Comparison of gluten peptides and potential prebiotic carbohydrates in old and modern Triticum turgidum ssp. genotypes

COMPARISON OF GLUTEN PEPTIDES AND POTENTIAL PREBIOTIC CARBOHYDRATES IN OLD AND MODERN *Triticum turgidum* ssp. GENOTYPES

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ABSTRACT

Old wheat genotypes are perceived by consumers as healthier than modern ones. The release of gluten peptides with *in vitro* digestion and the content of potentially prebiotic carbohydrates (i.e. resistant fraction of starch and cell-wall associated dietary fiber) were evaluated in tetraploid wheats, namely 9 old and 3 modern *Triticum turgidum* ssp. genotypes. Simulated digestion of wholemeal flours yielded 152 major peptides, 59 of which were attributed a sequence. Principal component analysis revealed that peptide profiles were variable in old genotypes, unlike in modern ones. Digestion of old genotypes generally yielded peptides in greater concentration. In particular, 5 peptides of γ-gliadin, known to trigger the adaptive immune reaction, and two peptides of α-gliadin, known to be toxic to celiac patients, were particularly abundant in some old varieties. Resistant starch (RS) was negligible in modern genotypes (< 0.6%), but it was remarkably abundant in some old varieties, reaching the highest value in Dauno III (8.5%, $P < 0.05$). Dauno III also presented the highest amount of soluble fiber (4.2%, $P < 0.05$). Pasta was made with an old and a modern genotype (Dauno III and PR22D89, respectively) with opposite RS content. Pasta making and cooking affected starch digestibility, overtaking differences between genotypes and yielding the same amount of RS for both the varieties (approx. 1.7%). The data herein presented suggest that the wholemeal flours of old tetraploid wheat genotypes could not boast particular claims associated to a lower exposure to gluten peptides and, if cooked, to a prebiotic potential.

KEYWORDS

Triticum turgidum ssp.; old wheat; modern wheat; dietary fiber; resistant starch; prebiotic; gluten; celiac disease;

ABBREVIATIONS

RS, resistant starch; CD, celiac disease; UPLC, Ultra Performance Liquid Chromatography; ESI, electrospray ionization; MS, mass spectrometry; PCA, Principal Component Analysis.

1. INTRODUCTION

Durum wheat (*Triticum turgidum*) is one of the most important crops in Italy and the exclusive raw material for pasta production for the Italian law. Native, local, and old *T. turgidum* ssp. genotypes have become obsolete for decades because of lower yields coupled with higher stature, but they have been recently reintroduced by farmers (Mangini et al. 2018). In fact, old landraces and varieties of wheats are perceived as safer, healthier, more tasteful and sustainable than the modern cultivars (Arzani & Ashraf, 2017; Shewry 2018), and there is an increasing interest of industries to grasp health claims, sometimes without consistent experimental evidence. In the present study it is presented a comparative study on 9 old and 3 modern varieties of tetraploid wheat, in terms of important health characteristics such as the release of gluten peptides involved and the content of potentially prebiotic carbohydrates.

In genetically susceptible individuals, the celiac disease (CD) is induced by gliadin peptides that resist digestion and trigger adverse innate immune response (CD-related toxic peptides) or adaptive immune reactions (immunogenic peptides) (Ciccocioppo, Di Sabatino, & Corazza, 2005; Cornell & Mothes, 1993; Hausch, Shan, Santiago, Gray, & Khosla, 2002). All the wheats, regardless they are old or modern, have gluten and are not safe for celiacs due to the release of toxic and immunogenic peptides (Boukid et al., 2017; Gelinas & McKinnon, 2016; Malalgoda, Meinhardt, & Simsek, 2018; Prandi, Mantovani, Galaverna, & Sforza, 2014). However, varieties yielding lower amounts of CD related peptides may retard the onset of the disease in predisposed people (Molberg et al., 2005). A previous study describes the CD-related gluten peptides generated by *in vitro* digestion of different old and modern *Triticum* wheats (*T. aestivum*, *T. dicoccum*, *T. monococcum*, *T. spelta*, and *T. turgidum*), but included only 2 old and 2 modern varieties of durum wheat (Prandi, Tedeschi, Folloni, Galaverna, & Sforza, 2017). The present study aims to extend the knowledge of the peptide profile to a greater set of durum varieties and to determine whether varietal selection operated in the last century affected the amount of peptides mediating the immune reaction.

Positive health effects deriving from cereal consumption could originate from the presence of prebiotic carbohydrates which can escape the digestion and absorption in the upper gastrointestinal tract, becoming carbon and energy sources for beneficial bacteria of gut microbiota. Most of the starch, the main carbohydrate of wheat, is hydrolyzed and absorbed during the passage through the small intestine, but a minor fraction is not digested and is referred to as resistant starch (RS) (Magallanes-Cruz, Flores-Silva, & Bello-Perez, 2017). RS is fermented in the colon, yielding short-chain fatty acids that exert many health promoting effects (LeBlanc et al., 2017). Other indigestible non-starch polysaccharides, occurring especially in wholemeal foods, are cell wall components referred to as dietary fiber (Duncan et al., 2016; Onipe, Jideani, & Beswa, 2015; Shewry et al. 2013). Previous studies of prebiotic components in wheat have focused mostly on bread varieties, revealing that differences among varieties can be ascribed to heritable and environmental factors, although old and modern groups differed little (Gebruers et al. 2010; Shewry 2018). Evidence has been recently provided that breeding during the 20th century had little impact on dietary fiber components of durum wheat genotypes as well (De Santis et al. 2018). However, data on the prebiotic potential of durum wheat remains scarce, especially on the content in RS, which has never been compared among old and modern varieties. Moreover, food-processing techniques, the variable proportion of glucose polymers, and their interaction with the other constituents are expected to influence the behavior and the digestibility of starch, but little information are available on carbohydrate changes during processing, in particular considering milling and pasta-making (Fares et al., 2008). In addition to gluten peptides, modern durum wheats were compared in the present study with respect to the content of cell-wall associated fiber and the resistant fraction of starch. The amount of RS was determined throughout different phases of pasta making and cooking. A comparative study of old and modern *T turgidum* genotypes would offer a scientific basis for an aware evaluation of health promoting features (if any) associated to consumption of old varieties.

2. MATERIALS AND METHODS

2.1 *Wheat genotypes and wholemeal flour production*

A tetraploid wheat (*T. turgidum* L., 2n = 4x = 28; AABB genome) collection, consisting of 12 accessions classified as ssp. *durum* (10) and ssp. *turanicum* (2), was chosen based on the year of release and the pedigree as reported in Table 1. The genetic materials were selected based on their diffusion in the Italian cereal areas and were classified into two groups: i) old genotypes, including nine landraces and obsolete cultivars released in Italy from the beginning of 1900s until 1960, with tall stature and still not crossed with the semi-dwarf *green revolution* wheats; ii) modern genotypes, that carry the Rht genes, containing two high yielding semi-dwarf cultivars released after 2004 and one breeding line, fixed as pure line in 2014, derived from a 'old × modern' cross.

Seeds of the CREA tetraploid wheats germplasm collection were collected from a plot field trail carried out during the growing seasons 2014-15 and 2015-16 (referred to as 2015 and 2016, respectively) at CREA Research Centre for Cereal and Industrial Crops (from here, CREA-CI) in Foggia, Italy (41°28′N, 15°32′E; 75 m a.s.l.). Standard agronomical management for the area was applied to the plots as previously described (De Vita, Colecchia, Pecorella, & Saia, 2017). Wholemeal flour was produced by milling 100 g of grain in a laboratory machine (Tecator Cyclotec 1093, International PBI, Milan, Italy) and passing through a fine-mesh, 500-micron, screen. All samples were kept at 4°C until analyses.

2.2 *Proteins and gluten index*

Protein content (N × 5.7, dry weight) was assayed using a micro-Kjeldahl method (AACC International, 1999a). Dry gluten and gluten index (%) were determined in triplicate using an automatic gluten washing apparatus Glutomatic 2020 (Perten, Sweden) according to the AACC International (1999b).

2.3 *In vitro protein digestion*

The wholemeal flour was subjected to simulated oral and gastrointestinal digestion, applying the conditions described by Bottari et al. (2017). Briefly, to simulate the oral digestion, 2.5 g of flour were mixed with 2.5 mL of simulated salivary fluid (i.e. 150 U/mL amylase and salivary salts) and incubated for 2 min at 37°C under reciprocating agitation. To simulate the gastric digestion, 5 mL of simulated gastric juice (*i.e*. 4000 U/mL pepsin, 0.02 M HCl, and gastric salts) were added, the pH was adjusted to 3.0 with HCl, and the mixture was incubated for 2 h at 37°C under reciprocating agitation. To simulate the intestinal digestion, 10 mL of simulated duodenal content (*i.e*. 200 U/mL pancreatin, 9 mg/mL bile, and minerals) were added to the mixture, the pH was adjusted to 7 with 1 M NaOH, and the mixture was incubated for 2 h at 37°C under reciprocating agitation. The sample was heated at 95°C for 15 min to stop the digestion and then cooled. For chromatographic analysis, the supernatant was clarified by centrifugation (45 min at 4°C at 3220 × *g*) and filtration through 0.45 µm membranes. 5 µL of an aqueous solution (1.6 mM) of the isotopically labeled peptide TQQPQQPF(d_5)PQQPQQPF(d_5)PQ were added, as the internal standard, to 295 µL of the supernatant.

2.4 *Separation and quantification of peptides by UPLC/ESI-MS*

The supernatants of the digests were analyzed in an UPLC/ESI-MS system (Waters, Milford, MA, USA) to separate and quantify the peptides generated by gluten digestion, as previously described (Prandi, Tedeschi, Folloni, Galaverna, & Sforza, 2017). The samples were analyzed in the Full Scan mode and the areas of the peptides and the internal standard were integrated with the MassLynx software. The semi-quantification value for all the peptides was obtained as the ratio peptide area/internal standard area (dimensionless number). For CD related peptides, the quantification was obtained multiplying the ratio peptide area/internal standard area

for the moles of internal standard, taking into account all the dilution factors occurred during the digestion. For these peptides, the results are expressed as ppm (mg of peptide per g of wheat sample).

2.5 *Peptide identification by LC-MS/MS techniques*

To determine the amino acid sequence of the peptides previously quantified, the digests were analyzed by both low and high resolution LC-MS/MS techniques, i.e. using HPLC/ESI-MS/MS (Waters) and μHPLC-LTQ-OrbiTRAP (Thermo Scientific, Waltham, MS, USA) systems, respectively. HPLC/ESI-MS/MS was operated aiming at the identification of short aminoacidic sequences (Prandi, Tedeschi, Folloni, Galaverna, & Sforza, 2017). The samples were first analyzed in Full Scan mode, to identify the characteristic ions and the retention time of the unknown peptides, and then in Daughter Scan mode using a variable collision energy on the basis of the mass and charge of the ion to be fragmented. The software FindPept (http://web.expasy.org/findpept/) was used to find the peptide sequences with the molecular weight matching the experimental data. The software Proteomics Toolkit (http://db.systemsbiology.net/proteomicsToolkit/FragIonServlet.html) was used to verify the correspondence between the theoretical MS/MS fragmentation and the obtained spectra.

μHPLC-LTQ-OrbiTRAP was operated as reported by Anzani et al. (2018), mainly directed to the identification of longer peptides. The acquisition consisted of event 1 was a high resolution full scan event followed by 4 data dependent scans targeting and fragmenting the most intense ions. Peptide and protein identification was carried out with the software Peaks (Bioinformatic Solutions Inc., Waterloo, ON, Canada), with the following parameters: tolerance on parent ion 5 ppm, tolerance on daughter ions 0.08 Da, decoy database search strict 0.01, decoy database relaxed 0.05.

2.6 *Available carbohydrates, dietary fiber, starch, and resistant starch (RS)*

The content of available carbohydrates (i.e. the carbohydrates digested and absorbed by the human small intestine, including D-glucose, D-fructose, sucrose, maltodextrins, and non-resistant starch), dietary fiber, starch, and RS was determined using Megazyme kits according to the manufacturer's instructions (Megazyme International Ireland Ltd, Bray, Ireland). In particular, available carbohydrates were determined by the 'Available Carbohydrates and Dietary Fibre' kit (K-ACHDF 06/14); soluble and insoluble dietary fiber by 'Total Dietary Fiber' (K-TDFR-100A/K-TDFR-200A 12/15); starch by 'Total Starch' (K-TSTA-50A/K-TSTA-100A 08/16); and RS by 'Resistant Starch' (K-RSTAR 10/15). All measurements were performed in triplicate.

2.7 *Pasta-making*

The old durum wheat genotype Dauno III and the modern one PR22D89 were chosen for semolina and pasta production. For semolina, seeds were conditioned at 16.5 % moisture and milled by a laboratory mill (MLU 202; Bühler Brothers, Uzwill, Switzerland) with a six-roller system, three breaking and three sizing passages, and an attached semolina purifier, for a fineness range of 200 - 315 μm. Semolina (5 Kg) was used for spaghetti by means of a pilot plant equipped with an extruder (60VR, Namad, Rome, Italy) and a dryer (SG600, Namad, Rome Italy). The extruder was equipped with a screw (30 cm in length, 5.5 cm in diameter) that ended with a bronze die (diameter of the hole: 1.70 mm), applying an extrusion pressure of 3.4 bar, and reaching a temperature in pasta of 26-28 °C. A sample of the extruded pasta was analyzed (fresh pasta). Other samples were dried at room temperature, or mechanically transferred into a drying chamber, where two different temperature/time cycles were applied. The first (80 °C dried pasta) was the following: i) 20 min at 60 °C and 65% moisture (external drying); ii) 130 min at 90 °C and 79% moisture (wrapping); iii) 150 min at 80 °C and 78% moisture (drying); iv) 160 min at 45 °C and 63% moisture (first cooling phase); v) 17 h and 20 min at 50 °C and 50% moisture (second cooling phase). The second cycle (50 °C dried pasta) was the following: i) 20 min at 55

°C and relative humidity of 62%; ii) 9 h and 40 min at 75 °C and relative humidity of 75%; iii) 40 min at 50 °C and relative humidity of 73%; iv) 20 min at 45 °C and relative humidity of 63%; v) 14 h at 40 °C and relative humidity of 50%. The final moisture content in all samples of dried pasta was 12.5%. Three independent production trials of pasta were performed. Pasta from each batch was cooked for 10 min according to Ficco et al. (2016). Samples of cooked pasta were dried in an oven linked to a vacuum pump (30 °C, vacuum 760 mm Hg), ground through a 0.5-mm screen (Tecator Cyclotec 1093), and kept at 25 °C until analysis.

2.8 *Statistical analysis*

Two-way analysis of variance (ANOVA), followed by Tukey's *post hoc* test, was used to evaluate differences between cultivation years and groups (old/modern) and between cultivation years and genotypes. Differences were considered significant for $P < 0.05$. Two Principal Component Analyses (PCA) of peptide profile after digestion were carried out using as inputs the semi-quantification values of all the peptides generated from *in vitro* digestion or the quantification values of CD related peptides. Statistical analyses were performed using SPSS software (version 17.0).

3. RESULTS

3.1 *Rainfall and temperature in the growing seasons*

Mean temperatures and rainfall distribution during the growing-seasons 2014-15 and 2015-16 are reported in Table S1. Quantity and distribution of rainfall were variable, as typical of Mediterranean climate (Table S1). In the years 2015 and 2016, cumulated rainfall in the growing cycle (November to May) was 192 mm and 226 mm, respectively (thirty-years average 342 mm). With respect to long-term rainfall data, the years 2015 and 2016 were characterized by a longer drought stress period. The 2015 presented the lowest rainfall, albeit recording for

January and March similar values to those of long-term . The 2016, instead, was characterized by abundant rainfall in November and May and fluctuating levels in the others. Respect to the long-term (thirty years) average records, the 2015 and 2016 years showed similar trends in terms maximum and minimum temperatures.

3.2 *Proteins and gluten in wholemeal flours*

The quantity and quality of gluten are considered the most important quality parameters of wholemeal flours. The gluten content was directly correlated to the grain protein, since gluten is the most conspicuous part of it. A positive correlation was generally observed between gluten and proteins, although a linear relationship could not be established ($R^2 = 0.61$). Total proteins in wholemeal flour ranged from 11.4 to 17.5% w/w, with gluten ranging from 7.3 to 13.8% (Fig. 1A). Flours produced with 2015 wheat grains generally contained a higher amount of proteins and gluten than 2016 (data not shown; $P < 0.05$). The old genotypes were richer in both proteins and gluten than the modern ones ($P < 0.05$). In the old genotypes, proteins accounted for at least 15.6% while gluten accounted for at least 9%. Old Saragolla, and Scorsonera were the richest in total proteins (> 17.0%; $P < 0.05$), while Cappelli was the richest in gluten (13.8 %; $P < 0.05$). The modern varieties, including the breeding line L14 (old × modern), contained less than 14% of proteins and less than 9% of gluten, PR22D89 being the poorest.

The opposite pattern was observed for the gluten index, ranging up to 78.5%, with the three modern durum varieties presenting generally stronger gluten properties than the old ones, despite the higher gluten concentration (Fig. 1B). Garigliano was the sole variety laying below the limit of detection (3%), whereas PR22D89 presented by far the highest value ($P < 0.05$).

3.5 *Peptide profile after in vitro digestion*

The peptides generated by simulated digestion of wholemeal flour obtained from old and modern varieties were analyzed by both high and low resolution LC/MS techniques in order to

determine the general peptide profile and to quantify specific peptides containing sequences recognized as immunogenic or toxic in CD. The general peptide profile of the flour digests determined by UPLC/ESI-MS encompassed 152 major peptides with MW ranging from 200 to 4339 Da and a degree of polymerization ranging from 2 to approx. 40 (Fig. S1). In the lack of a standard of each peptide, presumably exhibiting a different analytical response, the peak areas were integrated and utilized for semi-quantification with respect to the same internal standard. Although the absolute concentration of each peptide could not be determined, their relative amount could be compared across the wheat varieties. Of the 152 peptide ions detected, 42 presented different abundances in old and modern genotypes ($P < 0.05$), the vast majority of them (39) being more abundant in the old genotypes than in the modern ones.

The PCA model calculated on the whole dataset of peptide abundances did not reveal any cluster that could suggest a relationship between specific peptides and genotypes or growing-seasons (Fig. S2). Although old and modern varieties did not group in separate clusters, they were distributed along PC1, according to a lower abundance of most peptides in modern varieties.

3.6 *Identification of immunogenic and toxic peptides*

The amino acid sequence was assigned to a total of 59 peptides on the basis of their MW and fragmentation pattern (Table S2). Among them, five peptides deriving from γ-gliadin digestion and containing the DQ 2.5 restricted epitope DQ2.5-glia-γ4c (QQPQQPFPQ) were identified and referred to as immunogenic peptides (hereinafter referred to as IP): TQQPQQPFPQ (IP1), SQQPQQPFPQPQ (IP2), QAFPQQPQQPFPQ (IP3), TQQPQQPFPQQPQQPFPQ (IP4), and PQTQQPQQPFPQFQQPQQPFPQPQQP (IP5). Similarly, two peptides deriving from α-gliadin were identified as toxic peptides (hereinafter referred to as TP), relatively to the CD: LQPQNPSQQQPQ (TP1) and RPQQPYPQPQPQ (TP2).

IP and TP peptides, quantified using the deuterated analog of IP4 as internal standard, were more abundant in wholemeal flours obtained from 2015 harvest than from 2016 (data not shown; $P < 0.05$). The wholemeal flours from the different genotypes contained different amounts of IP and TP (Fig. 2; $P < 0.05$). Taking into account the mean values across the years, the amount of immunogenic peptides was in the range of 44-129 ppm for IP1, 37-168 ppm for IP2, 8-44 ppm for IP3, 28-220 ppm for IP4 and 3-81 ppm for IP5 (Fig. 2A). The highest amount of total IP was found in the old genotypes Cappelli, Dauno III, Old Saragolla, Scorsonera, and Timilia 'Reste Nere' (Fig. 2C). On the opposite, the lowest amounts of IP were found in the modern varieties (L14, PR22D89, and Sfinge), and in the old varieties Etrusco, Perciasacchi, and Russello. The amount of TP ranged from 2 to 79 ppm for TP1, and from 2 to 29 ppm for TP2 (Fig. 2B). Fluctuation in the amount of TP among the wheat varieties was lower compared to IP.

A PCA model restricted to the sole IP and TP revealed a tendency to form diverse clusters for old and modern varieties (Fig. 3). Modern varieties for most parameters group in the left side of the plot in correspondence with low values of PC1, due to minor amount of both CD-related IP and TP peptides.

3.2 *Content of digestible and indigestible carbohydrates in wholemeal flour samples*

The composition of the wholemeal flours obtained from old and modern varieties was investigated in order to compare the amount of indigestible carbohydrates which could escape human digestion and absorption (Fig. 4A). Low but significant differences between the 2 growing seasons were observed (always lower than 0.7%), with 2016 generally resulting in a higher amount of total, insoluble, and soluble dietary fiber than 2015 (data not shown; $P < 0.05$). In addition to the variance due to differences between the two years, significant differences in dietary fiber content were associated with the genotype. No clear trend of total, insoluble and soluble fiber was evident between the two groups of wheat genotypes (Fig. 4A). In particular, Etrusco, Russello, Old Saragolla, and Sfinge presented the lowest amount of total dietary fiber,

lying in the range of 11.5-12.4%, whereas Dauno III and Scorsonera the highest amount (15.2 and 14.9%, respectively; $P < 0.05$). Insoluble dietary fiber ranged between 9.9 and 12.4%, and they were always more abundant than the soluble ones, which ranged between 1.0 and 4.2%. The genotypes Etrusco, L14, Russello, and Sfinge contained the lowest amount of insoluble dietary fiber (9.9 and 10.4%; $P < 0.05$), and the varieties Cappelli, Scorsonera, and Timilia the highest (11.8 to 12.4%; $P < 0.05$). The genotypes Cappelli and Old Saragolla were characterized by the lowest amount of soluble dietary fiber (1.0-1.35%; $P < 0.05$), and Dauno III by the highest (4.2%; $P < 0.05$). Any relationship between total dietary fiber and the distribution between soluble and insoluble fraction could not be established ($R^2 = 0$ .46 and 0.47, respectively). Among the genotypes with the highest content of dietary fiber, Dauno III presented the highest amount of soluble dietary fiber and Scorsonera of insoluble ones.

The wholemeal flours bore an amount of available total carbohydrates and starch ranging from 65.4 to 75.5% and from 46.0 to 57.2%, respectively (Fig. 4B). Resistant starch ranged from 0.3 to 8.4%. A significant difference between the 2 growing seasons was observed, with 2015 resulting richer than 2016 in terms of both total carbohydrates and starch (data not shown; $P < 0.05$,), but poorer in resistant starch ($P < 0.05$). In addition to the variance due to years, differences were observed among the varieties. Total available carbohydrates and total starch were more concentrated in the group of the modern genotypes compared with the old (Fig. 4B), with the 3 modern genotypes L14, PR22D89, and Sfinge presenting the highest level of total carbohydrates (> 74.4%; $P < 0.05$). These modern genotypes contained also the highest amount of total starch (> 57.0%; $P < 0.05$), whereas the old ones bore lower amounts ($P < 0.05$). The opposite trend was observed for resistant starch, less abundant in modern varieties (< 0.6%; $P < 0.05$%), while all the old genotypes contained always > 1.2% ($P < 0.05$). Dauno III was the genotype presenting the far highest amount of resistant starch, 8.4%.

3.3 *Effect of processing on resistant starch in fresh, dried and cooked pasta*

The old Dauno III and the modern PR22D89 genotypes, contrasting for year of release, origin, and chemical features (soluble dietary fiber, RS and gluten index), were selected to produce pasta with the semolina obtained by a pilot milling plant. The content of resistant starch was determined in semolina, fresh pasta, and pasta dried at different time/ temperature, before and after cooking (Fig. 5). RS was much higher, more than 8-fold, in the semolina from Dauno III than in that from PR22D89 (6.0 *vs.* 0.7%; $P < 0.05$), and the same striking difference was observed in the corresponding fresh pasta ($P > 0.05$). A drying process at room temperature determined a slight reduction of RS in Dauno III pasta samples (from 6.0 to 5.1%), while significantly lower residual RS was recorded at higher drying temperatures (from 5.1% to, respectively, 1.6, 0.7% for pasta dried at 50°C and 80°C). A similar trend was observed also for PR22D89 pasta, although with a much lower difference between room temperature and higher temperature drying, and being the initial RS much lower. Pasta samples showed a different behavior after cooking (Fig. 5). Among the old and modern varieties, the content of RS became not statistically different (1.7 ± 0.2), regardless of the drying temperature of the original pasta sample. The old genotype showed a decrease of RS after cooking of both fresh and room temperature-dried pasta, while RS remained stable for pasta dried at 50 °C; it was significantly increased after cooking for pasta dried at high temperature (80 °C), although in both cases (50 and 80 °C drying) equal to the previous cooked samples (fresh and room temperature dried samples; Fig. 5). On the other hand, cooking caused a significant increase of RS for the modern cultivar PR22D89, equal between all samples, fresh and dried; and in cooked pastas from the modern genotype the resistant starch was not lower than in those made from the old one (Fig. 5).

4. DISCUSSION

This study shed light on features of wholemeal flours that can be associated to health-promoting properties. Beneficial features claimed for old genotypes such as the lower release of CD-related peptides and the higher amount of prebiotics are not sustainable with scientific

evidence, whereas modern varieties do not seem worse in terms of characters associated to health. On the other hand, processing and cooking can exert a major role on resistant starch amount, in both old and modern tetraploid wheats.

4.1 *Content of immunogenic and toxic peptides in old and modern varieties*

In order to determine whether old and modern durum wheat varieties behave differently in triggering CD, the peptide profile originating from simulated gastrointestinal digestion was analyzed and compared. The results clearly indicated that digestion of wholemeal flours of the old genotypes released higher amounts of peptides associated to CD than the modern ones, confirming previous information (Boukid et al., 2017; Prandi, Mantovani, Galaverna, & Sforza, 2014; Prandi, Tedeschi, Folloni, Galaverna, & Sforza, 2017) and extending it to a greater number of old Italian durum wheats. This result conflicts with the general perception of the consumers, but also with literature that hypothesized an increased amount of these epitopes due to wheat breeding practices (van den Broeck et al., 2010). In the present study, 5 peptides triggering the adaptive immune reaction were identified, deriving from γ-gliadin digestion and containing the restricted epitope DQ2.5-glia-γ4c (QQPQQPFPQ) (Sollid, Qiao, Anderson, Gianfrani, & Koning, 2012). For what concerns the innate reaction, two peptides exhibiting toxic activity in celiac patients were identified, deriving from α-gliadin (Cornell & Mothes, 1993; Mothes, Muhle, Muller, & Herkens, 1985).

The peptide profile after *in vitro* gastrointestinal digestion showed a higher variability in old genotypes, as demonstrated by the PCA analysis, while they were more similar within the modern varieties. The old genotypes were generally richer in proteins than the modern ones as a common effect of breeding (De Vita et al., 2007), partially justifying the observation that the old ones yielded peptides in greater concentration. However, the different abundance of peptides in the digest cannot be entirely ascribed to the diverse amount of proteins, since the ratio between protein content of the richest and the poorest genotypes (Old Saragolla *vs.* PR22D89) was 1.5,

whereas for the 58 peptides the ratio between the highest and the lowest abundance ($R_{max/min}$) ranged up to 15.4 (Fig. 4). Some difference among the genotypes in protein accessibility by digestive enzymes can be hypothesized (Mandalari et al., 2018) and may have arisen with the development of modern lines.

4.2 *Potentially prebiotic carbohydrates in wholemeal flours and pasta*

The prebiotic activity of wholemeal flours can be ascribed to the resistant fraction of starch and to cell-wall associated fiber. Despite the modern varieties resulted richer in total starch than the old ones, the RS fraction was negligible, always < 0.6%. On the contrary, the old genotypes contained a greater amount of RS, always > 1%, which reached 8.5% in Dauno III. Dauno III also presented the greatest amount of soluble fiber, and hence of total non-starch polysaccharides, in agreement with previous results (Ficco et al., 2017). The resistant starch found in wholemeal flour is expected to belong to RS1 and RS2 types, the former inaccessible to digestion owing to its entrapment within whole or partly milled grains or seeds and to the presence of intact cell walls in grains, the latter resisting to digestion because of the high crystalline structure of the granules (Raigond, Ezekiel, & Raigond, 2015). Since all the old and modern grains were milled in the same facility under the same conditions, the difference in RS1 and RS2 content between old and modern genotypes may reside in the evolution of the last ones which may have affected the structure of granules and/or the cell walls.

Since the old genotype Dauno III stood out for both soluble fiber and RS, it appeared a good candidate for further studies. Deeping inside to the process, the pasta made with semolina of the two genotypes with contrasting chemical characteristics, the old Dauno III and the modern PR22D89, were compared. For both the varieties, the drying process negatively affected the level of RS in pasta: the higher the temperature, the lower the amount of residual RS. The effect was particularly marked for Dauno III since it presented the highest initial level of RS, that decreased below 1% after drying at 80°C. The effect of the drying temperature on resistant starch

has been the subject of a few previous studies on starchy food matrices, none of which specifically focused on durum wheat pasta. Available information is controversial, since some studies report that starch crystallinity decreased as the drying temperature increased, causing it to become more accessible to human digestive enzymes, while others report the opposite trend, due to a decreased gelatinization (Aviara, Igbeka, & Nwokocha, 2010; Donlao & Ogawa, 2017; Wiset, Srzednicki, Wootton, Driscoll, & Blakeney, 2005; Yue, Rayas-Duarte, & Elias, 1999). It is likely that both these opposite mechanisms were involved in starch transformation during the drying phase, resulting in a change of RS content. It is remarkable that, after cooking, the level of RS settled at approx. 1.7% for both the genotypes, without significant differences with respect to the initial amount of RS and the drying temperature. As a whole, cooking caused an increase of RS in each pasta sample dried at 50 °C and 80 °C respect to the correspondent non-cooked pasta. This trend was more evident if the dry pasta contained a low amount of RS. Increase of RS after cooking was likely due to retrogradation of gelatinized starch during the cooling (Aravind, Sissons, Fellows, Blazek, & Gilbert, 2013; Wang, Li, Copeland, Niu, & Wang, 2015). It is also plausible that cooking pasta modifies starch directly by increasing the non-digestible fraction, with an effect depending mainly on amylose/amylopectin ratio, polymer chain length, and processing conditions (in particular, temperature and water content) (Sgrulletta, Scalfati, De Stefanis, & Conciatori, 2005). The present study highlights how processing and cooking are pivotal in determining the content of RS, thus affecting the potential prebiotic effect of durum wheat pasta. The effect of drying temperature requires deeper investigation and more attention by food technologists aiming to maintain or even improve the health promoting features.

5. CONCLUSIONS

This study shed light on features of wholemeal flours that can be associated to health-promoting properties, revealing that beneficial properties claimed for old genotypes are not always sustainable with scientific evidence, whereas modern varieties do not seem worse in

terms of characters associated to health. In particular, the wholemeal flours of old tetraploid wheat genotypes cannot detemine a lower exposure to gluten peptides and, in many cases, yield a greater amount of immunogenic and toxic peptides than modern varieties. With regard to the prebiotic potential ascribable to resistant starch, evidence is provided that differences among the varieties may be leveled by the drying and cooking of pasta .

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576 FIGURE LEGENDS

577

578 Fig. 1. Proteins and gluten in the wholemeal flour obtained from old (plain bars) and modern
579 (dashed bars) varieties of durum wheat. Panel A: percentage composition (w/w) of proteins
580 (purple) and gluten (cyan). Panel B: gluten index (green). Values are means ± SD (2 years
581 samples, each in triplicate). Within each series, means with different symbols/letters are
582 significantly different ($P < 0.05$). Means were compared with 2-way ANOVA (year ×
583 old/modern and year × variety) and Tukey's *post hoc* comparison. < *LOD*, lower than the limit
584 of detection of 3%.

585

586 Fig. 2. CD-related immunogenic and toxic peptides in the digests of wholemeal flour obtained
587 from old (plain bars) and modern (dashed bars) varieties of durum wheat. Panel A: immunogenic
588 peptides (IP1, IP2, IP3, IP4, and IP5 from left to right, colored in green from the darkest to the
589 lightest). Panel B: toxic peptides (TP1 and TP2 from left to right, colored in fuchsia from the
590 darkest to the lightest). Panel C: total immunogenic peptides (green), total toxic peptides
591 (fuchsia), and the sum of immunogenic and toxic peptides (grey). Values are means ± SD (2
592 years samples, each in triplicate). Within each series, means with different symbols/letters are
593 significantly different ($P < 0.05$). Means were compared with 2-way ANOVA (year ×
594 old/modern and year × variety) and Tukey's *post hoc* comparison.

595

596 Fig. 3. PCA plots (PC1 vs PC2, respectively accounting for 79 and 8% of total variance) of CD-
597 related immunogenic and toxic peptides in the wholemeal flour obtained from old and modern
598 varieties. The loading plot of the peptides (A) and the score plot of wheat samples, colored on
599 the basis of the variety (B), the crop year (C), and the old/modern classification (D), are reported.

600

Fig. 4. Percentage composition (w/w) of the wholemeal flour obtained from old (plain bars) and modern (dashed bars) varieties of durum wheat. Panel A: total (grey), insoluble (orange) and soluble dietary fiber (cyan). Panel B: available carbohydrates (green), total starch (navy blue), and resistant starch (red). Values are means ± SD (2 years samples, each in triplicate). Within each series, means with different symbols/letters are significantly different ($P < 0.05$). Means were compared with 2-way ANOVA (year × old/modern and year × variety) and Tukey's *post hoc* comparison.

Fig. 5. Content of resistant starch (w/w) in semolina (dashed blue), fresh and dried pasta (light blue), and cooked pasta (dark blue) made with Dauno III and PR22D89. Values are means (n = 3, SD always < 0.5% w/w). Means with a common letter are not significantly different ($P > 0.05$, ANOVA, Tukey's post hoc comparison). Abbreviation r.t. means room temperature.

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Table 1
Accession name, year of release, and origin (geographic or pedigree) of tetraploid wheat genotypes.

	Taxonomic classification	Accession	Year of release	Cultivar / landrace / Breeding line - Origin
Old genotypes	*T. turgidum* ssp. *turanicum**	Perciasacchi	< 1915	Landrace - Southern Italy
	T. turgidum ssp. *durum*	Old Saragolla	< 1915	Landrace - Sicily, Italy
	T. turgidum ssp. *durum*	Scorsonera	< 1915	Landrace - Southern Italy
	T. turgidum ssp. *durum*	Dauno III	1914	Landrace - Southern Italy
	T. turgidum ssp. *durum*	Cappelli	1915	Cultivar - Selection from Tunisian population ‘Jean Retifah’
	T. turgidum ssp. *durum*	Russello	1928	Landrace - Sicily, Italy
	T. turgidum ssp. *durum*	Timilia "reste nere"	1930	Landrace - Sicily, Italy
	T. turgidum ssp. *turanicum**	Etrusco	1930-40	Landrace - Central Italy
	T. turgidum ssp. *durum*	Garigliano	1950-60	Cultivar - Tripolino/Cappelli
Modern genotypes	*T. turgidum* ssp. *durum*	Sfinge	2004	Cultivar - Ofanto/Tavoliere//Doro
	T. turgidum ssp. *durum*	PR22D89	2005	Cultivar - Ofanto/Duilio//Ixos
	T. turgidum ssp. *durum*	L14	2014	Breeding line - Etrusco/Ofanto

615 * Perciasacchi and Etrusco genotypes are classified as *T. turgidum* ssp. *turanicum* on the basis of morphological characteristics.

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