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

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23 ABSTRACT

24 Old wheat genotypes are perceived by consumers as healthier than modern ones. The release of
25 gluten peptides with *in vitro* digestion and the content of potentially prebiotic carbohydrates (i.e.
26 resistant fraction of starch and cell-wall associated dietary fiber) were evaluated in tetraploid
27 wheats, namely 9 old and 3 modern *Triticum turgidum* ssp. genotypes. Simulated digestion of
28 wholemeal flours yielded 152 major peptides, 59 of which were attributed a sequence. Principal
29 component analysis revealed that peptide profiles were variable in old genotypes, unlike in
30 modern ones. Digestion of old genotypes generally yielded peptides in greater concentration. In
31 particular, 5 peptides of γ -gliadin, known to trigger the adaptive immune reaction, and two
32 peptides of α -gliadin, known to be toxic to celiac patients, were particularly abundant in some
33 old varieties. Resistant starch (RS) was negligible in modern genotypes (< 0.6%), but it was
34 remarkably abundant in some old varieties, reaching the highest value in Dauno III (8.5%, $P <$
35 0.05). Dauno III also presented the highest amount of soluble fiber (4.2%, $P <$ 0.05). Pasta was
36 made with an old and a modern genotype (Dauno III and PR22D89, respectively) with opposite
37 RS content. Pasta making and cooking affected starch digestibility, overtaking differences
38 between genotypes and yielding the same amount of RS for both the varieties (approx. 1.7%).
39 The data herein presented suggest that the wholemeal flours of old tetraploid wheat genotypes
40 could not boast particular claims associated to a lower exposure to gluten peptides and, if
41 cooked, to a prebiotic potential.

42

43 KEYWORDS

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

46 ABBREVIATIONS

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

49 1. INTRODUCTION

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

60 In genetically susceptible individuals, the celiac disease (CD) is induced by gliadin
61 peptides that resist digestion and trigger adverse innate immune response (CD-related toxic
62 peptides) or adaptive immune reactions (immunogenic peptides) (Ciccocioppo, Di Sabatino, &
63 Corazza, 2005; Cornell & Mothes, 1993; Hausch, Shan, Santiago, Gray, & Khosla, 2002). All
64 the wheats, regardless they are old or modern, have gluten and are not safe for celiacs due to the
65 release of toxic and immunogenic peptides (Boukid et al., 2017; Gelinias & McKinnon, 2016;
66 Malaloda, Meinhardt, & Simsek, 2018; Prandi, Mantovani, Galaverna, & Sforza, 2014).
67 However, varieties yielding lower amounts of CD related peptides may retard the onset of the
68 disease in predisposed people (Molberg et al., 2005). A previous study describes the CD-related
69 gluten peptides generated by *in vitro* digestion of different old and modern *Triticum* wheats (*T.*
70 *aestivum*, *T. dicoccum*, *T. monococcum*, *T. spelta*, and *T. turgidum*), but included only 2 old and
71 2 modern varieties of durum wheat (Prandi, Tedeschi, Folloni, Galaverna, & Sforza, 2017). The
72 present study aims to extend the knowledge of the peptide profile to a greater set of durum
73 varieties and to determine whether varietal selection operated in the last century affected the
74 amount of peptides mediating the immune reaction.

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

100

101 2. MATERIALS AND METHODS

102

103 2.1 *Wheat genotypes and wholemeal flour production*

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

113 Seeds of the CREA tetraploid wheats germplasm collection were collected from a plot
114 field trial carried out during the growing seasons 2014-15 and 2015-16 (referred to as 2015 and
115 2016, respectively) at CREA Research Centre for Cereal and Industrial Crops (from here,
116 CREA-CI) in Foggia, Italy (41°28’N, 15°32’E; 75 m a.s.l.). Standard agronomical management
117 for the area was applied to the plots as previously described (De Vita, Colecchia, Pecorella, &
118 Saia, 2017). Wholemeal flour was produced by milling 100 g of grain in a laboratory machine
119 (Tecator Cyclotec 1093, International PBI, Milan, Italy) and passing through a fine-mesh, 500-
120 micron, screen. All samples were kept at 4°C until analyses.

121

122 2.2 *Proteins and gluten index*

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

127

128 2.3 *In vitro* protein digestion

129 The wholemeal flour was subjected to simulated oral and gastrointestinal digestion,
130 applying the conditions described by Bottari et al. (2017). Briefly, to simulate the oral digestion,
131 2.5 g of flour were mixed with 2.5 mL of simulated salivary fluid (i.e. 150 U/mL amylase and
132 salivary salts) and incubated for 2 min at 37°C under reciprocating agitation. To simulate the
133 gastric digestion, 5 mL of simulated gastric juice (i.e. 4000 U/mL pepsin, 0.02 M HCl, and
134 gastric salts) were added, the pH was adjusted to 3.0 with HCl, and the mixture was incubated
135 for 2 h at 37°C under reciprocating agitation. To simulate the intestinal digestion, 10 mL of
136 simulated duodenal content (i.e. 200 U/mL pancreatin, 9 mg/mL bile, and minerals) were added
137 to the mixture, the pH was adjusted to 7 with 1 M NaOH, and the mixture was incubated for 2 h
138 at 37°C under reciprocating agitation. The sample was heated at 95°C for 15 min to stop the
139 digestion and then cooled. For chromatographic analysis, the supernatant was clarified by
140 centrifugation (45 min at 4°C at $3220 \times g$) and filtration through 0.45 µm membranes. 5 µL of
141 an aqueous solution (1.6 mM) of the isotopically labeled peptide
142 TQQPQQPF(*d*₅)PQQPQQPF(*d*₅)PQ were added, as the internal standard, to 295 µL of the
143 supernatant.

144

145 2.4 Separation and quantification of peptides by UPLC/ESI-MS

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

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

156

157 *2.5 Peptide identification by LC-MS/MS techniques*

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

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

177

178 *2.6 Available carbohydrates, dietary fiber, starch, and resistant starch (RS)*

179 The content of available carbohydrates (i.e. the carbohydrates digested and absorbed by
180 the human small intestine, including D-glucose, D-fructose, sucrose, maltodextrins, and non-
181 resistant starch), dietary fiber, starch, and RS was determined using Megazyme kits according to
182 the manufacturer's instructions (Megazyme International Ireland Ltd, Bray, Ireland). In
183 particular, available carbohydrates were determined by the 'Available Carbohydrates and Dietary
184 Fibre' kit (K-ACHDF 06/14); soluble and insoluble dietary fiber by 'Total Dietary Fiber' (K-
185 TDFR-100A/K-TDFR-200A 12/15); starch by 'Total Starch' (K-TSTA-50A/K-TSTA-100A
186 08/16); and RS by 'Resistant Starch' (K-RSTAR 10/15). All measurements were performed in
187 triplicate.

188

189 *2.7 Pasta-making*

190 The old durum wheat genotype Dauno III and the modern one PR22D89 were chosen for
191 semolina and pasta production. For semolina, seeds were conditioned at 16.5 % moisture and
192 milled by a laboratory mill (MLU 202; Bühler Brothers, Uzwil, Switzerland) with a six-roller
193 system, three breaking and three sizing passages, and an attached semolina purifier, for a
194 fineness range of 200 - 315 μm . Semolina (5 Kg) was used for spaghetti by means of a pilot
195 plant equipped with an extruder (60VR, Namad, Rome, Italy) and a dryer (SG600, Namad, Rome
196 Italy). The extruder was equipped with a screw (30 cm in length, 5.5 cm in diameter) that ended
197 with a bronze die (diameter of the hole: 1.70 mm), applying an extrusion pressure of 3.4 bar, and
198 reaching a temperature in pasta of 26-28 °C. A sample of the extruded pasta was analyzed (fresh
199 pasta). Other samples were dried at room temperature, or mechanically transferred into a drying
200 chamber, where two different temperature/time cycles were applied. The first (80 °C dried pasta)
201 was the following: i) 20 min at 60 °C and 65% moisture (external drying); ii) 130 min at 90 °C
202 and 79% moisture (wrapping); iii) 150 min at 80 °C and 78% moisture (drying); iv) 160 min at
203 45 °C and 63% moisture (first cooling phase); v) 17 h and 20 min at 50 °C and 50% moisture
204 (second cooling phase). The second cycle (50 °C dried pasta) was the following: i) 20 min at 55

205 °C and relative humidity of 62%; ii) 9 h and 40 min at 75 °C and relative humidity of 75%; iii)
206 40 min at 50 °C and relative humidity of 73%; iv) 20 min at 45 °C and relative humidity of 63%;
207 v) 14 h at 40 °C and relative humidity of 50%. The final moisture content in all samples of dried
208 pasta was 12.5%. Three independent production trials of pasta were performed. Pasta from each
209 batch was cooked for 10 min according to Ficco et al. (2016). Samples of cooked pasta were
210 dried in an oven linked to a vacuum pump (30 °C, vacuum 760 mm Hg), ground through a 0.5-
211 mm screen (Tecator Cyclotec 1093), and kept at 25 °C until analysis.

212

213 2.8 Statistical analysis

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

221

222 3. RESULTS

223

224 3.1 Rainfall and temperature in the growing seasons

225 Mean temperatures and rainfall distribution during the growing-seasons 2014-15 and
226 2015-16 are reported in Table S1. Quantity and distribution of rainfall were variable, as typical
227 of Mediterranean climate (Table S1). In the years 2015 and 2016, cumulated rainfall in the
228 growing cycle (November to May) was 192 mm and 226 mm, respectively (thirty-years average
229 342 mm). With respect to long-term rainfall data, the years 2015 and 2016 were characterized by
230 a longer drought stress period. The 2015 presented the lowest rainfall, albeit recording for

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

235

236 *3.2 Proteins and gluten in wholemeal flours*

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

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

253

254 *3.5 Peptide profile after in vitro digestion*

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

257 determine the general peptide profile and to quantify specific peptides containing sequences
258 recognized as immunogenic or toxic in CD. The general peptide profile of the flour digests
259 determined by UPLC/ESI-MS encompassed 152 major peptides with MW ranging from 200 to
260 4339 Da and a degree of polymerization ranging from 2 to approx. 40 (Fig. S1). In the lack of a
261 standard of each peptide, presumably exhibiting a different analytical response, the peak areas
262 were integrated and utilized for semi-quantification with respect to the same internal standard.
263 Although the absolute concentration of each peptide could not be determined, their relative
264 amount could be compared across the wheat varieties. Of the 152 peptide ions detected, 42
265 presented different abundances in old and modern genotypes ($P < 0.05$), the vast majority of
266 them (39) being more abundant in the old genotypes than in the modern ones.

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

272

273 *3.6 Identification of immunogenic and toxic peptides*

274 The amino acid sequence was assigned to a total of 59 peptides on the basis of their MW
275 and fragmentation pattern (Table S2). Among them, five peptides deriving from γ -gliadin
276 digestion and containing the DQ 2.5 restricted epitope DQ2.5-glia- γ 4c (QQPQQPFPQ) were
277 identified and referred to as immunogenic peptides (hereinafter referred to as IP):

278 TQQPQQPFPQ (IP1), SQQPQQPFPQPQ (IP2), QAFPQQPQQPFPQ (IP3),

279 TQQPQQPFPQQPQQPFPQ (IP4), and PQTQQPQQPFPQFQQPQQPFPQPQQP (IP5).

280 Similarly, two peptides deriving from α -gliadin were identified as toxic peptides (hereinafter

281 referred to as TP), relatively to the CD: LQPQNPSQQQPQ (TP1) and RPQQPYPQPQPQ

282 (TP2).

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

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

298

299 3.2 Content of digestible and indigestible carbohydrates in wholemeal flour samples

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

309 lying in the range of 11.5-12.4%, whereas Dauno III and Scorsonera the highest amount (15.2
310 and 14.9%, respectively; $P < 0.05$). Insoluble dietary fiber ranged between 9.9 and 12.4%, and
311 they were always more abundant than the soluble ones, which ranged between 1.0 and 4.2%. The
312 genotypes Etrusco, L14, Russello, and Sfinge contained the lowest amount of insoluble dietary
313 fiber (9.9 and 10.4%; $P < 0.05$), and the varieties Cappelli, Scorsonera, and Timilia the highest
314 (11.8 to 12.4%; $P < 0.05$). The genotypes Cappelli and Old Saragolla were characterized by the
315 lowest amount of soluble dietary fiber (1.0-1.35%; $P < 0.05$), and Dauno III by the highest
316 (4.2%; $P < 0.05$). Any relationship between total dietary fiber and the distribution between
317 soluble and insoluble fraction could not be established ($R^2 = 0.46$ and 0.47 , respectively).
318 Among the genotypes with the highest content of dietary fiber, Dauno III presented the highest
319 amount of soluble dietary fiber and Scorsonera of insoluble ones.

320 The wholemeal flours bore an amount of available total carbohydrates and starch ranging
321 from 65.4 to 75.5% and from 46.0 to 57.2%, respectively (Fig. 4B). Resistant starch ranged from
322 0.3 to 8.4%. A significant difference between the 2 growing seasons was observed, with 2015
323 resulting richer than 2016 in terms of both total carbohydrates and starch (data not shown; $P <$
324 0.05), but poorer in resistant starch ($P < 0.05$). In addition to the variance due to years,
325 differences were observed among the varieties. Total available carbohydrates and total starch
326 were more concentrated in the group of the modern genotypes compared with the old (Fig. 4B),
327 with the 3 modern genotypes L14, PR22D89, and Sfinge presenting the highest level of total
328 carbohydrates ($> 74.4\%$; $P < 0.05$). These modern genotypes contained also the highest amount
329 of total starch ($> 57.0\%$; $P < 0.05$), whereas the old ones bore lower amounts ($P < 0.05$). The
330 opposite trend was observed for resistant starch, less abundant in modern varieties ($< 0.6\%$; $P <$
331 0.05), while all the old genotypes contained always $> 1.2\%$ ($P < 0.05$). Dauno III was the
332 genotype presenting the far highest amount of resistant starch, 8.4%.

333

334 *3.3 Effect of processing on resistant starch in fresh, dried and cooked pasta*

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

356

357 4. DISCUSSION

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

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

364

365 4.1 *Content of immunogenic and toxic peptides in old and modern varieties*

366 In order to determine whether old and modern durum wheat varieties behave differently
367 in triggering CD, the peptide profile originating from simulated gastrointestinal digestion was
368 analyzed and compared. The results clearly indicated that digestion of wholemeal flours of the
369 old genotypes released higher amounts of peptides associated to CD than the modern ones,
370 confirming previous information (Boukid et al., 2017; Prandi, Mantovani, Galaverna, & Sforza,
371 2014; Prandi, Tedeschi, Folloni, Galaverna, & Sforza, 2017) and extending it to a greater number
372 of old Italian durum wheats. This result conflicts with the general perception of the consumers,
373 but also with literature that hypothesized an increased amount of these epitopes due to wheat
374 breeding practices (van den Broeck et al., 2010). In the present study, 5 peptides triggering the
375 adaptive immune reaction were identified, deriving from γ -gliadin digestion and containing the
376 restricted epitope DQ2.5-glia- γ 4c (QQPQQPFPQ) (Sollid, Qiao, Anderson, Gianfrani, &
377 Koning, 2012). For what concerns the innate reaction, two peptides exhibiting toxic activity in
378 celiac patients were identified, deriving from α -gliadin (Cornell & Mothes, 1993; Mothes,
379 Muhle, Muller, & Herkens, 1985).

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

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

391

392 *4.2 Potentially prebiotic carbohydrates in wholemeal flours and pasta*

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

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

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

434

435 5. CONCLUSIONS

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

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

444

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566

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575

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 \pm 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 \times
583 old/modern and year \times 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 \pm 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 \times
594 old/modern and year \times 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

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

608
609 Fig. 5. Content of resistant starch (w/w) in semolina (dashed blue), fresh and dried pasta (light
610 blue), and cooked pasta (dark blue) made with Dauno III and PR22D89. Values are means ($n =$
611 3 , SD always $< 0.5\%$ w/w). Means with a common letter are not significantly different ($P > 0.05$,
612 ANOVA, Tukey's *post hoc* comparison). Abbreviation r.t. means room temperature.

613

614

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

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

616