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

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

Massimiliano Rinaldi[a*], Maria Paciulli[a], Augusta Caligiani[a], Francesca Scazzina[a], Emma Chiavaro[a*]

[a] Dipartimento di Scienze degli Alimenti, Università degli Studi di Parma, Parco Area delle Scienze 47/A, 43124 Parma, Italy

Corresponding authors:

* phone: +39 (0521) 905846 fax: +39 (0521) 906028 e-mail massimiliano.rinaldi@unipr.it

* phone: +39 (0521) 905888 fax: +39 (0521) 906028 e-mail emma.chiavaro@unipr.it

Abstract

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

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

1. Introduction

Celiac disease (CD), an immune-mediated enteropathy caused by the ingestion of gluten in genetically susceptible individuals, is one of the most common lifelong disorders; people suffering of this disease 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 consumers who avoid gluten for lifestyle reasons, follow a gluten-free diet (Masure et al., 2016). For this reason, the market of gluten-free foods in the last decades has grown together with the products on the shelves (Global Gluten-Free Products Market Research Report, 2016). Notwithstanding, the improvement of gluten-free products appears as a big challenge for the food technology and a relevant issue in the scientific literature, as demonstrated by the great number of published studies in the last years (Capriles and Arêas, 2014; Houben et al., 2012; Masure et al., 2016; Matos and Rosell, 2015).

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 shelf-life, probably as a consequence of the lack of the viscoelastic network formed by gluten (Houben et al., 2012). Thus, improvement of both technological and nutritional quality of gluten-free breads is 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, 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 received more and more attention due to its nutritional and health benefits for gluten-free bread 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 al., 2004). It was recently reported that the addition of chestnut flour in bread-making increases antioxidant capacity and fiber content of wheat bread (Dall'Asta et al., 2013) as well as reduces

42 moisture losses in both crust and crumb and could slow the staling process (Rinaldi et al., 2015).
43 Regarding gluten-free breads, Demirkesen et al. (2010) studied the effects of different levels of its
44 addition on a simple rice-based gluten-free formulation reporting that elevated amounts of chestnut
45 flour led to some deterioration in quality parameters. This fact limits the actual nutritional improvement
46 of gluten-free breads. More recently, Paciulli et al. (2016) studied the effects of chestnut flour addition
47 to two commercial gluten-free mixtures for producing technologically and nutritionally improved
48 breads. Similarly, the authors found that the addition of chestnut flour influenced the characteristics of
49 breads just after baking and during storage but allowed improving total antioxidant capacity and fiber
50 content.
51 The use of sourdough (a dough containing a *Lactobacillus* culture in symbiotic combination with
52 yeasts) was reported to be a potential strategy for improving quality of both soft and hard wheat breads
53 (Rinaldi et al., 2015). Its use to overcome defects in gluten-free breads with reduced need for expensive
54 additives and higher acceptance from consumers was also reported (Moroni et al., 2009).
55 Exopolysaccharides (EPS) formed from sucrose during sourdough fermentation can improve the
56 technological properties of gluten-free breads and potentially replace hydrocolloids (Galle et al., 2012).
57 Aguilar et al. (2016) studied spontaneously fermented chestnut flour sourdough and evaluated its effect
58 in gluten-free breads based on corn starch and chestnut flour during 7 days of storage: chestnut flour
59 sourdough improved bread specific volume, rendered breads with lighter crusts, reduced crumb
60 hardness at day 0 and day 7 and reduced pH. However, chestnut flour sourdough had no effect on
61 yeasts and molds growth during 7 days of bread storage and did not influence sensory characteristics
62 perceived by consumers.
63 By a nutritional point of view, the potential of sourdough application to reduce the predicted glycemic
64 index on gluten-free breads was also investigated by Wolter et al. (2014). In addition, the degradation
65 of cereal proteins during sourdough fermentation markedly affects the overall quality of baked goods.

66 The acidification and the reduction of disulfide bonds of gluten by hetero-fermentative lactobacilli
67 promote the primary activity of cereal proteases, which lead to the liberation of various sized
68 polypeptides, many of them considered as bioactive or biogenic peptides (Gobbetti et al., 2014).
69 Sourdough fermentation is also considered to be one of the most suitable biotechnology for the
70 manufacture of baked goods rich sources in dietary fibre; moreover, it is reported to stimulate the
71 activity of grain endogenous phytase that could decrease the phytate content of whole flours (Leenhardt
72 et al., 2005).
73 In this contest, the application of the sourdough technology represents the new frontier for the
74 production of high quality gluten-free bread. This strategy could also be applied to satisfy the consumer
75 demands for clean labels, natural products and for a reduced use of additives. Anyway, only few
76 attempts were reported for producing and characterizing gluten-free sourdoughs and the functional
77 properties of the breads thereof (Moroni et al., 2009). Thus, the aim of the present work was to evaluate
78 the effects of sourdough addition, also in combination with chestnut flour, on chemico-physical,
79 thermal and nutritional properties of a commercial gluten-free bread formulation during 5 days of shelf-
80 life.
81

2. Materials and Methods

2.1. Materials and chemicals

Gluten-free bread mixture, chestnut flour, salt, compressed yeast were purchased from the local market. Deuterium Oxide (D_2O), chloroform-d $CDCl_3$, pepsin from porcine gastric mucosa, pancreatin from porcine pancreas and Amyloglucosidase from *Aspergillus niger* were obtained from Sigma Aldrich (St. Louis, Missouri, USA). The rest of other chemicals are of analytical grade unless and otherwise specified.

2.2. Samples, breadmaking and storage

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

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 g/100g), 6.3g/100g, 9.4 g/100g and 3.6 g/100g, respectively.

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

- 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 (7.1) compressed yeast (2.9);
- MS (mixture+sourdough): mixture (100.0) water (95.8) sunflower oil (5.2) sourdough (20.8);
- MCS (mixture+chestnut flour+sourdough): mixture (100.0) chestnut flour (45.4) water (136.4) sunflower oil (7.6) sourdough (30.3).

106 These chestnut commercial gluten-free flour ratios were selected based on previous results (Paciulli et
107 al., 2016) and preliminary experimentations.
108 Before the breadmaking process, gluten-free sourdough was refreshed at least three times by mixing
109 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
110 28°C and then the refreshed sourdough was added to the other ingredients.
111 A domestic bread maker machine (Moulinex, Groupe Seb Italia S.p.A., Milano, Italy) was used for
112 breadmaking, with the rapid program for samples M and MC: stirring + kneading + rising, 80 min;
113 baking, 55min at 210 °C. On the contrary, samples MS and MCS presented a proofing step of 3 hours
114 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*
124 The protein content (g/kg) was determined by the Kjeldhal method using 1g of ground sample, as
125 previously reported (Dall'Asta et al., 2013). A correction factor of 5.7, typical of flour mixtures, was
126 used for the calculation. Fat content (g/kg) was determined utilizing a Soxhlet extractor (Velp
127 Scientifica, Monza-Brianza, Italy) on 5 g of ground sample, and diethyl ether as solvent. Analyses were
128 carried out at day 0 of storage.

129 pH was measured on each bread sample at time 0 according to Plessas et al. (2005) by placing 15 g of
130 breadcrumb and 100 ml of distilled water in a clean dry container, which was sealed and stirred until
131 the bread dispersed into a semi-liquid mixture, and measured using a pH meter (Jenway 3510, Bibby
132 Scientific, Staffordshire, UK).
133 For organic acids analysis, 200 mg of sourdough at the beginning of the fermentation as well as cooked
134 breads were dissolved in 1ml of D_2O and blended with magnetic stirring for 1h at room temperature.
135 To ensure a complete removal of the apolar component, 100 µl of $CDCl_3$ were added. After
136 centrifugation at 10000 rpm, 600 µl of supernatant were taken for the analysis according to Caligiani et
137 al. (2007). Triplicate analyses were carried out on each sample.
138
139 *2.4. Specific bulk volume, crumb grain characteristic and moisture content*
140 Specific bulk volume of breads was determined according to the AACC Approved Method 10-05.01
141 (AACC, 2000) and expressed as the weight/volume ratio of cooked bread (mg/L).
142 Crumb grain was evaluated by means of a digital image analysis system, as reported previously
143 (Dall'Asta et al., 2013). Images of three central slices (20 mm thickness) of each loaf were acquired
144 with a scanner (Hewlett Packard, Palo Alto, CA, USA) at 600 dots per inch (dpi) taking squares (40x40
145 mm) from the centre of the images after calibration, standardization and optimization by means of
146 Image-Pro Plus 4.5 (Media Cybernetics Inc., USA) software. The number of pores (expressed as
147 percentage of the total number) was obtained according to pre-selected dimensional classes based on
148 their area. Selected classes were: class-1: 0.005-0.099 mm^2; class-2: 0.01-0.99 mm^2; class.3: 1-10 mm^2;
149 class-4: > 10 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.

153

154 *2.5. Physical and thermal analysis*

155 Texture analysis was performed on crust and crumb using a TA.XT2 Texture Analyzer equipped with a
156 25 kg load cell (Stable Micro Systems, Godalming, UK) and Texture Expert for Windows software
157 (version1.22) for data analysis on each loaf. Crust hardness was measured by means of a puncture test
158 using a 3 mm diameter stainless steel probe and a test speed of 2 mm/s. Maximum peak force (N) was
159 measured from the penetration curve and taken as crust hardness. Measurements were taken on five
160 preselected points of the crust.

161 Crumb evaluation was carried out on ten cube of 20×20×20 mm extracted from two central slices of the
162 samples. A TPA test was performed with a 35 mm diameter cylindrical aluminium probe by means of a
163 double compression with a speed of 1mm/s up to the 50% of the original sample height. The textural
164 parameters considered were hardness (maximum peak force of the first compression cycle, N),
165 cohesiveness (ratio of positive force area during the second compression to that during the first
166 compression area, dimensionless), resilience (area during the withdrawal of the penetration, divided by
167 the area of the first penetration, dimensionless), and chewiness (product of hardness x cohesiveness x
168 springiness, N) (Bourne, 1978). In addition, crumb hardness increase was calculated as the percent
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),
171 hermetically sealed and analyzed with a DSC Q100 (TA Instruments, New Castle, DE). Indium
172 (melting temperature 156.6 °C, ΔH_f 28.45 J/g) and *n*-dodecane (melting temperature –9.65 °C, ΔH_f
173 216.73 J/g) were used to calibrate the instrument, and an empty pan was used as reference. Samples
174 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
175 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 cm^3/min. Thermograms were analyzed with Universal Analysis Software, Version 3.9A (TA

177 Instruments, New Castle, DE), and enthalpy (ΔH, 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.
180 Colour was determined on ten pre-selected locations of the crust and crumb of each bread loaf. The
181 analyses were performed using a Minolta Colorimeter (CM 2600d, Minolta Co., Osaka, Japan)
182 equipped with a standard illuminant D65 and a 10° position of the standard observer. The instrument
183 was calibrated before each analysis with white and black standard tiles. *L** (lightness), *a** (redness) and
184 *b** (yellowness) were quantified on each sample using the Spectramagic software (Ver. 3.6).
185
186 *2.6. Starch hydrolysis*
187 Breads were tested *in vitro* to determine the rate of starch hydrolysis. *In vitro* digestions were
188 performed as previously described by Paciulli et al. (2016) for gluten free breads: about 8 g of sample
189 was suspended in phosphate buffer (20 mmol/L) and incubated at 37 °C stepwise with human saliva,
190 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
191 transferred into 20 cm dialysis tubing strips (12 kDa molecular weight cutoff) with 100 mg of
192 pancreatin from porcine pancreas (3xUSP), sealed with plastic clamps, and incubated for 5 h at pH 6.9
193 into 1000 mL sealed containers containing 500 mL of phosphate buffer. Two aliquots (0.5 ml) from the
194 dialyzed solution were removed for analysis at time 0, every 15 min during the first hour and every 30
195 min until 5 hour digestion. The aliquots were used to determine the number of glucose monomers of
196 the permeated fragments. To this purpose, each aliquot sample was hydrolyzed using 20 µl of 0.5%
197 amyloglucosidase solution (200 U) at pH 5.6 and the glucose concentration was determined with a
198 glucose analyzer (2900 Biochemisty Analyzer, YSI Inc., Yellow Springs, USA). All analyses were
199 performed in triplicate.

200

201 *2.7. Statistical analysis*

202 Means and standard deviations were calculated with SPSS (Version 23.0, SPSS Inc., Chicago, USA)
203 statistical software. SPSS was used to perform one way (ANOVA) to evaluate the effect of sourdough
204 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.

206 **3. Results and Discussion**

207 *3.1. Chemical analyses of dough and bread*

208 A dough pH value of 4.3 ± 0.2 was measured. Lactic and acetic acid contents were 4.14 ± 0.15 and
209 1.67 ± 0.05 g/kg respectively, in the range reported for sourdough breads (Corsetti, 2012), with a
210 fermentation quotient (molar ratio between lactic and acetic acids) of 1.65, slightly lower than the
211 optimum range 2.0 - 2.7 (Hammes and Gänzle, 1998).

212 Lactic acid content in MCS samples (Table 1) resulted the highest followed by MS and by M and MC,
213 as expected. The high content of sugar in chestnut flour may have probably favoured the growth of
214 sourdough microflora and caused a higher production of organic acids. Acetic acid content was almost
215 four folds higher in samples with sourdough MS and MCS (Table 1), if compared to breads with
216 compressed yeast (M and MC). The content of organic acids influenced final pH values of breads, as
217 expected; samples with sourdough (MCS and MS) presented significantly lower values than samples
218 with compressed yeast (M and MC). The higher pH value of MCS samples compared to MS was in
219 contrast with the higher content of organic acids found in the latter sample. It could be hypothesized
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.

224 The percentages of the main chemical components are also reported in Table 1. Breads without
225 chestnut flour (M and MS) presented significantly lower content of protein and fat due to the
226 composition of the ingredients: gluten free mixture contained 1.8 and 0.9 g/100g of protein and fat vs.
227 6.3 and 3.6 g/100g of chestnut flour. No significant differences were observed in total carbohydrate
228 content among samples. Otherwise, the addition of chestnut flour reduced the starch content as it
229 contains more simple sugars (Table 1) in comparison to the mixture.

230

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

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

241 Crumb grain characteristic of all breads at time 0 is reported in Figure 1. The addition of chestnut flour
242 (MC) caused a coarser but more homogenous structure (higher pores of class 3) in comparison with M
243 breads that showed a significantly higher number of little holes but also of cells of large sizes (class 2
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.
248 Similarly, Demirkesen et al. (2016) reported a heterogeneous crumb structure with high amounts of
249 open pores for rice-based gluten free breads. The addition of sourdough (MS) led to a significant
250 reduction of the pores belonging to the highest class (class 4) in comparison to M ones and thus the
251 studied level of sourdough addition contributed to reduce the heterogeneity of M crumb, in accordance
252 with Demirkesen et al. (2016). Finally, breads with the simultaneous addition of chestnut flour and
253 sourdough (MCS) presented the coarsest structure with the highest percentages of class 3 and 4 and

254 lowest of class 1 and 2 among all the breads, as consequence, probably for the interconnection between
255 all gas cells (open pores). Demirkesen et al. (2016) reported an increase in average size of pore due to a
256 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
258 showed an increase in pores belonging to class 3, and a simultaneous decrease of pores belonging to
259 class 2. No variations were observed for the remaining classes, in agreement with Rinaldi et al. (2015).
260 Probably, this variation was due to the moisture migration from the crumb to the crust that caused the
261 drying of the grain walls of the crumb with a reduction in their thickness and an increase in pore area,
262 as consequence (Gray and Bemiller, 2003). In addition, larger pores in MCS samples at time 0 day
263 might have caused the loss of more moisture or faster water redistribution, leading to an increase in
264 crumb coarseness during shelf life (Demirkesen et al., 2016). MCS crumb showed the highest loss in
265 the first day of shelf life (-5.2%) (Figure 2), if compared to all the other samples that presented similar
266 value (about 2.1 %).
267
268 *3.3. Moisture content*
269 Moisture content trends of all samples for crust, under-crust layer and crumb during 5 days of storage
270 are reported in Figure 2.
271 All samples presented an increase of crust moisture during storage, as expected, due to the migration of
272 water from crumb to crust (Gray and Bemiller, 2003). Among samples, MCS (Figure 2, panel D)
273 showed significant highest values of crust moisture content, while M (Figure 2, panel A) exhibited the
274 lowest content. The presence of chestnut flour fibres and products of sourdough, probably EPS, may
275 have increased the ability to retain water in MCS. In addition, the coarser structure of this bread type
276 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

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

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

302 hardness since day 1 (58, 107 and 126 % for MC, MCS and MS, respectively vs. 22% for M).
303 Conversely, M bread presented a significantly lower increase during shelf life with the highest value
304 only at the end of shelf-life (177 % at day 5). The gelling and thickening agents of the gluten free
305 mixture may have retained water during storage leading to a softer crust. Moreover, the observed trend
306 could be also due to the crumb grain characteristic for MC and MCS breads that presented a coarser
307 crumb in comparison to the M ones (Figure 1), in relation to the increase of the moisture migration
308 during storage (Figure 2, panel A) and to the acidification that reduced the water holding capacity for
309 MS samples. However, at time 5 days, M samples presented a dramatic increase in crust hardness with
310 a value almost two-fold compared to other samples.
311 Crumb textural data are reported in Table 2. Sourdough and/or chestnut flour addition caused a
312 significant increase in crumb hardness probably due to the lower development of the final bread (loaf
313 collapse) and the lower softness of the crumb, as already stated in paragraph 3.2 in accordance with
314 Mariotti at al. (2013). No significant differences were observed in crumb hardness among chestnut or
315 sourdough added samples (MC, MS, MCS) at time 0 as also observed for specific volume data (Table
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
318 sourdough was in contrast with Demirkesen et al. (2016) who reported a reduction of firmness up to
319 20% of sourdough addition. Galle et al. (2012) studied the influence of EPS on dough rheology and
320 quality of gluten-free sorghum bread and reported that EPS formed during 10% sourdough
321 fermentation led to a softer crumb in the fresh and stored sorghum bread. During shelf life, breads
322 added with chestnut flour (MC) and sourdough (MS) presented a higher staling rate, expressed as
323 percentage increase of hardness, at time 1 day in comparison to M and MCS. In particular, 113.9 and
324 130.7 % crumb hardness increasing were observed for MS and MC, while 82.3 and 88.7 % were
325 obtained for M and MCS, respectively. At time 3 days a higher staling rate (expressed as percent

326 hardness increase) was observed for MS, while no significant differences were measured among the
327 remaining samples. Finally, at time 5 days M samples gave the highest hardness value and staling rate
328 followed by MS and, finally, by MC and MCS (Table 2). Cohesiveness was an indicator of the internal
329 cohesion of the material: generally, breads with low cohesiveness are susceptible to fracture and
330 crumble (Onyango, Mutungi, Unbehend & Lindhuaer, 2010) and are not desirable. M samples showed
331 the highest cohesiveness values both at time 0 and during shelf life, probably thank to the higher
332 concentration of thickening and gelling agents from the gluten free mixture. Sourdough and/or chestnut
333 flour addition may have caused a dilution of gluten free mixture and consequently a reduction of the
334 additives able to retain water and maintain the freshness of bread. Similar results were obtained by
335 adding chestnut flour to a commercial gluten free mixture (Paciulli et al., 2016). Resilience values
336 (Table 2) showed a trend similar to that of cohesiveness, as a reduction in resilience was reported to
337 cause loss of elasticity and tendency to crumble (Onyango et al., 2010). Sourdough and chestnut added
338 samples (MS, MC and MCS) exhibited significantly lower values of resilience ($p<0.05$) during shelf-
339 life. Finally, chewiness values of M bread, an indication of the energy required to masticate a solid
340 food prior to swallow, were significantly lower in comparison to the other samples (Table 2) at all
341 times of analysis. MS samples gave the highest values meaning hard break of these breads in the mouth
342 probably due to the effect of acidity on thickening or gelling agents of the mixture.
343 Amylopectin retrogradation enthalpies (ΔH), monitored and quantified by DSC, are reported in Table
344 2. All the samples showed a significant increase of ΔH during storage, as expected, and as already
345 observed by Demirkesen et al. (2014) for gluten free breads. Sourdough seemed to show a significant
346 effect in delaying retrogradation measured by means of DSC in accordance with Corsetti et al. (2000),
347 even if crumb hardness of MSC breads was lower than M only at day 5. Similar results were reported
348 by Moroni et al. (2011) studying different levels of sourdough addition in gluten free breads prepared
349 with buckwheat flour; also in this case, sourdough addition caused a reduction in volume and an

350 increase in crumb hardness and positive effects in delaying macroscopic staling phenomenon were
351 observed only after 5 days of storage.
352 Colorimetric parameter measured on both crumb and crust of tested breads are reported in Table 3.
353 Crust of MC and MCS samples presented lower *L** and higher *a** values compared to the other samples
354 at time 0, due to the darkening effect of chestnut flour previously observed (Paciulli et al., 2016). The
355 lower crust lightness (*L**) of sourdough breads (MS and MCS) was probably related to the first phases
356 of Maillard reactions, which was reported to be more consistent under sourdough bread-making with a
357 greater concentration of all compounds involved in non-enzymatic browning (Torrieri et al. 2014).
358 These differences remained unaltered during storage (Table 3). Crust of MC also presented the highest
359 *b** values due to a higher percentage of chestnut flour in the recipe than MCS sample.
360 Crumb colorimetric data (Table 3) are aligned with those of the crust. Chestnut flour addition deeply
361 influenced colour with a darkening effect by lowering *L** and increasing both *a** and b* values, as
362 already reported (Paciulli et al., 2016). In general, sourdough fermentation did not produce significant
363 differences in bread colorimetric parameters with the exception of *b** value in MS samples that
364 significantly decreased in comparison with M. The higher fermentation time of MS (180 vs. 40 min)
365 probably favoured a higher lipoxygenase activity leading to a partial oxidation of the carotenoid
366 pigments (Leenhardt et al., 2006). During storage, *L** and *a** values tended to decrease in M, probably
367 due to the water loss from the cell walls, which could increase opacity making crumb darker from the
368 instrumental measurement. *a** values also significantly decreased due to the sourdough addition (MS)
369 during storage. Crumb colour remained substantially unaltered in MC and MCS samples during shelf-
370 life in accordance with Paciulli et al. (2016) reporting that added chestnut flour better preserved the
371 gluten free bread discoloration during shelf life.
372 *3.5. Starch hydrolysis*
373 To better characterize the nutritional properties of the breads prepared within this study, the starch

374 digestibility was assessed over 5 hours by enzymatical hydrolysis: the percentage of hydrolysed starch
375 permeated through the dialysis tube as well as the total areas under the curves (AUC) during a 5 h in
376 vitro digestion are considered. After 5h hydrolysis, the digested starch fractions of the total starch were
377 60.9 ± 1.2 % for M, 61.7 ± 2.3 % for MC, 56.5 ± 1.7 % for MS, 54.3 ± 1.0 % for MCS. A significant
378 reduction of the digested starch was achieved by means of sourdough fermentation as previously
379 observed by De Angelis et al. (2009) and due the presence of lactic and acetic acids that limited the
380 starch bioavailability.
381 The area under curve (AUC) values were 10885 ± 289, 11206 ± 349, 9573 ± 124 and 9691 ± 333 (mg
382 min/dL), for M, MC, MS and MCS, respectively. The addition of sourdough allowed a reduction of
383 AUC with a presumable reduction of glycaemic index and a nutritional improvement of the gluten free
384 breads. In a previous study, the sourdough leavening technique in bread production was able to
385 significantly reduce glucose response in healthy subjects respect to the corresponding products
386 leavened with *S. cerevisiae* (Scazzina et al, 2009). In accordance with Wolter et al. (2014), the
387 reduction in AUC values was not related to an increase in resistant starch due to sourdough
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 ± 0.05 and 1.92 ± 0.05, respectively). The decrease of AUC
390 in sourdough fermented gluten-free breads may be related to a different mechanism than the presence
391 of organic acids and the formation of resistant starch (Fardet et al., 2006). Indigenous factors of the
392 food matrix (starch susceptibility, protein and lipid contents) as well as the macroscopic structure of the
393 food (botanical integrity of ingredients, physical texture) and starch characteristics (native structure,
394 physical encapsulation, degree of gelatinisation and retrogradation of the starch granules, as well as by
395 the proportion of damaged granules) might have affected the starch hydrolysis, too. This aspect needs
396 to be further investigated.
397

4. Conclusions

This work describes the effects of the combination of chestnut flour (40%) and sourdough (20%) 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 increase in crumb firmness due to excessive acidification. Volume of all breads prepared with chestnut flour and/or sourdough resulted lower compared to the control but the combination of chestnut flour and sourdough contributed to reduce crumb grain heterogeneity. Sourdough and/or chestnut flour addition caused a significant increase in crumb hardness probably due to the lower volume. During storage, a significant reduction of the staling phenomenon measured as crumb hardness increase was observed only after 5 days, even if amylopectin fusion enthalpy was lowered. From a nutritional point of view, the percentage of hydrolysed starch during in vitro digestion was significantly reduced by sourdough fermentation with a presumable lower glycaemic index.

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.

418 **References**

419 AACC, American Association of Cereal Chemists. (2000). Approved Methods of the AACC (10th ed.)
420 St. Paul, USA: Am Assoc Cereal Chem.

421 Aponte, M., Boscaino, F., Sorrentino, A., Coppola, R., Masi, P., & Romano, A. (2013). Volatile
422 compounds and bacterial community dynamics of chestnut-flour-based sourdoughs. *Food Chemistry*,
423 141, 2394-2404.

424 Bourne, M.C. (1978) Texture profile analysis. *Food Technology*, 32, 62-66.

425 Caligiani, A slowing of the aging process, Acquotti, D., Palla, G., & Bocchi, V. (2007). Identification and
426 quantification of the main organic components of vinegars by high resolution 1H NMR spectroscopy.
427 *Analita Chimica Acta* 585, 110-119.

428 Capriles, V.D., & Arêas, J.A.. (2014). Novel approaches in gluten-free breadmaking: Interface between
429 food science, nutrition, and health. *Comprehensive Reviews in Food Science and Food Safety*, 13, 871-
430 890.

431 Corsetti, A. (2012). Technology of sourdough fermentation and sourdough applications. In Gobbetti,
432 M., & Gänzle, M. (Eds.). *Handbook on sourdough biotechnology* (pp 85-100). New York: Springer
433 Science & Business Media,

434 Corsetti, A., Gobbetti, M., De Marco, B., Balestrieri, F., Paoletti, F., Russi, L., & Rossi, J. (2000).
435 Combined effect of sourdough lactic acid bacteria and additives on bread firmness and staling. *Journal*
436 *of Agricultural and Food Chemistry*, 48, 3044-3051.

437 Dall'Asta, C., Cirlini, M., Morini, E., Rinaldi, M., Ganino, T., & Chiavaro, E. (2013). Effect of
438 chestnut flour supplementation on physico-chemical properties and volatiles in bread making. *LWT-*
439 *Food Science and Technology,* 53, 233–239.

De Angelis, M., Damiano, N., Rizzello, C. G., Cassone, A., Di Cagno, R., & Gobbetti, M. (2009). Sourdough fermentation as a tool for the manufacture of low-glycemic index white wheat bread enriched in dietary fibre. *European Food Research and Technology*, 229, 593-601.

Demirkesen, I., Campanella, O. H., Sumnu, G., Sahin, S., & Hamaker, B. R. (2014). A study on staling characteristics of gluten-free breads prepared with chestnut and rice flours. Food and Bioprocess technology, 7, 806-820.

Demirkesen, I., Mert, B., Sumnu, G., & Sahin, S., (2010). Utilization of chestnut flour in gluten-free bread formulations. Journal of Food Engineering 101, 329–336.

Demirkesen, I., Puchulu-Campanella, E., Kelkar, S., Campanella, O. H., Sumnu, G., & Sahin, S. (2016). Production and characterisation of gluten-free chestnut sourdough breads. *Quality Assurance and Safety of Crops & Foods*, 1-10.

Fardet, A., Leenhardt, F., Lioger, D., Scalbert, A., & Rémésy, C. (2006). Parameters controlling the glycemic response to breads. *Nutrition research reviews*, 19, 18-25.

Galle, S., Schwab, C., Dal Bello, F., Coffey, A., Gänzle, M. G., & Arendt, E. K. (2012). Influence of in-situ synthesized exopolysaccharides on the quality of gluten-free sorghum sourdough bread. *International Journal of Food Microbiology*, 155, 105-112.

Global Gluten-Free Products Market Research Report, 2016: http://www.gosreports.com/global-gluten-free-products-market-research-report-2016/# (accessed 10/07/2016).

Gobbetti, M., De Angelis, M., Di Cagno, R., & Rizzello, C.G. (2008). Sourdough lactic/acid bacteria IN E.K. Arendt, F. Dal Bello (Eds.), *Gluten-free Cereals Products and Beverages*, (pp. 267–288), London: Academic Press.

Gobbetti, M., Rizzello, C. G., Di Cagno, R., & De Angelis, M. (2014). How the sourdough may affect the functional features of leavened baked goods. *Food Microbiology*, 37, 30-40.

463 Hammes, W.P., & Gänzle, M.G. (1998) Sourdough breads and related products. In BJB Woods (Ed.),
464 *Microbiology of fermented foods*, 2nd ed. (pp 199–216), London: Blackie Academic/Professional.
465 Houben, A., Höchstötter, A., & Becker, T. (2012). Possibilities to increase the quality in gluten-free
466 bread production: an overview. *European Food Research and Technology*, 235, 195-208.
467 Leenhardt, F., Levrat-Verny, M. A., Chanliaud, E., & Rémésy, C. (2005). Moderate decrease of pH by
468 sourdough fermentation is sufficient to reduce phytate content of whole wheat flour through
469 endogenous phytase activity. *Journal of Agricultural and Food Chemistry*, 53, 98-102.
470 Leenhardt, F., Lyan, B., Rock, E., Boussard, A., Potus, J., Chanliaud, E., & Remesy, C. (2006). Wheat
471 lipoxygenase activity induces greater loss of carotenoids than vitamin E during breadmaking. *Journal*
472 *of Agricultural and Food Chemistry*, 54, 1710-1715.
473 Mariotti, M., Pagani, M.A., & Lucisano, M. (2013). The role of buckwheat and HPMC on the
474 breadmaking properties of some commercial gluten-free bread mixtures. *Food Hydrocolloids*, 30, 393-
475 400.
476 Masure, H.G., Fierens, E., & Delcour, J.A. (2016). Current and forward looking experimental
477 approaches in gluten-free bread making research. *Journal of Cereal Science*, 67, 92-111.
478 Matos, M.E., & Rosell, C.M. (2015). Understanding gluten-free dough for reaching breads with
479 physical quality and nutritional balance. *Journal of the Science of Food and Agriculture*, 95, 653-661.
480 Moroni, A.V., Dal Bello, F., & Arendt E.K. (2009). Sourdough in gluten-free bread-making: An 423
481 ancient technology to solve a novel issue? *Food Microbiology*, 6, 676-684.
482 Moroni, A.V., Dal Bello, F., Zannini, E., & Arendt, E.K. (2011). Impact of sourdough on buckwheat
483 flour, batter and bread: biochemical, rheological and textural insights. *Journal of Cereal Science*, 54,
484 195-202.

485 Onyango, C., Mutungi, C., Unbehend, G., & Lindhuaer, M.G. (2010). Batter rheology and bread
486 texture of sorghum-based gluten-free formulations modified with native or pregelatinized cassava
487 starch and α-amylase. *International Journal of Food Science and Technology*, *45,* 1228–1235.
488 Paciulli, M., Rinaldi, M., Cirlini, M., Scazzina, F., & Chiavaro, E. (2016) Chestnut flour addition in
489 commercial gluten-free bread: A shelf-life study. *LWT - Food Science and Technology*, 70, 88-95.
490 Plessas, S., Pherson, L., Bekatorou, A., Nigam, P., & Koutinas, A. A. (2005). Bread making using kefir
491 grains as baker's yeast. *Food Chemistry*, 93, 585-589.
492 Rinaldi, M., Paciulli, M., Caligiani, A., Sgarbi, E., Cirlini, M., Dall'Asta, C., & Chiavaro, E. (2015a).
493 Durum and soft wheat flours in sourdough and straight-dough bread-making. *Journal of Food Science*
494 *and Technology,* 1-12.
495 Rinaldi, M., Paciulli, M., Dall'Asta, C., Cirlini, M., & Chiavaro, E. (2015b). Short-term storage
496 evaluation of quality and antioxidant capacity in chestnut–wheat bread. *Journal of the Science of Food*
497 *and Agriculture*, 95, 59-65.
498 Sacchetti, G., Pinnavaia, G.G., Guidolin, E., & Dalla-Rosa, M. (2004). Effects of extrusion temperature
499 and feed composition on the functional, physical and sensory properties of chestnut and rice flour-
500 based snack-like products. *Food Research International*, 37, 527–534.
501 Scazzina, F., Del Rio, D., Pellegrini, N., & Brighenti, F. (2009). Sourdough bread: Starch digestibility
502 and postprandial glycemic response. *Journal of Cereal Science*, 49, 419-421.
503 Tamani, R. J., Goh, K. K. T., & Brennan, C. S. (2013). Physico-Chemical Properties of Sourdough
504 Bread Production Using Selected Lactobacilli Starter Cultures. *Journal of Food Quality*, 36, 245-252.
505 Torrieri, E., Pepe, O., Ventorino, V., Masi, P., & Cavella, S. (2014). Effect of sourdough at different
506 concentrations on quality and shelf-life of bread. *LWT-Food Science and Technology* 56, 508-516.

507 Wolter, A., Hager, A. S., Zannini, E., & Arendt, E. K. (2014). Influence of sourdough on in vitro starch
508 digestibility and predicted glycemic indices of gluten-free breads. *Food & Function*, 5, 564-572.
509

510 **Captions for figures**

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

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

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

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

	pH	*Acetic acid*	*Lactic acid*	*Carbohydrate*	*Starch*	*Fat*	*Protein*	*Volume (mg/L)*
M	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 ($p < 0.05$).
527

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

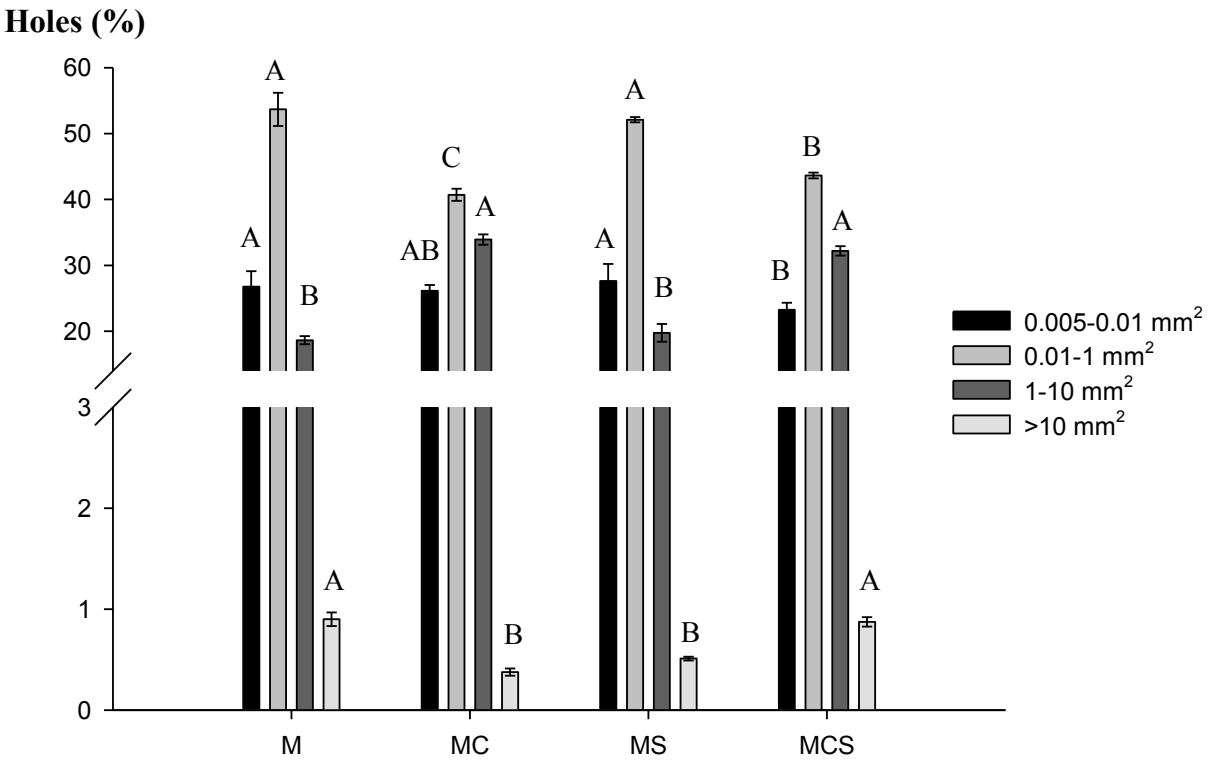
	Hardness (N)	*Cohesiveness*	*Resilience*	*Chewiness (N)*	*ΔH (J/g$_{solid}$)*	*Hardness (N)*	*Cohesiveness*	*Resilience*	*Chewiness (N)*	*ΔH (J/g$_{solid}$)*
		M					***MC***			
t0	2.55 (0. 98) bD	0.82 (0.05) aA	0.49 (0.06) bA	2.33 (0.81) bC	-	4.39.(0.88) aB	0.79 (0.05) bA	0.52 (0.03) abA	3.48 (0.69) aB	-
t1	4.68 (0.54) cC	0.73 (0.05) aA	0.45 (0.05) aA	3.11 (0.54) cB	0.983 (0.059) bA	10.13 (1.41) aA	0.55 (0.07) cB	0.32 (0.05) cB	4.96 (0.21) bA	1.172 (0.001) aA
t3	7.66 (2.1) bB	0.57 (0.08) aB	0.30 (0.05) aB	3.93 (0.72) cAB	1.368 (0.044) bB	11.68 (2.01) aA	0.42 (0.05) cC	0.22 (0.05) bC	4.33 (1.21) bA	2.286 (0.098) aB
t5	14.80 (3.3) aA	0.43 (0.06) abC	0.20 (0.02) bcC	4.80 (0.81) bA	2.371 (0.197) bC	11.65 (2.46) bA	0.43 (0.03) abC	0.23 (0.03) bC	4.78 (1.18) bA	3.475 (0.153) aC
		MS					***MSC***			
t0	4.31 (1.08) aC	0.83 (0.04) aA	0.55 (0.04) aA	3.41 (0.94) aB	-	4.47 (0.73) aC	0.78 (0.04) bA	0.51 (0.03) abA	3.36 (0.55) aB	-
t1	9.07 (1.50) bB	0.66 (0.05) bB	0.38 (0.03) abB	5.52 (0.76) aA	0.385±0.066 dA	8.22 (1.22) bB	0.67 (0.04) bB	0.42 (0.04) bB	5.11 (0.48) aA	0.699 (0.011) cA
t3	13.13 (1.91) aA	0.50 (0.06) bC	0.27 (0.03) abC	6.02 (0.96) aA	1.058±0.033 cB	11.92 (2.12) aA	0.43 (0.04) cC	0.22 (0.02) bC	4.83 (0.78) bA	0.881 (0.121) cB
t5	14.56 (1.93) aA	0.48 (0.06) aC	0.25 (0.02) aC	6.66 (1.20) aA	1.310±0.012 cC	12.24 (1.23) bA	0.36 (0.02) bD	0.18 (0.01) cD	3.90 (0.90) cAB	1.141 (0.015) cC

[a] n=10 for texure parameters and n=3 for enthalpy, 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.

535 **Table 3**. Crumb and crust colorimetric parameters for analysed breads. [a]

	crumb			*crust*		
	L*	***a****	***b****	***L****	***a****	***b****
M						
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

536 [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
537 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.

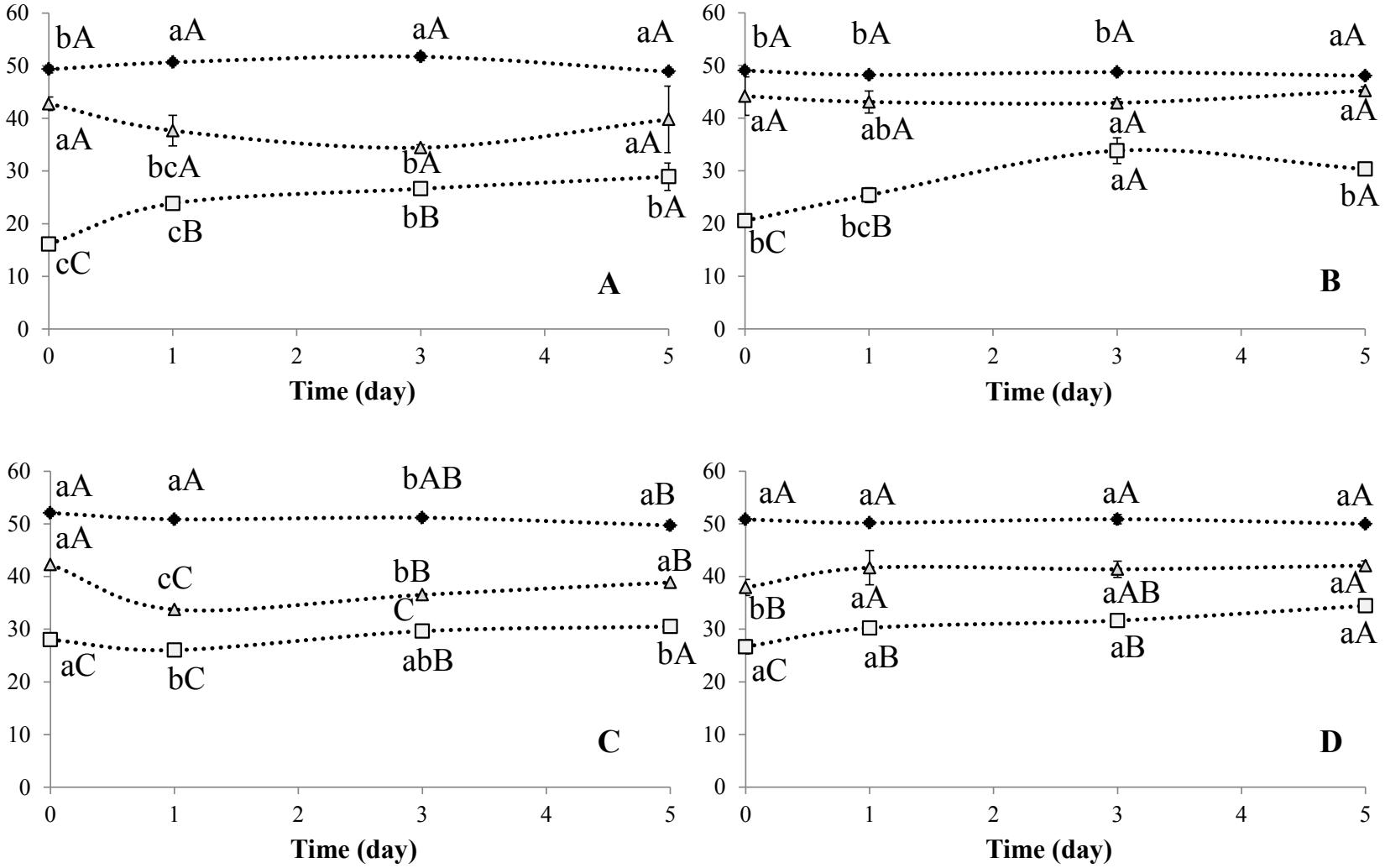


539

	Sourdough (A)	Chestnut (B)	A x B
Class 1	**n.s.**	*	**n.s.**
Class 2	**n.s.**	*	*
Class 3	**n.s.**	*	*
Class 4	**n.s.**	**n.s.**	*

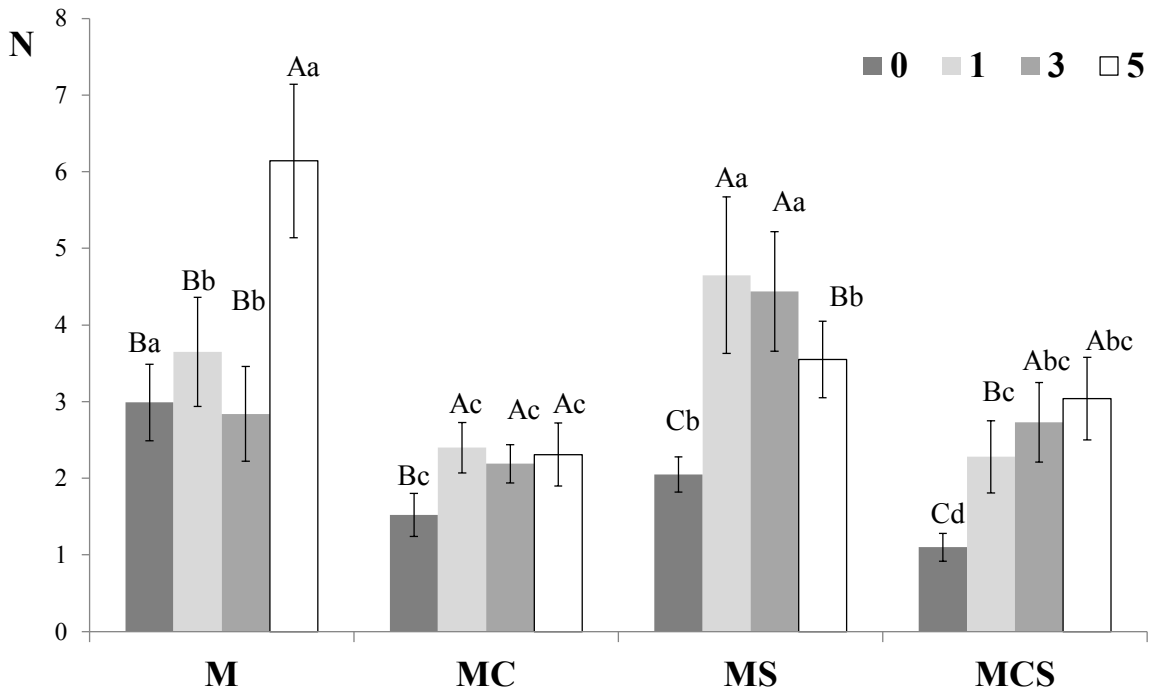
540

541 **Fig. 1**


60
50
40
30
20
10
0
0
1
2
3
4
5
Time (day)
bA
aA
aA
aA
aA
bcA
bA
aA
cB
bB
bA
cC
A
bA
bA
bA
aA
aA
abA
aA
aA
bcB
aA
bA
bC
B
aA
aA
bAB
aB
aA
cC
bB
aB
C
bB
aC
bC
abB
bA
C
aA
aA
aA
aA
bB
aA
aAB
aA
aC
aB
aB
aA
D

542

543 **Fig. 2**



Sourdough (A)	Chestnut (B)	A x B
*	*	n.s.

Fig. 3