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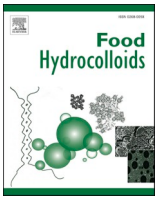

Starch characteristics and baking quality of chilled ready-to-eat sandwich bread made with *starch branching enzyme II* mutant wheat flour

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ABSTRACT

Suitability of high-amylose *starch branching enzyme II* (*sbeII*) flour for industrial processing of wheat convenience foods (i.e., ready-to-eat chilled sandwich bread) is not known, specifically its impacts on bread quality and starch digestibility over chilled storage. Here we evaluated *sbeII* wheat quality in an industrial pilot plant using Chorleywood bread processing. Industrially-made *sbeII* bread showed lower volume upon production, and after chilled storage had lower starch digestibility (~4% difference of starch digested at 90 min) and more resilient crumb texture compared to a wildtype (WT) control. *sbeII* breads made in a laboratory scale using an optimised AACC method also showed lower starch digestibility when analysed fresh and after chilled storage. Short-range molecular orderring (an indicator of starch crystallinity measured by ^{13}C solid-state NMR) was lower for both fresh and stored bread, which suggested that the enzyme-resistant structures in *sbeII* bread were independent of starch retrogradation induced by storage.

Author contributions

Marina Corrado: Conceptualization, funding acquisition, investigation, methodology, project administration, data curation and formal analysis, writing – original draft preparation, visualization, writing – review & editing. **Todor T. Koev:** investigation, methodology, visualization, writing – review & editing. **George M. Savva:** methodology, data curation, formal analysis, writing – review & editing. **Yaroslav Z. Khimyak:** conceptualization, funding acquisition, methodology, supervision, writing – review & editing. **Brittany A. Hazard:** Conceptualization, funding acquisition, methodology, supervision, writing – review & editing.

1. Introduction

Structural modification of starch *in planta* is a valuable method for developing *ad-hoc* starch phenotypes with applications in a variety of industries, from food production to packaging. While the use of high-amylose wheat flour from *starch branching enzyme II* (*sbeII*) mutants in breadmaking is a potentially viable alternative to conventional high-glycaemic wheat bread (Corrado, et al., 2022), more information is required to understand its behaviour in commercial applications.

Commercial wheat-based products made from medium or high moisture doughs such as sandwich bread can be sold freshly baked or refrigerated (chilled). Chilled sandwiches typically remain on sale for up to three days after production after which they are discarded. Therefore, understanding the impact of novel wheat flours on starch and texture characteristics of ready-to-eat sandwich bread requires studying bread on production day and within 72 h of baking, when stored at fridge temperature. A previous study showed that starch in high-amylose *sbeII* bread is consistently less digestible than a wildtype (WT) control when the bread is freshly baked as well as after chilled storage, and is less prone to storage-induced changes in crumb texture (Corrado, et al., 2023). It was also shown that resistant starch (RS) content in breads does not change during storage, suggesting that starch that is resistant to digestion may be formed independent of storage and amylopectin retrogradation (Li & Gidley, 2022). This shows potential to produce chilled ready-to-eat sandwiches with a lower glycaemic impact bread than conventional white wheat bread.

However, in our previous study, bread was produced using a modified straight-dough method resembling home-baking bread rather than industrially produced sandwich bread. In the UK, industrially produced sandwich bread is obtained using the Chorleywood bread process (CBP),

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Abbreviations

AACC	American Association of Cereal Chemists (Cereals & Grains Association)
CBP	Chorleywood Bread Process
CP/MAS	cross-polarisation magic angle spinning
CPSP/MAS	cross-polarisation-single pulse magic angle spinning
RS	Resistant Starch
RVA	Rapid Visco Analysis
QIB	Quadram Institute Bioscience
WT	Wildtype

a rapid method relying on a high speed mixing under pressure controlled conditions, a quick fermentation (or proving) and baking in metal tins.

Other authors showed that depending on the bread making method, texture of the final product can vary. For example, Li and colleagues showed that freshly baked high-amylose bread (with amylose content similar to that in our study) made using a kitchen bread-maker and CBP had similar firmness to the control bread. Comparing the texture obtained from the two bread making methods, breads made with the bread-maker showed greater firmness compared to the CBP (Li, Dhital, & Gidley, 2022).

In this study, we determined starch characteristics and product quality of bread made with *sbeII* wheat flour on production day (P0) and after 48 h of chilled storage (P2 at +4 °C to +5 °C), compared to a control bread from WT wheat flour.

To investigate the presence of starch that is resistant to digestion and formed independently of retrogradation during storage of freshly baked bread, sandwich bread was produced on a laboratory scale using an AACC optimised method for CBP. We measured starch susceptibility to amylolysis, in freshly baked bread (P0) and after chilled storage (P2), where starch retrogradation is accelerated. We then probed the ordered double-helical content and molecular mobility of starch in the breads by solid-state ^{1}H–^{13}C cross-polarisation magic angle spinning nuclear magnetic resonance (CP/MAS NMR).

We also investigated the micro- and macrostructure of CBP sandwich bread made from *sbeII* wheat flour compared to a WT control in an industrial pilot plant; the plant reproduces accurately industrial CBP bread production but on a smaller scale. Rheological and pasting properties of flour were used to optimise the bread making method aiming to achieve an acceptable quality of bread according to industry standards. We then measured the texture of the baked product to determine its quality when fresh and after chilled storage, and starch digestibility.

2. Materials

A *sbeII* mutant bread wheat and a WT control bread wheat (*Triticum aestivum* L. ssp. *Aestivum* cv. Lassik) were sown in 2019 in a field trial at the John Innes Centre Church Farm field station (Bawburgh, UK), using a randomised block design. The *sbeII* bread wheat mutant used was generated in the hexaploid wheat cultivar Lassik and previously described by Schönhofen, Hazard, Zhang, and Dubcovsky (2016). Wheat grains were debranned and milled on a Bühler mill with feeding rate of 100 g/min at ADM milling (Bristol, UK) with ~16.4% humidity.

3. Methods

3.1. Study design

Harvested grains were combined and milled into one batch of flour. The following analyses refer to samples taken from a single batch of flour and independently measured.

Starch characteristics (total starch, amylose content) were measured on raw flour or starch isolated from raw flour, while dough rheological and pasting properties were determined on flour after adding water to form a dough.

A portion of this flour was allocated to make industrial CBP bread, where one single dough was mixed and portioned into six loaves of ~450 g per genotype; three loaves were used to measure crumb texture and starch digestibility at Quadram Institute Bioscience (QIB) and two loaves were used to determine bread making performance of flour according to industry parameters (volume, density, overall texture and crumb structure).

Another portion of the same flour was used to make bread at QIB using an adapted AACC CBP method. For each wheat flour genotype, three batches of dough were made on three consequent days for a total of nine bread loaves. On each day the three loaves were allocated to a position in the oven (A, B or C). Bread A was analysed on production day (P0), bread B was analysed at P2, after 48 h of chilled storage, and bread C was used for NMR analysis. Breads were matched by proofing, baking and storage position to reduce variability due to the processing. Each analysis was carried out with three independent replicates (loaves). Digestibility and bread texture experiments were carried out with two and four technical replicates respectively, per bread loaf.

3.2. Total starch quantification

The proportion of total starch in flour was determined using a 'Total starch kit' (KTSTA-100A DMSO format, AOAC 996.11, Megazyme International, Wicklow, Ireland), $n = 4$. An estimate of starch content (by difference) is also reported in the proximate analysis of flour.

3.3. Starch isolation and amylose quantification

Starch isolation and amylose determination ($n = 3$) were carried out on *sbeII* and WT control flours as described previously, (Corrado, et al., 2020).

3.4. Dough rheological properties

Mixolab® was used to measure rheological properties of dough under dual stress of mixing and increasing and decreasing temperature between 20 and 90 °C. Briefly, flour was added to the MixoLab bowl and mixed with water. To measure viscoelasticity, dough mixing started at 30 °C with constant speed of 190 rpm, and was then heated to 90 °C over 7.5 min at the rate of 8 °C/min. Sample dough was held at 90 °C for 6 min, cooled over 5 min to 50 °C at the rate of −8 °C/min and finally held at 50 °C for 4 min.

Pasting properties of samples were measured using a Rapid Visco Analyser 4800 (SN 216HT1−48A) according to the standard procedure in AACC method 76−21. Flour samples were weighed into aluminium RVA canisters followed by addition of distilled water. Samples were dispersed by spinning at 960 rpm for 10 s, and then held at 50 °C for 1 min, heated to 95 °C at ~10 °C/min, then held at 95 °C for 3 min. Samples were then cooled to 50 °C at ~ −10 °C/min and held at 50 °C for 2 min.

3.5. Industrially made CBP

A standard CBP method using a pressure mixer was used in an industrial pilot plant to produce CBP bread loaves. Mixing time was determined based on consistency of the dough. Formulation and process are summarised in Table 1. Industrially made loaves were used to determine starch digestibility. During product development, the quality tests reported below (3.6 Bread quality on industrially-made loaves) were carried out on the industrially-made loaves to ensure a correct dough processing and an optimised end-product.

Table 1
Formulation and processing conditions for industrially-made CBP bread.

Formulation for 6 loaves g, (% flour basis)	WT control	*sbell*
Flour	2500	2500
Water	1580 (63.2%)	1920 (76.8%)
Salt	40.0 (1.6%)	40.0 (1.6%)
Yeast	83.0 (3.3%)	83.0 (3.3%)
Improver	50.0 (2.0%)	50.0 (2.0%)
Preservative	5.0 (0.2%)	5.0 (0.2%)
Emulsifier	11.0 (0.44%)	11.0 (0.4%)
Procedure		
Mixing time (sec)	245	238
Proving time (min)	52:00	52:00
Bake time (min)	30:00	30:00
Bake temperature (°C)	250	250

The bread formulation is reported on a flour-basis.

3.6. Bread quality on industrially-made loaves

3.6.1. Bread volume and density

The specific volume of bread loaves was determined as the vol/wt ratio using a TexVol Instruments BVM-L450 following manufacturer instructions.

3.6.2. Overall texture

Crumb quality was evaluated externally according to industry standards. For texture, a TA-XT2i was used; hardness and elasticity were calculated from the force used to compress two slices of bread per loaf, twice. No other indicators were measured here as industry standards were based on the hardness (g) as indicator of bread firming and elasticity (percentage of sample height recovery after compression obtained by measuring the height of the sample before and after the compression) indicating resilience to touch or slicing.

3.6.3. Crumb structure

A C-Cell Baking Quality Analyser (C-Cell Version 2, Model CC.200.06) was used to evaluate bread slices for size, shape, colour and internal structural parameters. Following manufacturer instructions, bread slices were analysed for slice area, average slice height, wall thickness (wall of cells in the slice thickness), cell diameter, total concavity, average cell elongation, slice brightness, cell density, average top shoulder, and number of cells.

3.7. AACC adapted method for CBP bread making

To mimic high speed mixing used in the CBP method, a Magimix blender was used to form the dough instead of a traditional mixer with hook attachment. All ingredients described in Table 2 were mixed at once, mixing time is reported in Table 2. The formulation was adjusted to match the breads for starch content (~50 g) allowing for a direct comparison of starch characteristics described later. During the fermentation step a series of punches were carried out as described by the standard AACC method 10–10.03 (AACC International, 1999b), at 52, 25, 13 min, respectively. Loaves were shaped and transferred to a greased baking tin before the final proof, then baked as described in Table 2. Baked loaves were cooled for 2h at room temperature (19–21 °C) before packaging.

Table 2
Formulation and processing conditions for AACC adapted bread.

Formulation to produce 3 loaves (g, % flour basis)	WT	*sbeII*
Flour	216.2	229.0
Water	134.0 (62%)	168.0 (73%)
Yeast	5.4 (2.5%)	5.7 (2.5%)
Salt	3.2 (1.5%)	3.4 (1.5%)
Shortening	6.5 (3%)	6.9 (3%)
Improver	2.2 (1%)	2.3 (1%)
Sugar	7.6 (3.5%)	8.0 (3.5%)
Procedure		
Fermentation time (min)[a]	90	90
Proving time (min)[b]	20	20
Bake time (min)	20	20
Bake temperature (°C)	185–190	185–190

The bread formulation is reported on a flour-basis.
[a] 40 °C, 80% relative humidity.
[b] 40 °C, 100% relative humidity.

Sampling was carried out as follows. **For texture and digestibility analyses,** the bread crust was removed from each loaf and the crumb was sliced into four 25 × 25 × 25 mm cubes used for texture analysis. Immediately after texture analysis, the cubes of crumb were ground using a Kenwood Minichopper and sieved using a 1 mm sieve. The fraction below 1 mm was used for starch digestibility; the fraction above 1 mm was used for moisture analysis. Moisture content of bread crumb samples was measured by the air-oven drying (AACC 44-15 A), one stage procedure (AACC International, 1999a).

Considering the small amount of material required for **NMR analysis** (~120 mg), P0 and P2 samples were taken from the same bread (C). To this end, samples were taken from the loaf core by removing a section of crust from the bottom. The crust section was then reapplied and the loaf was packed in a sealed bag and stored in the fridge. At P2, the same proceedure was performed to take another sample from the core.

3.8. Texture analysis

Crumb texture was measured on bread crumb on production day (P0) and after 2 days of chilled storage (P2), of loaves produced according to the adapted AACC CBP method. Texture was measured instrumentally using a 'two-bite test' for 3 independent loaves per genotype and storage (P0, P2) on a TA-XT2 Texture Analyser (Stable Micro Systems, Godalming, UK), equipped with a 5-kg load cell using a modified AACC method 74-09. The Texture Analyser was equipped with a 50 mm diameter compression plate (P50); a uniaxial compression with crosshead speed of 100 mm/min was applied to 25 × 25 × 25 mm samples to mimic mastication, with crumb hardness corresponding to the force (N) required for 40% compression. Exponent (version 6.0, Stable Micro Systems, Godalming, UK) software for texture profile analysis was used to assess the following texture parameters: *hardness*, *springiness*, *cohesiveness, gumminess*, *chewiness* and. The parameter gumminess will not be discussed here as it does not apply to solid foods but semi-solid only.

3.9. Starch digestibility

Starch digestibility was measured as previsously described by Corrado et al., 2022.

Sieved bread crumb was weighed in a tube to achieve 5.4 mg/mL starch in Phosphate-Buffered Saline (PBS,~pH 7.4). Where improver containing starch was used, the improver starch content was accounted for when sampling. Samples were incubated at 37 °C with end-over mixing and 2 U/mL of porcine pancreatic α-amylase per incubation mix were added to start the assay, after taking a baseline measurement of endogenous maltose (Y0). Samples (100 μl) were taken at 0 (before adding the enzyme), 3, 6, 9, 12, 15, 18, 21, 25, 30, 35, 45, 60, 75, 90 min of incubation and added to tubes containing 100 μL of Na_2CO_3 to stop the reaction. Reducing sugars obtained from α-amylase hydrolysis of starch were measured using 'PAHBAH' (p-hydroxybenzoic acid hydrazide) colorimetric method, (Edwards, Cochetel, Setterfield, Perez-Moral, & Warren, 2019). Samples were centrifuged at 15,000×*g* for 5 min; the supernatant was diluted 1:10 with deionised water to 100 μL and incubated in a boiling water bath for 5 min with 1 mL of PAHBAH reagent. For the standard curve, 1 mM maltose in water was used to make up standard solutions (0–1 mM) and incubated with PAHBAH reagent with the digestion samples.

Absorbance was read in a microplate reader (VersaMax Microplate Reader, Molecular Devices, LLC., CA, USA) at 405 nm and maltose

equivalent concentration in samples were calculated using the maltose standard curve.

3.10. Solid-state NMR

Solid-state $^{1}H–^{13}C$ cross-polarisation magic angle spinning (CP/MAS) NMR experiments were carried out for the bread samples using a Bruker Avance III 300 MHz spectrometer, equipped with an HX 4-mm probe, operating at a^{13}C frequency of 75.54 MHz, and MAS rate of 6 kHz. Approximately 120 mg of each bread sample (fresh and stored) were packed directly inside a 4-mm zirconium oxide rotor with a Kel-F end cap. The $^{1}H–^{13}C$ CP and CP/SP MAS NMR experimental acquisition parameters were $\pi/2$ ^{1}H rf pulse of 3.20 μs and $\pi/2$ ^{13}C rf pulse of 4.40 μs, a contact time of 2000 μs, a recycle delay of 5 s, with a minimum of 5120 number of scans. The ^{13}C chemical shifts were referenced externally with respect to tetramethylsilane (TMS). The spectra were measured at *ca.* 22 °C. Fresh bread samples were packed in the rotors within an hour of baking, whereas stored bread samples were packed following a 48-h storage period at 4 °C. Molecular mobility across all ^{13}C environments was calculated as shown below (Equation (1)).

$$\%\ Mobility = \frac{I_{CPSP} - I_{CP}}{I_{CPSP}} \times 100 \quad (1)$$

where I_{CPSP} and I_{CP} are the ^{13}C peaks' normalised intensity values in their CPSP (cross-polarisation-single pulse) and CP/MAS NMR spectra, respectively (Koev, Muñoz-García, Iuga, Khimyak, & Warren, 2020).

3.11. Statistical analysis

3.11.1. Bread quality

C-Cell parameters were compared between groups (genotype) using a independent *t*-test with 95% CI. Results are reported as the mean of n = 3 independent replicates (loaves).

Texture parameters were compared across groups using a mixed-effects model per 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 'days since production', the main effect of genotype and the interaction between 'days since production' and genotype as well as a random intercept to account for having multiple data points from each loaf.

Amylolysis curves were fitted to a first order equation (Edwards, et al., 2019) using a non-linear regression model ($Starch \sim (C\infty * (1 - exp(-k * Time)))$). The first order rate constant (k) and predicted starch digested at the end of reaction (C_∞) were estimated from the model, after subtracting the 'endogenous' maltose detected before the start of the reaction (Y0) from the subsequent timepoints. The experimental endpoint C_{90} and the incremental Area Under the fitted Curve (iAUC) are reported as additional observed descriptors of the susceptibility to hydrolysis.

Amylolysis parameters (C_{90} and iAUC, k and C_∞) were compared between bread groups with a linear regression model with main effects of days since production and genotype and an interaction term of days since production by genotype for CBP industrial bread. Parameters k and C_∞ were log transformed, then a mixed-effects model was used for each amylolysis parameter of AACC optimised bread method, using the lmerTest R package (version 3.1.2), with days since production and genotype as fixed effects along with an interaction term of days since production by genotype, and bread batch as a random effect.

3.11.2. Predictive model, short-range order and mobility calculation

Short-range starch molecular ordering was estimated using the method described by (Flanagan, Gidley, & Warren, 2015). In brief, the $^{1}H–^{13}C$ CP/MAS NMR spectra were subjected to partial least squares (PLS) fit using a large library of experimental $^{1}H–^{13}C$ CP/MAS NMR spectra of both raw granular and processed starches of various botanical origins, featuring all crystalline polymorphs (A-, B- and V-type). The short-range ordering of the samples was obtained from the fit. Estimation of mobility levels across all peaks of interest was calculated as in Koev et al. (2020). Short range-order percent and mobility percent were compared across groups (for genotype and storage) using a mixed model, using the lmerTest R package (version 3.1.2), with days since production and genotype as fixed effects along with an interaction term of days since production by genotype, and bread batch as a random effect.

Annotated code and source data are available as Supplementary material.

4. Results

4.1. Flour quality

Milling parameters, flour proximate analysis and solvent retention capacity are reported in electronic supplementary information (ESI), Tables 2–4

The total starch content of the *sbeII* flour (65.5% ± 1.5%) was slightly lower than the WT control flour (69.4% ± 1.0%) (mean ± SEM, n = 4). As expected, the apparent amylose proportion measured by iodine-binding method on starch isolated from flour was greater for *sbeII* starch (43.5 ± 0.4% of total starch, mean ± SEM, n = 3) than the WT control (27.2 ± 2.0% of total starch, mean ± SEM, n = 3). Moisture content of the flour measured at the time of starch analyses at QIB was ~15.3% (*sbeII*) and ~14.6% (WT).

Fig. 1A shows the RVA profile, while Fig. 1B shows the dough performance under stress obtained from the Mixolab. The RVA analysis of *sbeII* flour showed a lower average viscosity compared to the WT flour indicating lower retrogradation. The peak viscosity (~835 cP) was reached at 6.13 min for the *sbeII* flour, compared to ~1618 cP at 6.07 min for WT flour, as the higher proportion of amylose in the *sbeII* flour required increased temperature for starch granules to swell completely. The breakdown value (calculated as the difference between peak viscosity and holding strength, the lowest value reached during the holding stage) was 26 cP for *sbeII* flour compared to 494 cP for the WT control, indicating lower resistance to shear force for the *sbeII* paste. The gel stability of the *sbell* flour did not vary during the holding stage suggesting a good gel strength, unlike the WT control where viscosity dropped possibly because of lower protein strength. The total setback (calculated as the difference between final and peak viscosity) of *sbeII* flour was ~8-times lower than the WT control, suggesting lower retrogradation due to lower viscosity observed during heating, however this was only estimated as the final viscosity was not reached during the standard analysis time.

The viscoelasticity profile from the Mixolab showed that dough made from both flour types formed correctly following water absorption, in the first mixing stage. This was followed by a decrease in torque after ~5 min of mixing, indicating protein weakening. The minimum torque was reached in the range of 77–79 °C, the *sbeII* flour sample showed slightly more protein weakening at this stage than the WT control, (minimum torque = 0.411 Nm and 0.480 Nm, *sbeII* and WT control respectively).

During the following phase of the Mixolab analysis, the temperature increase caused starch to gelatinise resulting in torque increase for both flour types (peak viscosity = 1.328 Nm and 2.078 Nm, *sbeII* and WT control respectively), as the doughs became more elastic. Higher peak viscosities are expected for flour producing higher rise during baking, however the *sbeII* flour reached a lower peak viscosity than the WT control. Peak viscosity was followed by a decrease in torque due to starch granule breakdown. We observed a good stability during the hot gel stage and gradual increase in viscosity in *sbeII* dough, suggesting greater resistance to heat and shear stress. The cooling phase is indicative of dough consistency; the lower torque readings of *sbeII* dough

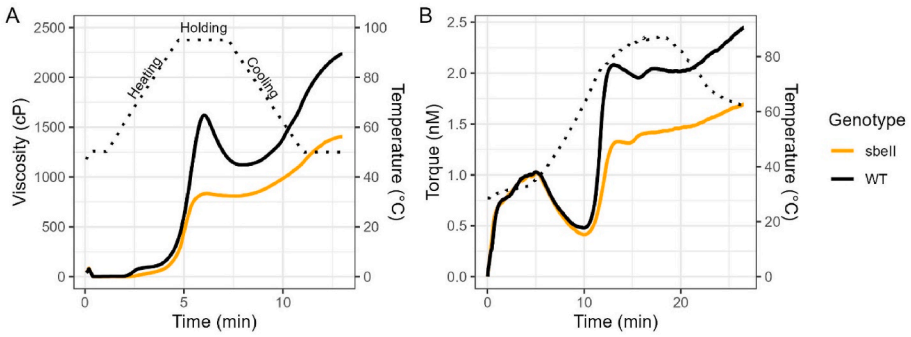

Fig. 1. A. RVA. B. Mixolab. Dotted lines are the dough temperature profile during the analysis.

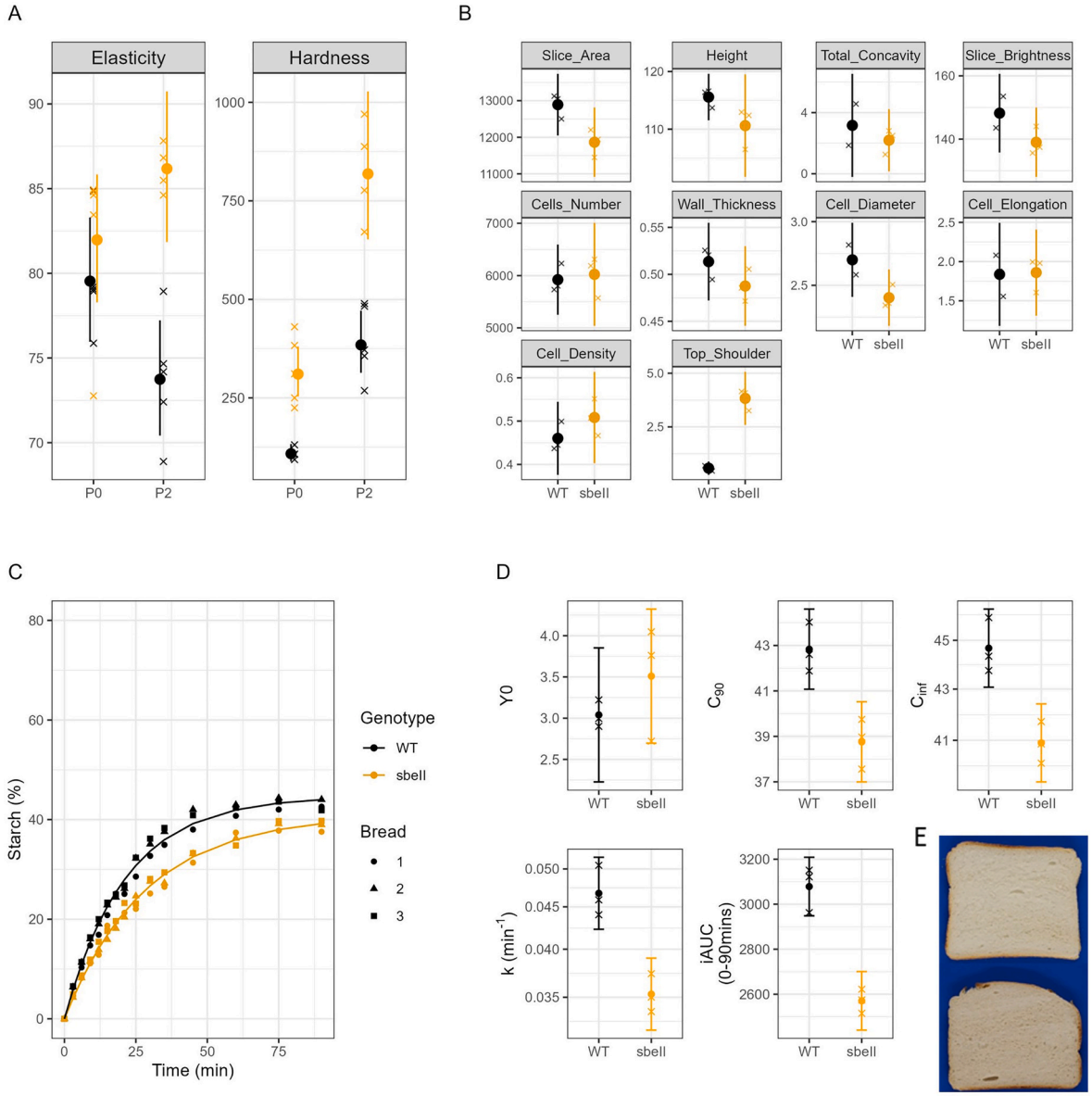

Fig. 2. **A.** Texture parameters measured instrumentally by Texture Analyser, P0 representing bread analysed fresh after baking, P2 representing bread analysed after chilled storage. **B.** C-Cell parameters for crumb structure analysed on freshly baked bread only as indicator of processing acceptability (e.g., bread rise in the oven). **C.** Starch susceptibility to amylolysis curves, sbeII (yellow) and WT control (black) bread samples, experimental data points represent independently treated samples, n = 3 loaves analysed after chilled storage (P2) only due to logistic constraints. 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.** Grouped means of parameters obtained from digestibility curves, C_{90}, C_∞, k, iAUC, error bars represent the 95% CI (n = 3) obtained from the mixed-effects model. **E.** DigiEye images of WT (top) and *sbeII* (bottom) bread slices from freshly baked bread.

suggest a firmer product in applications, compared to the WT control.

4.2. Industrially made CBP bread

Dough mixing time was determined by the dough temperature. To achieve a final dough temperature of 27.8 °C, the total mixing time was 4:08 min for the WT control flour and 3:97 min for the *sbeII* flour.

Total starch content of bread produced industrially were 65.2% ± 1.8% and 63.6% ± 4.4% (as is) with a moisture content of 46.8% ± 1.8% and 50.2% ± 0.2%, for WT and *sbeII* bread loaves (n = 3 independent samples, mean ± sd). Loaves produced were approx. 472.5 g and 494.2 g unbaked (WT and *sbeII* respectively) and 444.8 g and 452.6 g once baked, (WT and *sbeII* respectively). Loaves were stored packed in airtight plastic bags leading to a 4.2%–4.6% moisture loss during storage in the fridge.

Bread characteristics measured at P0 showed differences in the baked products. The specific volume (vol/wt ratio) of *sbeII* bread was 3.9 cm^3/g, determined as the vol/wt ratio of baked breads, compared to the WT bread (5.05 cm^3/g). The *sbeII* bread was firmer than the WT control (hardness = 310.4 ± 29.6, 108.3 ± 10.3 respectively, p = <0.001) however, crumb elasticity was not significantly different between bread types at P0 (WT = 79.5 ± 1.7, *sbeII* = 81.9 ± 1.7, p = 0.3). With storage, crumb hardness increased in both bread types as expected (WT = 384.4 ± 36.6, *sbeII* = 818.6 ± 87.3, at P2, p = <0.001) and the *sbeII* crumb showed higher elasticity than the WT control (WT = 73.7% ± 1.5%, *sbeII* = 86.1% ± 2.0%, p = 0.001). Overall, the difference in crumb hardness between *sbeII* and WT breads was not significantly affected by storage (storage by genotype interaction effect on hardness, p = 0.1); while differences in crumb elasticity varied significantly depending on storage (storage by genotype interaction effect on elasticity p = 0.01), Fig. 2A. Data is reported as estimated marginal means ± SEM.

The *sbeII* bread was characterised by a higher top shoulder (*sbeII* = 3.8, WT = 0.5, Welch's T-test., p = 0.005) and lower slice area (*sbeII* =

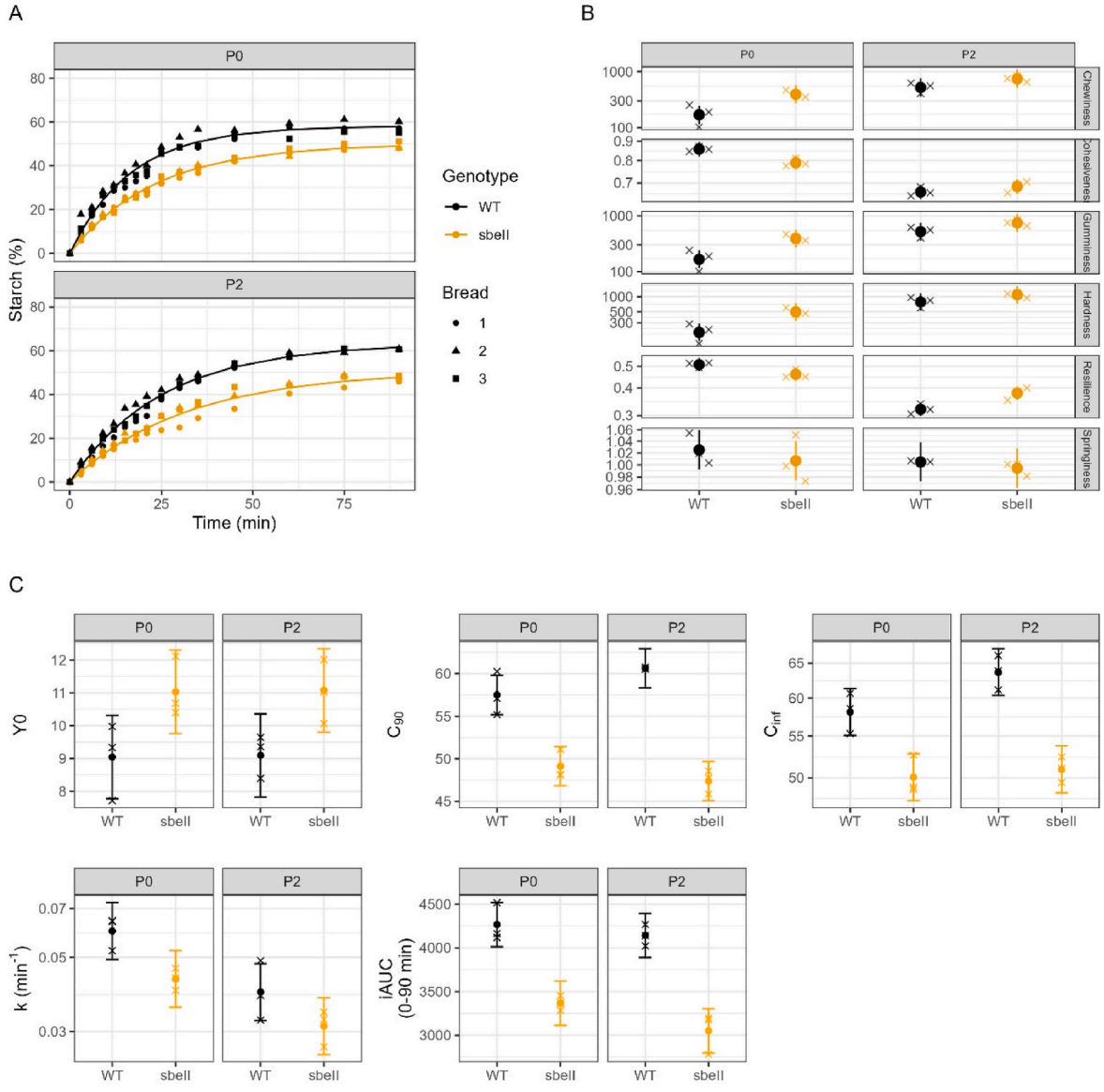

Fig. 3. A. Starch susceptibility to amylolysis curves, *sbeII* (yellow) and WT control (black) bread samples, experimental data points represent independently treated samples, n = 3 loaves. Experimental data (replicate datapoints) are shown by fitting a first-order equation based on the estimates of k and C_∞ values (n = 3 independent samples) obtained from a non-linear regression model. **B.** Texture parameters measured instrumentally by Texture Analyser, shown as mean ± 95% CI and experimental data points (n = 3 independent loaves). **C.** Grouped means of parameters obtained from digestibility curves, C_{90}, C_∞, k, iAUC, error bars represent the 95% CI (n = 3). P0 indicates loaves analysed on production day, P2 indicates loaves analysed after 48h (2 days) of chilled storage.

11864.2, WT = 12891.7, Welch's T-test., p = 0.02). Cell structure was similar, cell diameter was marginally smaller in *sbeII* crumb compared to the WT control but cell elongation and density were similar between breads (Welch's T-test., p = 0.02, p = 0.9, p = 0.2, respectivey), Fig. 2B, E.

Starch digestibility in industrially made bread was analysed after two days of chilled storage (P2), this is shown in Fig. 2C. After 90 min incubation with α-amylase, a lower percentage of starch was digested in *sbeII* bread compared to the WT control (C_{90}, mean difference = 4.1%, p = 0.01). Starch in *sbeII* bread was digested at a slower rate (k) (mean difference = 0.28 min^{-1}, p = 0.005) resulting in a lower iAUC, compared to the WT control (mean difference = 508, p = 0.002), Fig. 2D.

4.3. AACC bread

A total of nine loaves for each genotype were produced for the following analyses, in three subsequent batches.

Moisture of breads after baking was 43.1% for WT loaves and 45.0% for *sbeII* loaves (n = 3 loaves). Processing and bread characteristics are described in ESI Table 1.

Starch digestibility was lower in *sbeII* bread compared to the WT control, as expected (C_{90}, mean difference = 8.4%, p < 0.001, AUC mean difference = 898, p < 0.001), (Fig. 3A), after correcting for the amount of endogenous reducing sugars, which were slightly higher in the *sbeII* bread compared to the WT control (mean difference = 2%, p = 0.04). Amylolysis parameters (Fig. 3C) indicated a lower rate and extent of starch digestion in *sbeII* bread compared to the WT control, when compared by genotype (*k*, mean difference = 0.33, p = 0.02 and $C\infty$ mean difference = 0.15, p = 0.002), and when compared by storage (fresh vs chilled for 2 days), (*k*, mean difference = 0.42, p = 0.006 and $C\infty$ mean difference = 0.09, p = 0.04). There was no evidence of effect of a storage by genotype interaction, when considering modelled parameters *k* and $C\infty$ (p = 0.4 and p = 0.2 respectively) but we observed a significant interaction between storage and genotype when considering C_{90}, explaining 4.9%, difference in reducing sugars measured at 90 min (mean difference p = 0.03), resulting from starch digested.

Starch short-range order (a reflection of the local organization of starch molecules into crystalline arrays) in the flour samples was 12.1 and 22.8% for *sbeII* and WT control respectively. Once baked into bread, the starch in *sbeII* bread was characterised by a significantly lower short-range order compared to the WT control bread, this was 7.01% and 15.5% at P0 and 16.1% and 28.2% at P2 for *sbeII* and WT control, respectively, (genotype effect, p = 0.0002, storage effect, p = 0.0002). No evidence of storage by genotype interaction was observed here (p = 0.24). Comparison of $^{1}H–^{13}C$ CP and CPSP/MAS NMR spectra for breads showed domains of local mobility in C-1, C-2,3,5 and C-6 environments. Starch chains in *sbeII* bread were characterised by a lower mobility compared to the WT control bread, this was 71.0% and 73.5% at P0 and 65.5% and 65.8% at P2 for *sbeII* and WT control, respectively, (genotype effect, p = 0.01, storage effect, p = <0.001). When considering chain mobility, there was some evidence of storage by genotype interaction (p = 0.04). Fig. 4 A1 and A2 show an example of NMR spectra obtained from one independent replicate (one flour sample and one loaf), B and C show the proportion of short-range ordering and molecular mobility observed in bread freshly baked and after chilled storage. All bread NMR spectra are included in ESI Figs. 1 and 2.

Bread produced by AACC optimised method showed similarities with the CBP industrially made bread. The *sbeII* bread was firmer than the WT control at P0 (hardness p = 0.002) when fresh. Chilled storage also had

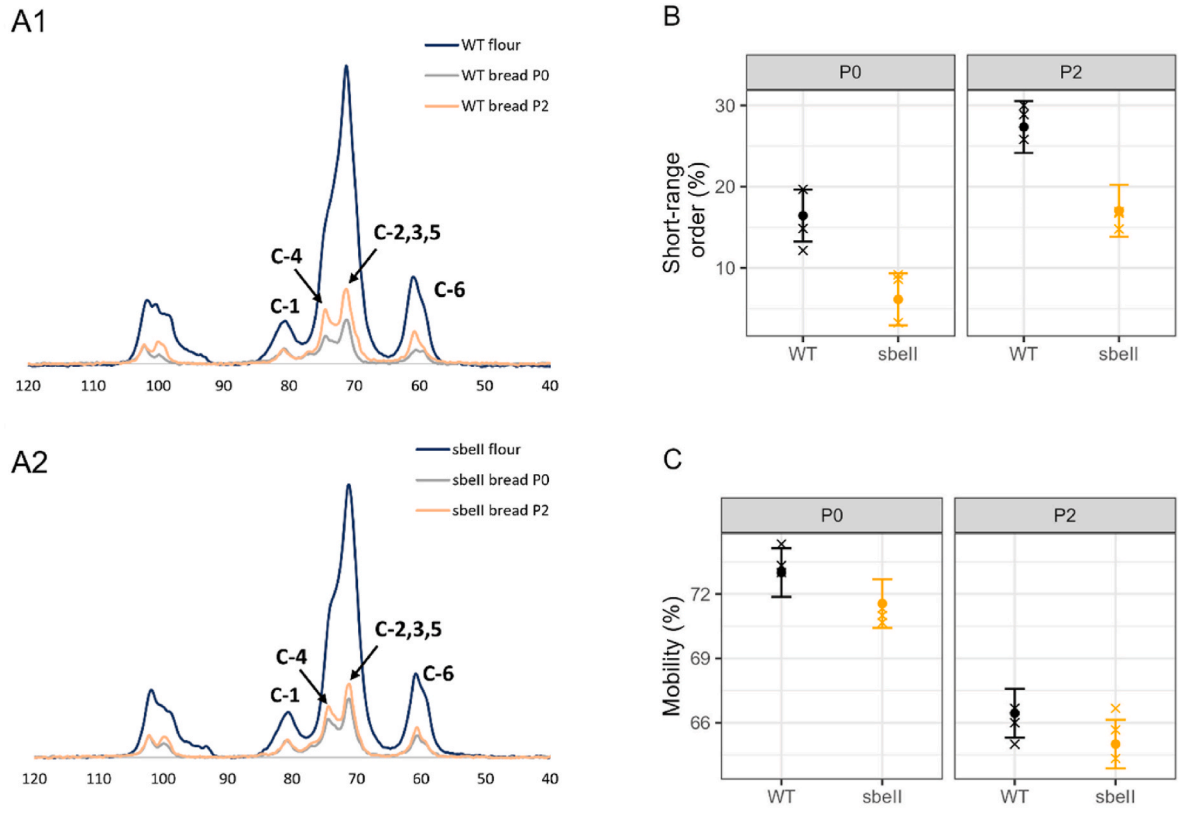

Fig. 4. A1 and A2. $^{1}H–^{13}C$ CP and CPSP/MAS NMR Spectra of WT control and *sbeII* flour, P0 bread (freshly baked) and P2 bread (after 48 h of chilled storage) **B.** Short-range order (%) calculated from $^{1}H–^{13}C$ CP/MAS NMR spectra, shown as mean ± 95% CI of n = 3 independent loaves. **C.** Molecular mobility (%) calculated from $^{1}H–^{13}C$ CP and CPSP/MAS NMR spectra, and reported as mean ± 95% CI of n = 3 independent loaves. P0 indicates loaves analysed on production day, P2 indicates loaves analysed after 48 h (2 days) of chilled storage, *sbeII* (yellow) and WT control (black) bread samples.

an effect on bread hardness which was higher compared to when fresh (P2 vs P0, p = <0.001), however evidence of a combined effect of storage and genotype on bread hardness (storage × genotype interaction) was not strong (p = 0.059). Resilience was higher in *sbeII* bread at P2 compared to fresh bread (P0), (p = <0.001) with evidence of an interaction effect between storage and genotype (p = 0.002). Fig. 3B shows the texture parameters, estimated marginal means and 95% confidence intervals obtained from the model of starch and texture parameters are reported in ESI, Table 5.

Comparing starch amylolysis parameters measured on CBP and AACC bread after 48 h of chilled storage (P2), we observed an effect of genotype for all amylolysis parameters, as expected (ESI Fig. 3). There was a difference between breads made with CBP and AACC methods in the amount of endogenous maltose detected before starting the digestion (Y0, P = <0.001) but no interaction between processing method and genotype was observed (p = 0.2). This may be due to the different improver used in the two formulations; one containing added starch (AACC bread), which was then removed from the improver formulation used in the CBP bread to avoid interferences with starch analyses. A minor effect of the processing method was observed on measurement of C_{90} and $C\infty$ (p = 0.01, p = 0.016, respectively), after correcting for the presence of endogenous maltose at time 0 (Y0), however no significant interaction was found between processing and genotype (p = 0.2, p = 0.4, C_{90} and $C\infty$ respectively). The rate of digestion k obtained from the model and the AUC calculated from the experimental data were not different between processing methods.

5. Discussion

The use of *sbeII* wheat flour to produce CBP sandwich bread resulted in bread with lower starch digestibility compared to the WT control, freshly baked and after two days of chilled storage, as shown by the digestion parameters comparison presented. Breads made using the adapted AACC method and industrial-made CBP were overall comparable. We observed some variation in C_{90} and $C\infty$ digestion parameters across different methods, likely due to different production setting or batches (oven, equipment, manual handling etc.), which can be expected in a less controlled bakery setting, although the differences between genotypes were consistent across methods.

Starch digestibility in *sbeII* CBP bread was significantly lower than the WT control, regardless of where it was produced suggesting that the *sbeII* trait is resilient to processing and may be used in an array of baked products, particularly breads, without altering its starch digestibility. While both storage and genotype contributed to lower the digestibility of starch in *sbeII* bread, there was no significant interaction effect between storage and genotype for most indicators, suggesting that the lower digestibility of starch in *sbeII* bread was not due to the chilled storage. Therefore, the lower digestibility of *sbeII* starch in bread may be independent of structures formed with storage and linked to retrogradation, confirming our previous findings (Corrado, et al., 2023).

Considering the processing characteristics, the primary difference between the *sbeII* and WT bread formulations was the water behaviour, the *sbeII* flour had a higher water absorption so the amount of water was adjusted accordingly to obtain quality loaves. The greater water content in *sbeII* dough was expected to provide plasticization and promote retrogradation (Li & Gidley, 2022; Nivelle, Beghin, Vrinten, Nakamura, & Delcour, 2020) and increase the overall short-range order, measured in this study by NMR (Bogracheva, Wang, & Hedley, 2001; Bogracheva, Wang, Wang, & Hedley, 2002). Starch short-range ordering in *sbeII* bread remained lower than WT control bread, which was previously shown to be associated with lower digestibility (Koev, Harris, Kiamehr, Khimyak, & Warren, 2022). This was the case for both stored and freshly baked bread. The index of molecular mobility, as measured by $^{1}H–^{13}C$ CPSP/MAS NMR (ESI Fig. 2) was shown to decrease on storage by *ca.* 8–13%, where lower degree of molecular mobility has previously been associated with lower degree of digestibility (Koev, et al., 2022). This supports the hypothesis that enzyme-resistant structures may be present in *sbeII* wheat that are independent of retrogradation during storage.

For bread quality, the industrially made *sbeII* bread did not reach full rise during baking, as shown by the image analysis on baked bread (top shoulder parameter) but this could be improved by optimising the formulation and the use of the improver. While *sbeII* was characterised by lower bread volume and cell diameter in the crumb, cell elongation and density were similar to the WT control showing potential for improvement.

RVA and Mixolab analyses showed a lower peak viscosity but good hot gel strength of *sbeII* flour, higher in amylose proportion, compared to the WT control. Sestili et al. (2010) also observed a negative correlation between amylose and viscosity and similar RVA profile for lines with 43.5% amylose content (as ours) and likewise Schönhofen, Zhang, and Dubcovsky (2017) observed lower viscosity for the *sbeII* mutant with 45.2% amylose content, compared to their control. The same behaviours were also observed by Li, Dhital, Gilbert, and Gidley (2020), where the swelling ability of starches was negatively associated with amylose content. Compared to their study, the setback region of *sbeII* flour here was closer to the WT control, a possible consequence of the incomplete swelling of *sbeII* starch granules. This may be due to the higher temperature requirements of high amylose starch granules to gelatinise compared to conventional wheat starch or other non-starch components (e.g., protein) inhibiting the development of viscosity (Li, et al., 2020). A greater proportion of long chains with higher mobility, as observed here, would suggest potential to form domains with some thermostable molecular ordering stable at high temperature and, at the temperature used in this analysis, it is likely that only a partial gelatinisation occurred. A limitation of this study was the duration of the RVA analysis, chosen according to industry standards, and not high-temperature RVA analysis. The *sbeII* wheat flour used in this study had a good content of protein, compared to other flours for bread making, but the protein quality and gluten strength were not investigated. From the Mixolab analysis we also observed slightly higher protein weakening in *sbeII* sample compared to the WT control. This may indicate a weaker gluten network in the *sbeII* dough requiring increased levels of improvers to help gas retention and rise during baking.

Furthermore, based on the dough consistency at the end of the heat/mixing stress phase of the Mixolab test, we expected a firmer baked bread from *sbeII* flour, compared to the WT control which is consistent with the hardness measured instrumentally. Interestingly, compared to our previous study using a home-baking method, we observed opposite effects on firming; previously we found that 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. It should be noted that the use of improvers containing emulsifiers, like in this case, can have a significant effect on bread firming by delaying starch retrogradation and bread staling (Eduardo, Svanberg, & Ahrné, 2016). In the current study we observed that *sbeII* bread was firmer and more resilient after fridge storage, resilience is a desirable quality characteristic associated with bread freshness that suggests potential for use in chilled sandwich breads.

6. Conclusions

Novel high amylose wheat lines have shown great potential to be used in bakery products. In this study we showed that *sbeII* flour is versatile and can be used instead of conventional wheat flour in white sandwich bread making to achieve wheat products with lower glycaemic potency. However, further studies are required to optimise the formulation and achieve higher end-product quality. Improving the formulation may involve taking into account other flour components such as the protein, quality and quantity, or modifying improver components to enhance flour performance. It is also important to note that various *sbeII* mutants have been reported leading to a range of amylose levels, which may impact the extent of effects on bread quality and digestibility

parameters (Botticella, et al., 2018; Slade et al., 2012).

At the meso- and microstructural levels, we were able to investigate starch molecular order/crystallinity using NMR on a complex matrix like bread. This type of analysis was fundamental to separate the effects of processing on starch characteristics from those linked to genetic modification in planta. NMR analysis provided insight in the proportion of ordered material as well as the mobile fraction within a processed food matrix, which were associated with the breads' degree of susceptibility to amylolysis. Here, we observed enzyme-resistant structures that may arise primarily from the structural characteristics of the starch, rather than the processing, making *sbeII* wheat bread suitable for consumption freshly baked or after being stored without losing its lower starch digestibility, expected to lead to lower glycaemic response.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Annotated code and source data are available as Supplementary.

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Appendix A. Supplementary data

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