and endo-protease) activities were confirmed by enzymatic assays performed on the lysate strains. Furthermore, fermentation parameters, that are crucial for the outcome of the fermentation process, were not change by the inoculum of select LAB.

The studies present in this thesis represent a methodological basis for improving our current understanding of the evolution of the structure/function relationship of macromolecules, and how these relationships affect the technological, nutritional and sensory properties of food matrices. From a biochemical point of view, studies carried out in this PhD thesis described at molecular level the evolution of food systems, providing numerous information necessary for an even greater understanding of the overall role of proteins in influencing the macroscopic properties of food systems. Furthermore, the increasing demand for healthy and functional foods, as well as for food product with nutritional and health-beneficial properties, is pushing the food industry in searching new proteins to innovate the traditional food and produce new products. This represent a big challenge, in which studies similar to those reported in this thesis will be helpful to apply (and improve) the actual knowledge in order to develop new

technologies, trying to preserve and improve all the beneficial properties of food for the customer health.

5.1 References

Bresciani, A., Emide, D., Saitta, F., Fessas, D., Iametti, S., Barbiroli, A., Marti, A., 2022. Impact of Thermal Treatment on the Starch-Protein Interplay in Red Lentils: Connecting Molecular Features and Rheological Properties. Molecules. 27, 1266.

Bresciani, A., Iametti, S., Emide, D., Marti, A., Barbiroli, A., 2021. Molecular features and cooking behavior of pasta from pulses. Cereal Chem. 00, 1–5.

Emide, D., 2022. The complexity of protein network in foods: insight in the protein structure in cereal products. In: Proceeding of the 26th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, Università di Torino (Asti, Italy), September 19-21.

Marengo, M., Mamone, G., Ferranti, P., Polito, L., Iametti, S., Bonomi, F., 2019. Topological features of the intermolecular contacts in gluten-forming proteins: Exploring a novel methodological approach based on gold nanoparticles. Food Res. Int. 119, 492–498.

Sadat, A., Corradini, M. G., Joye, I. J., 2019. Molecular spectroscopy to assess protein structures within cereal systems. Curr. Opin. Food Sci. 25, 42–51.

6. SCIENTIFIC PRODUCTION

6.1 Supplementary materials

6.1.1 Section 4.4

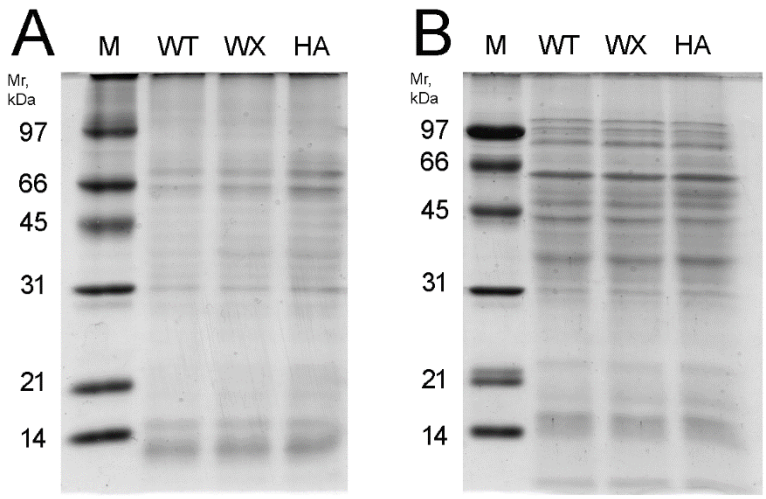


Figure S1: SDS-PAGE of wild type (WT), waxy (WX) and high amylose (HA) Cadenza lines in non-reducing (A) and reducing (B) conditions. M: molecular weight markers.

6.1.2 Section 4.6

Bresciani, A., **Emide, D.,** Saitta, F., Fessas, D., Iametti, S., Barbiroli, A., Marti, A., 2022. Impact of Thermal Treatment on the Starch-Protein Interplay in Red Lentils: Connecting Molecular Features and Rheological Properties. Molecules. 27,1266.

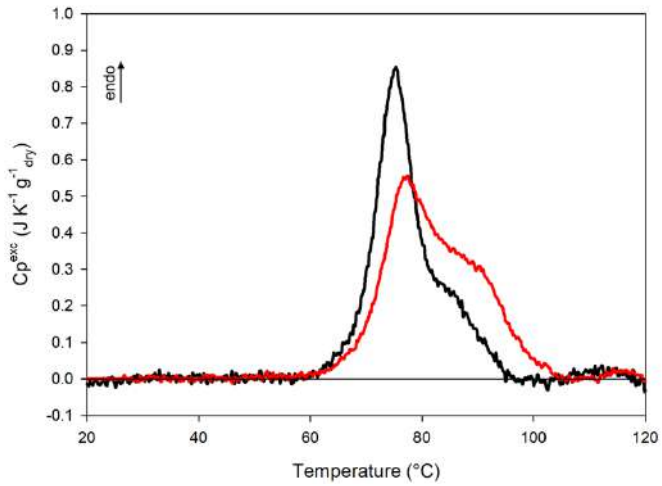


Figure S1. DSC thermogram obtained for a highly hydrated sample of treated flour from red lentils (black trace) that was stored at 4°C for 48 hours before the measurement for evaluating the presence of starch retrogradation (72% humidity; closed pans; 2°C/min scanning rate). For the sake of comparison, the DSC profile obtained for treated flour of red lentils (already reported in Figure 1A in the main text) is also shown as a red trace (60% humidity; closed pans; 2°C/min scanning rate).

Raw and heat treated flours show no differences in their overall gelatinization enthalpy (ΔH_{gel} = 10.5 ± 0.5 J/g_{dry} for both systems).

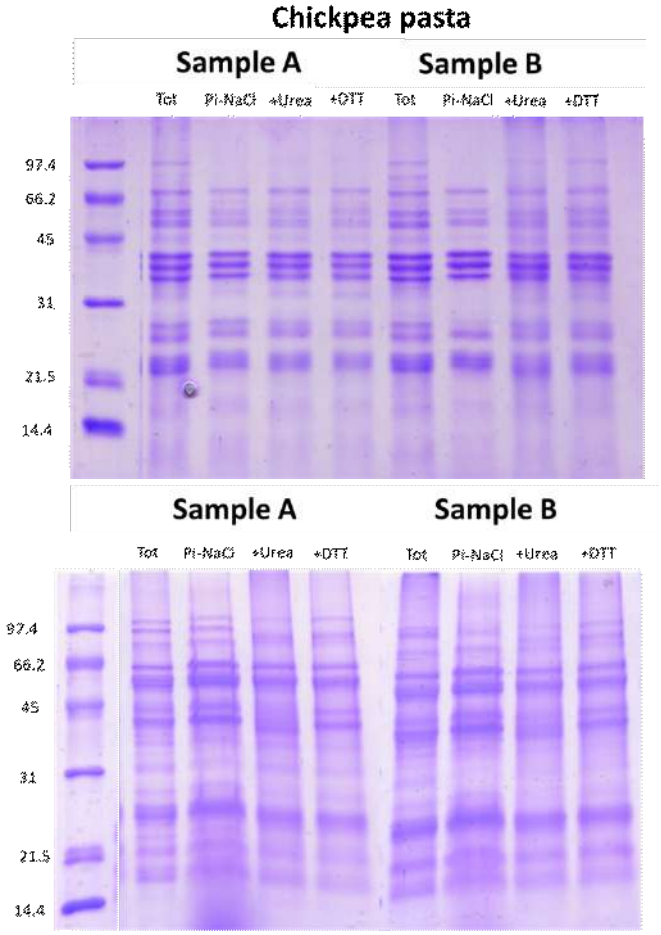
An additional DSC measurement was carried out on heat treated flour by choosing a different experimental approach as to favour the occurrence of any possible starch retrogradation. In details, a highly hydrated sample (water amount above 70%) was stored at 4°C for 48 hours before the DSC measurement. The DSC thermogram (Figure S1, black trace) show no starch retrogradation despite the favourable experimental conditions. Indeed, if any starch retrogradation occurred, it would have been visible as an endothermic contribution at about 40°C [23].

Although the experimental methods applied were different, it is possible to observe that the water content affects the kinetics of the gelatinization, since the amount of water immediately available for the process is different [22]. Specifically, the higher the water content, the higher the gelatinization rate and the amount of gelatinized starch at the first step (first gelatinization peak). On the other hand, we may notice that the gelatinization onset

temperature does not undergo any variation since it only relies on the native starch composition and structure [22]. Moreover, the enthalpy of gelatinization kept the same values at both moisture levels.

6.1.2 Section 4.7

Bresciani, A., Iametti, S., **Emide, D.,** Marti, A., & Barbiroli, A., 2021. Molecular features and cooking behavior of pasta from pulses. Cereal Chem. 99, 270– 274.



Supplementary Figure 1. SDS-PAGE of proteins extracted from chickpea (upper panel) and red lentil (lower panel) pasta in differential solubility study. Tot: whole sample; Pi NaCl: protein soluble in saline buffer; +Urea: protein soluble in saline buffer added of 4M urea; +DTT: protein soluble in saline buffer added of 4M urea and 10 mM DTT.

Supplementary Table 1. Carbohydrate and protein content of commercial chickpea and red lentil pasta as reported in the label. Values are expressed on 100 g of pasta.

	Chickpea pasta		Red lentil pasta	
	Sample A	Sample B	Sample A	Sample B
Carbohydrate (g)	57	45.1	55	47.4
of which sugars (g)	2.2	2.9	1.2	1.8
Protein (g)	21	21	23	25

6.1.2 Section 4.11

Dea Korcari, Alberto Fanton, Giovanni Ricci, Noemi Sofia Rabitti, Monica Laureati, Johannes Hogenboom, Luisa Pellegrino, **Davide Emide**, Alberto Barbiroli, Maria Grazia Fortina. Fine cocoa fermentation with selected Lactic Acid Bacteria: fermentation performance and impact on chocolate composition and sensory properties

Table S1 List of sensory descriptors used to describe the samples with relevant definitions and reference standards.

Sensory dimension	Sensory Descriptor	Definition	Reference Standard
Appearance	Brown color	Characteristic perceived through the sense of sight referring to the brown color of chocolate	Minimum of the scale (score 1): milk chocolate (Lindt & Sprungli S.p.A.) Maximum of the scale (score 9): 100% criollo chocolate (Esselunga S.p.A)
Odor	Cocoa	Characteristic aroma of cocoa perceived orthonasally with the sense of smell	Cocoa powder Perugina (Nestlé Italiana S.p.A.)
	Red Fruits	Characteristic aroma of red fruits perceived orthonasally with the sense of smell	NeroNero extra dark chocolate with raspberries and almond granules (Novi S.p.A.)
	Roasted	Characteristic aroma of roasted/burnt perceived orthonasally with the sense of smell	100% criollo chocolate (Esselunga S.p.A)
	Honey	Characteristic aroma of honey perceived orthonasally with the sense of smell	Excellence dark with honey and almonds (Lindt & Sprungli S.p.A.)
Taste	Sweet	One of the basic tastes caused by sweet compounds (e.g. sugar) perceived in the oral cavity	Extra dark chocolate Perugina (Nestlé Italiana S.p.A.)
	Bitter	One of the basic tastes caused by bitter compounds (e.g. caffeine) perceived in the oral cavity	100% criollo chocolate (Esselunga S.p.A)
	Sour	One of the basic tastes caused by sour compounds (e.g. lemon) perceived in the oral cavity	100% criollo chocolate (Esselunga S.p.A)
Flavour	Cocoa	Characteristic flavour of cocoa perceived retronasally when swallowing	Cocoa powder Perugina (Nestlé Italiana S.p.A.)
	Red Fruits	Characteristic flavour of red fruit perceived retronasally when swallowing	NeroNero extra dark chocolate with raspberries and almond granules (Novi S.p.A.)
	Roasted	Characteristic aroma of roasted/burnt perceived retronasally when swallowing	100% criollo chocolate (Esselunga S.p.A)
	Honey	Characteristic flavour of honey perceived retronasally when swallowing	Excellence dark with honey and almonds (Lindt & Sprungli S.p.A.)
Mouthfeel sensations	Hardness	Degree of compression between the incisor teeth obtained prior to the breaking of the product	Minimum of the scale (score 1): milk chocolate (Lindt & Sprungli S.p.A.) Maximum of the scale (score 9): 100% criollo chocolate (Esselunga S.p.A)
	Astringency	Tactile sensation of dryness and puckering perceived in the oral cavity	100% criollo chocolate (Esselunga S.p.A)

Table S2. Amounts in ppm of volatile compounds measured from cocoa mass and respective flavour descriptors. A: inoculated with *L. fabifermentans* at t_0; B: inoculated with *L. fabifermentans* at t_0 and t_{48}; C: inoculated with *F. rossiae* at t_0; D: inoculated with *F. rossiae* at t_0 and t_{48}; E: inoculated with both strains at t_0 and t_{48}.

Volatile compounds	Samples						Descriptor
	Control	A	B	C	D	E	
	Volatile compounds (ppm)						
Methyl acetate	0.086	0.085	0.068	0.08	0.104	0.074	Green, fruity, fresh, rum and whiskey-like
2,3-Butandion	1.556	4.631	5.179	1.145	0.824	0.921	Sweet, buttery, creamy, milky
Acetic acid	231.979	276.219	314.842	130.573	67.649	88.608	Pungent, sour, overripe fruit
3-Methyl butanal	1.704	1.582	1.758	1.157	0.645	0.774	Fruity, green, chocolate, nutty, leafy, cocoa
2-Methyl butanal	0.565	0.402	0.629	0.463	0.196	0.211	Musty, rummy, nutty, cereal, caramel, fruity
Pentanal	0.378	0.437	0.541	0.107	0.11	0.257	Winey, bready, cocoa, chocolate notes
Hexanal	0.067	0.208	0.109	0.03	0.037	0.163	Green, woody, apple, grassy, orange
3-Methyl butyric acid	4.46	6.701	4.442	5.633	5.052	4.61	Cheesy, dairy, creamy, sweet, berry
2-Methyl butyric acid	2.403	3.709	2.566	3.605	3.829	3.571	Fruity, acidic, dairy, buttery
2-Heptanone	3.291	9.884	6.016	2.799	1.515	3.413	Cheese, fruity, coconut, waxy, green
2,3-Dimethyl pyrazine	0.066	0.073	0.066	0	0	0	Nutty, nut skin, cocoa, coffee, walnut
Butyrolactone	0.243	0.217	0.203	0.1	0.081	0.074	Milky, creamy with fruity afternotes
Benzaldehyde	0.57	0.733	0.8	0.545	0.419	0.425	Sweet, oily, almond, cherry, nutty, woody
Trimethyl pyrazine	0.402	0.391	0.357	0.345	0.29	0.31	Toasted, nutty, earthy, chocolate, coffee
Benzyl acetaldehyde	0.539	0.177	0.206	0.525	0	0.369	Green, melon, fruity, citrus
Tetramethyl pyrazine	5.22	3.788	2.962	5.926	5.26	3.638	Nutty, musty, cocoa, peanut, coffee notes
2-Nonanone	1.837	4.093	3.061	1.318	1.556	2.213	Cheesy, green, fruity, dairy, dirty, buttery
Nonanal	0.277	0.285	0.354	0.184	0.149	0.158	Citrus, cucumber, and melon rind
2-Phenylethanol	6.088	6.258	7.83	4.771	4.357	4.961	Floral, sweet, rosy and bready
Phenethyl acetate	4.857	3.161	4.248	2.298	1.898	1.843	Sweet, honey, floral, rosy

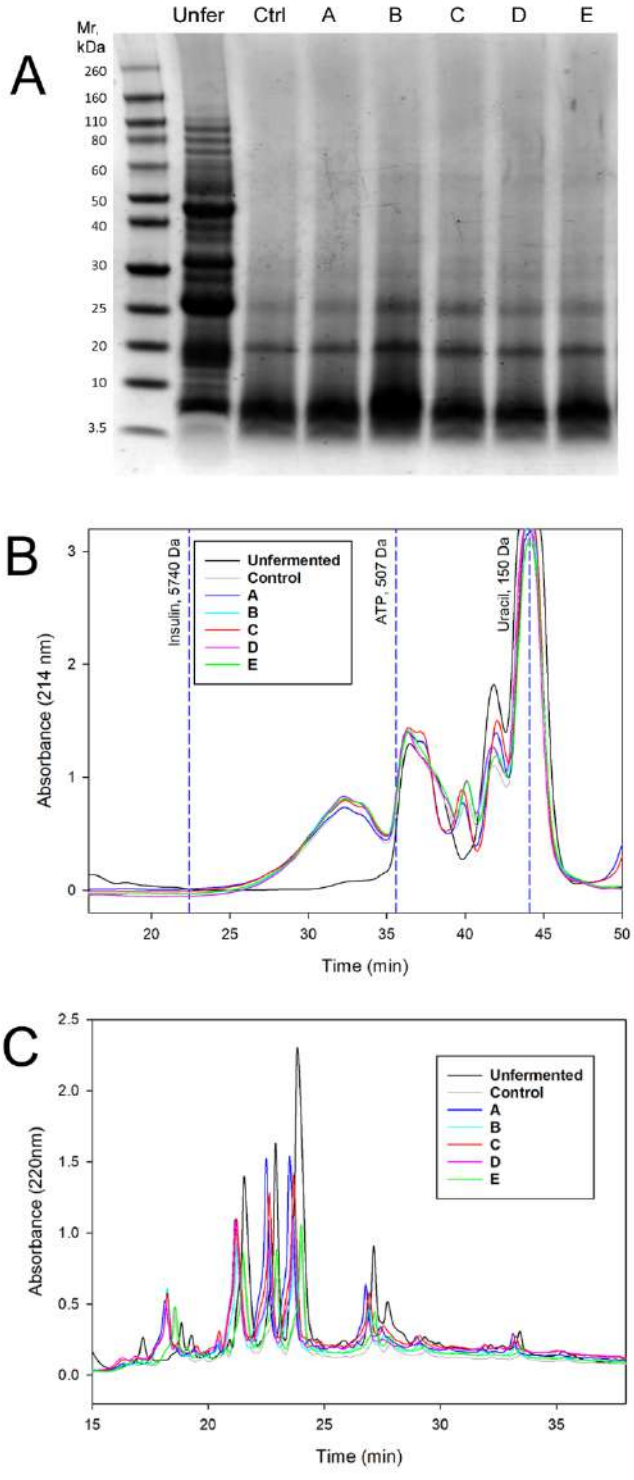
Table S3. Effect of samples (6), judges (10), and replicates (2) on the 14 sensory descriptors considered (*** significant at p<0.001; ** significant at p<0.01; * significant at p<0.05; n.s. not significant).

Sensory dimension	**Sensory Descriptor**	**Samples**	**Judges**	**Replicates**	**J X S**	**S X R**	**J X R**
Appearance	Brown color	93,81***	1,83n.s.	0,10n.s.	1,83*	0,57n.s.	1,83n.s.
Odor	Cocoa	13,45***	2,26*	0,00n.s.	1,52n.s.	0,38n.s.	0,75n.s.
	Red Fruits	3,15*	6,84***	3,31n.s.	1,97*	1,39n.s.	0,97n.s.
	Roasted	27,59***	7,05***	1,79n.s.	2,27**	0,62n.s.	1,46n.s.
	Honey	4,97**	1,77n.s.	0,73n.s.	1,18n.s.	0,36n.s.	0,45n.s.
Taste	Sweet	10,08***	2,31*	0,00n.s.	1,45n.s.	2,91*	1,56n.s.
	Bitter	30,31***	8,09***	0,06n.s.	1,14n.s.	1,15n.s.	1,53n.s.
	Sour	15,47***	8,65***	2,06n.s.	1,62n.s.	2,23n.s.	0,47n.s.
Flavour	Cocoa	10,04***	3,71**	0,24n.s.	1,12n.s.	0,24n.s.	1.95n.s.
	Red Fruits	11,25***	5,03***	6,41*	1,40n.s.	1,01n.s.	1,47n.s.
	Roasted	44,90***	2,42*	0,13n.s.	1,03n.s.	0,39n.s.	1,18n.s.
	Honey	33,47***	4,46***	0,06n.s.	1,13n.s.	1,98n.s.	2,01n.s.
Mouthfeel sensations	Hardness	9,85***	4,76***	0,53n.s.	1,49n.s.	0,50n.s.	0,80n.s.
	Astringency	17,30***	11,19***	0,69n.s.	1,16n.s.	0,46n.s.	2,04n.s.

Table S4 Mean ratings (on a 9-point rating scale) obtained for the 6 dark chocolate samples for each sensory descriptor (*** significant at p<0.001; ** significant at p<0.01; * significant at p<0.05). Different letters by row indicate significant differences (p<0.05) according to LSD post hoc test. A: inoculated with *L. fabifermentans* at t_0; B: inoculated with *L. fabifermentans* at t_0 and t_{48}; C: inoculated with *F. rossiae* at t_0; D: inoculated with *F. rossiae* at t_0 and t_{48}; E: inoculated with both strains at t_0 and t_{48}.

Sensory dimension	**Sensory descriptor**	**Chocolate samples**					
		A	B	C	D	E	Control
Appearance	Brown color ***	6,8d	5,2a	6,0c	5,8b	6,0c	7,0e
Odor	Cocoa ***	6,6b	5,9a	6,5b	5,9a	6,4b	6,6b
	Red fruits *	4,5bc	4,5bc	4,6bc	4,4ab	4,7c	4,2a
	Roasted ***	6,4c	5,4a	5,7b	5,6ab	6,2c	6,4c
	Honey **	4,8a	5,5b	4,7a	4,7a	4,5a	4,6a
Taste	Sweet ***	5,4c	5,4c	5,4c	5,5c	4,8a	5,1b
	Bitter ***	5,6c	5,4b	4,8a	4,6a	5,6c	5,6c
	Sour ***	5,6a	5,6a	6,2b	5,6ab	5,6a	6,0b
Flavor	Cocoa ***	6,5b	6,0a	6,3b	6,3b	6,3b	6,8c
	Red fruits ***	4,8c	4,0a	3,8a	3,8a	4,5bc	4,3b
	Roasted ***	6,0b	5,6a	5,6a	6,2c	7,0d	6,8d
	Honey ***	4,0a	5,6d	4,4b	4,8c	3,8a	3,8a
Mouthfeel	Hardness ***	6,4b	6,0a	6,4b	6,3b	6,5b	6,8c
	Astringency ***	4,8a	4,8a	4,8a	5,5b	5,3b	4,9a

Figure S1. Protein and peptide pattern of unfermented and fermented samples. Panel A: SDS-PAGE of total protein extracts; Unfer: unfermented cocoa beans; Ctrl: spontaneous fermentation; A: inoculated with *L. fabifermentans* at t_0; B: inoculated with *L. fabifermentans* at t_0 and t_{48}; C: inoculated with *F. rossiae* at t_0; D: inoculated with *F. rossiae* at t_0 and t_{48}; E: inoculated with both strains at t_0 and t_{48}. Panel B: SE-HPLC chromatograms of peptide extracted from cocoa beans. Panel C: RP-HPLC chromatograms of peptide extracted from cocoa beans.



6.2 Publications

molecules

MDPI

Article

Impact of Thermal Treatment on the Starch-Protein Interplay in Red Lentils: Connecting Molecular Features and Rheological Properties

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Abstract: Thermal treatments are widely applied to gluten-free (GF) flours to change their functionality. Despite the interest in using pulses in GF formulations, the effects of thermal treatment at the molecular level and their relationship with dough rheology have not been fully addressed. Raw and heat-treated red lentils were tested for starch and protein features. Interactions with water were assessed by thermogravimetric analysis and water-holding capacity. Finally, mixing properties were investigated. The thermal treatment of red lentils induced a structural modification of both starch and proteins. In the case of starch, such changes consequently affected the kinetics of gelatinization. Flour treatment increased the temperature required for gelatinization, and led to an increased viscosity during both gelatinization and retrogradation. Regarding proteins, heat treatment promoted the formation of aggregates, mainly stabilized by hydrophobic interactions between (partially) unfolded proteins. Overall, the structural modifications of starch and proteins enhanced the hydration properties of the dough, resulting in increased consistency during mixing.

Keywords: pulses; legumes; heat treatment; starch; proteins

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1. Introduction

Pulses are key to the Mediterranean diet pattern [1] due to several health benefits associated with their consumption [2], such as a reduction in the risk of cardiovascular diseases and type 2 diabetes. In addition, pulse consumption is expanding in response to sustainability and food security issues [3]. Despite some differences among countries, most dietary guidelines recommend an adherence to a plant-based diet [4]. Among plant species, pulses are a good source of proteins, in terms of both protein content (17–30%) and amino acid composition, in particular lysine [5]. When combined with cereal proteins, pulses sustainably provide balanced nutrition [6].

Pulses are mainly consumed as grains, but this entails long preparation and cooking times. Moreover, pulses may contain antinutrients such as phytic acid, trypsin inhibitors, and some non-digestible oligosaccharides, which are related, among others, to some digestive disorders [7].

One approach to increase the consumption of pulses is to use them in the form of flour, which can be added to formulations of cereal-based products [8,9]. Specifically, pulses (or their fractions) enhance the nutritional value of gluten-free products due to their high protein content (21–25%) and dietary fiber (12–20%) [10].

The production of gluten-free products, such as bread, cookies, and pasta, requires the use of flours that have been subjected to a thermal treatment able to modify starch properties.

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NOTE

Molecular features and cooking behavior of pasta from pulses

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Abstract

Background and objectives: Pulse pasta is one of the latest responses of the food industry to meet the consumers request for healthy and sustainable foods. Among pulses, red lentils and chickpeas are the preferred raw materials for making 100% pulse pasta. This study aimed at addressing starch and protein features in commercial pulse pasta to provide an insight on how their molecular organization may affect cooking behavior.

Findings: Differences in starch pasting profile and in protein overall organization were found among commercial pasta samples. Considering the same pulse, the best performing pasta showed a protein network characterized by a more compact structure. Regardless of the producer, lentils gave pasta with the best cooking behavior (low cooking loss and high firmness).

Conclusions: Cooking quality of pulse pasta depends on both the type of pulse (chickpeas or red lentils) and pasta-making process.

Significance and novelty: This study lays some molecular groundwork as for elucidating the role of individual pulses and of the pasta-making process in determining the quality of pulse pasta.

KEYWORDS

chickpea, legumes, pasta, protein aggregation, red lentil

1 | INTRODUCTION

The production of 100% pulse pasta represents the most recent innovation in the pasta industry in response to the consumer request for healthy and sustainable food products (Lascialfari et al., 2019; Tucci et al., 2021). Pasta from 100% lentils, beans, green peas, and chickpeas is currently available on the market; and differences in nutritional traits and cooking behavior have been reported for a few commercial products (Turco et al., 2019). Such differences are related to differences in raw materials and in processing conditions, as both are known to drive the molecular rearrangements affecting the quality of pasta from both wheat (Bock et al., 2015; Bonomi et al., 2012) and gluten-free cereals (Barbiroli et al., 2013; Marti et al., 2010). However, no information is available on the relation between starch and protein features and cooking behavior in pulse pasta. In this

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ORIGINAL ARTICLE

Journal of Applied Microbiology JAM

Exploration of *Lactiplantibacillus fabifermentans* and *Furfurilactobacillus rossiae* as potential cocoa fermentation starters

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Abstract

Aims: To investigate the characteristics of two minority autochthonous LAB species, with particular regard to those properties that could be exploited in an improved cocoa fermentation process from a quality and safety point of view.

Methods and Results: Bacterial, yeast and mould strains characteristic of spontaneously fermented Dominican cocoa beans were isolated and identified by 16S or 26S rRNA gene sequencing. The potential of two autochthonous strains of LAB belonging to the species *Lactiplantibacillus fabifermentans* and *Furfurilactibacillus rossiae* were investigated. The two selected LAB strains were able to utilize glucose and fructose, produced mainly D-L lactic acid and had a good ability to resist to cocoa-related stress conditions such as low pH, high temperature and high osmotic pressure, as well as to grow in sterile cocoa pulp. The strains did not inhibit the growth of yeasts and acetic acid bacteria, that are essential to the cocoa fermentation process, and possessed a complex pool of peptidases especially active on hydrophobic amino acids. The strains also showed antifungal activity against mould species that can be found at the final stages of cocoa fermentation, as *Aspergillus tamarii, A. nidulans, Lichtheimia ornata* and *Rhizomucor pusillus*.

Conclusions: The tested strains are good candidates for the design of starter cultures for a controlled cocoa fermentation process.

Significance and Impact of the Study: This research showcases the potential of two alternative LAB species to the dominating *Lactiplantibacillus plantarum* and *Limosilactibacillus fermentum* as cocoa fermentation starters, with an interesting activity in improving the safety and quality of the process.

KEYWORDS

antifungal activity, Dominican cocoa bean, fermentation, LAB, proteolysis, starter cultures, stress resistance

6.3 Oral and poster communication to national and international congress

D. Emide, F. Bonomi, S. Iametti, A. Barbiroli., From proteins to bread: novel tools for the molecular description of protein-protein interactions (and their evolution) in baked products, IUBMB-FEBS-PABMB 2022 Congress, 9 - 14 July, Lisbon.

D. Emide, A. Barbiroli, F. Bonomi, S. Iametti, Nuovi approcci metodologici allo studio delle interazioni tra proteine in matrici complesse: applicazioni al network proteico di cereali, 12° Convegno AISTEC, CEREALI E SCIENZA: ambiente, globalizzazione e comunicazione, 15 – 17 June, Portici (NA), Italy.

D. Emide, S. Iametti, A. Barbiroli, Thiolomics and proteomics approaches to study gluten structure, Lake Como School of Advanced Studies: “Novel approaches to the food-health relationship: from molecules to sociotypes”, 19-22 October 2021 – Como, Italy.

D. Emide, FIGHTING FOR WATER: STARCH vs PROTEINS, Short Cycle School on Thermal Analysis, 3 – 9 October 2021, Cyprus.

D. Emide, C. Magni, S. Iametti, A. Marti, F. Sestili, D. Lafiandra, E. Botticella and A. Barbiroli, Molecular information for addressing climate changes (and consumers demands): wheat proteins, 61st SIB Congress, 2021, Virtual Meeting (Poster, selected for an oral presentation).

D. Emide, F. Bonomi, C. Nitride, P. Ferranti, L. Polito, S. Iametti, A. Barbiroli, Assessing the geometrical features of protein-protein interactions in gluten, International Conference on FOODOMICS, 6th edition, Cesena, Italy (Oral communication).

D. Emide, Novel biochemistry approach to study protein network in complex matrices, Workshop on the Developments in the Italian PhD Research on Food Systems, Milan, September 15th, 2020.

D. Emide, Novel thiolomics approach to study the biochemical features of protein network in cereal-based complex matrices, 1st Telematic Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology Palermo, September 14th – 15th, 2021.

D. Emide, The complexity of protein network in foods: insight in the protein structure in cereal products, 26th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, Asti, September 19-21, 2022.