Impact of storage on starch digestibility and texture of a high-amylose wheat bread

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Abstract

Staling is a complex process that determines the shelf-life of baked products like bread. Breads made using high-amylose flour may elicit a lower glycaemic response, with benefits for health, however the impact of storage on novel high-amylose wheat foods structure are not known.

We investigated the staling behaviour of high-amylose bread made from a *starch branching enzyme II* (*sbeII*) wheat mutant compared to a wild-type (WT) control, by measuring starch digestibility (susceptibility to amylolysis) and bread texture over time in different storage conditions. Breads prepared from *sbeII* and WT control wheat flours were subjected to fresh, refrigerated and frozen storage, and starch digestibility and crumb texture were measured up to three days. Starch from *sbeII* flour was characterised by a larger proportion of long chains resulting in increased amylose content, typical of *sbeII* mutant wheat. Starch in *sbeII* bread was less susceptible to amylolysis when freshly baked (~17% difference) and after storage (26%-28% difference, depending on the storage condition), compared to the WT control. Texture of freshly baked *sbeII* bread was similar to the WT control; storage conditions affected the progression of crumb firming and resilience to touch for both breads, but changes in crumb texture were less pronounced in *sbeII* bread. Overall, *sbeII* bread was less prone to staling than conventional WT bread during the first three days of storage, particularly when stored in the fridge or at room temperature.

Introduction

Bread made from white wheat flour is a staple food in many countries, however, its high glycaemic potency can potentially harm cardiometabolic health over time (Livesey, et al., 2019).

The glycaemic response to bread reflects the digestibility of starch, the main dietary carbohydrate, so developing wheat starch with greater resistance to digestive enzymes (such as α-amylase) may lead to

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a viable healthier alternative to conventional white wheat bread, if product quality and shelf-life can be preserved.

One promising approach is the use of novel types of wheat with increased amylose content and lower starch digestibility (Hallström E., Sestili F., Lafiandra D., Björck I., & Östman E., 2011; Roman L. & Martinez M. M., 2019). *Starch branching enzyme II* (*sbeII*) wheat mutants obtained by Targeting Induced Local Lesions in Genomes (TILLING), a non-transgenic technology (McCallum, Comai, Greene, & Henikoff, 2000), can produce starch with higher amylose content than conventional wheat starch (Schonhofen A., Zhang X., & Dubcovsky J., 2017). Foods made from *sbeII* wheat flour have lower starch digestibility and could therefore be used to lower glycaemic responses to starch-rich staple foods (Corrado M., et al., 2022; Corrado M., et al., 2020; Sissons M., Sestili F., Botticella E., Masci S., & Lafiandra D., 2020).

The use of high-amylose foods to replace conventional high glycaemic ones requires studies of their aging behaviour, a determinant of shelf-life. Starch structure can change during storage, affecting physicochemical properties which underpin not only digestibility but also bread texture and quality.

The bread making process affects both the starch and protein fractions in the flour while hydration, shear, and heat determine gluten development and starch gelatinisation. During cold storage, moisture loss and starch retrogradation cause increased hardness over time, mainly due to recrystallization of the starch fraction (Ribotta P.D., Leon A.E., & Anon M.C., 2003). Starch retrogradation involves the formation of new ordered structures in both solubilised amylose (20%-30% of wheat starch) and amylopectin (70%-80% of wheat starch) within the gelatinised granule (Miles M. J., Morris V. J., Orford P. D., & Ring S. G., 1985). The molecular structure of these two polymers determines starch physicochemical characteristics and functionality during processing (Vamadevan & Bertoft, 2018). Considering starch retrogradation, differences in the molecular structure affect this dynamic process greatly. Amylose is characterised by infrequent branches and can retrograde quickly, while amylopectin, a highly branched polymer, requires more time to form double helical structures (Kohyama K., Matsuki J., Yasui T., & Sasaki T., 2004; Roman L., Campanella O., & Martinez, 2019).

Bread aging (staling) and consequently shelf-life are determined by texture firming (network plasticization), moisture migration and starch retrogradation (Gray J.A. & Bemiller J.N., 2003). Changes in starch molecular structure, the major component of wheat flour, can affect starch physicochemical properties and the progression of staling, however, previous studies reported conflicting results on the effect of flour replacement with modified starches on bread quality and staling rate. While some studies reported increased firmness and starch retrogradation in bread with added resistant starch

compared to conventional bread (Rosell C. M. & Santos E., 2010), others reported no effects or, in one case, a decreased rate of starch retrogradation of bread with added resistant starch (from modified pea starch), (Sanz-Penella J.M., Wronkowska M., Soral-Śmietana M., Collar C., & Haros M., 2010). This was likely due to the source and type of modification to resistant starch, or the formulation and processing used for breadmaking. Improving the understanding of the physicochemical transformations of high-amylose *sbeII* wheat starch during baking and storage are of interest as they can affect texture and ultimately, palatability and shelf-life of this food product. In a recent study, bread made from high-amylose wheat flour had a higher content of resistant starch and similar initial firmness to the control bread. During storage, high amylose bread showed greater stability than the control bread (Li C. & Gidley M.J., 2022).

In this study, we investigated the effect of different conditions commonly used to store bread on moisture loss, crumb texture and starch susceptibility to amylolysis in a *sbeII* bread compared to a wild-type (WT) control bread. Breads were produced using a modified straight dough method which resembles a traditional home-baking process and analysed freshly baked (0h) and after storage at room temperature (RT), in the fridge and in the freezer for up to 72 hours as at lower storage temperature starch is expected to retrograde faster with evident changes in crumb firmness (Bosmans G. M., Lagrain B., Fierens E., & Delcour J. A., 2013). Structural differences between *sbeII* mutant starch and the WT control were measured using size exclusion chromatography (SEC) and starch crystallinity differences between *sbeII* and WT control flour and breads were explored using light microscopy, in raw flour, in freshly baked bread to identify differences due to the baking process and after freezer storage (7 days) as indicator of bread ageing.

Materials and methods

Wheat materials and bread making process

A field trial at the John Innes Centre Field Station (Norfolk, UK) was used to supply wheat grains of a high-amylose *sbeII* mutant and WT control (Schonhofen A., et al., 2017). Grains were milled to produce refined white flour which was used for making bread rolls with approximately 75 g of total starch per roll. The starch characteristics of the flour used in this study have been described previously (Corrado M., et al., 2022). Briefly, the total starch content of the *sbeII* and WT control flours was similar while, as expected, the apparent amylose proportion measured on starch isolated from flour was greater in *sbeII* starch (39 ± 1.1% of total starch, mean ± SEM, n = 3) than the WT control (25.7 ± 0.7% of total starch, mean ± SEM, n = 3).

95 Bread rolls were baked from *sbeII* and WT flours as described previously (Corrado M., et al., 2022) to
96 achieve a similar starch content (~75 g) based on the total starch content of the flour. The same
97 process as used to produce *sbeII* and WT control doughs, later portioned into rolls and baked. Using a
98 Kenwood mixer (KM300, Kenwood UK) with a hook attachment, flour (WT = 57%, *sbeII* = 55%), caster
99 sugar (WT and *sbeII* = 2%), Allison's dry yeast (WT and *sbeII* = 2%) and water (WT = 37%, *sbeII* = 39%)
100 were mixed at low speed for one minute, after which Willow vegetable shortening (WT and *sbeII* = 2%)
101 was added. After three minutes of mixing, salt (WT and *sbeII* = 1%) was added and the dough was
102 mixed at increasing speed for five more minutes.

103 The dough was then fermented for two hours at ambient conditions (21℃, 41% Relative Humidity
104 (RH)), then rolls were shaped and proofed for 15 minutes at 40℃, 100% RH using a steam oven (DG
105 6001 GourmetStar, Miele, UK). Rolls were sprayed with water and baked at ~185℃ for 20 minutes in a
106 pre-heated convection oven (Hotpoint HAE60P, Whirlpool UK) with a tray of water to provide steam,
107 to reach a core temperature of ~95℃. Batches were prepared under identical conditions and rolls (3
108 per batch) were paired by their baking position in the oven to ensure even baking. After baking, rolls
109 were left at room temperature to cool for two hours.

110 **Allocation to storage conditions and sample preparation**

111 After 2h cooling at room temperature, bread rolls were either analysed immediately (fresh, 0h) or
112 after 24h, 48h, and 72h of storage at room temperature (RT, +19 to +21°C), freezer temperature (-18
113 to -20°C) and fridge temperature (+3 to +5°C), n = 3 per condition. Samples stored in the fridge and
114 freezer were allowed to return to RT before analysis. A roll of ~165 g required approximately 3h to
115 reach RT at the core after freezer storage, and approximately 2h after fridge storage.

116 Figure 1 shows sample preparation of each bread genotypes for the different analyses.

117 **For starch digestibility analysis**, the structure of the bread was not preserved as the starch
118 characteristics were analysed at microstructural level.

119 Three bread rolls per bread type (*sbeII* and WT control) were produced, with a sample from each roll
120 subjected to each storage condition. After 2h of cooling at RT, the crust was removed and the crumb
121 was ground using a Kenwood mini-chopper food processor and sieved using a 1 mm sieve. The fraction
122 below 1 mm was used to determine starch digestibility *in vitro*. Starch susceptibility to amylolysis was
123 measured on fresh bread and bread stored for 24h, 48h, 72h at RT, fridge and freezer conditions, as
124 described above.

125 All samples were weighed out from fresh bread and then stored in the allocated conditions, with three
126 independent samples (from three rolls) per condition, Figure 1A. For samples stored in cold

temperature (fridge and freezer), tubes were allowed to return to RT before analysis. Because of the small amount of sample required for this type of analysis (~63 mg) compared to the weight of a whole roll (~165 g), thawing time was proportionally adjusted. Samples from the fridge reached RT in 20 min and samples from the freezer reached RT in 45 minutes.

For texture and moisture analyses, the macrostructure of the rolls was preserved as the crust plays a role in moisture loss and crumb firming. Therefore, bread rolls were produced over three subsequent days and assigned to a storage condition (n = 3 rolls per condition). Each roll was stored in fridge, freezer or at RT in individual sealed bags.

Each analysis was carried out on three independent rolls, from different batches of dough to capture batch-to-batch variability and ensure fair comparison between *sbeII* and WT control breads. After storage and ahead of analysis, each roll was weighed then the crust was discarded, the core of the roll was cut into four 5x5x5 cm cubes and was analysed immediately (leading to four technical replicates for each measure), Figure 1B. Moisture by air-oven method AACC (44-15A), one stage procedure (AACC International, 1999) was used to measure moisture in samples from the same rolls used for texture analysis, using the remaining bread crumb (n = 3 independent samples per condition).

For microscopy, three rolls were produced per bread type (*sbeII* and WT control), samples of crust and crumb (from the core of the roll) were taken when fresh (2h after baking) after which the rolls were stored in the freezer for 7 days. After 7 days, each roll was left at RT for approx. 3h to reach ambient temperature (+19 °C). After thawing, crust and crumb samples were taken from the core of the roll and mixed with water to disrupt the crumb matrix and imaged.

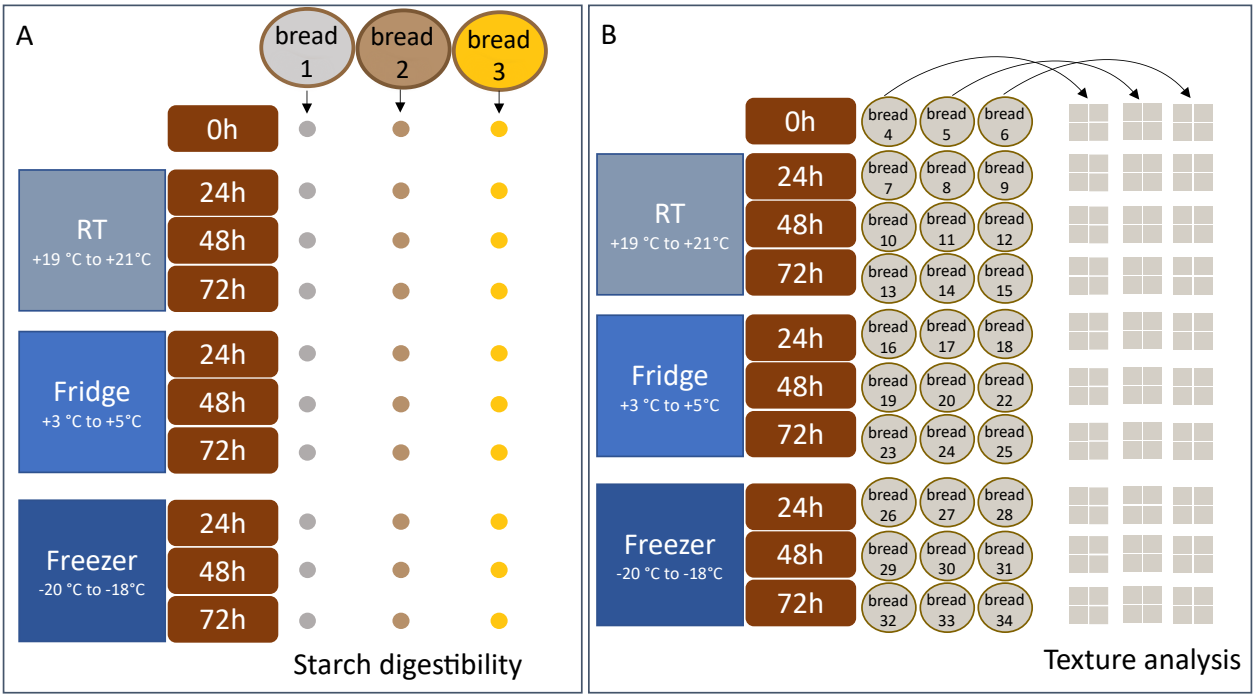


***Figure 1**. Bread sampling for starch digestibility (A), texture analysis (B). For starch digestibility, seven samples per genotype (sbeII and WT) were taken from each bread roll (●) after baking and allocated to a storage condition (n = 3 per storage condition). Isolated crumb from each roll was ground and sieved using 1 mm sieve. The fraction below 1 mm was used for in vitro starch susceptibility to amylolysis; the fraction above 1 mm was used for moisture analysis. Samples for the starch digestibility assay and moisture analysis were weighed out at the same time and either analysed or stored at the storage conditions described above. For texture analysis, three rolls per genotype (sbeII and WT) were assigned to each storage condition. For the analysis, the crust of the bread was removed to isolate the core crumb which was sliced into four 5x5x5 cm cubes (□) and used for texture analysis (TPA).*

Analytical Methods

Moisture of bread crumb during storage

Moisture content of bread crumb samples was measured by the air-oven drying (AACC 44-15A), one stage procedure (AACC International, 1999). Samples were weighed out in metal tins immediately after slicing to prevent moisture loss. These were then placed in the oven for exactly 16 hours, then removed from the oven and left to cool for one hour in a desiccator before weighing the tins again. Moisture content was calculated by subtracting the weight of the sample after drying in the oven to that of the fresh sample for three independent replicates (one from each roll).

Moisture content of bread rolls during storage was also determined by weighing the rolls before and after storage.

Texture analysis of bread crumb during storage

168 Crumb texture was determined using a two-bite test to obtain *Hardness*, *Cohesiveness*, *Chewiness*,
169 *Gumminess*, *Springiness*, and *Resilience* of bread on a TA-XT2 Texture Analyser (Stable Micro System,
170 Godalming, UK) equipped with a 5-kg load cell and 50 mm compression plate (P50). Uniaxial
171 compression with 100 mm min-1 crosshead speed was applied to a 5 x 5 x 5 cm sample with crumb
172 hardness corresponding to the force *N* required for a 40% compression. Parameters of interest were
173 obtained using Exponent software (6.0, Stable Micro System, Godalming, UK), (Corrado M., et al.,
174 2022).

175 **Starch susceptibility to amylolysis**

176 Starch susceptibility to amylolysis was measured on bread crumb. Bread samples were incubated at 37
177 ˚C with end-over mixing with porcine pancreatic α-amylase and reducing sugars obtained from
178 hydrolysis were measured at 0, 3, 6, 9, 12, 15, 18, 21, 25, 30, 35, 45, 60, 75, 90 minutes of incubation
179 and quantified using 'PAHBAH' (p-hydroxybenzoic acid hydrazide) colorimetric method, (Edwards C.H.,
180 Cochetel N., Setterfield L., Perez-Moral N., & Warren F.J., 2019).

181 **Starch chain-length distribution**

182 Starch isolated from *sbeII* and WT wheat flour was used to determine starch chain length distribution
183 by size exclusion chromatography (SEC). Starch was isolated as described in (Corrado M., et al., 2020)
184 and debranched using iso-amylase prior to analysis by HPLC-SEC as described by Fahy *et al.* 2022
185 (Fahy B., et al., 2022). The relative peak heights corresponding to amylopectin and amylose chains
186 were calculated as defined by Hanashiro *et al.* 1996 (Hanashiro, Abe, & Hizukuri, 1996) and Vilaplana
187 *et al.* 2012 (Vilaplana, Hasjim, & Gilbert, 2012). No cross-sample normalisation was applied as
188 parameters were calculated within sample.

189 **Microscopy**

190 Images of starch were obtained using an Olympus BX60 upright brightfield and widefield
191 fluorescent microscope with colour camera, using a brightfield and polarised light filter to visualise the
192 birefringence pattern, objective x10. Images were captured using the ProgRes CT3 Colour 3.15 MP
193 cMOS camera: 3.2 µm2 pixel size, 2048 x 1536 image size.

194 Samples of crust and crumb were mixed with water briefly to disrupt the bread matrix. The suspension
195 was laid on microscope slides 1.0 – 1.2 mm with glass cover n. 1.5, 22 x 50mm, (SuperFrost®, VWR®)
196 and imaged immediately.

197 **Statistical analysis**

198 Amylolysis curves were fitted to a first order equation previously described (Edwards C.H., et al., 2019)
199 using a non-linear regression model. The first order rate constant (*k*) and endpoint (C_∞) were
200 estimated from the equation, after subtracting the 'endogenous' maltose detected before the start of
201 the reaction (Y_0) from the subsequent timepoints. The experimental endpoint C_{90} and the Area Under
202 the Curve (AUC) are reported as additional descriptors of the susceptibility to hydrolysis.

203 Amylolysis parameters (C_{90} and AUC, *k* and C_∞) were compared across groups with mixed-effects
204 models using the *lmerTest* R package (version 3.1.2), (Bates, Mächler, Zurich, Bolker, & Walker, 2014;
205 Kuznetsova A., Brockhoff P.B., & Christensen R.H.B., 2017) with storage and genotype as fixed effects
206 and an interaction term of storage condition by genotype.

207 A separate mixed-effects model was estimated for each texture parameter following log-
208 transformation. Upon visual inspection, log-transformation stabilised the variances and resulted in
209 model residuals normally distributed. Each model included fixed effects of every combination of
210 storage condition (storage duration by storage temperature), the main effect of genotype and the
211 interaction between storage condition and genotype as well as a random intercept to account for
212 having multiple data points from each roll. With regard to springiness, data was difficult to model
213 because of variability between replicates, so no statistical model was fitted for this outcome
214 (Supplementary Figure 2).

215 The effect of storage on bread moisture was estimated using a linear regression model, with main
216 effects of storage condition and genotype and an interaction term of storage condition by genotype.

217 Chain-length distribution of debranched starch was analysed as described by Fahy and colleagues
218 (Fahy B., et al., 2022) using a mixture model where the distribution components were estimated using
219 constrained values of degree of polymerisation (DP) previously described by Hanashiro *et al.* 1996
220 (Hanashiro, et al., 1996) and Vilaplana *et al.* 2012 (Vilaplana, et al., 2012). The *mixdist* (version 0.5-5) R
221 package was used to estimate mixture models (Macdonald P. & Du J., 2018).

222 Specific contrasts and marginal means were obtained from mixed models with Satterthwaite
223 approximations for degrees of freedom, using the *emmeans* R package (version1.7.2), following back-
224 transformation, if required (Lenth R., 2020).

225 Datasets were curated in R Core (R version 4.1.1), (R Core Team, 2021). The AUC was integrated using
226 the *mosaicCalc* (version 0.5.1), (Kaplan D.T., Pruim R., & Horton N.J., 2020) package. Annotated code
227 and source data are available as Supplementary.

228 **Results**

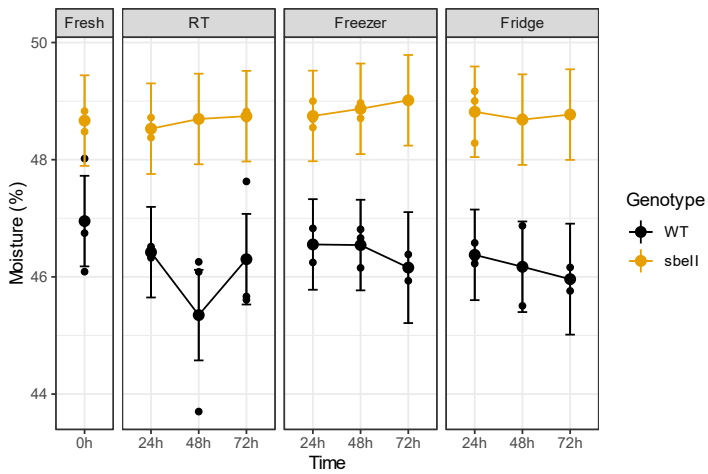
229 Dough formulation was similar for *sbeII* and WT control breads, however *sbeII* flour required
230 additional water (39% for *sbeII* and 37% for WT control) to produce a workable dough. After mixing,
231 the dough was assessed visually and found to be well developed however, the WT control dough
232 appeared to be slightly stickier than the *sbeII* dough, in all batches produced. Both roll types
233 developed and baked well, reaching a core temperature of 95°C.

234 Rolls were made to deliver an equal starch content; the *sbeII* flour was characterised by a lower total
235 starch content, therefore the *sbeII* rolls were ~10 g larger than the WT control rolls (161.26 g ± 0.16 g,
236 155.73 g ± 0.40 g, respectively, mean ± SEM, n=3).

237 **Moisture**

238 Moisture changes are shown in Figure 2, data is shown in Supplementary Table 1. During baking,
239 moisture loss of bread rolls made with *sbeII* flour was 11.9% compared to 10.3% in WT controls.

240 Freshly baked *sbeII* bread rolls had a small but higher moisture content (48.7%) compared to WT
241 breads (47%; $p = 0.003$). The moisture difference between genotypes was maintained consistently
242 over storage, regardless of the storage conditions (interaction between genotype and storage $p = 0.3$,
243 0.1, 0.1 for RT, Freezer and Fridge).



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245 ***Figure 2.*** *Estimated marginal means of moisture during storage, n = 3 with error bars representing the 95%CI.*

246 **Starch digestibility in bread**

247 Starch digestibility was defined using the following parameters: the extent of digestibility within 90
248 min of incubation (C_{90}), the predicted starch digested at the end of reaction (C_{∞}), the digestibility rate
249 constant (k) and area under the curve (AUC), Figure 2D-G.

250 Overall, starch in *sbeII* fresh bread was significantly less digested than the WT control, as shown by the
251 C_{90} and AUC (p = 0.005, p = <0.001, respectively), regardless of storage, Supplementary Table 2.

252 Compared to freshly baked bread, storage led to increased starch digested at 90 minutes (C_{90}, p =
253 <0.001, for all conditions), in both bread genotypes, but there was no evidence of an interaction effect
254 between storage and genotype on starch digested (C_{90}, p = 0.3), showing that the effect difference
255 between *sbeII* and WT control bread is maintained throughout storage.

256 Starch digestibility was 51.7% in *sbeII* freshly baked bread compared to 61.4%, 61.7% and 60.4% when
257 *sbeII* bread was stored for 72h in the freezer, fridge and RT respectively. In freshly baked WT control
258 bread, starch digestibility was 62.4% and increased to 78.6%, 78.5% and 82.1% after 72h freezer,
259 fridge and RT storage, respectively. For samples stored at RT, some evidence of interaction between
260 storage and bread genotype was observed (p = 0.03). Here, C_{90} was higher after 48h storage at RT
261 compared to fresh bread.

262 These changes are relative to the starch digested within 90 minutes of incubation however, the model
263 suggests that digestion of *sbeII* bread is slower compared to the WT control, and that at the end of the
264 reaction the differences between the breads would be negligible (as evidenced by estimates of the C_∞
265 parameter), (Figure 3E).

266 The digestion rate (k) of starch in *sbeII* bread was overall lower compared to starch in WT control
267 bread (p = <0.001) and no effect of interaction between genotype and storage was found (p = 0.1).
268 Compared to fresh bread, RT and fridge storage affected the rate of starch digestion in both bread (p =
269 <0.001, both conditions) but not in breads stored in the freezer (p = 0.2), Figure 3A-C.

270 Considering the AUC of digestibility curves, besides the significant effect of the genotype (p = <0.001),
271 there was a significant effect of the fridge and freezer storage on AUC, compared to fresh bread (p =
272 0.002 and p = <0.001, respectively). There was some evidence of an interaction effect between storage
273 and genotype on AUC however, the magnitude of the effect is relatively small as the AUC summarises
274 rate (k) and extent of digestibility (C_{90}), (p = 0.0007).

275 The estimated marginal means with 95% CI for all storage conditions are reported in Supplementary
276 Table 2.

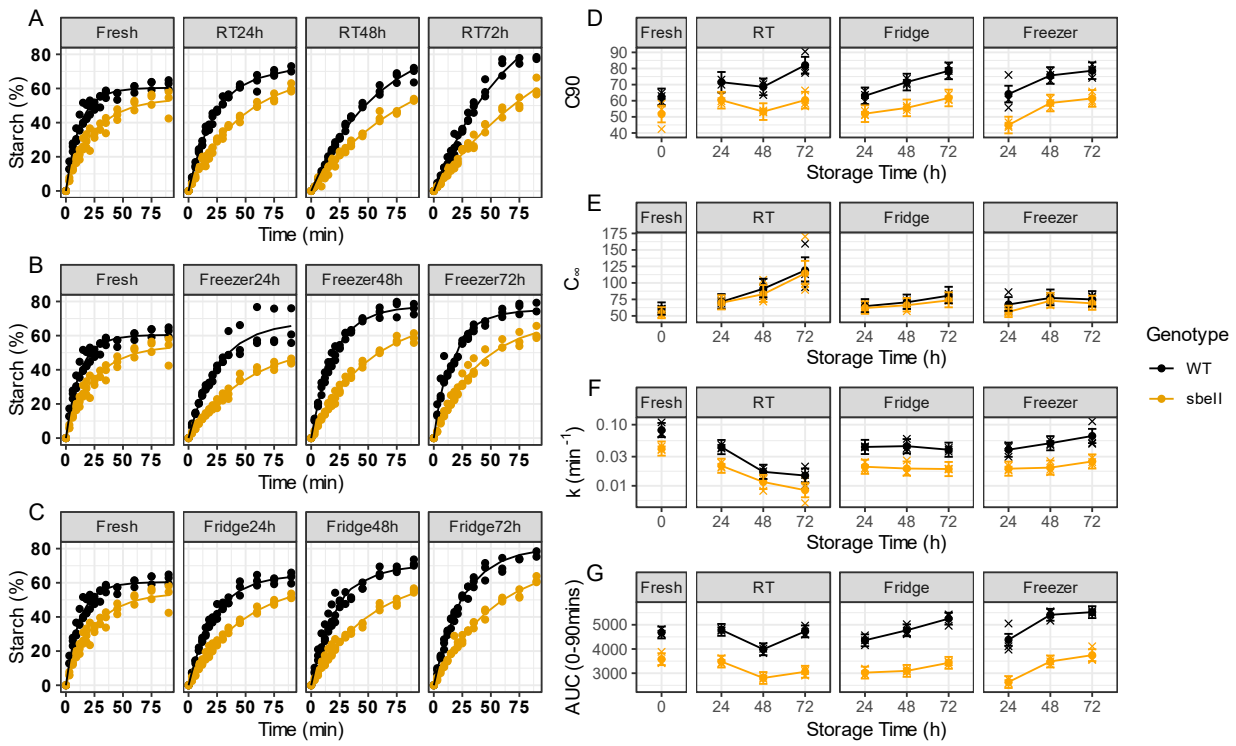

Figure 3 A-C. *Starch digestibility curves of sbeII (yellow) and WT control (black) bread samples, experimental data points represent independently treated samples, n = 3 per condition. Experimental data (replicate datapoints) are shown by fitting a first-order equation based on the estimates of k and C_∞ values (n = 3 independent samples) obtained from a non-linear regression model.* ***D-G.*** *Grouped means of parameters obtained from digestibility curves, (D) C_{90}, (E) C_∞, (F) k, (G) AUC, error bars represent the 95%CI (n = 3).*

Texture analysis

Macrostructure of bread crumb was analysed instrumentally for textural differences between bread types, during storage. Estimated means of all characteristics following each storage conditions are reported in Supplementary Table 3 and are showed in Figure 4.A.

Freshly baked *sbeII* bread was similar to the WT control for hardness (*sbeII* = 373 g and WT = 306 g, p = 0.1), cohesiveness (*sbeII* = 0.859%, and WT = 0.906 g, p = 0.3), resilience (*sbeII* = 0.62% and WT = 0.64%, p = 0.4) but showed lower chewiness compared to the WT control (*sbeII* = 516 and WT = 734, p = 0.01).

With storage, increased crumb firming and decreased resilience was observed in both bread genotypes. Firming was less pronounced in *sbeII* breads than in the WT control while loss of resilience was more marked in *sbeII* bread than the WT control (p = <0.001, both parameters). Overall, the effect of storage on crumb hardness and resilience varied by genotype (storage by genotype interaction effect on hardness p = 0.001, effect on resilience p = <0.001) but not when considering bread chewiness and cohesiveness.

297 There was less effect on bread hardness with freezing, with no difference in the effect of freezing
298 between genotypes.

299 Following storage, the *sbeII* bread appeared slightly chewier than the WT, with the most marked
300 difference after 72h in the fridge or at RT (*sbeII* fridge 72h = 634.5, WT fridge 72h = 1088, $p = 0.0002$,
301 *sbeII* RT 72h = 593.9, WT RT 72h = 888.9, $p = 0.004$).

302 Resilience of *sbeII* breads was marginally lower than the WT control after 24h of freezer storage (*sbeII*
303 freezer 24h = 0.52, WT freezer 24h = 0.57, $p = 0.003$) but slightly higher than the WT control after
304 fridge storage (*sbeII* fridge 24h = 0.51% and WT fridge 24h = 0.47, $p = 0.004$).

305 ***Chain length distribution***

306 Analysis of starch chain-length distribution showed differences in the molecular structure of amylose
307 and amylopectin from *sbeII* mutant wheat compared to the WT control.

308 The proportion of long amylopectin chains (DP 37 to 100) was higher in *sbeII* starch (19% ± 0.3%)
309 compared to the WT control (10.9% ± 0.7%) and presented two distinct peaks (Figure 4, B), in contrast
310 to a slight shoulder peak in WT starch. The *sbeII* starch was also characterised by a larger proportion of
311 amylose chains (DP 100 - 1600) compared to the WT control (26.3% ± 3.4, 15.8% ± 2.7%, respectively).
312 The percentage of short chains (DP<25) in mutants was lower in the *sbeII* mutant compared to the WT
313 control (Figure 4, C).

314 Chain length proportions per DP fraction are reported as Mean ± SD, n = 3. All fractions can be found
315 in Supplementary Table 4.

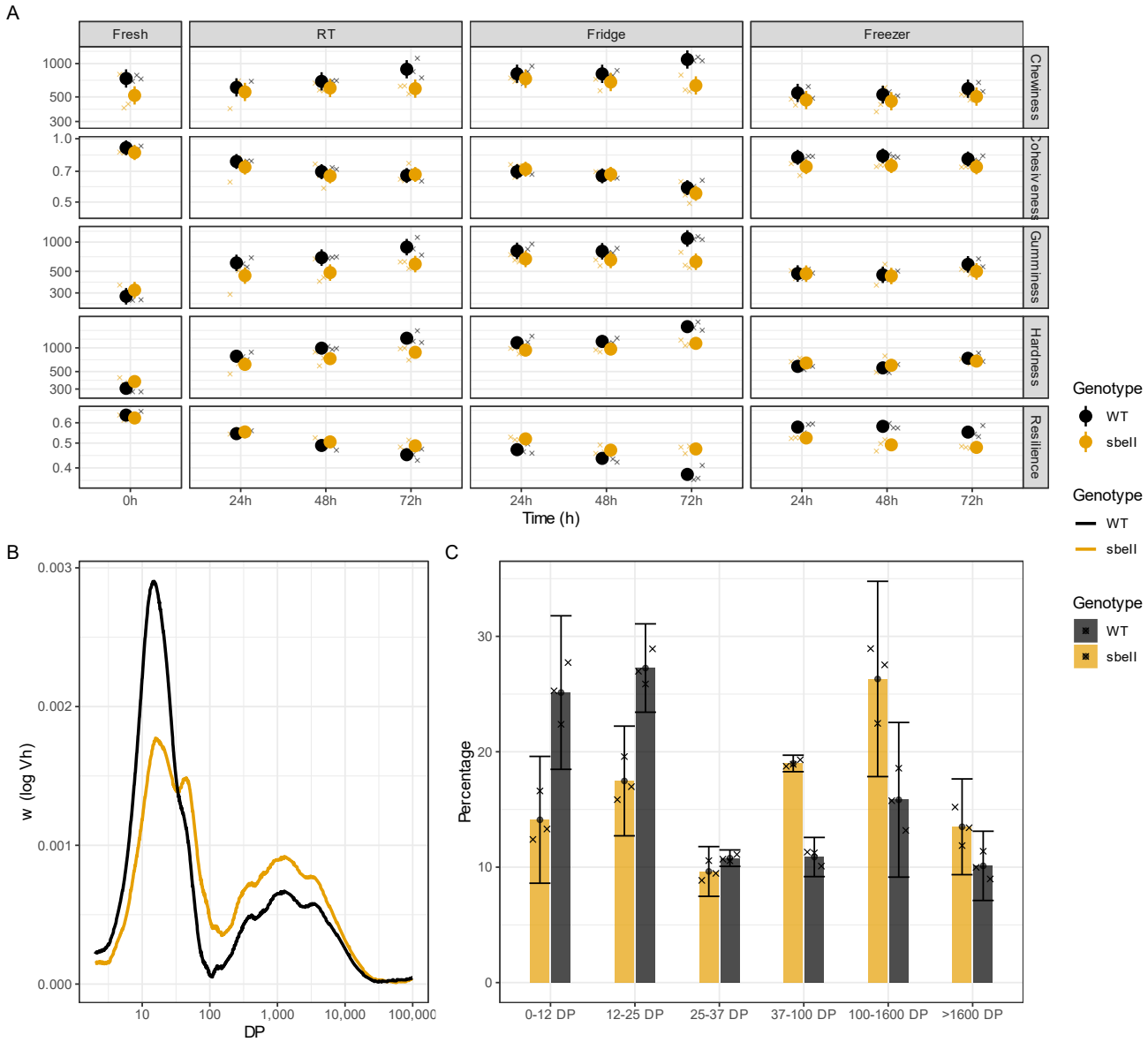

Figure 4. A. *Texture profile of WT and sbeII breads, data points represent the mean value with error bars = ± 95% CI, n = 3 independent bread rolls were analysed per condition, with four technical replicates per bread roll.* ***B.*** *Chain-length distribution of debranched sbeII and WT control starch determined by SEC, average of n = 3 independent replicates.* ***C****, Fractions grouped, with error bars = 95% CI, based on DP as described by Hanashiro et al.(Hanashiro, et al., 1996) and Vilaplana et al. (Vilaplana, et al., 2012) where amylopectin short chains are 0-37 DP, amylopectin long chains are 37-100 DP, amylose short chains are 100-1600 DP and amylose long chains are >1600 DP.*

Microscopy

Micrographs of starch in water using regular brightfield microscopy complemented by polarised-light microscopy were used to visually assess starch crystallinity in raw flour and in bread.

Starch in raw *sbeII* flour showed a typical birefringence pattern ('Maltese cross') however, this was less defined in starch granules with altered morphology Figure 5.A.

328 In freshly baked bread, only few starch granules in both WT and *sbeII* crumb were visibly birefringent
329 suggesting loss of starch semi-crystalline structure during baking (Figure 5.B).

330 Breads were then stored for 7 days in the freezer and thawed once. A larger number of granules with a
331 birefringent pattern was observed in the crumb WT control bread, while almost no granules with such
332 pattern were visible in the crumb of frozen *sbeII* bread, Figure 5.C.

333 The crust of breads was also included in the analysis. Here, the birefringence was less evident in *sbeII*
334 starch granules from fresh crust compared to the WT control granules, possibly due to different levels
335 of gelatinisation and/or crystallinity (Supplementary Figure 2.D). Granules with birefringent pattern
336 were also observed in the crust of frozen bread, both mutant *sbeII* and WT control, however to a
337 lower extent (Supplementary Figure 2.E).

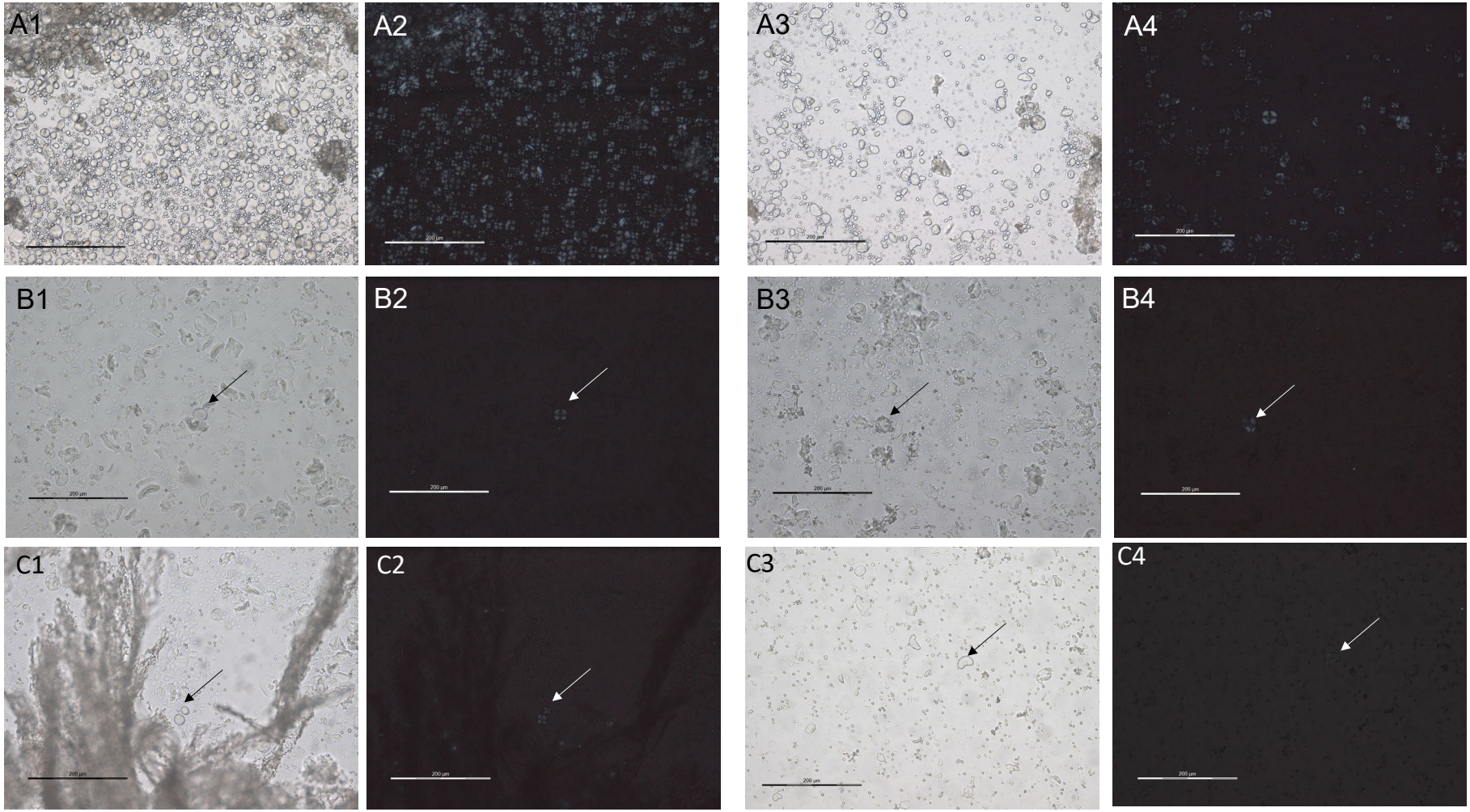

Figure 5. *Micrographs of starch in water using brightfield (BF) and polarised light filter (P) highlighting birefringence of starch granules.* ***1.*** *WT control starch under BF;* ***2.*** *WT control starch under P;* ***3.*** *sbeII mutant starch under BF;* ***4.*** *sbeII mutant starch under P;* ***A.*** *starch from flour;* ***B.*** *Starch from fresh bread crumb;* ***C.*** *Starch from bread crumb after 7 days of freezer storage. Scale bar in white or black represent 200 µm.*

342 **Discussion**

343 The use of high-amylose flour in the production of starch-based foods has the potential to elicit a
344 lower glycaemic response compared to conventional starch-based foods (Ang K., et al., 2020;
345 Granfeldt Y., Drews A., & Björck I., 1995; Hallström E., et al., 2011; Hoebler C., Karinthi A., Chiron H.,
346 Champ M., & Barry J.L., 1999). In this study we investigated the macro- and microstructural changes in
347 bread made from a high-amylose wheat flour during three days of storage. The *sbeII* starch in flour
348 had a higher proportion of intermediate chains (DP 37-100) compared to the WT control, as a result of
349 the reduced activity of SBEII enzymes during starch biosynthesis. This is consistent with results from
350 other studies of crops with mutations in *starch branching enzyme* genes (Jane J., et al., 1999; Regina
351 A., et al., 2015).

352 Starch in white bread made from *sbeII* mutant wheat flour was found to be less susceptible to
353 amylolysis when fresh and after storage, and it was characterised by a consistently slower digestion
354 rate (*k*) leading to lower starch digested (AUC). Therefore, the lower starch digestibility observed in
355 *sbeII* fresh and frozen breads may be due to enzyme-resistant structures already present after baking
356 and linked to the amylose content rather than structures formed during storage.

357 Considering the different digestibility parameters, we have shown that the percentage of starch
358 digested in *sbeII* bread at 90 mins is consistently lower than the starch digestibility from WT control
359 bread, but the rate starch hydrolysis can be affected by the storage condition.

360 Overall, the effect of the storage condition can affect *sbeII* starch digestibility early on during storage
361 compared to the WT control, however the difference between *sbeII* bread and WT control is
362 maintained during storage. This could be due to the increased amylose proportion in *sbeII* starch and
363 altered retrogradation behaviour compared to the WT control starch, which has a higher proportion of
364 amylopectin and may be potentially slower at retrograding. This was also observed in microscopy
365 images, where *sbeII* starch granules in fresh bread and after 7 days of storage in freezer, did not show
366 the birefringence pattern typical of recrystallised granules, as in the WT control.

367 A recent study measured RS content of bread during storage and reported a similar mechanism in
368 bread made from partial substitution (50%) of conventional white flour with high-amylose wheat flour
369 and stored in the fridge for up to 5 days (Li C., et al., 2022). Interestingly, they did not observe an
370 increase in RS content of bread during storage. We showed that starch digestibility in bread stored in
371 the fridge increased over time compared to fresh bread however, the starch in *sbeII* bread remained
372 less susceptible to amylolysis compared to the WT control. When left at RT, the rate of starch
373 hydrolysis of both bread types decreased within three days of baking, compared to fresh bread. The

374 single enzyme assay used to determine starch digestibility here is a sensitive method and may be able
375 to detect subtle differences compared to the RS AOAC method by producing several experimental
376 parameters to characterise the digestibility curves providing accurate estimates of digestion. The
377 percentage of starch hydrolysed by α-amylase, as reported in this study (C_{90}), has been shown to
378 correlate well with glycaemic index measurements in human participants (Edwards C.H., et al., 2019),
379 suggesting that the lower starch digestibility of *sbeII* starch in bread has potential for applications in
380 frozen bread, as shown by a previous study (Corrado M., et al., 2022), but also when bread is
381 consumed fresh, or after storage in the fridge or at room temperature.

382 Based on the texture measurements reported in this study, macrostructural changes to the crumb
383 texture set in earlier during storage in *sbeII* bread compared to the WT control. Overall, texture
384 changes in *sbeII* bread crumb were limited compared to the WT control crumb, suggesting a more
385 stable structure over storage. Crumb ageing was mitigated by the greater water holding capacity of
386 *sbeII* flour, as seen in the hardness and resilience parameters.

387 Similarly, Li and Gidley (Li C., et al., 2022) showed that substitution of conventional wheat white flour
388 with 50% high-amylose flour in bread making does not lead to a noticeable increase in bread firmness
389 over storage in the fridge. Barros *et al.,* reported that added resistant starch in bread making delayed
390 bread staling by decreasing the rate of starch recrystallisation, without a negative impact on bread
391 quality (Barros J. H. T., Telis V. R. N., Taboga S., & Franco C. M. L., 2018). Evident changes in crumb
392 firmness have been shown to be linked to amylopectin retrogradation, especially at freezer
393 temperatures, (Bosmans G. M., et al., 2013) and independent from the moisture loss (Ribotta P.D., et
394 al., 2003).

395 These studies support the findings reported here; considering the limited micro- and macrostructural
396 changes in bread made from *sbeII* flour during storage, this high-amylose flour could be used to make
397 bread with lower glycaemic potency for commercial use thanks to the limited staling.

398 Interestingly, instrumentally measured resilience (a measure of elasticity) of *sbeII* bread crumb was
399 found to be slightly higher compared to the WT control after storage in the fridge but a little lower
400 than the control after storage in the freezer, suggesting higher stability of refrigerated *sbeII* bread
401 compared to conventional bread.

402 This is promising for potential use of this wheat in production of sandwich bread as it is common to
403 use dough improvers to decrease the progression of staling and reduce bread firming (Hug-Iten,
404 Escher, & Conde-Petit, 2003). In this study, the formulation, particularly the amount of water used to
405 hydrate the flour and form the dough, may have played a role in promoting bread softness over time.

In a recent publication (Arp C.G., Correa M.J., & Ferrero C., 2020) it was reported that in breads with intermediate content of resistant starch (10% and 20% high-amylose flour replacement), the good initial quality and slower dehydration rate of the matrix counteracted the negative effects of starch retrogradation on bread texture. While no major water adjustments were made to form the *sbeII* dough, the WT control dough was slightly stickier than the *sbeII* dough, so a slightly lower water content may have produced a better WT control dough, for this type of processing. It is noteworthy that the bread making method used here was closer to home baking than the industrial Chorleywood method, so bread texture may differ when scaling up the formulation for large industrial production of convenience bread. Based on results from this study, the *sbeII* wheat flour shows promise for home and artisan bread making with lower starch digestibility, however other studies on dough development and machinability are required.

Conclusions

Bread made from *sbeII* wheat flour was found to be more stable during storage and less prone to staling than the WT control, representing conventional white bread. This is likely due to the increase in amylose proportion in *sbeII* starch. Despite requiring more water to form a well-developed dough compared to conventional wheat white flour, as shown by previous research, the starch digestibility of *sbeII* bread remained consistently lower than the WT control over storage. At the texture level, *sbeII* bread was less prone to firming compared to WT control bread.

This shows potential for *sbeII* bread to be used to develop freshly prepared and refrigerated wheat bread with a lower glycaemic potency and similar or, in some cases, better ageing than conventional white bread.

Authors contribution

Marina Corrado: Conceptualization, investigation, methodology, project administration, data curation and formal analysis, writing – original draft preparation, writing – review & editing, Petros Zafeiriou: investigation, methodology, writing – review & editing, Jennifer Ahn-Jarvis: investigation, methodology, writing – review & editing, George M. Savva: methodology, data curation, visualisation, formal analysis, writing – review & editing, Cathrina H. Edwards: conceptualization, methodology, supervision, writing – review & editing, Brittany A. Hazard: conceptualization, funding acquisition, methodology, supervision, writing – review & editing.

435 **Conflicts of interest**

436 The authors declare no conflicts of interest.

437

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