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1 **Technological quality and nutritional value of two durum wheat varieties depend on both**

2 **genetic and environmental factors**

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15

16 **Abstract**

17 Durum wheat (*Triticum turgidum* L. subsp. *durum* (Desf.) Husn) is a major food source in the
18 Mediterranean countries since it is utilized for the production of pasta, leavened and unleavened
19 breads, couscous and other traditional foods. The technological and nutritional properties of wheat
20 flour depend mainly on the type of gluten proteins and on their amount which is a genotype and
21 environment dependent trait influenced by the cross talk between endosperm and embryo proteins
22 and enzymes. Gluten proteins are also responsible for celiac disease (CD), an autoimmune
23 enteropathy with a prevalence of about 0.7-2% in the human population. At this purpose, two Italian
24 durum wheat cultivars, Saragolla and Cappelli, currently used for monovarietal pasta, were chosen
25 to compare: i) the reserve and embryo proteome, ii) the free and conjugated phenolics, antioxidant
26 activity, and amino acid composition and iii) the content of immunogenic peptides produced after a
27 simulated gastrointestinal digestion. The results obtained from two years of field cultivation, in
28 average showed a higher amount of gluten proteins, amino acids and immunogenic peptides in
29 Cappelli. Saragolla showed a higher abundance in antioxidant enzymes and stress response proteins
30 in line with its higher antioxidant activity. However, the impact of the year of cultivation, largely
31 depending on varying rainfall regimes through the wheat growth cycle, was significant for most of
32 the parameters investigated. Differences in technological and nutritional characteristics observed
33 between the two cultivars are discussed in relation to the influence of genetic and environmental
34 factors.

35 *Keywords:* Durum wheat flour, embryo-proteins, gluten, *in vitro* digestion, comparative proteomics,
36 polyphenols, antioxidant activity

37

38 **Introduction**

39 Durum wheat (*Triticum turgidum* L. subsp. durum (Desf.) Husn), the 10th most important crop
40 worldwide is grown mainly in the Mediterranean region; it is an integral component of the
41 Mediterranean diet, since it is used for the production of different kinds of pasta, leavened and
42 unleavened breads, couscous and other traditional foods. Global climate changes increasing
43 atmospheric carbon dioxide and the earth surface solar radiation, rising temperatures, and
44 unpredictable devastating precipitation are expected to affect *T. turgidum* ssp. *durum* development
45 and yield. In particular, high temperatures and drought negatively affect wheat growth, reducing
46 yield. [1,2]

47 In Mediterranean environments, soil water availability is crucial in ensuring crop performance of
48 fall-sown cereals: those genotypes that are not capable of rapidly reaching the most critical stage in
49 grain yield determination are heavily affected by terminal drought events.[3] Water stress, often
50 associated with high temperatures in the second half of wheat biological cycle, is one of the main
51 factors influencing quantity and quality of durum wheat production.[4] In the post-flowering phase,
52 the availability of water plays a decisive role: water stress (and high-temperatures) reduces
53 photosynthesis and the source-to-sink transportation of photosynthates in the caryopsis, affecting
54 the formation of the seed proteome. On the contrary, excessive humidity increases yield by
55 increasing the concentration of starch in the caryopsis and decreasing proteins content.

56 An important factor in determining flour technological quality is the seed protein content and its
57 composition.[5] Environmental factors influence the composition of the reserve proteome in a way
58 that may vary with the genotype. Seed storage proteins are classified into two major families,
59 according to differences in solubility: i) globulins and prolamins i.e. gliadins and glutenins (gluten
60 proteins) which are soluble in a mix of salt-/alcohol-/alcohol and reducing agents; ii) water-soluble
61 non prolamins, (i.e.. albumins, enzymes and polypeptide inhibitors).[6] Globulins and prolamins are
62 the major components of the wheat endosperm and influence the viscoelastic properties of flour,
63 affecting processing and rheological properties. The second group plays important functions in

64 grain metabolism, development and response to environmental cues.[7] Seeds contain also embryonic
65 proteins, which play important roles in germination and subsequently in plant growth and
66 development.[8,9] Regulation of enzymatic activities in the embryo is also important for generating
67 high-quality flours,[10] since the quantity and quality of the reserve proteins in the endosperm is
68 largely affected by molecular events occurring during grain development.[7] It is known that non-
69 prolamins influence the processing and rheological properties of wheat flours and that wheat
70 genotypes of different quality showed differences in proteomic profiles of non-prolamin proteins,
71 including albumins and globulins; approximately 80% of these proteins were identified in the
72 developing grain as enzymes with different physiological functions.[11]
73 In addition to reserve and embryo proteins, whole grains are well known to contain high amounts of
74 dietary fibers, various minerals and vitamins and a variety of bioactive compounds, most of which
75 are antioxidants, with an important role in disease prevention and health promotion.[12] Antioxidants
76 of wheat grains are phytochemicals with small molecular weight, which includes carotenoids,
77 tocopherols, flavonoids, and phenolic acids[13] unevenly distributed across the grain kernel.[14]
78 Approximately 99% of total phenolic compounds in durum wheat are attributed to bran and only
79 1% to flour.[15] A full characterization of the health-beneficial compounds present in the whole grain
80 is important for the branding and the marketing of wheat.[16]
81 However, the gluten proteins may be responsible of clinical conditions in predisposed subjects,
82 since their amino acid composition makes them highly resistant to gastric and pancreatic proteases.
83 The partially digested polypeptides move from the stomach into the intestine, where they are only
84 partially hydrolyzed by the pancreatic proteases and by the exopeptidases of the brush border
85 membrane. The antigenicity of these peptides is intensified by deamination of selected glutamine
86 residues by tissue transglutaminase 2, which increases their affinity to the human leukocyte antigen
87 (HLA)-DQ2 or HLA-DQ8.[17] The peptide-DQ2 complex enhances the inflammatory gut mucosa
88 response, with an increase of Th1 cells, which ultimately leads to painful chronic erasure of
89 intestinal microvilli in patients with active celiac disease.[18]

90 Moving from the old to the modern durum wheat varieties, height was reduced and heading
91 anticipated determining a yield increase, and preserving or improving the grain quality traits. The
92 increase in grain proteins concentration, associated with the incorporation into recent cultivars, of
93 favorable glutenin alleles determined the improvement in pasta making quality of the recent
94 genotypes.[18,19]
95 In this work, we performed a comparative analysis of two *T. turgidum* ssp. *durum* cvs, Cappelli
96 (old) and Saragolla (modern), which are widely cultivated in Italy and utilized for the production of
97 mono-varietal flours for pasta. We have compared, along with two years of cultivation: i) the
98 proteomic profile of storage and embryo proteins; ii) the antioxidant properties and the levels of
99 polyphenolic compounds as health related traits and iii) the amount of peptides known to trigger
100 celiac disease in predisposed subjects produced by a simulated gastrointestinal digestion. The
101 differences in technological and nutritional characteristics observed in the two cvs were discussed
102 in relation to the influence of genetic and environmental factors.

103 **Materials and Methods**

104 *Plant growth and grain sampling*

105 Two different durum wheat (*Triticum turgidum* L. subsp. *durum* (Desf.) Husn.) cvs were utilized in
106 this study: Cappelli, the first Italian durum wheat cultivar obtained by pure line selection in
107 Southern Italian Environments of North African landraces by Nazareno Strampelli (1930, Italy,
108 Strampelli selection from Jennah Khetifa) and Saragolla (2004, by Syngenta, formerly PBS-
109 Produttori Sementi Bologna), a modern cv of unrelated pedigree. Their main characteristics are
110 shown in Table S1.
111 Plants were grown in the field at Council for Agricultural Research and Economics - Research
112 Centre for Cereal and Industrial Crops (CREA-CI), Foggia (Italy, 41° 28′ N, 15° 32′ E and 75 m
113 a.s.l.) on a clay-loam soil, in two consecutive growing-seasons (2015–2016 and 2016–2017),
114 indicated from now on as 2016 and 2017, respectively. Seeds were harvested at physiological
115 maturity (13% RH).

116 *Environmental conditions*

117 Rainfall distribution and maximum and minimum ten-day period mean temperatures during the
118 2016 (a) and 2017 (b) cropping seasons are reported in Fig. 1. Excluding January, higher and more
119 evenly distributed rainfall occurred in the first crop season as compared with the second. This trend
120 was more evident during vegetative period and during grain filling when the amount of rainfall that
121 occurred from the 2nd ten-day period of April (flowering) to the 2nd ten-day period of June
122 (harvest) was higher in the first crop season (181 mm vs 55 mm).

123 *Protein extraction and quantification*

124 *Seed storage proteins extraction.* Thirty-five grams of grains from each cultivar were milled with
125 Knifetec™ 1095 (Foss, Hillerød, Denmark) to obtain a fine powder. Gluten proteins (gliadins,
126 HMW-GS and LMW-GS), were extracted from wheat flour (30 mg) as a previously described.[20]

127 *Embryo proteins extraction.* 150 mg of mature embryos were manually dissected from seeds of
128 each cvs, ground to a fine powder in liquid nitrogen and the powder was then suspended in cold
129 acetone containing 10% (w/v) trichloroacetic acid (TCA) and 0.07% (v/v) β-mercaptoethanol, 0.4%
130 proteinase inhibitor cocktail (Sigma Aldrich, Milan, Italy), vortexed and kept at -20°C overnight.
131 Each sample was centrifuged at 20,000 *g* for 15 min at 4°C and the resulting pellet was washed
132 twice by re-suspending in cold acetone containing 0.07% (v/v) β-mercaptoethanol, 0.4% proteinase
133 inhibitor cocktail (Sigma Aldrich) for 1 hour each at -20°C before further centrifugation at 20,000 *g*
134 for 15 min at 4°C. The resulting pellet was vacuum dried for 30 min, and solubilized in freshly-
135 prepared buffer containing 7 M urea, 2 M thiourea, 4% (w/v) 3-[(3-cholamidopropyl) dimethyl-
136 ammonio]-1-propanesulfonate (CHAPS), 18 mM TrisHCl pH 8, 0.4% proteinase inhibitor, 14 mM
137 dithiothreitol (DTT) and incubated 20 min on ice.

138 Each sample was then centrifuged at 35,000 *g* for 25 min at 4°C and the supernatant was re-
139 centrifuged at 35,000 *g* for 25 min at 4°C. The supernatant containing protein samples was
140 quantified and aliquots of 500 μg precipitated in 4 volumes of cold acetone and left at -20°C
141 overnight.

142 *SDS-PAGE protein separation and densitometry analysis of gliadins*

143 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a Mini-
144 PROTEAN Tetra Cell (Bio-Rad) using 12% of acrylamide gels. Aliquots (20 μg) of dried gliadins
145 were suspended in 20 μL of loading buffer containing 20 g L^{-1} SDS, 0.2 g L^{-1} bromophenol blue, 1
146 mL L^{-1} β-mercaptoethanol, 0.05 mol L^{-1} Tris HCl (pH 6.8) and 100 mL L^{-1} glycerol and boiled at
147 95°C for 5 min before loading onto the gels. An Amersham Low Molecular Weight Calibration Kit
148 for SDS Electrophoresis (MW 14,400- 97,000 Da) (GE, Healthcare, Chicago, Illinois, USA) was
149 used to detect gliadins. After electrophoretic separation at 40 mA, the gels were fixed in 70 mL L^{-1}
150 acetic acid and 400 mL L^{-1} methanol, and stained with Coomassie Brilliant Blue R-250 Staining
151 Solution (Bio-Rad). Image Lab 4.5.1 software (Bio-Rad) was used for the relative quantification of
152 the gliadin subunits on each gel. Gliadins were divided into three classes (ω-, α/β, and γ-) based on
153 their molecular weight according to Tosi et al.[21]

154 *Two-dimensional gel electrophoresis (2D-GE)*

155 *Gliadin proteins*. Samples were purified by ReadyPrepTM 2-D Cleanup Kit (Bio-Rad®) and 75 μg
156 of proteins were suspended in a rehydration buffer containing 7 M urea, 2 M thiourea, 4% (w/v)
157 CHAPS, 0.2% (w/v) ampholytes ReadyStrip™ 100× pH 7–10 Buffer (Bio-Rad®), 50 mM DTT
158 (Sigma-Aldrich), 1.6% DeStreak Reagent (GE Healthcare) and 1% (w/v) bromophenol blue. For the
159 first dimension, iso-electric focusing (IEF) was performed using ReadyStrip™ IPG Strips 7-10 (7
160 cm) (Bio-Rad®): the rehydration step was carried out for 16 hours at 50 V. After active rehydration,
161 IEF was carried out at 14,000 V/h in a Protean i12 IEF System (Bio-Rad®). Then strips were
162 equilibrated 15 min in equilibration buffer containing 6 M urea, 2% (w/v) SDS, 1.5 M mM Tris-
163 HCl pH 8.8, 20% (v/v) glycerol, 1% (w/v) bromophenol blue with 1% (w/v) DTT, and then in the
164 same buffer containing 2.5% (w/v) iodoacetamide for an additional 15 min. For the second
165 dimension, the strips were transferred onto Mini-PROTEAN Tetra Cell (Bio-Rad®) using 12%
166 acrylamide gels and Low Molecular Weight Calibration Kit for SDS Electrophoresis (MW 14,400-

167 97,000 Da) (GE, Healthcare). The electrophoretic separation conditions were previously reported.
168 After separation, gels were stained with QC Colloidal Coomassie (Bio-Rad®) for 16 h, destained for
169 2 hours with distilled water before image acquisition. Three technical replicates were performed for
170 each sample.
171 *Embryo proteins*. ReadyStrip™ IPG Strips 4-7 and 3-10 (11 cm) (Bio-Rad®) were rehydrated in a
172 buffer containing: 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.4% (w/v) ampholytes 40%
173 BioLyte® 3/10 Ampholyte (Bio-Rad®), 50 mM DTT (Sigma-Aldrich), 1.6% DeStreack Reagent
174 (GE Healthcare), and 1% (w/v) bromophenol blue. For each sample, 175 µg of proteins were loaded
175 on one strip. After active rehydration, IEF was carried out at 35,000 V/h. For the second dimension,
176 strips were equilibrated and loaded on the top of a 12% acrylamide Criterion XT precast gel for 11
177 cm strips. After separation, gels were stained with QC Colloidal Coomassie (Bio-Rad®) for 16 h,
178 destained for 2 hours with distilled water before image acquisition. Three technical replicates were
179 performed for each sample.
180 *Proteins digestion and ORBITRAP analysis*
181 Protein spots were removed from the 2D-GE with a razor blade and destained in a solution 1:1 (v/v)
182 of 100 mM ammonium bicarbonate/acetonitrile (ACN) overnight. In-gel digestion was performed
183 with 13 ng μL^{-1} of trypsin for embryo spots or chymotrypsin for gliadins. After digestion, peptides
184 were injected into the mass analyzer. LC/ESI-MS/MS, using the system LTQ Orbitrap XL (Thermo
185 Fisher Scientific, Waltham, MA, USA), as previously reported.[8]
186 *Determination of Free and Conjugated Phenolic Content*
187 *Free Phenolic Acids*. The assay was performed as previously described[22] with some modifications.
188 Kernels were milled with Knifetec™ 1095 (Foss, Hillerød, Denmark) to obtain a fine powder and
189 the extracts were obtained by mixing 1 g of flour in 15 mL methanol 80%, stirred and sonicated for
190 45 minutes. The mixes were centrifuged at 2,500 *g* for 10 minutes at 4°C and the supernatants
191 recovered, dried up to vacuum by Savant™ SpeedVac™ SPD1010 (Thermo Fisher Scientific,
192 USA), and stored at -80°C.

193 *Conjugated Phenolic Acid.* After methanol extraction, the pellet was hydrolyzed with 10 ml of
194 sodium hydroxide (2M). The mixture, with 0.5g of flour, was mixed for 4 h and the pH was
195 adjusted to 2 with a 6M HCl. Diethyl ether (50 ml) was added to the mixture, the container was
196 inverted 15 times and then centrifuged at 1000g for 10 min. The supernatant was removed and the
197 process was repeated with 37.5 ml of diethyl ether. The supernatants were pooled, dried to vacuum
198 by Savant™ SpeedVac™ SPD1010 (Thermo Fisher Scientific, USA), and stored at -80°C.
199 Phenolic content (free and conjugated) was determined spectrophotometrically by microplate using
200 a modified Folin-Ciocalteu assay: 10 µL of sample was added to 95 µL of Folin-Ciocalteu reagent
201 (Sigma-Aldrich) previously diluted 1:10, then the reaction was neutralized with sodium carbonate
202 20%. After 5 minutes of incubation at 22°C and 30 seconds of agitation, the absorbance was read at
203 750 nm using the iMark™ microplate reader (Bio-Rad, Hercules, California, USA). Gallic acid was
204 used as standard (6.25-100 µg mL^{-1}), and the total phenolic content was expressed as µg g^{-1} of
205 gallic acid equivalent (GAE).
206 *Antioxidant activity determination*
207 The 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonate) radical cation ($ABTS^{\bullet+}$) scavenging
208 capacity was performed, to determine the antioxidant activity.[23] Briefly, $ABTS^{\bullet+}$ was generated by
209 adding 2.45 mM potassium persulfate to 7 mM $ABTS^{\bullet+}$ solution and allowing the mixture standing
210 to react in the dark at room temperature for 16 h. Then the solution was diluted 1:80 with methanol
211 to obtain an absorbance of 0.70 ± 0.02 at 735 nm. Fresh $ABTS^{\bullet+}$ solution was prepared for each
212 assay. One hundred µL of sample was added to 1 mL of $ABTS^{\bullet+}$ solution (A_{735nm} 0.70 ± 0.02) and
213 the A_{735nm} was read after 5 minutes. Trolox was used as standard (25-250 µM), and the
214 determination of antioxidant activity was expressed as µM g^{-1} of Trolox equivalent (TE).
215 *Simulated gastrointestinal digestion*
216 One gram of ground wheat kernels was *in vitro* digested following an internationally recognized
217 consensus static method.[24] At the end of the digestion, to inactivate the enzymes, the samples were
218 heated at 95°C for 10 min. Then, to eliminate insoluble compounds, samples were centrifuged at

219 3,220 *g* at 4°C for 45 min. To quantify the peptides which contain sequences recognized by the
220 immune system of celiac patients, 295 μL of each sample supernatant were spiked with 5 μL of
221 deuterated standard solution (TQQPQQPF(d_5)PQQPQQPF(d_5)PQ, 1.6 mM), as previously
222 reported.[25] Peptides present in the digested wheat samples were quantified with reverse phase liquid
223 chromatography coupled to mass spectrometry (RP-UPLC/ESI-MS), as previously described.[26]
224 Total amino acids composition of the wheat kernels was determined after acid hydrolysis as
225 previously described.[27]

226 *Bioinformatics and Statistical analysis*

227 PDQuest 8.0 2D Analysis Software (Bio-Rad®) was used to compare 2D raw gels (three biological
228 replicates) of the gliadins and of the embryo proteins from Saragolla and Cappelli grown in 2016
229 and 2017. After run, gels were analysed by ChemiDoc™ MP System (Bio-Rad®) with Image Lab
230 Software 4.0 (Bio-Rad®) to produce a master map, one for each sample. To highlight qualitative and
231 quantitative differences between the master maps of the gliadins and of the embryo proteins, the
232 highest level of matching was chosen. The spot intensity in each master map is proportional to the
233 amount of proteins in replicate gels and is normalized to the total protein fraction. For a quantitative
234 analysis, the software was set to consider the spots whose abundance was at least double in Cappelli
235 with respect to Saragolla (C/S ratio (r) r <0,5 or r> 2) as significantly different (Student's *t* test).
236 Raw data obtained by LTQ ORBITRAP XL were submitted to Proteome Discoverer (Thermo
237 Fisher Scientific) against *Viridiplantae* database. The search parameters were as follows:
238 chymotrypsin (gliadins) or trypsin (embryo proteins) as digestion enzyme, with five missed
239 cleavages; carbamido-methylation of cysteines (delta mass: 57.0215) as static side chain
240 modification; oxidation of methionine (delta mass, 15.9994) and de-amination of asparagine,
241 glutamine and arginine, as dynamic side chain modification (delta mass, 0.9840); precursor mass
242 tolerance: 10 ppm; fragment mass tolerance: 0.8 Da. Further bioinformatics analyses were
243 performed using the BLAST program (https://blast.ncbi.nlm.nih.gov) and the UniProt database
244 (www.uniprot.org) to classify proteins and perform the Gene Ontology analysis.

245 Statistical analyses were performed using the software PAST 3.x.[28] The contents of gluten protein
246 fractions in grains at maturity as well as the amount of IP and TP peptides, phenolics, antioxidant
247 activity, amino acids were subjected to one-way ANOVA.

248 RESULTS AND DISCUSSION

249 *General climatic conditions*

250 As reported in Fig. 1, the two growing seasons had very different weather conditions, with lower
251 rainfall and higher temperatures in 2017 as compared to 2016. In 2017, during the grain filling
252 period, the temperatures exceeded 30 °C, while in 2016 they were below 27 °C. Furthermore, in
253 2017 the pre- and post-anthesis periods had low rainfall (20 mm) respect to 2016 (40 mm). The new
254 cv Saragolla and the old cv Cappelli could face these environmental conditions in a genotype
255 dependent manner, in terms of seed development and maturation. It is well known that high
256 temperatures during seed development may constitute a stressful condition which affect the duration
257 and rate of grain filling.[4,29] Water availability during vegetative growth is positively associated with
258 plant development but an excess of water during grain filling reduces gluten proteins amount.

259 *Characterization of gluten proteins*

260 Saragolla had the typical allelic configuration HMW-GS 6+8 (Table S1), as previously reported.[30,31]
261 It has been suggested that this HMW-GS configuration contributes, favorably, to the gluten
262 technological quality. As expected, Cappelli exhibited the HMW-GS 20 allele (Table S1). Shewry
263 et al.[32] used wheat transformation to show that HMW-GS 20 allele is associated with lower dough
264 strength, due to a minor density of intermolecular disulfide bonds (fewer cysteine residues at the N-
265 terminal), with a lower polymeric protein content.

266 Both cvs showed the typical configuration LMW-2 at the Glu-B3 locus (Table S1).[31] This
267 configuration is characterized by a 42 kDa LMW-GS, the most abundant GS of this class. Gluten
268 quality rests on the genetic characteristics of each cv, but environmental factors may have as well a
269 role in regulating the relative amount of each gluten fraction. Therefore, composition of the reserve

270 proteome of each *T. turgidum* ssp. *durum* variety cultivated in 2016 and 2017 was analyzed by the
271 sequential extraction of gliadins and glutenins, both HMW and LMW-GS. As reported in Table 1,
272 the gliadins fraction is the most abundant component followed by the LMW-GS and by the less
273 abundant HMW-GS. In Saragolla, the gliadins fraction had a significant lower amount ($p < 0.01$) in
274 2017 (40.96%) respect to 2016 (47.11%). Cappelli did not show significant variations between 2016
275 and 2017 (50.02% and 50.41% respectively).
276 The content of LMW-GS increased significantly in Cappelli in 2017 (25.78%) as compared to 2016
277 (15.82%), whereas it remained stable in Saragolla. The content of HMW-GS was significantly
278 higher in 2016 respect to 2017, in both Cappelli and Saragolla.
279 The proportions of gliadins and glutenins subunits were significantly influenced by both genotype
280 and crop season factors, and were characterized by a significant 'genotype x year' interaction ($p <$
281 0.01) as reported in other *T. turgidum* ssp. *durum* genotypes.[31].
282 The values of two important technological parameters, gliadin/glutenin and HMW-GS/LMW-GS
283 ratio[20] are reported in Table 1. In Saragolla, the gliadin/glutenin ratio significantly decreased in the
284 2017 harvest respect to 2016 (0.69 and 0.89, respectively; $p < 0.01$). Moreover, the HMW/LMW
285 ratio significantly decreased in 2017 as compared to 2016 in both varieties ($p < 0.01$); also Koga and
286 colleagues[33] showed that the composition of gluten proteins and the gliadin/ glutenin and HMW-
287 GS/LMW-GS ratios, were affected by the climatic conditions and in particular by desiccation.
288 Figure S1 shows the SDS-PAGE profile of the gliadin fraction (60-70% of total gluten) of both cvs
289 in the two years. Seven main bands were identified on the gel: one corresponding to a 66-kDa ω-
290 gliadin and six – in the 45-30 kDa range – which represent γ-gliadins and α/β-gliadins. This profile
291 was conserved in all samples but with differences in abundance, in relation with cvs and year of
292 cultivation, as shown by the densitometry analysis (Fig. S1, Table 1). In 2017, the relative quantity
293 of ω-type gliadins increased in both cvs but only in Cappelli the difference was significant ($p <$
294 0.01), while the α/β-type gliadins increased significantly in both cvs ($p < 0.01$). Differently, γ-type
295 gliadins in 2017 significantly decreased in both cvs ($p<0.01$) (Table 1).

296 Accumulation of gluten proteins is a complex process involving spatial and temporal regulation and
297 is affected by environmental and abiotic signaling.[34] Environmental conditions determine changes
298 in gluten fractions and high temperatures influence also yield by altering the duration and rate of the
299 grain filling period.[29] Under high temperature regimens, transcription from many gene families
300 started slightly earlier, but the period of transcripts accumulation was shorter.[35] Indeed high
301 temperatures affect the transcription of gliadin genes, thereby altering the gliadin to glutenin ratio
302 and affecting dough properties.[36]
303 The rate of grain filling increases in some varieties with temperature up to 30°C, which might
304 reflect an increased enzyme activity and active metabolic processes.[29]
305 Several studies showed that storage proteins are upregulated by high temperature, suggesting how
306 these proteins were directly or indirectly affected by stress conditions.[5,37]
307 In our experiment, Cappelli synthesized highest amount of storage proteins in 2017, with more
308 stressful conditions (Fig.1). This observation fits with some features of this cv, since Cappelli
309 displays a water conserving behavior (isohydric plant).[38]
310 Considering the different gluten fractions, our results agree with those reported in *T. durum* by other
311 authors.[31,39] In response to high temperatures, the proportions of ω-gliadins (low S-protein), HMW-
312 GS and α-gliadins (low to medium S-proteins) increased, while LMW-GS and γ-gliadins (high S-
313 proteins) remained steady or decreased. This fits with the observation that high temperatures
314 promote gliadin synthesis and that genes encoding for α-gliadins contain heat-shock responsive
315 elements in their promoter.[35,40] At variance, γ-gliadins were less abundant in Saragolla and Cappelli
316 during 2017 (a period of high temperatures), in agreement with previous results which firstly
317 reported a decrease of γ-gliadins in conditions of high temperature. [36]
318 Several studies agree on a decreased dough strength and gluten quality in plants exposed to
319 extremely high temperatures, low relative humidity and increased levels of grain nitrogen, during
320 the grain filling period.[34,39,41]
321 *2D gel electrophoresis analysis of gliadin protein*

322 The proteomic master map of gliadins (Fig. S2) contained 15 spots, with molecular weights from 30
323 to 44 kDa. All spots were isolated from the gels and characterized (Table 2). A comparative
324 analysis within the two years showed that most spots were equally abundant in both varieties in
325 2016; while an α-gliadin (spot 7301) was present only in Cappelli and a α/β-gliadin (spot 7102) was
326 more abundant in Saragolla ($p < 0.01$). In 2017, a γ-gliadin (spot 6501) and a α–gliadin (spot 7801)
327 were present only in Cappelli; four spots more abundant ($p < 0.01$) in Saragolla corresponded to γ-
328 gliadins (spots 4903, 6901), and α–gliadins (spots 4301 and 2201) and two spots more abundant (p
329 < 0.01) in Cappelli corresponded to α–gliadins (spots 7301 and 8801).
330 During 2017, when higher temperatures were measured during the grain-filling period, several
331 gliadins were differentially modulated in both cvs, however this modulation did not affect the
332 proportion of the different fractions as reported in Table 1.
333 *Comparative analysis of 2-DE of wheat embryo proteins*
334 In addition to gluten proteins, that are the major protein components of wheat seeds, also albumins,
335 globulins and embryo proteins play a role determining the nutritional/anti-nutritional properties.
336 Moreover, some of these proteins can cause allergic reactions in sensitive individuals.[42] Albumins
337 and globulins are distributed mainly in the outer layers of wheat kernels, and thus are important
338 components in whole-wheat flours and semolina, which are encountering an increasing consumer
339 interest, because of their high content of fibers, proteins, and other functional constituents.[5]
340 The embryonal proteins of Saragolla and Cappelli were separated in the pH ranges 3-10 and 4-7. In
341 total 343 and 171 spots were identified in 2016 and 2017 respectively; of these 97 and 36, which
342 were differentially abundant (Fig. 2), were identified by MS (Table S2). In 2016, 11 spots were
343 present only in Cappelli and five only in Saragolla; moreover, 40 spots were more abundant in
344 Cappelli and 35 in Saragolla. In 2017, only one spot was present in Saragolla but not in Cappelli
345 and 28 spots were more abundant in Saragolla and 8 spots in Cappelli. Since the durum wheat
346 genome annotation is not publicly available yet, several proteins matched with the corresponding
347 proteins of *T. aestivum* (51 proteins), *T. urartu* (21 proteins) and *T. dicoccoides* (2 proteins). In

348 other cases, the closest homologs from other species were identified, such as *A. tauschii* 5 proteins,
349 *H. vulgare* 10 proteins, *O. sativa* 9 proteins, *Z. mays* 6 proteins and *A. thaliana* 13 proteins (Table
350 S2). Differentially abundant proteins, were classified according to biological process, molecular
351 function and cellular component GO classes (Table S2). As far as biological process, the proteins
352 identified have been cataloged in 16 GO classes (Fig. 3) whereas some proteins could not be
353 catalogued, and are shown as "not classified".
354 The most represented GO classes were 'stress response' and 'oxidation-reduction processes' in both
355 years.
356 *Stress response*
357 Several spots were assigned to the 'stress response' GO class. They are Heat Shock Proteins (HSPs)
358 and Late Embryogenesis Abundant (LEA) proteins, whose function is to protect the embryo from
359 abiotic stresses, such as oxidative stress and the water deficit occurring at the final stage of seed
360 maturation. Three spots (12, 38, and 16) were identified as HSP70, HSP90 and HSP 18.1, and five
361 spots (17, 21, 50, 72 and 116) attributed to LEA proteins. The abundance of these proteins,
362 increased in Saragolla during both years of cultivation (Fig. 2).
363 Water deficit induces the accumulation of protecting proteins as chaperone (HSP), LEA proteins,
364 proteinase inhibitors but also of a large number of proteins with unknown functions.[43,44] Members
365 of the HSP family play key roles in response to high temperatures, in particular members of the
366 HSP70 gene family are components of the cellular network of molecular chaperones and are
367 essential to normal cell function.[8,45] HSP70 is highly conserved in plants, it is developmentally
368 regulated: abundant in dry mature seeds but disappears during germination,[9] and it has been
369 demonstrated that overexpression of HSP70 and HSP90 increases thermotolerance.[46] LMW HSPs
370 are accumulated in seeds during maturation and in various stress conditions.[5] It was reported that
371 LMW HSPs are up-regulated in heat-tolerant cvs during mid-grain development.[47] They can form
372 large multimeric structures and display a wide range of cellular functions, as well as being able to
373 act as molecular chaperones, in particular HSP18.1 exhibits chaperone activity to protect proteins

374 from irreversible aggregation and proteolysis.[48] Thus, the increased abundance of the HSPs in
375 Saragolla may have increased the protection against high temperatures. LEA proteins in both plants
376 and animals are associated with tolerance to water stress resulting from desiccation or cold.[49] A
377 LEA protein was present only in Cappelli in 2016 (spot 72), others were more abundant in
378 Saragolla (spots 17, 21 and 50). In 2017, a LEA was more abundant in Saragolla (spot 116).

379 *Oxidative-reductive processes*

380 Several proteins were classified in 'oxidative stress response' GO class. Among these, we identified
381 enzymes such as glutaredoxin, thiol-specific peroxidase, NADH-ubiquinone oxidoreductase,
382 succinate-semialdehyde dehydrogenase, peroxiredoxin, glutathione peroxidase and superoxide
383 dismutase (Fig. 2, spots 10, 9, 77, 43, 41, 94, 103). The amount of these proteins increased in
384 Saragolla mainly in 2017 (Fig. 3), reflecting their relevance in protecting seeds against a desiccation
385 stress due to the active oxygen species (ROS) which are produced during seed development.[8] Plant
386 tissues typically contain several peroxidase isoforms varying in isoelectric point from acidic to
387 basic.[50] Several spots (Fig. 2 spots 52, 63, 75 and 111) were identified as peroxidase 1 according to
388 their molecular mass but they have different isoelectric points and abundance. Peroxidases in wheat
389 grains varies with the developmental stage, and peroxidase 1 might be relevant through a
390 differential expression pattern or through its phosphorylated modification.[50]

391 Superoxide dismutase catalyzes the first step in the scavenging of active oxygen species. This
392 protein was more abundant in Saragolla in 2017 as compared to Cappelli, showing how the two
393 genotypes react differently to high temperatures. A balance of oxidation-reduction in wheat grain is
394 important for the polymerization of glutenins subunits, an important step in the formation of the
395 gluten protein network with influence upon final gluten quality.[51]

396 The protein super-families of thiol oxidoreductases, thioredoxins, and their functional analogues,
397 glutaredoxins, play a key role in maintaining the intracellular SS/SH redox balance they catalyze
398 the reaction of dithiol−disulfide exchange or the removal of glutathione from the mixed disulfide

399 protein−GSH.[52] Thus, the different modulation of such proteins in Saragolla respect to Cappelli, in
400 the two years of cultivation, could influence also grain quality.

401 **Antioxidant analysis**

402 The free phenolics content (μg GAE g^{-1}) determined in the two cvs in the two years are presented in
403 Table 3. In 2016, the content of free polyphenols was 27.65 ± 4.52 μg GAE g^{-1} and 28.75 ± 4.17 μg
404 GAE g^{-1} in Cappelli and Saragolla respectively; the content of conjugated polyphenols was 191.20
405 ± 1.80 and 180.09 ± 8.66 μg GAE g^{-1} in Cappelli and Saragolla respectively.

406 In 2017, the amount of free polyphenols was 30.06 ± 5.00 and 33.02 ± 3.7μg GAE g^{-1} in Cappelli
407 and Saragolla respectively, higher than in 2016. The content of conjugated polyphenols was 195.65
408 ± 5.91 and 176.92 ± 6.48 μg GAE g^{-1} in Cappelli and Saragolla respectively, however these values
409 were not statistically significant respect to those of 2016 (Table 3). These results confirm that the
410 wheat phenolic compounds primarily exist in a conjugated form associated with cell wall
411 materials.[53]

412 The antioxidant potential of Cappelli was 157.81 ± 0.32 μM TE g^{-1} in 2016 and 195.79 ± 0.24 μM
413 TE g^{-1} in 2017 (Table 3). In Saragolla, higher values were measured: 209.71 ± 0.21 μM TE g^{-1} in
414 2016 and 232.05 ± 0.16 μM TE g^{-1} in 2017. The observed variations were statistically significant in
415 relation to both cultivation year and cvs ($p< 0.01$). Several authors reported the significant impact
416 of both genotype and environment on the antioxidant activity and phenolic acids content [12,54] it was
417 also shown that the rainfall level before heading period may be a more relevant factor than total
418 precipitation.[55] In our study, the rainfalls were abundant in 2016 before heading period, differently
419 from 2017 in which they were nearly absent (Fig. 1). A higher antioxidant activity in Saragolla may
420 balance for the damaging effects of ROS which are produced under unfavorable environmental
421 conditions, and which cause oxidative damage to chloroplasts and other cell structures.[56]

422

423 *In vitro digestion of gluten peptides and characterization*

424 Gluten proteins are considered as the main external stimulus of celiac disease. A comparative
425 proteomic analysis showed how much the gluten protein composition is affected both by the
426 genotype (Saragolla vs Cappelli) and by the cropping year (2016 vs 2017), in terms of total gluten
427 amount and of distribution of the protein sub-classes (α/β-gliadins, γ-gliadins, ω-gliadins, LMW-
428 glutenins, and HMW-glutenins). These variations in gluten composition may impact the
429 immunogenic potential of wheat for people genetically predisposed to celiac disease. Anyway, since
430 the triggering stimulus are actually some peptide sequences arising from the incomplete digestion of
431 gluten, in order to carefully assess the potential of the different samples to elicit a celiac response,
432 Saragolla and Cappelli semolina were *in vitro* digested as to generate the peptides, which are likely
433 present in the gastrointestinal tract. Those peptides containing sequences able to trigger an
434 immunogenic response like in celiac disease, were quantified with LC-MS techniques, dividing
435 them in the two subclasses of peptides known to elicit an adaptive and an innate immune response.
436 Table 4 reports the amount of each of these peptides for the two cvs and in the two different
437 cropping years. The amount of peptides, which contained sequences able to trigger the adaptive
438 immune response (IP) was lower in the grains harvested in 2016 than in 2017. In 2017, the amount
439 of all these peptides increased: the increase was limited in Cappelli but well-marked in Saragolla. In
440 2016, in general, Saragolla had a lower amount of IPs than Cappelli, but the difference was not
441 significant in 2017. Since the IPs arise mainly from γ-gliadins, which were present in a lower
442 percentage in 2017 than in 2016, the amount of IPs as emerging after the *in vitro* digestion must be
443 affected also by other factors (protein structure, starch-protein interactions and other matrix
444 influences on gluten digestibility). The crop year effect is more evident when looking at peptides,
445 which contain sequences able to trigger the innate immune response (“TP”): these peptides were
446 present in higher amount in 2017, respect to 2016, especially in Saragolla, consistently with a
447 higher amount of α/β-type gliadins in 2017.
448 The analysis of the amino acid composition of Saragolla and Cappelli (Table 5) generally reflects
449 the trend observed for peptides, with an increase of most of the amino acids in Saragolla harvested

450 in 2017 compared to the same cv harvested in 2016. As observed for peptides, the increase in
451 Cappelli was more limited than in Saragolla.
452 In conclusion, our results have confirmed that seed proteome composition is strongly influenced by
453 stressful environmental conditions especially when occurring during grain filling. We have also
454 shown that the old Cappelli and the modern Saragolla wheat cvs counteract the environmental
455 trigger in different ways. The crop season 2017 was characterized by lower rainfalls and higher
456 temperatures than 2016. In this condition, in Saragolla, the gliadins fraction had a significant lower
457 amount, in particular due to a lower amount of ω-gliadins and γ-gliadins. Overall, Cappelli
458 synthesized the highest amount of reserve proteins in 2017.
459 Furthermore, during the driest season (2017), Saragolla was characterized by a higher amount of
460 proteins involved in abiotic stress response (HSPs and LEAs) and of enzymes involved in
461 oxidation-reduction processes, associated to a higher antioxidant capacity, symptoms of a better
462 response to environmental constraints.
463 Similarly, for what concerns peptides deriving from gluten digestion, Saragolla and Cappelli
464 behaved markedly differently in the two years, and Saragolla showed a significantly lower amount
465 of peptides involved in celiac disease in 2016 than in 2017. In general, we observed that Saragolla,
466 the modern variety, was more influenced by environmental conditions than Cappelli, and this may
467 affect gluten content and its relative immunogenic potential along with nutritional characteristics
468 particularly in a stressful environment.
469 **ABBREVIATIONS USED**
470 2D-GE, two-dimensional polyacrylamide gel electrophoresis;
471 HLA, human leukocyte antigen;
472 HMW-GS, high molecular weight glutenin;
473 LMW-GS, low molecular weight glutenin;
474 TCA, trichloroacetic acid;
475 CHAPS, 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propanesulfonate;

476 DTT, dithiothreitol;

477 SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis;

478 IEF, iso-electric focusing;

479 ACN, acetonitrile;

480 LC/ESI-MS/MS, liquid chromatography electrospray ionization tandem mass spectrometric; GAE,

481 gallic acid equivalent;

482 $ABTS^{\bullet+}$, 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonate) radical cation;

483 TE, trolox equivalent;

484 RP-UPLC/ESI-MS, reverse phase liquid chromatography coupled to mass spectrometry;

485 GO, gene ontology;

486 LEA, late embryogenesis abundant;

487 HSP, heat shock protein; ROS, active oxygen species;

488 IP, adaptive immune response;

489 TP, innate immune response

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498

499 The authors declare no competing financial interest.

500

501 **SUPPORTING INFORMATION**

502 Main agronomic traits and grain quality of durum wheat cultivars Saragolla and Cappelli
503 (Supplementary Table S1);
504 Separation on SDS-PAGE (12% acrylamide) of gliadin components extracted from Cappelli and
505 Saragolla cultivated in 2015-2016 (1, 2) and in 2017-2018 (3, 4) (Figure S1);
506 2D master map of gliadin proteins extracted from Saragolla and Cappelli cultivated in 2017. (Figure
507 S2);
508 List of proteins identified by ORBITRAP analysis, in the two cvs in two years. Each of them were
509 classified respect the function and the GO category (Table S2).

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Figure captions:

Figure 1. Rainfalls distribution and ten-day max and min mean temperatures in the 2015-2016 (a) and 2016-2017 (b) crop seasons. Thin arrows indicate the sowing date. Black and white arrows indicate the heading date of Saragolla and Cappelli respectively.

Figure 2. Embryo proteins analyzed by 2D-GE. Proteins extracted in 2016 and 2017 were focused on ReadyStrip™ IPG Strips 3-10 (A, B) and 4-7 (C, D). Differentially abundant spots between Saragolla and Cappelli are indicated with numbers. Empty circles are protein spots present only in Cappelli.

Figure 3. GO annotation terms for the set of differentially expressed proteins in the 2016 (A) and 2017 (B) from Cappelli and Saragolla.

Table 1. Reserve proteome composition and densitometry analysis of gliadin subunits of Cappelli and Saragolla cultivated in 2016 and 2017

		gluten protein					gliadin densitometry analysis (%)		
cultivar	**year**	**GLI %**	**HMW-GS %**	**LMW-GS %**	**gli/glu**	**HMW-GS/ LMW-GS**	**ω**	**γ**	**α/β**
Cappelli	**2016**	50.02a	34.16a	15.82b	1.00a	2.16a	7.55b	43.40a	49.05b
	2017	50.41a	23.81b	25.78a	1.02a	0.92b	11.95a	16.45b	71.60a
Saragolla	**2016**	47.11a	31.60a	21.29 a	0.89a	1.48a	5.97a	55.67a	38.36b
	2017	40.96b	28.55b	30.49 a	0.69b	0.94b	6.79a	16.70b	76.52a

γ gliadin = sum of different γ gliadin subunits; α/β gliadin = sum of different α/β gliadin subunits.

Different letters in the same column correspond to statistically different values ($p < 0.01$ one way ANOVA, post doc Tukey's test).

Table 2. Identification of gliadin proteins obtained by LC-MS/MS derived from 2D gel spots and characterization of their abundance in Saragolla and Cappelli in 2016 and 2017.

							spot abundance Saragolla/Cappelli	
n° spot	**accession number**	**protein name**	**MW (KDa) theor**	**pI theor**	**coverage %**	**score %**	**2016**	**2017**
1901	D5FPE1	LMW γ-gliadin like Glu B3-3	44.5	8.3	51.1	77.6	+/+	+/+
2901	R4JDM5	LMW γ-gliadin like Glu B3-3	37.8	8.1	68.4	154.8	+/+	+/+
1301	A0A1D5S346	γ-gliadin	32.6	8.5	68.4	77.3	+/+	+/+
4903	R9XV62	γ-gliadin	34.4	7.3	16.5	15.3	+/+	**/+
5901	M9TK56	γ-gliadin	41.2	8.1	44.7	149.2	-/+	+/+
6501	M9TLK0	γ-gliadin	33.8	8.1	35.3	34.6	+/+	-/+
4301	A0A0E3Z673	α-gliadin	34.1	7.6	38.0	179.9	+/+	**/+
2201	A0A1K0ISX1	α-gliadin	33.0	7.4	45.1	43.9	+/+	**/+
6901	M9TG60	γ-gliadin	38.8	7.9	30.9	73.6	+/+	**/+
7301	A5JSA5	α-gliadin	32.8	7.7	62.4	82.1	-/+	+/**
6401	B2ZRD1	α-gliadin	33.4	7.0	65.1	109.3	+/+	+/+
7801	A0A1P8DSL3	α-gliadin	32.2	7.0	50.9	64.7	+/+	-/+
8801	K7WV47	α-gliadin	37.3	8.2	27.1	45.6	+/+	+/**
7102	Q41533	α/β-gliadin	30.0	6.6	56.7	70.2	**/+	+/+
9601	I0IT55	α/β-gliadin	33.5	7.7	49.8	110.7	+/+	+/+

+ = present; - = absent; ** = more abundant protein ($p < 0.01$)

Table 3. Free and conjugated phenolic and antioxidant properties of Cappelli and Saragolla in 2016 and 2017.

genotype	year	free phenolics (µg GAE g^{-1}	conjugated phenolics (µg GAE g^{-1}	antioxidant capacity TEAC (µM TE g^{-1})
Cappelli	2016	27.65 ± 4.52a	191.20 ± 1.80a	157.81 ± 0.32a
	2017	30.06 ± 5.00a	195.65 ± 5.91a	195.79 ± 0.24b
Saragolla	2016	28.76 ± 4.17a	180.09 ± 8.66a	209.71 ± 0.21c
	2017	33.02 ± 3.70a	176.92 ± 6.48a	232.05 ± 0.16d

Different letters in the same column correspond to statistically different values (one way ANOVA, post doc Tukey's test)

Table 4. Peptides generated by *in vitro* digestion which contain sequences, know to trigger the immune system of celiac patients. Results are expressed in ppm (µg of peptide per gram of sample). Peptides, which contain sequences able to trigger the adaptive immune response, are indicated with the code "IP", while peptides, which contain sequences able to trigger the innate immune response, are indicated with the code "TP".

cultivar	year	IP1	IP2	IP3	IP4	IP5	IP6	IP7	TP1	TP2	TP3	IPT	TPT	TT
Cappelli	2016	167±4b	170±21b	57±4b	505±82b	296±2b	78±31b	88±9b	29±6a	80±3b	16±1a	1363±86b	126±2b	1488±84b
	2017	121±21a	137±12b	92±1c	709±44c	571±33c	158±16c	274±19c	245±1b	300±12c	120±13b	2062±122c	665±3c	2727±124c
Saragolla	2016	95±4a	65±16a	27±2a	170±27a	86±50a	15±2a	18±7a	10±0a	24±2a	3±2a	477±76a	37±0a	513±76a
	2017	106±0a	94±6a	98±5c	630±4bc	576±3c	105±2b	254±10c	234±17b	335±7d	186±20c	1862±7c	755±10d	2618±17c

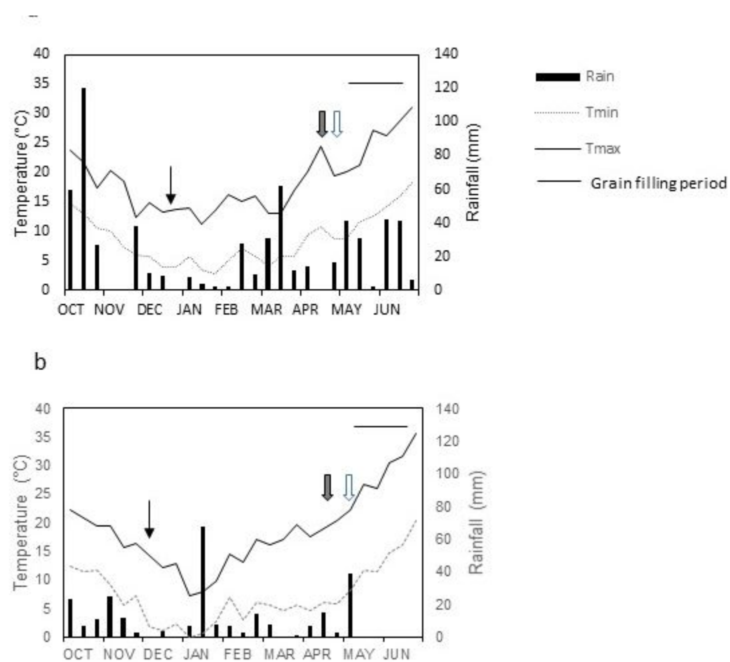
IP1: TQQPQQPFPQ; IP2: SQQPQQPFPQPQ; IP3: QAFPQQPQQPFPQ; IP4: TQQPQQPFPQQPQQPFPQ; IP5: PQTQQPQQPFPQFQQPQQPFPQPQQP;
IP6: FPQQPQLPFPQQPQQPFPQPQQPQ; IP7: QQPQQPFPQPQQTFPQQPQLPFPQQPQQPF; TP1: LQPQNPSQQQPQ; TP2: RPQQPYPQPQPQ;
TP3: LQPQNPSQQQPQEQVPL; IPT: sum of peptides, which contain sequences able to trigger adaptive immune response; TPT: sum of peptides, which contain sequences able to trigger innate immune response; TT: sum of peptides, which contain sequences able to trigger immune response in celiac people.
Different letters on the same column, correspond to statistically different values (one way ANOVA, post doc Duncan's test).

Table 5. Amino acid composition of Cappelli and Saragolla harvested in 2016 and 2017. %AA indicate the sum of quantified amino acids expressed as % on weight of semolina.

cultivar	year	Asp	Ser	Glu	Gly	His	Thr	Ala	Pro	Tyr	Val	Met	Ile	Leu	Phe	% AA
Cappelli	2016	5.9^{b}	6.9^{b}	40.7^{bc}	5.1^{b}	3.9^{b}	3.9^{b}	4.3^{a}	13.4^{b}	2.6^{a}	6.3^{b}	2.0^{a}	4.6^{bc}	7.6^{b}	6.7^{bc}	11.4^{bc}
	2017	6.8^{c}	7.3^{b}	44.6^{c}	5.4^{d}	3.9^{b}	4.3^{c}	4.6^{a}	14.2^{b}	3.1^{a}	6.6^{b}	2.0^{a}	4.8^{c}	7.9^{b}	7.0^{bc}	12.2^{c}
Saragolla	2016	4.8^{a}	5.2^{a}	28.8^{a}	3.5^{a}	2.8^{a}	3.1^{a}	3.7^{a}	10.4^{a}	2.5^{a}	5.1^{a}	1.7^{a}	3.3^{a}	5.9^{a}	5.0^{a}	8.6^{a}
	2017	6.8^{c}	6.6^{b}	38.4^{b}	4.5^{c}	3.3^{ab}	4.0^{bc}	4.6^{a}	12.6^{b}	2.4^{a}	5.7^{ab}	1.6^{a}	4.1^{b}	7.1^{b}	6.0^{b}	10.8^{b}

Different letters in the same column correspond to statistically different values (one way ANOVA, post hoc Duncan's test).

Figure 1



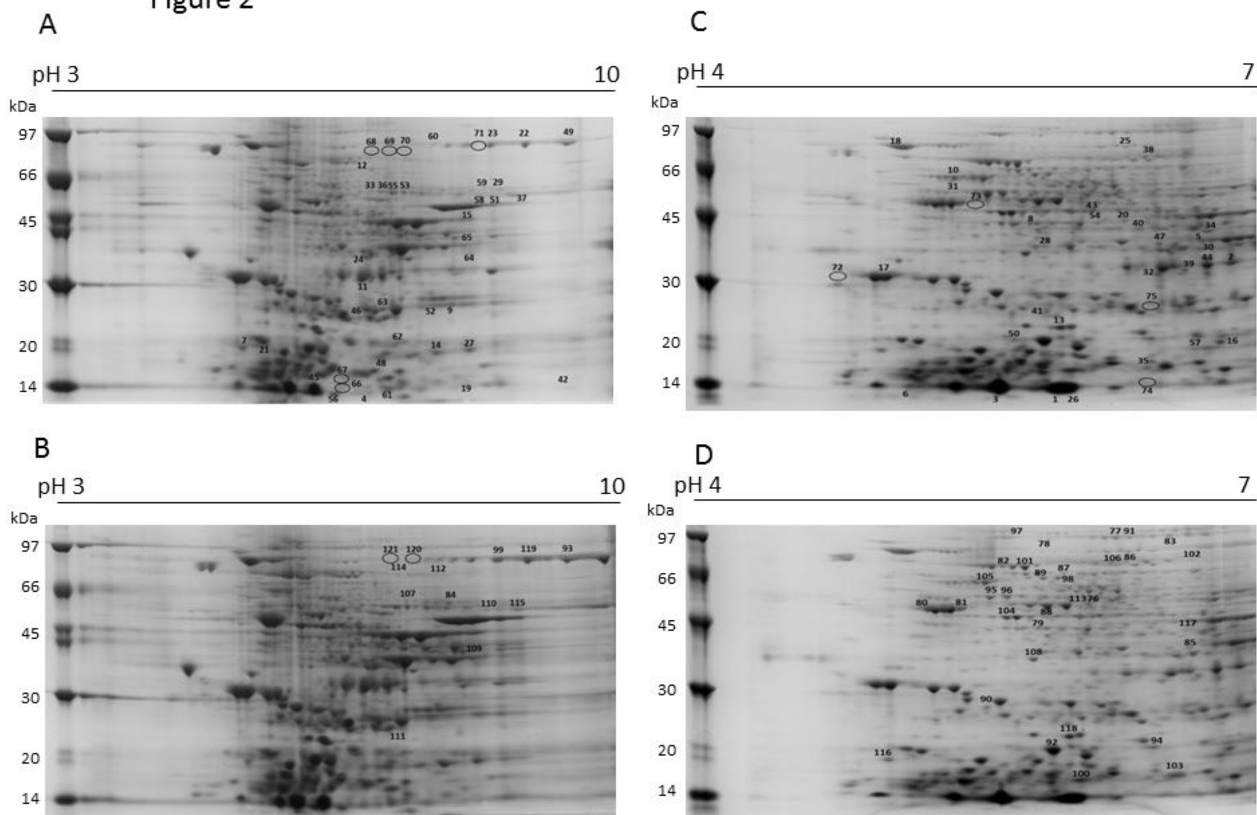


Figure 2
A
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D
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Figure 3