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(Article begins on next page)

Sourdough Fermentation and Chestnut Flour in Gluten-free Bread: a shelf-life evaluation

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1 Abstract

Effects of sourdough fermentation combined with chestnut flour addition were investigated for 2 improving technological and nutritional quality during shelf life of gluten-free bread. Sourdough 3 fermentation by itself and with chestnut flour caused a reduction in final volume of loaves, but reduced 4 heterogeneity in crumb grain characteristic. Sourdough technology allowed increasing crumb moisture 5 content in comparison with control breads with no significant variations during 5 days shelf life. 6 7 Chestnut flour darkened both crumb and crust colours while no significant effects were observed for sourdough. Sourdough and/or chestnut flour addition caused a significant increase in crumb hardness at 8 time 0 while, during storage, a significant reduction of staling was observed only at 5 days, even if a 9 decrease in amylopectin fusion enthalpy was observed. From a nutritional point of view, the percentage 10 of hydrolysed starch during in vitro digestion was significantly reduced by sourdough fermentation 11 12 with a presumable lower glycaemic index.

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15 Keywords: sourdough, chestnut flour, gluten-free bread, physical analysis, shelf-life

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18 **1. Introduction**

Celiac disease (CD), an immune-mediated enteropathy caused by the ingestion of gluten in genetically 19 susceptible individuals, is one of the most common lifelong disorders; people suffering of this disease 20 21 need to exclude these storage proteins of certain cereals from their diet. Not only celiac disease patients, but also people who suffer from nonceliac gluten sensitivity and an increasing share of 22 consumers who avoid gluten for lifestyle reasons, follow a gluten-free diet (Masure et al., 2016). For 23 this reason, the market of gluten-free foods in the last decades has grown together with the products on 24 the shelves (Global Gluten-Free Products Market Research Report, 2016). Notwithstanding, the 25 improvement of gluten-free products appears as a big challenge for the food technology and a relevant 26 issue in the scientific literature, as demonstrated by the great number of published studies in the last 27 years (Capriles and Arêas, 2014; Houben et al., 2012; Masure et al., 2016; Matos and Rosell, 2015). 28

29 Among gluten-free foods, bread is the most important and gluten-free breads are generally reported to show crumbling texture, poor colour, not satisfying taste and low specific volume as well as a short 30 shelf-life, probably as a consequence of the lack of the viscoelastic network formed by gluten (Houben 31 32 et al., 2012). Thus, improvement of both technological and nutritional quality of gluten-free breads is 33 now highly debated in literature. Several approaches have been reported, starting from the investigation of different gluten-free flours and starches, new additives and novel technologies (Matos and Rossell, 34 35 2015; Capriles and Arêas, 2014) to the addition of ingredients with a high nutritional value (reduced fat, complex carbohydrates, dietary fibre, vitamins and minerals). In the last years, chestnut flour 36 received more and more attention due to its nutritional and health benefits for gluten-free bread 37 38 improvement. This flour contains essential amino acids (4-7%), dietary fibre (4-10%), low amount of fat (2–4%) and also vitamin E, vitamin B group, potassium, phosphorous, and magnesium (Sacchetti et 39 al., 2004). It was recently reported that the addition of chestnut flour in bread-making increases 40 antioxidant capacity and fiber content of wheat bread (Dall'Asta et al., 2013) as well as reduces 41

42 moisture losses in both crust and crumb and could slow the staling process (Rinaldi et al., 2015).

Regarding gluten-free breads, Demirkesen et al. (2010) studied the effects of different levels of its 43 addition on a simple rice-based gluten-free formulation reporting that elevated amounts of chestnut 44 45 flour led to some deterioration in quality parameters. This fact limits the actual nutritional improvement of gluten-free breads. More recently, Paciulli et al. (2016) studied the effects of chestnut flour addition 46 to two commercial gluten-free mixtures for producing technologically and nutritionally improved 47 breads. Similarly, the authors found that the addition of chestnut flour influenced the characteristics of 48 breads just after baking and during storage but allowed improving total antioxidant capacity and fiber 49 content. 50

The use of sourdough (a dough containing a Lactobacillus culture in symbiotic combination with 51 yeasts) was reported to be a potential strategy for improving quality of both soft and hard wheat breads 52 53 (Rinaldi et al., 2015). Its use to overcome defects in gluten-free breads with reduced need for expensive additives and higher acceptance from consumers was also reported (Moroni et al., 2009). 54 Exopolysaccharides (EPS) formed from sucrose during sourdough fermentation can improve the 55 56 technological properties of gluten-free breads and potentially replace hydrocolloids (Galle et al., 2012). Aguilar et al. (2016) studied spontaneously fermented chestnut flour sourdough and evaluated its effect 57 in gluten-free breads based on corn starch and chestnut flour during 7 days of storage: chestnut flour 58 59 sourdough improved bread specific volume, rendered breads with lighter crusts, reduced crumb hardness at day 0 and day 7 and reduced pH. However, chestnut flour sourdough had no effect on 60 yeasts and molds growth during 7 days of bread storage and did not influence sensory characteristics 61 62 perceived by consumers.

By a nutritional point of view, the potential of sourdough application to reduce the predicted glycemic
index on gluten-free breads was also investigated by Wolter et al. (2014). In addition, the degradation
of cereal proteins during sourdough fermentation markedly affects the overall quality of baked goods.

The acidification and the reduction of disulfide bonds of gluten by hetero-fermentative lactobacilli promote the primary activity of cereal proteases, which lead to the liberation of various sized polypeptides, many of them considered as bioactive or biogenic peptides (Gobbetti et al., 2014). Sourdough fermentation is also considered to be one of the most suitable biotechnology for the manufacture of baked goods rich sources in dietary fibre; moreover, it is reported to stimulate the activity of grain endogenous phytase that could decrease the phytate content of whole flours (Leenhardt et al., 2005).

In this contest, the application of the sourdough technology represents the new frontier for the 73 production of high quality gluten-free bread. This strategy could also be applied to satisfy the consumer 74 demands for clean labels, natural products and for a reduced use of additives. Anyway, only few 75 attempts were reported for producing and characterizing gluten-free sourdoughs and the functional 76 77 properties of the breads thereof (Moroni et al., 2009). Thus, the aim of the present work was to evaluate the effects of sourdough addition, also in combination with chestnut flour, on chemico-physical, 78 79 thermal and nutritional properties of a commercial gluten-free bread formulation during 5 days of shelf-80 life.

82 2. Materials and Methods

2.1. Materials and chemicals 83

Gluten-free bread mixture, chestnut flour, salt, compressed yeast were purchased from the local market. 84 Deuterium Oxide (D₂O), chloroform-d CDCl₃, pepsin from porcine gastric mucosa, pancreatin from 85 porcine pancreas and Amyloglucosidase from Aspergillus niger were obtained from Sigma Aldrich (St. 86 Louis, Missouri, USA). The rest of other chemicals are of analytical grade unless and otherwise 87 specified. 88

89

2.2. Samples, breadmaking and storage 90

A commercial (NT FOOD S.p.A., Altopascio, Lucca, Italy) gluten-free bread mixture was purchased 91 on the market with ingredients, as reported on label, as follows: corn, rice cream soup, tapioca starch, 92 sugar, vegetable fibres, salt, thickening agents: guar flour and hydroxypropylmethylcellulose, 93 flavourings. The proximate composition of the mixture was the following: moisture 9.7g/100g, 94 carbohydrate 85.7g/100g, fibers 1.9g/100g, protein 1.8g/100g, fat 0.9g/100g, salt 1.9g/100g. 95

96 Similarly, a chestnut flour (C) obtained from Italian chestnuts was purchased on the market and it showed moisture, carbohydrate (sugar), protein, fiber and fat contents of 14.0 g/100g, 76.1 g/100g (24 97 g/100g), 6.3g/100g, 9.4 g/100g and 3.6 g/100g, respectively. 98

99 Four bread samples were prepared from these flours with the following formulations on mixture basis:

- 100
 - M (only mixture): mixture (100.0) water (80.0) sunflower oil (5.0) compressed yeast (2.0); _
- MC (mixture+chestnut flour): mixture (100.0) chestnut flour (42.9) water (124.2) sunflower oil 101 _ 102 (7.1) compressed yeast (2.9);
- MS (mixture+sourdough): mixture (100.0) water (95.8) sunflower oil (5.2) sourdough (20.8); 103
- MCS (mixture+chestnut flour+sourdough): mixture (100.0) chestnut flour (45.4) water (136.4) 104 sunflower oil (7.6) sourdough (30.3). 105
 - 6

These chestnut commercial gluten-free flour ratios were selected based on previous results (Pacialli etal., 2016) and preliminary experimentations.

Before the breadmaking process, gluten-free sourdough was refreshed at least three times by mixing with rice flour (1:1; w:w) and water (1:0.4; w:w) at 22/24°C with intervals of 4 hours, incubated at 28°C and then the refreshed sourdough was added to the other ingredients.

A domestic bread maker machine (Moulinex, Groupe Seb Italia S.p.A., Milano, Italy) was used for breadmaking, with the rapid program for samples M and MC: stirring + kneading + rising, 80 min; baking, 55min at 210 °C. On the contrary, samples MS and MCS presented a proofing step of 3 hours in a climatic chamber at 28°C and 75 % relative humidity in addition to the machine program.

115 Cooking losses after baking were measured and ranged from 8.6 g/100g for M samples that presented 116 the lowest absolute percentage of water in the recipe (48.7 g/100g) to about 12% for all the other 117 breads that presented similar water content each other (about 52 g/100g). The cooked breads were 118 cooled at room temperature, packaged in alcohol-sprayed sealed air-tight plastic bags and stored in a 25 119 °C temperature-controlled chamber in the dark (ISCO 9000, Milan, Italy). Samples were analysed at 0, 120 1, 3 and 5 days of shelf life. Three loaves were used for the characterization of the breads at each 121 storage time for a total of 12 loaves for each bread type.

122

123 *2.3. Chemical analysis*

The protein content (g/kg) was determined by the Kjeldhal method using 1g of ground sample, as previously reported (Dall'Asta et al., 2013). A correction factor of 5.7, typical of flour mixtures, was used for the calculation. Fat content (g/kg) was determined utilizing a Soxhlet extractor (Velp Scientifica, Monza-Brianza, Italy) on 5 g of ground sample, and diethyl ether as solvent. Analyses were carried out at day 0 of storage. pH was measured on each bread sample at time 0 according to Plessas et al. (2005) by placing 15 g of
breadcrumb and 100 ml of distilled water in a clean dry container, which was sealed and stirred until
the bread dispersed into a semi-liquid mixture, and measured using a pH meter (Jenway 3510, Bibby
Scientific, Staffordshire, UK).

For organic acids analysis, 200 mg of sourdough at the beginning of the fermentation as well as cooked breads were dissolved in 1ml of D_2O and blended with magnetic stirring for 1h at room temperature. To ensure a complete removal of the apolar component, 100 µl of CDCl₃ were added. After centrifugation at 10000 rpm, 600 µl of supernatant were taken for the analysis according to Caligiani et al. (2007). Triplicate analyses were carried out on each sample.

138

139 2.4. Specific bulk volume, crumb grain characteristic and moisture content

Specific bulk volume of breads was determined according to the AACC Approved Method 10-05.01
(AACC, 2000) and expressed as the weight/volume ratio of cooked bread (mg/L).

Crumb grain was evaluated by means of a digital image analysis system, as reported previously 142 143 (Dall'Asta et al., 2013). Images of three central slices (20 mm thickness) of each loaf were acquired with a scanner (Hewlett Packard, Palo Alto, CA, USA) at 600 dots per inch (dpi) taking squares (40x40 144 mm) from the centre of the images after calibration, standardization and optimization by means of 145 146 Image-Pro Plus 4.5 (Media Cybernetics Inc., USA) software. The number of pores (expressed as percentage of the total number) was obtained according to pre-selected dimensional classes based on 147 their area. Selected classes were: class-1: 0.005-0.099 mm²; class-2: 0.01-0.99 mm²; class.3: 1-10 mm²; 148 149 class-4: $> 10 \text{ mm}^2$.

150 The moisture content (g/100g) within the bread loaves was evaluated following the AACC standard

151 method, 44-15.02 (AACC, 2000). The crust, under-crust layer, and central crumb were examined at

152 each shelf-life time for each bread type.

154 *2.5. Physical and thermal analysis*

Texture analysis was performed on crust and crumb using a TA.XT2 Texture Analyzer equipped with a 25 kg load cell (Stable Micro Systems, Godalming, UK) and Texture Expert for Windows software (version1.22) for data analysis on each loaf. Crust hardness was measured by means of a puncture test using a 3 mm diameter stainless steel probe and a test speed of 2 mm/s. Maximum peak force (N) was measured from the penetration curve and taken as crust hardness. Measurements were taken on five preselected points of the crust.

Crumb evaluation was carried out on ten cube of $20 \times 20 \times 20$ mm extracted from two central slices of the 161 samples. A TPA test was performed with a 35 mm diameter cylindrical aluminium probe by means of a 162 double compression with a speed of 1mm/s up to the 50% of the original sample height. The textural 163 164 parameters considered were hardness (maximum peak force of the first compression cycle, N), cohesiveness (ratio of positive force area during the second compression to that during the first 165 compression area, dimensionless), resilience (area during the withdrawal of the penetration, divided by 166 167 the area of the first penetration, dimensionless), and chewiness (product of hardness x cohesiveness x springiness, N) (Bourne, 1978). In addition, crumb hardness increase was calculated as the percent 168 169 difference between the samples at time 0 and other times referred to the hardness of the time 0 ones. 170 Samples of bread crumb (8–10 mg) were weighed in stainless steel pans (Perkin Elmer, USA), hermetically sealed and analyzed with a DSC Q100 (TA Instruments, New Castle, DE). Indium 171 (melting temperature 156.6 °C, ΔH_f 28.45 J/g) and *n*-dodecane (melting temperature -9.65 °C, ΔH_f 172

216.73 J/g) were used to calibrate the instrument, and an empty pan was used as reference. Samples
were equilibrated at 30 °C for 5 min, cooled to -80 °C at a rate of 2 °C/min, equilibrated at -80 °C for 3
min and then re-heated to 130 °C at a rate of 10 °C /min. Dry nitrogen was purged in the DSC cell at 50

¹⁷⁶ cm³/min. Thermograms were analyzed with Universal Analysis Software, Version 3.9A (TA $_9$

177 Instruments, New Castle, DE), and enthalpy (Δ H, J/g). Retrograded amylopectin (J/g sample) was 178 obtained by the integration of the endothermic peak in the 50–80 °C temperature range. At least 179 triplicate analyses were carried out per sample.

Colour was determined on ten pre-selected locations of the crust and crumb of each bread loaf. The analyses were performed using a Minolta Colorimeter (CM 2600d, Minolta Co., Osaka, Japan) equipped with a standard illuminant D65 and a 10° position of the standard observer. The instrument was calibrated before each analysis with white and black standard tiles. L^* (lightness), a^* (redness) and b^* (yellowness) were quantified on each sample using the Spectramagic software (Ver. 3.6).

185

186 2.6. Starch hydrolysis

Breads were tested in vitro to determine the rate of starch hydrolysis. In vitro digestions were 187 performed as previously described by Paciulli et al. (2016) for gluten free breads: about 8 g of sample 188 was suspended in phosphate buffer (20 mmol/L) and incubated at 37 °C stepwise with human saliva, 189 for 2 min at pH 6.9, and porcine pepsin (2500 U), for 2 h at pH 2.0-2.5. Each sample was then 190 191 transferred into 20 cm dialysis tubing strips (12 kDa molecular weight cutoff) with 100 mg of pancreatin from porcine pancreas (3xUSP), sealed with plastic clamps, and incubated for 5 h at pH 6.9 192 into 1000 mL sealed containers containing 500 mL of phosphate buffer. Two aliquots (0.5 ml) from the 193 dialyzed solution were removed for analysis at time 0, every 15 min during the first hour and every 30 194 min until 5 hour digestion. The aliquots were used to determine the number of glucose monomers of 195 the permeated fragments. To this purpose, each aliquot sample was hydrolyzed using 20 µl of 0.5% 196 amyloglucosidase solution (200 U) at pH 5.6 and the glucose concentration was determined with a 197 glucose analyzer (2900 Biochemisty Analyzer, YSI Inc., Yellow Springs, USA). All analyses were 198 performed in triplicate. 199

201 *2.7. Statistical analysis*

202 Means and standard deviations were calculated with SPSS (Version 23.0, SPSS Inc., Chicago, USA)

- statistical software. SPSS was used to perform one way (ANOVA) to evaluate the effect of sourdough
- and chestnut flour addition at a significance level of 0.05 (p < 0.05). A Tukey-Kramer post-hoc test at a
- 205 95% confidence level was also applied using the same software to verify the differences among groups.

3. Results and Discussion

207 *3.1. Chemical analyses of dough and bread*

A dough pH value of 4.3 ± 0.2 was measured. Lactic and acetic acid contents were 4.14 ± 0.15 and 1.67 \pm 0.05 g/kg respectively, in the range reported for sourdough breads (Corsetti, 2012), with a fermentation quotient (molar ratio between lactic and acetic acids) of 1.65, slightly lower than the optimum range 2.0 - 2.7 (Hammes and Gänzle, 1998).

Lactic acid content in MCS samples (Table 1) resulted the highest followed by MS and by M and MC, 212 as expected. The high content of sugar in chestnut flour may have probably favoured the growth of 213 sourdough microflora and caused a higher production of organic acids. Acetic acid content was almost 214 four folds higher in samples with sourdough MS and MCS (Table 1), if compared to breads with 215 compressed yeast (M and MC). The content of organic acids influenced final pH values of breads, as 216 expected; samples with sourdough (MCS and MS) presented significantly lower values than samples 217 with compressed yeast (M and MC). The higher pH value of MCS samples compared to MS was in 218 contrast with the higher content of organic acids found in the latter sample. It could be hypothesized 219 220 that the buffering properties of chestnut proteins could have limited the pH decrease. Similarly, Aponte 221 et al. (2013) observed higher pH values for sourdoughs composed of 40 % chestnut flour mixed with 222 wheat or rice flour compared to those composed of only wheat or rice with no correlation between pH 223 and total titratable acidity (TTA) values.

The percentages of the main chemical components are also reported in Table 1. Breads without chestnut flour (M and MS) presented significantly lower content of protein and fat due to the composition of the ingredients: gluten free mixture contained 1.8 and 0.9 g/100g of protein and fat vs. 6.3 and 3.6 g/100g of chestnut flour. No significant differences were observed in total carbohydrate content among samples. Otherwise, the addition of chestnut flour reduced the starch content as it contains more simple sugars (Table 1) in comparison to the mixture.

231 *3.2 Crumb grain characteristics and specific bulk volume of bread*

Specific volumes measured at 0 day of storage are reported in Table 1: control gluten free bread (M) 232 presented the highest volume at time 0 and during the whole shelf life (data not shown), remained also 233 unchanged. No differences were observed among the other sample at time 0 and during storage. The 234 addition of chestnut flour and/or sourdough influenced the loaf development by reducing the 235 effectiveness of gelling and thickening agents of the gluten free mixture, as previously stated by 236 Paciulli et al. (2016) and Demirkesen et al. (2010). On the contrary, Demirkesen et al. (2016) reported 237 no influence on specific volume with 20% of sourdough addition. In the present work, about 10 % of 238 sourdough was added and the detrimental effect on specific volume could be due to the differences in 239 mixture composition. 240

241 Crumb grain characteristic of all breads at time 0 is reported in Figure 1. The addition of chestnut flour (MC) caused a coarser but more homogenous structure (higher pores of class 3) in comparison with M 242 breads that showed a significantly higher number of little holes but also of cells of large sizes (class 2 243 244 and class 4). This finding is in disagreement with data presented by Paciulli et al. (2016) who reported 245 a significant increase of the pores of the greatest dimension related to the addition of chestnut flour. 246 Similarly, Mariotti, Pagani and Lucisano (2013) found a significantly higher alveolate area to total area 247 ratio and coarse crumb grain appearance due to buckwheat flour addition to gluten-free mixtures. Similarly, Demirkesen et al. (2016) reported a heterogeneous crumb structure with high amounts of 248 open pores for rice-based gluten free breads. The addition of sourdough (MS) led to a significant 249 250 reduction of the pores belonging to the highest class (class 4) in comparison to M ones and thus the studied level of sourdough addition contributed to reduce the heterogeneity of M crumb, in accordance 251 with Demirkesen et al. (2016). Finally, breads with the simultaneous addition of chestnut flour and 252 sourdough (MCS) presented the coarsest structure with the highest percentages of class 3 and 4 and 253 13

lowest of class 1 and 2 among all the breads, as consequence, probably for the interconnection between
all gas cells (open pores). Demirkesen et al. (2016) reported an increase in average size of pore due to a
sourdough incorporation of 20% in gluten free breads with a chestnut/rice ratio up to 30/100.

257 During storage, no significant differences were observed for all breads with the exception of MCS that showed an increase in pores belonging to class 3, and a simultaneous decrease of pores belonging to 258 class 2. No variations were observed for the remaining classes, in agreement with Rinaldi et al. (2015). 259 Probably, this variation was due to the moisture migration from the crumb to the crust that caused the 260 drying of the grain walls of the crumb with a reduction in their thickness and an increase in pore area, 261 as consequence (Gray and Bemiller, 2003). In addition, larger pores in MCS samples at time 0 day 262 might have caused the loss of more moisture or faster water redistribution, leading to an increase in 263 crumb coarseness during shelf life (Demirkesen et al., 2016). MCS crumb showed the highest loss in 264 the first day of shelf life (-5.2%) (Figure 2), if compared to all the other samples that presented similar 265 value (about 2.1 %). 266

267

268 *3.3. Moisture content*

269 Moisture content trends of all samples for crust, under-crust layer and crumb during 5 days of storage270 are reported in Figure 2.

All samples presented an increase of crust moisture during storage, as expected, due to the migration of water from crumb to crust (Gray and Bemiller, 2003). Among samples, MCS (Figure 2, panel D) showed significant highest values of crust moisture content, while M (Figure 2, panel A) exhibited the lowest content. The presence of chestnut flour fibres and products of sourdough, probably EPS, may have increased the ability to retain water in MCS. In addition, the coarser structure of this bread type might increase the migration of water vapour during cooking from crumb to crust.

277 Moisture content of under-crust layer did not show significant differences during storage for M and 14

MC (Figure 2, panels A and B, respectively). On the contrary, MCS breads (Figure 2, panel D) showed a constant increasing trend during shelf life due to the water migration from crumb to crust, starting from significantly lower values than all the other samples. MS showed (Figure 2, panel C) a significant decrease at time 1 and a further increase until time 5 days. In the first day of storage, crust absorbed moisture from the under-crust layer more quickly than the simultaneous absorption from the crumb to the under-crust layer.

MS and MCS samples showed significantly higher crumb moisture content values at time 0 (Figure 2): 284 this fact could be due to the metabolite products of fermentation such as EPS that are reported to be 285 able in the water retention (Taman et al., 2013). Crumb moisture content didn't vary significantly 286 during storage, with the exception of MS samples that showed a significant decrease of the crumb 287 moisture content, but only at the end of shelf life (day 5). A similar behaviour was already reported 288 from Taman et al. (2013) by adding 10% of sourdough to wheat bread. Similarly, Galle et al. (2012) 289 observed a decrease in water holding capacity and an increase in crumb firmness in sorghum gluten 290 free dough and breads induced by organic acids and enzymes released during sourdough fermentation. 291 292 Probably, this effect was observed only in MS samples and not in MCS thanks to the positive effect of 293 chestnut fibres in the latter.

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295 *3.4. Physical and thermal analyses*

Gluten free breads' crust hardness values (Figure 3) at time 0 day are in accordance with moisture content. M samples, which exhibited the lowest moisture content (Figure 2, panel A) also presented the highest value of crust hardness. On the other side, MCS showed the lowest hardness due to the high moisture content in the crust (Figure 2, panel D). During storage (day 1, 3 and 5), MC showed the lowest values in accordance with the trend in crust moisture content. Generally, samples obtained by means of sourdough fermentation and/or containing chestnut flour presented a rapid increase in crust

hardness since day 1 (58, 107 and 126 % for MC, MCS and MS, respectively vs. 22% for M). 302 Conversely, M bread presented a significantly lower increase during shelf life with the highest value 303 only at the end of shelf-life (177 % at day 5). The gelling and thickening agents of the gluten free 304 305 mixture may have retained water during storage leading to a softer crust. Moreover, the observed trend could be also due to the crumb grain characteristic for MC and MCS breads that presented a coarser 306 crumb in comparison to the M ones (Figure 1), in relation to the increase of the moisture migration 307 during storage (Figure 2, panel A) and to the acidification that reduced the water holding capacity for 308 MS samples. However, at time 5 days, M samples presented a dramatic increase in crust hardness with 309 a value almost two-fold compared to other samples. 310

Crumb textural data are reported in Table 2. Sourdough and/or chestnut flour addition caused a 311 significant increase in crumb hardness probably due to the lower development of the final bread (loaf 312 collapse) and the lower softness of the crumb, as already stated in paragraph 3.2 in accordance with 313 Mariotti at al. (2013). No significant differences were observed in crumb hardness among chestnut or 314 sourdough added samples (MC, MS, MCS) at time 0 as also observed for specific volume data (Table 315 316 1). Crumb hardness increase was already reported by Paciulli et al. (2016) and Demirkesen et al. (2010) 317 for gluten free breads due to the chestnut flour incorporation. Crumb hardness increase due to sourdough was in contrast with Demirkesen et al. (2016) who reported a reduction of firmness up to 318 319 20% of sourdough addition. Galle et al. (2012) studied the influence of EPS on dough rheology and quality of gluten-free sorghum bread and reported that EPS formed during 10% sourdough 320 fermentation led to a softer crumb in the fresh and stored sorghum bread. During shelf life, breads 321 322 added with chestnut flour (MC) and sourdough (MS) presented a higher staling rate, expressed as percentage increase of hardness, at time 1 day in comparison to M and MCS. In particular, 113.9 and 323 130.7 % crumb hardness increasing were observed for MS and MC, while 82.3 and 88.7 % were 324 obtained for M and MCS, respectively. At time 3 days a higher staling rate (expressed as percent 325 16

hardness increase) was observed for MS, while no significant differences were measured among the 326 remaining samples. Finally, at time 5 days M samples gave the highest hardness value and staling rate 327 followed by MS and, finally, by MC and MCS (Table 2). Cohesiveness was an indicator of the internal 328 329 cohesion of the material: generally, breads with low cohesiveness are susceptible to fracture and crumble (Onyango, Mutungi, Unbehend & Lindhuaer, 2010) and are not desirable. M samples showed 330 the highest cohesiveness values both at time 0 and during shelf life, probably thank to the higher 331 concentration of thickening and gelling agents from the gluten free mixture. Sourdough and/or chestnut 332 flour addition may have caused a dilution of gluten free mixture and consequently a reduction of the 333 additives able to retain water and maintain the freshness of bread. Similar results were obtained by 334 adding chestnut flour to a commercial gluten free mixture (Paciulli et al., 2016). Resilience values 335 (Table 2) showed a trend similar to that of cohesiveness, as a reduction in resilience was reported to 336 cause loss of elasticity and tendency to crumble (Onyango et al., 2010). Sourdough and chestnut added 337 samples (MS, MC and MCS) exhibited significantly lower values of resilience (p<0.05) during shelf-338 life. Finally, chewiness values of M bread, an indication of the energy required to masticate a solid 339 340 food prior to swallow, were significantly lower in comparison to the other samples (Table 2) at all times of analysis. MS samples gave the highest values meaning hard break of these breads in the mouth 341 342 probably due to the effect of acidity on thickening or gelling agents of the mixture.

Amylopectin retrogradation enthalpies (Δ H), monitored and quantified by DSC, are reported in Table 2. All the samples showed a significant increase of Δ H during storage, as expected, and as already observed by Demirkesen et al. (2014) for gluten free breads. Sourdough seemed to show a significant effect in delaying retrogradation measured by means of DSC in accordance with Corsetti et al. (2000), even if crumb hardness of MSC breads was lower than M only at day 5. Similar results were reported by Moroni et al. (2011) studying different levels of sourdough addition in gluten free breads prepared with buckwheat flour; also in this case, sourdough addition caused a reduction in volume and an increase in crumb hardness and positive effects in delaying macroscopic staling phenomenon wereobserved only after 5 days of storage.

Colorimetric parameter measured on both crumb and crust of tested breads are reported in Table 3. 352 Crust of MC and MCS samples presented lower L^* and higher a^* values compared to the other samples 353 at time 0, due to the darkening effect of chestnut flour previously observed (Paciulli et al., 2016). The 354 lower crust lightness (L^*) of sourdough breads (MS and MCS) was probably related to the first phases 355 of Maillard reactions, which was reported to be more consistent under sourdough bread-making with a 356 greater concentration of all compounds involved in non-enzymatic browning (Torrieri et al. 2014). 357 These differences remained unaltered during storage (Table 3). Crust of MC also presented the highest 358 b^* values due to a higher percentage of chestnut flour in the recipe than MCS sample. 359

Crumb colorimetric data (Table 3) are aligned with those of the crust. Chestnut flour addition deeply 360 influenced colour with a darkening effect by lowering L^* and increasing both a^* and b^* values, as 361 already reported (Paciulli et al., 2016). In general, sourdough fermentation did not produce significant 362 differences in bread colorimetric parameters with the exception of b^* value in MS samples that 363 364 significantly decreased in comparison with M. The higher fermentation time of MS (180 vs. 40 min) probably favoured a higher lipoxygenase activity leading to a partial oxidation of the carotenoid 365 pigments (Leenhardt et al., 2006). During storage, L^* and a^* values tended to decrease in M, probably 366 367 due to the water loss from the cell walls, which could increase opacity making crumb darker from the instrumental measurement. a^* values also significantly decreased due to the sourdough addition (MS) 368 during storage. Crumb colour remained substantially unaltered in MC and MCS samples during shelf-369 370 life in accordance with Paciulli et al. (2016) reporting that added chestnut flour better preserved the gluten free bread discoloration during shelf life. 371

372 *3.5. Starch hydrolysis*

³⁷³ To better characterize the nutritional properties of the breads prepared within this study, the starch

digestibility was assessed over 5 hours by enzymatical hydrolysis: the percentage of hydrolysed starch permeated through the dialysis tube as well as the total areas under the curves (AUC) during a 5 h in vitro digestion are considered. After 5h hydrolysis, the digested starch fractions of the total starch were $60.9 \pm 1.2 \%$ for M, $61.7 \pm 2.3 \%$ for MC, $56.5 \pm 1.7 \%$ for MS, $54.3 \pm 1.0 \%$ for MCS. A significant reduction of the digested starch was achieved by means of sourdough fermentation as previously observed by De Angelis et al. (2009) and due the presence of lactic and acetic acids that limited the starch bioavailability.

The area under curve (AUC) values were 10885 ± 289 , 11206 ± 349 , 9573 ± 124 and 9691 ± 333 (mg 381 min/dL), for M, MC, MS and MCS, respectively. The addition of sourdough allowed a reduction of 382 AUC with a presumable reduction of glycaemic index and a nutritional improvement of the gluten free 383 breads. In a previous study, the sourdough leavening technique in bread production was able to 384 significantly reduce glucose response in healthy subjects respect to the corresponding products 385 leavened with S. cerevisiae (Scazzina et al, 2009). In accordance with Wolter et al. (2014), the 386 reduction in AUC values was not related to an increase in resistant starch due to sourdough 387 388 fermentation: MCS presented the lowest percentage of resistant starch (1.29 ± 0.03) followed by MC 389 (1.54 ± 0.06) and by both MS and M $(1.86 \pm 0.05 \text{ and } 1.92 \pm 0.05, \text{ respectively})$. The decrease of AUC in sourdough fermented gluten-free breads may be related to a different mechanism than the presence 390 391 of organic acids and the formation of resistant starch (Fardet et al., 2006). Indigenous factors of the food matrix (starch susceptibility, protein and lipid contents) as well as the macroscopic structure of the 392 food (botanical integrity of ingredients, physical texture) and starch characteristics (native structure, 393 394 physical encapsulation, degree of gelatinisation and retrogradation of the starch granules, as well as by the proportion of damaged granules) might have affected the starch hydrolysis, too. This aspect needs 395 to be further investigated. 396

399 4. Conclusions

This work describes the effects of the combination of chestnut flour (40%) and sourdough (20%) 400 401 fermentation on chemical, technological and nutritional attributes of gluten free breads. Chestnut flour limited acidification of both dough and breads limiting the decrease in water holding capacity and the 402 increase in crumb firmness due to excessive acidification. Volume of all breads prepared with chestnut 403 flour and/or sourdough resulted lower compared to the control but the combination of chestnut flour 404 and sourdough contributed to reduce crumb grain heterogeneity. Sourdough and/or chestnut flour 405 addition caused a significant increase in crumb hardness probably due to the lower volume. During 406 storage, a significant reduction of the staling phenomenon measured as crumb hardness increase was 407 observed only after 5 days, even if amylopectin fusion enthalpy was lowered. From a nutritional point 408 409 of view, the percentage of hydrolysed starch during in vitro digestion was significantly reduced by sourdough fermentation with a presumable lower glycaemic index. 410

In conclusion, the sourdough fermentation could be useful to improve chestnut flour gluten free bread characteristics even if further activities are required for obtaining an actual reduction in the staling process and an acceptable volume development. It has been also remarkable that the gluten free mixture used in this study is commercially available and sourdough was propagated under technological conditions similar to those used for the production, allowing the research findings being adapted to industrial gluten free bread production.

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510 **Captions for figures**

Figure 1: Number of pores as percentage of the total number of pores for the selected dimensional classes at time 0 day. Error bars represent +/- 1 standard deviation, (n = 9, sample size = 3 for each bread type). Bars of histograms with the same capital letters are not significantly different (p < 0.05).

Figure 2: Moisture content at crust (white symbol), near crust (grey symbol) and crumb (black symbol) for M (panel A), MC (panel B), MS (panel C) and MCS (panel D) breads during storage. Error bars represent +/- 1 standard deviation, (n = 9, sample size = 3 for each bread type). Different capital letters indicate significant differences (p < 0.05) among different times for the same bread while different lowercase letters indicate significant differences (p < 0.05) among the four types of bread at the same storage time.

Figure 3: Crust hardness at different time of storage for M1 and M1C (panel A) and M2 and M2C (panel B) breads. Error bars represent +/- 1 standard deviation, (n = 10, sample size = 3 for each bread type). Different capital letters indicate significant differences (p < 0.05) among different times for the same bread while different lowercase letters indicate significant differences (p < 0.05) among the four types of bread at the same storage time.

Table 1. Chemical parameters (g/100g) and specific bulk volume (mg/L) of analysed breads at time 0 day.^a

	рН	Acetic acid	Lactic acid	Carbohydrate	Starch	Fat	Protein	Volume (mg/L)
Μ	5.17±0.03 a	0.011±0.001 b	0.018±0.002 d	47.1±0.4 a	44.0±0.8a	2.40±0.14b	1.22±0.02b	2.4±0.24 a
MC	5.21±0.07 a	0.047±0.003 a	0.026±0.004 c	46.2±0.5 a	39.7±0.5b	2.86±0.04a	1.96±0.03a	1.82±0.18 b
MS	3.84±0.04 c	0.013±0.001 b	0.100±0.007 b	44.9±0.3 a	40.5±0.4b	2.10±0.06c	0.93±0.03c	1.62±0.16 b
MCS	4.58±0.02 b	0.044±0.001 a	0.236±0.001 a	44.8±0.4 a	37.8±0.5c	2.71±0.05ab	1.69±0.01b	1.74±0.16 b

526 a n=3, sample size =9 for each type of bread. Means in columns followed by different letter differed significantly (p < 0.05).

	Hardness (N)	Cohesiveness	Resilience	Chewiness (N)	$\Delta H \left(J/g_{solid} \right)$	Hardness (N)	Cohesiveness	Resilience	Chewiness (N)	$\Delta H (J/g_{solid})$	
		М			МС						
t0	2.55 (0. 98)	0.82 (0.05)	0.49 (0.06)	2.33 (0.81) bC	-	4.39.(0.88)	0.79 (0.05)	0.52 (0.03)	3.48 (0.69)	-	
	bD	aA	bA			aB	bA	abA	aB		
t1	4.68 (0.54)	0.73 (0.05)	0.45 (0.05)	3 11 (0 54)	0.983 (0.059)	10.13 (1.41)	0.55 (0.07)	0.32 (0.05)	4.96 (0.21)	1.172 (0.001)	
	cC	aA	aA	cB	bA	aA	cB	cB	bA	aA	
t3	7.66 (2.1)	0.57 (0.08)	0.30 (0.05)	3.93 (0.72) cAB	1.368 (0.044)	11.68 (2.01)	0.42 (0.05)	0.22 (0.05)	4.33 (1.21)	2.286 (0.098)	
	bB	aB	aB		bB	aA	cC	bC	bA	aB	
	14.80 (3.3)	0.43 (0.06)	0.20 (0.02)	4.80 (0.81) bA	2.371 (0.197)	11.65 (2.46)	0.43 (0.03)	0.23 (0.03)	4.78 (1.18)	3.475 (0.153)	
15	aA	abC	bcC		bC	bA	abC	bC	bA	aC	
		MS				MSC					
	4.31 (1.08)	0.83 (0.04)	0.55 (0.04)	3.41 (0.94)	-	4.47 (0.73)	0.78 (0.04)	0.51 (0.03)	3.36 (0.55)	-	
to	aC	aA	aA	aB		aC	bA	abA	aB		
	9.07 (1.50)	0.66 (0.05)	0.38 (0.03)	5.52 (0.76)	0.385±0.066	8.22 (1.22)	0.67 (0.04)	0.42 (0.04)	5.11 (0.48)	0.699 (0.011)	
tl	bB	bB	abB	aA	dA	bB	bB	bB	aA	cA	
t3	13.13 (1.91)	0.50 (0.06)	0.27 (0.03)	6.02 (0.96)	1.058±0.033	11.92 (2.12)	0.43 (0.04)	0.22 (0.02)	4.83 (0.78)	0.881 (0.121)	
	aA	bC	abC	aA	cB	aA	cC	bC	bA	cB	
t5	14.56 (1.93)	0.48 (0.06)	0.25 (0.02)	6.66 (1.20)	1.310±0.012	12.24 (1.23)	0.36 (0.02)	0.18 (0.01)	3.90 (0.90)	1.141 (0.015)	
	aA	aC	aC	aA	cC	bA	bD	cD	cAB	cC	

Table 2. Crumb textural profile analysis (TPA) parameters and amylopectin enthalpy of fusion for analysed breads.^a

 $\frac{a = 10 \text{ for texure parameters and } n=3 \text{ for enthalpy, sample size } =3 \text{ for each bread type at each storage time. Means in column followed by different capital letters significantly differ (p < 0.05) among different times for the same bread-Means followed by different lowercase letters significantly differ (p < 0.05) among the four types of bread at the same storage time.$

		crumb				
	L^*	<i>a*</i>	<i>b*</i>	L*	<i>a</i> *	b *
Μ				•		
0	86.6±0.7 aCB	-0.36±0.11 cA	12.7±0.8 bA	87.9±1.2 aB	0.83±0.42 bA	12.1±0.6 bA
1	87.9±1.6 aB	-0.57±0.11 cB	12.0±1.2 bA	90.4±0.4 aA	0.42±0.22 bAB	10.2±0.3 cB
3	89.7±0.7 aA	-0.47±009 dAB	12.0±0.5 bA	89.7±0.9 aA	0.57±0.17 bAB	10.4±0.2 cB
5	85.8±3.1 aC	-0.44±0.19 cAB	12.4±0.5 bA	90.0±1.3 aA	0.27±0.22 bC	10.3±0.5 cB
MC						
0	56.9±1.4 bA	7.64±0.54 aA	16.9±0.9 aA	74.3±2.3 cA	4.35±0.20 aA	18.7±1.0 aA
1	60.7±1.2 bA	7.22±0.32 aA	16.4±1.0 aB	73.7±1.4 cA	4.41±0.28 aA	18.8±0.5 aA
3	60.6±1.5 cA	7.13±026 aA	16.2±0.7 aB	74.4±1.0 cA	4.72±0.39 aA	19.0±1.0 aA
5	57.7±2.5 cA	6.81±0.87 aB	17.0±3.0 aA	75.5±1.7 cA	4.24±0.40 aA	16.2±1.1 aB
MS						
0	83.9±0.9 aB	-0.46±0.10 cC	9.2±0.5 cB	83.4±0.9 bB	0.16±0.08 bB	10.8±1.1 cB
1	85.0±1.2 aAB	-0.19±0.10 cB	9.2±0.5 cB	80.9±1.0 bC	0.25±0.04 bAB	12.7±1.2 cA
3	86.1±1.6 aA	-0.11±0.08 cA	9.4±0.4 cB	82.4±3.6 bBC	0.39±0.05 bA	10.1±1.5 cB
5	85.2±1.6 aAB	-0.24±0.06 cB	10.0±0.4 cA	86.0±1.9 bA	0.14±0.14 bB	8.7±1.46 dC
MCS						
0	60.6±1.2 bA	5.78±0.52 bA	15.5±1.0 aA	74.0±2.36 cB	4.39±0.31 aB	13.6±0.7 bB
1	61.7±1.3 bA	5.79±0.26 bA	14.8±0.6 aA	77.4±0.5 cA	4.91±0.30 aA	14.6±0.7 bA
3	61.1±1.6 bA	6.14±0.31 bA	15.3±0.9 aA	77.8±5.2 cA	4.61±1.23 aA	13.2±1.0 bBC
5	61.8±2.6 bA	6.07±0.36 bA	15.6±1.4 aA	73.2±1.4 cB	4.60±0.51 aB	12.4±0.8 bC

Table 3. Crumb and crust colorimetric parameters for analysed breads.^a

a n=10, sample size =3 for each bread type at each storage time. Means in column followed by different capital letters significantly differ (p < 0.05) among different times for the same bread- Means followed by different lowercase letters significantly differ (p < 0.05) among the four types of bread at the same storage time whether for

538 crust or crumb.



Fig. 1



Fig. 2

