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

Corrado M.[1], Zafeiriou P.[1], Ahn-Jarvis J.H.[1], Savva G.M.[1], Edwards C.H.[‡1] and Hazard B.A.[*‡1, 2]

* Corresponding author Brittany.Hazard@quadram.ac.uk

[‡]These authors contributed equally

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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 of starch digested at 90 min, C_{90}) and after storage (26%-28% C_{90} 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 pancreatic α-amylase)

[1] Food Innovation and Health, Quadram Institute Bioscience, Norwich Research Park, UK
[2] Designing Future Wheat and Molecules from Nature, John Innes Centre, Norwich Research Park, UK

may lead to 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 ageing behaviour, a determinant of shelf-life. Starch structure determines its physicochemical properties during storage, 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 storage, moisture loss and starch retrogradation and gluten network changes contribute to staling causing increased hardness over time. This is due to recrystallization of the starch fraction (Ribotta P.D., Leon A.E., & Anon M.C., 2003), water migration from crumb to crust and between protein and starch favouring network plasticisation and increased stiffness (Bosmans G. M., Lagrain B., Fierens E., & Delcour J. A., 2013). Starch, of which 20%-30% is amylose and 70%-80% is amylopectin in wheat, can retrograde by forming new ordered structures 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 (re-)crystallisation during storage, differences in the molecular structure affect this dynamic process greatly. Amylose is characterised by infrequent branches and can crystallise quickly, while amylopectin, a highly branched polymer, requires more time to form new double helical structures (Kohyama K., Matsuki J., Yasui T., & Sasaki T., 2004; Roman L., Campanella O., & Martinez, 2019).

Bread ageing (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 the molecular structure of starch, the major component of wheat flour, can affect starch physicochemical properties and the progression of staling depending on the degree of the modification. For instance, Arp *et al.* showed that 10% and 20% flour replacement with high amylose

maize starch (Hi-Maize 260™) in bread led to similar firming behaviour as the control while 30% replacement led to higher firmness (Arp C.G., Correa M.J., & Ferrero C., 2020). Only few studies have investigated the functionality of high amylose starch in bread obtained by starch structural modification *in planta*. In two recent studies, bread made from high-amylose wheat flour (51.5%) had similar texture to the control bread (Li C., Dhital S., & Gidley M. J., 2022). During fridge storage, high amylose bread was found to be less prone to ageing than the control bread (Li C. & Gidley M.J., 2022). 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 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 (after 2h of cooling corresponding to the moment breads can be packaged and stored, later referred to as '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., et al., 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 by iodine-binding method 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. Proximate nutritional content of breads is
110 reported in Supplementary Table 1.

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

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

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

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

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

126 All samples were weighed out from fresh bread and then stored in the allocated conditions, with three
127 independent samples (from three rolls) per condition, Figure 1A. For samples stored in cold
128 temperature (fridge and freezer), tubes were allowed to return to RT before analysis. Because of the
129 small amount of sample required for this type of analysis (~63 mg) compared to the weight of a whole
130 roll (~165 g), thawing time was proportionally adjusted. Samples from the fridge reached RT in 20 min
131 and samples from the freezer reached RT in 45 minutes.

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

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

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

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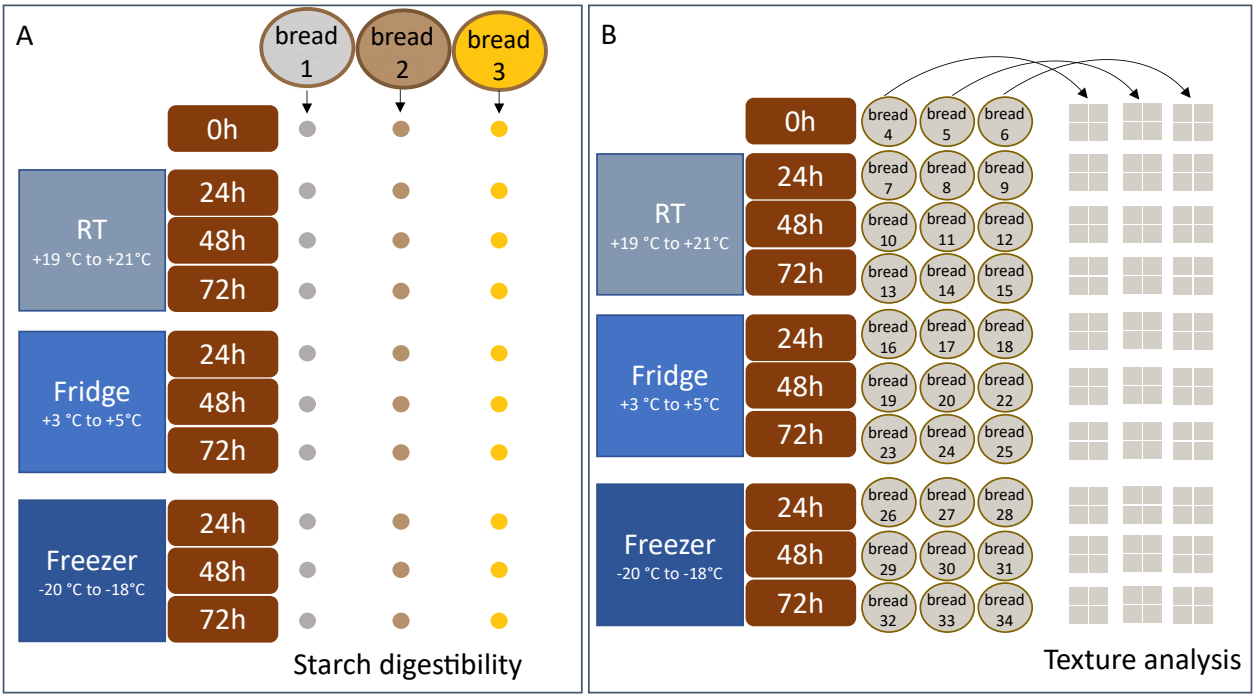


***Figure 1**. Bread sampling for starch digestibility (A), texture analysis (B). For starch digestibility, seven samples per genotype (sbeII and WT) were taken from each bread roll (●) after baking and allocated to a storage condition (n = 3 per storage condition). Isolated crumb from each roll was 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

169 Crumb texture was determined using a two-bite test to obtain *Hardness*, *Cohesiveness*, *Chewiness*,
170 *Gumminess*, *Springiness*, and *Resilience* of bread on a TA-XT2 Texture Analyser (Stable Micro System,
171 Godalming, UK) equipped with a 5-kg load cell and 50 mm compression plate (P50). Uniaxial
172 compression with 100 mm min-1 crosshead speed was applied to a 5 x 5 x 5 cm sample with crumb
173 hardness corresponding to the force *N* required for a 40% compression. Parameters of interest were
174 obtained using Exponent software (6.0, Stable Micro System, Godalming, UK), (Corrado M., et al.,
175 2022).

176 **Starch susceptibility to amylolysis**

177 Starch susceptibility to amylolysis was measured on bread crumb. Sieved bread crumb was weighed in
178 a tube to achieve 27 mg of starch in 5 mL of Phosphate-Buffered Saline (PBS). Samples were incubated
179 at 37˚C with end-over mixing and 2U/mL of porcine pancreatic α-amylase per incubation mix were
180 added to start the assay, after taking a baseline measurement of endogenous maltose (Y0). Samples of
181 incubations mix (100 µl) were taken at 0 (before adding the enzyme), 3, 6, 9, 12, 15, 18, 21, 25, 30, 35,
182 45, 60, 75, 90 minutes of incubation and added to tubes containing 100 µl of Na_2CO_3 to stop the
183 reaction. Reducing sugars obtained from hydrolysis were measured using 'PAHBAH' (p-hydroxybenzoic
184 acid hydrazide) colorimetric method, (Edwards C.H., Cochetel N., Setterfield L., Perez-Moral N., &
185 Warren F.J., 2019). Samples were centrifuged 15,000 x g for 5 minutes; the supernatant was diluted
186 1:10 with deionised water to 100 µL and incubated in a boiling waterbath for 5 minutes with 1 mL of
187 PAHBAH reagent. For the standard curve, 1 mM maltose in water was used to make up the standard
188 solutions (0 to 1 mM) and incubated with PAHBAH reagent with the digestion samples.

189 Absorbance was read in a microplate reader (VersaMax Microplate Reader, Molecular Devices, LLC.,
190 CA, USA) at 405 nm and maltose equivalent concentration in samples were calculated using the
191 maltose standard curve.

192 **Starch chain-length distribution**

193 Starch isolated from *sbeII* and WT wheat flour was used to determine starch chain length distribution
194 by size exclusion chromatography (SEC). For the starch isolation, ~5g of flour were mixed with 30 mL
195 of deionised water then filtered through a layer of Miracloth (475855, Calbiochem®). The solution was
196 centrifuged (2000 x g for 5 min) and the recovered pellet was washed once with 30 mL of 2% Sodium
197 Dodecyl Sulfate (SDS) and then centrifuged. This process was repeated twice using 5 mL of 2% SDS.
198 The resulting starch pellet was washed three times with 5 mL of acetone and left to dry overnight to
199 ensure complete evaporation of the solvent (Corrado M., et al., 2020; Verhoeven, Fahy, Leggett,
200 Moates, & Denyer, 2004). Starch (8 mg/mL) was solubilised in DMSO containing 0.5% (w/w) LiBr

201 followed by enzymatic debranching with isoamylase (Wu, Li, & Gilbert, 2014), freeze-dried, and
202 analysed by HPLC-SEC. Samples and standards were resolubilised in 4 and 2 mg/mL, respectively with
203 DMSO+0.5% (w/w), and stored at 80°C overnight prior to analysis. The HPLC-SEC system for peak
204 resolution was a Waters Alliance e2695 HPLC (Milford, MA, USA) equipped with refractive index
205 detector (RI), autosampler (40 °C), and column heater (90°C), as described by (Fahy B., Gonzalez O.,
206 Savva G. M., et al., 2022).

207 The isocratic mobile phase was prepared with DMSO with 0.5% LiBr (w/w) at 0.500 mL/min flow rate.
208 Two analytical columns (10 um; 300 Å followed by 30 Å; 8 x 300 mm, GRAM; Polymer Standard
209 Service, Mainz, DE) with a guard column (8 x 50 mm, GRAM; Polymer Standard Service, Mainz, DE)
210 were used as stationary phase. The total run time was 65 minutes and injection volume for each
211 sample was 50 µL. Calibration curves were generated using pullulan standards (PSS-pulkit, Polymer
212 Standard Service, Mainz, DE) with peak molecular weights ranging from 342 to 708,000 Da and
213 correlation coefficients of R2 = 0.9993 ± 0.0005. The relationship between elution volume and
214 hydrodynamic radius (Vh) for the linear glucans was determined using calibration curves described
215 similarly by Cave, *et al.*(Cave, Seabrook, Gidley, & Gilbert, 2009*)*. The debranched starch samples RI
216 elution profiles were converted to SEC weight distributions as described in detail by Perez-Moral, *et al.*
217 (Perez-Moral, Plankeele, Domoney, & Warren, 2018). The relative peak heights corresponding to
218 amylopectin and amylose chains were calculated as defined by Hanashiro *et al.* 1996 (Hanashiro, Abe,
219 & Hizukuri, 1996) and Vilaplana *et al.* 2012 (Vilaplana, Hasjim, & Gilbert, 2012).

220 **Microscopy**

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

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

228 **Statistical analysis**

229 Amylolysis curves were fitted to a first order equation previously described (Edwards C.H., et al., 2019)
230 using a non-linear regression model. The first order rate constant (*k*) and predicted starch digested at
231 the end of reaction (C_∞) were estimated from the equation, after subtracting the 'endogenous'
232 maltose detected before the start of the reaction (Y_0) from the subsequent timepoints. The

experimental endpoint C_{90} and the incremental Area Under the Curve (iAUC) are reported as additional descriptors of the susceptibility to hydrolysis.

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

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

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

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

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

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

Results

Dough formulation was similar for *sbeII* and WT control breads, however *sbeII* flour required additional water (39% for *sbeII* and 37% for WT control) to produce a workable dough. After mixing,

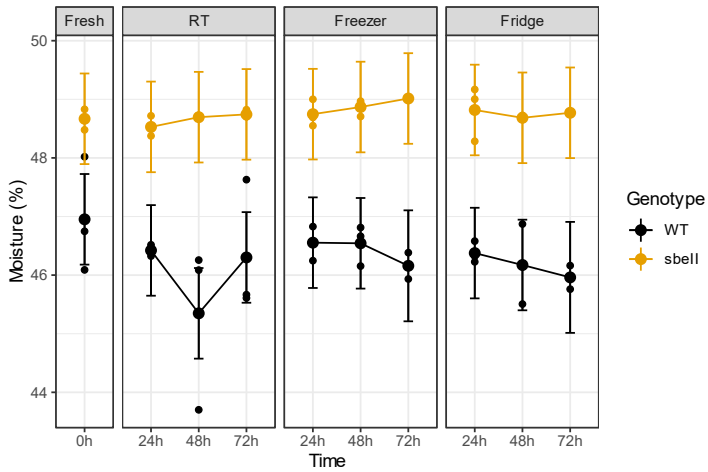
264 the dough was assessed visually and found to be well developed. Both roll types developed and baked
265 well, reaching a core temperature of 95°C.

266 Rolls were made to deliver an equal starch content; the *sbeII* flour was characterised by a lower total
267 starch content, therefore the *sbeII* rolls were ~10 g larger than the WT control rolls (161.26 g ± 0.16 g,
268 155.73 g ± 0.40 g, respectively, mean ± SEM, n=3).

269 **Moisture**

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

272 Freshly baked *sbeII* bread rolls had a small but higher moisture content (48.7%) compared to WT
273 breads (47%; $p = 0.003$). The moisture difference between genotypes was maintained consistently
274 over storage, regardless of the storage conditions (interaction between genotype and storage $p = 0.3$,
275 0.1, 0.1 for RT, Freezer and Fridge).



276
277 ***Figure 2.*** *Estimated marginal means of moisture during storage, n = 3 with error bars representing the 95%CI.*

278 **Starch digestibility in bread**

279 Starch digestibility was defined using the following parameters: the extent of digestibility within 90
280 min of incubation (C_{90}), the predicted starch digested at the end of reaction (C_{∞}), the digestibility rate
281 constant (k) and incremental area under the curve (iAUC), Figure 3D-G.

282 Overall, starch in *sbeII* fresh bread was significantly less digested than the WT control, as shown by the
283 C_{90} and iAUC ($p = 0.005$, $p = <0.001$, respectively), regardless of storage, Supplementary Table 3.

284 Compared to freshly baked bread, storage led to increased starch digested at 90 minutes (C_{90}, $p =$
285 <0.001, for all conditions), in both bread genotypes, but there was no evidence of an interaction effect

286 between storage and genotype on starch digested (C_{90}, p = 0.3), showing that the effect difference
287 between *sbeII* and WT control bread is maintained throughout storage.

288 The extent of starch digestibility (C_{90}) was 51.7% in *sbeII* freshly baked bread compared to 61.4%,
289 61.7% and 60.4% when *sbeII* bread was stored for 72h in the freezer, fridge and RT respectively. In
290 freshly baked WT control bread, starch digestibility was 62.4% and increased to 78.6%, 78.5% and
291 82.1% after 72h freezer, fridge and RT storage, respectively. For samples stored at RT, some evidence
292 of interaction between storage and bread genotype was observed (p = 0.03). Here, C_{90} was higher
293 after 48h storage at RT compared to fresh bread.

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

298 The digestion rate (k) of starch in *sbeII* bread was overall lower compared to starch in WT control
299 bread (p = <0.001) and no effect of interaction between genotype and storage was found (p = 0.1).
300 Compared to fresh bread, RT and fridge storage affected the rate of starch digestion in both bread (p =
301 <0.001, both conditions) but not in breads stored in the freezer (p = 0.2), Figure 3A-C.

302 Considering the iAUC of digestibility curves, besides the significant effect of the genotype (p = <0.001),
303 there was a significant effect of the fridge and freezer storage on iAUC, compared to fresh bread (p =
304 0.002 and p = <0.001, respectively). There was some evidence of an interaction effect between storage
305 and genotype on iAUC however, the magnitude of the effect is relatively small as the iAUC summarises
306 rate (k) and extent of digestibility (C_{90}), (p = 0.0007).

307 The estimated marginal means with 95% CI for all storage conditions are reported in Supplementary
308 Table 3.

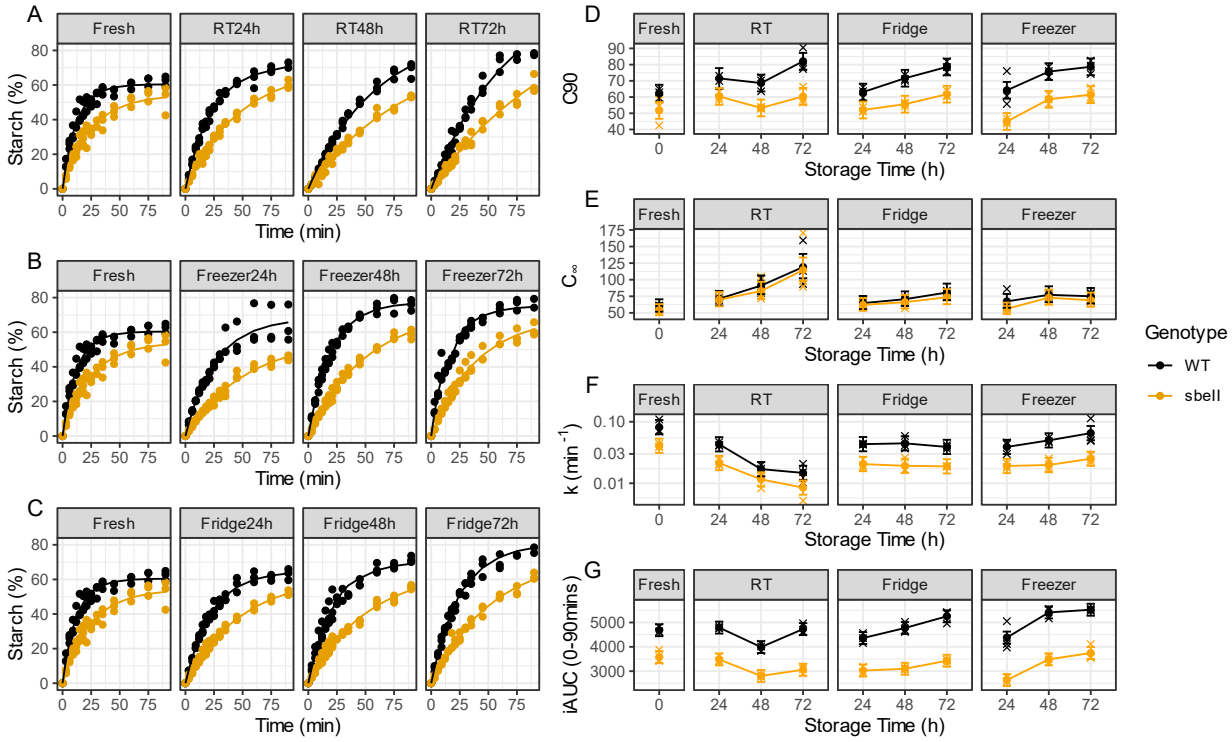


Figure 3 A-C. *Starch digestibility curves of sbeII (yellow) and WT control (black) bread samples, experimental data points represent independently treated samples, n = 3 per condition. Experimental data (replicate datapoints) are shown by fitting a first-order equation based on the estimates of k and 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) iAUC, 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 4 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.

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

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

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

337 ***Chain length distribution***

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

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

346 Chain length proportions per DP fraction are reported as Mean ± SD, n = 3. All fractions can be found
347 in Supplementary Table 5.

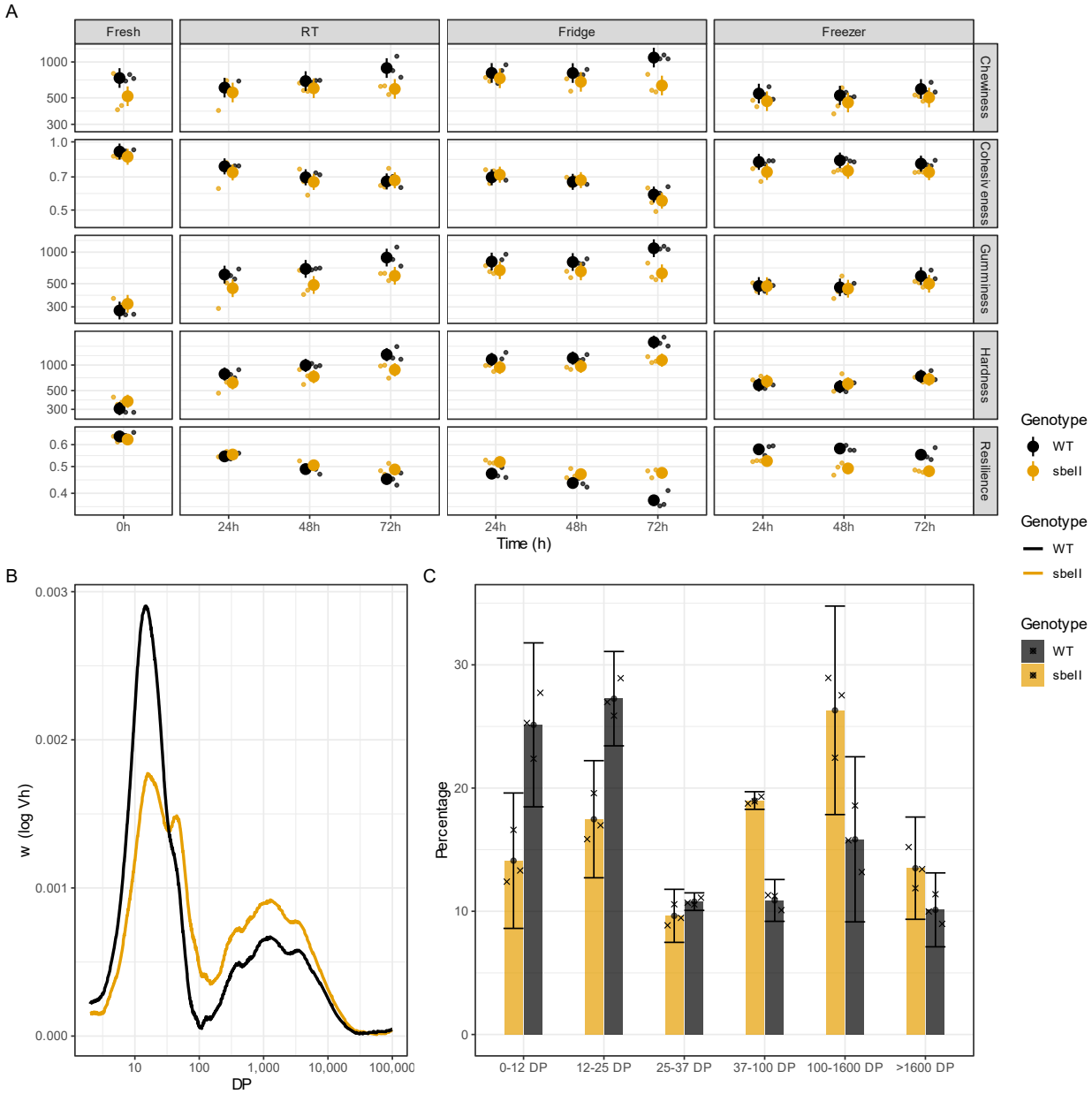


Figure 4. A. *Texture profile of WT and sbeII breads, data points represent the mean value with error bars = ± 95% CI, n = 3 independent bread rolls were analysed per condition, with four technical replicates per bread roll.* ***B.*** *Chain-length distribution of debranched sbeII and WT control starch isolated from flour 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 indirectly assess starch crystallinity in raw flour and in bread.

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

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

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

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

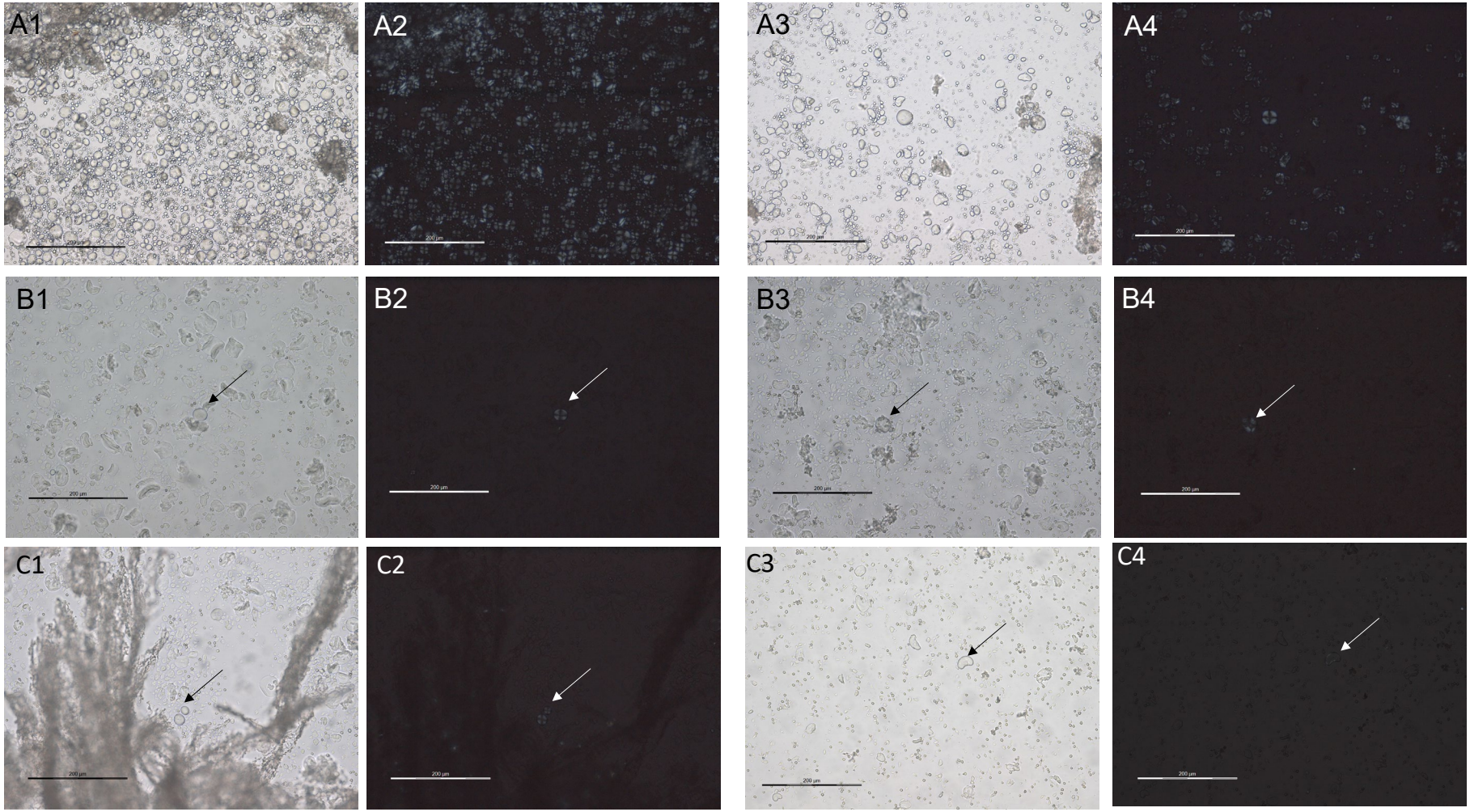


Figure 5. *Micrographs of starch in water using brightfield (BF) and polarised light filter (P) highlighting birefringence of starch granules.* ***1.*** *WT control starch under BF;* ***2.*** *WT control starch under P;* ***3.*** *sbeII mutant starch under BF;* ***4.*** *sbeII mutant starch under P;* ***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.*

374 **Discussion**

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

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

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

392 Overall, the effect of the storage condition can affect *sbeII* starch digestibility early on during storage
393 compared to the WT control, however the difference between *sbeII* bread and WT control is
394 maintained during storage. This could be due to the increased amylose proportion in *sbeII* starch
395 promoting faster retrogradation compared to the WT control. This was also observed in microscopy
396 images, where *sbeII* starch granules in fresh bread and after 7 days of storage in freezer, did not show
397 the birefringence pattern typical of recrystallised granules, as in the WT control.

398 A recent study measured RS content of bread during storage and reported that bread made from
399 partial substitution (50%) of conventional white flour with high-amylose wheat flour reaching 51.5%
400 amylose content, and stored in the fridge for up to 5 days (Li C. & Gidley M.J., 2022). Interestingly,
401 they did not observe an increase in RS content of bread during storage. We observed the starch in
402 *sbeII* bread remained less susceptible to amylolysis compared to the WT control. When left at RT, the
403 rate of starch hydrolysis of both bread types decreased within three days of baking, compared to fresh
404 bread while that starch digestibility (C_{90} and iAUC) in bread stored in the fridge increased over time
405 compared to fresh bread however, this may be due to variation in the moisture content of samples

406 stored. The study design did not allow to determine moisture on ground bread crumb samples at each
407 storage timepoint. This was due to time constraints in carrying out the digestibility experiments over
408 several days with the different storage conditions and a sufficient number of replicates. Therefore,
409 moisture was measured on fresh samples only (when bread sampling). This could have led to
410 overestimation of starch digestibility for the samples stored and it is a limitation of the study.
411 However, since both *sbeII* and WT control breads have been treated the same way, the effect size
412 measured in this study is not affected. The single enzyme assay used to determine starch digestibility
413 here is a sensitive method and may be able to detect subtle differences compared to the RS AOAC
414 method by producing several experimental parameters to characterise the digestibility curves
415 providing accurate estimates of digestion. The percentage of starch hydrolysed by α-amylase, as
416 reported in this study (C_{90}), has been shown to correlate well with glycaemic index measurements in
417 human participants (Edwards C.H., et al., 2019), suggesting that the lower starch digestibility of *sbeII*
418 starch in bread has potential for applications in frozen bread, as shown by a previous study (Corrado
419 M., et al., 2022), but also when bread is consumed fresh, or after storage in the fridge or at room
420 temperature.

421 The higher hardness of *sbeII* fresh bread compared to the WT control is has been attributed to the
422 presence of increased amylose content in *sbeII* starch (Goesaert, Slade, Levine, & Delcour, 2009) and
423 its ability to form double helices or aggregates of helices. Research in gluten free breads using high
424 amylose rice flour has shown that lower amylose content rice types led to softer crumb (Roman,
425 Reguilon, Gomez, & Martinez, 2020), like the softer freshly baked WT control bread in this study when
426 compared to the freshly baked *sbeII* bread. Future research studies investigating structural
427 organisation of starch chains may help clarify this.

428 Based on the texture measurements reported in this study, macrostructural changes to the crumb
429 texture set in earlier during storage in *sbeII* bread compared to the WT control. Overall, texture
430 changes in *sbeII* bread crumb were limited compared to the WT control crumb, suggesting a more
431 stable structure over storage. This may be due partly to the water, as a crystallized rigid starch
432 network formed during staling can trap bound water favouring crumb firmness (Hemdane, et al.,
433 2017). While moisture of bread as a whole did not vary significantly during storage, migration of water
434 from the protein to the starch fraction may have led to the strengthening of the starch network,
435 favouring water retention and greater crumb firmness (Bosmans G. M., Lagrain B., Ooms N., Fierens E.,
436 & Delcour J. A., 2013), though water migration was not examined in detail in this study. However, the
437 marginally higher water content of *sbeII* breads, due to the known higher water holding capacity of
438 the *sbeII* flour, may have contributed to the limited crumb firming within the first three days of
439 storage. As previously shown, moisture levels between 40%-50%, as the breads in this study, are ideal

for starch crystallisation as it promotes network plasticisation and favouring retrogradation (Zeleznak & Hoseney, 1986). Similarly, Li and Gidley (Li C. & Gidley M.J., 2022) showed that substitution of conventional wheat white flour with 50% high-amylose (51.5%) flour in bread making does not lead to a noticeable increase in bread firmness over storage in the fridge. Barros *et al.,* reported that 10%-20% wheat flour replacement with HI-MAIZE 260® in bread making delayed bread staling by decreasing the rate of starch recrystallisation, without a negative impact on bread quality (Barros J. H. T., Telis V. R. N., Taboga S., & Franco C. M. L., 2018). Evident changes in crumb firmness have been shown to be linked to amylopectin retrogradation, especially at freezer temperatures, (Bosmans G. M., Lagrain B., Fierens E., et al., 2013) and independent from the moisture loss (Ribotta P.D., et al., 2003).

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

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

This is promising for potential use of this wheat in production of sandwich bread as it is common to use dough improvers to decrease the progression of staling and reduce bread firming (Hug-Iten, Escher, & Conde-Petit, 2003). In this study, the formulation, particularly the amount of water used to 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., et al., 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. The dough produced with both flour types were well developed. 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.

488 **Conflicts of interest**

489 The authors declare no conflicts of interest.

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