Development of a high-throughput starch digestibility assay reveals wide variation among the A. E. Watkins wheat landrace collection

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Abstract

Breeding for less digestible starch in wheat can improve the health impact of bread and other wheat foods. Based on an established *in vitro* starch digestibility assay by Edwards et al. (2019) we developed a high-throughput assay to measure starch digestibility in hydrothermally processed samples for use in forward genetic approaches. Digestibility of purified starch from maize and wheat was measured using both methods and produced comparable results. Using the high-throughput assay, we estimated starch digestibility of 118 wheat landraces from the core Watkins collection and found wide variation across lines and elite UK varieties, (20% to 40% and 31% to 44% starch digested after 90 minutes respectively). Sieved flour fractions and purified starch for selected lines showed altered starch digestibility profiles compared with wholemeal flour, suggesting that matrix properties of flour rather than intrinsic properties of starch granules conferred the low starch digestibility observed.

Keywords: wheat, landrace, starch digestibility, natural variation, high-throughput, retrograded starch, starch structure, screening tool, pipetting tool, breeding

51 ## Introduction

52 Wheat (*Triticum aestivum* L.) is one of the most widely consumed crops worldwide and provides up
53 to 50% of the calories in the human diet, so improving its nutritional quality could deliver health
54 benefits to a large number of people (Shewry and Hey, 2015, Hazard et al., 2020). On average, a
55 mature wheat grain contains about 60-70% (w/w) starch, which is the greatest contributor to calories,
56 10-15% (w/w) protein and 11-15% (w/w) dietary fiber (Shewry et al., 2013, Delcour and Hoseney,
57 2010).

58 Starch is produced in the endosperm of wheat during grain filling and is composed of two distinct α-
59 glucan polymers, amylose (linear α-1,4-linked chains) and amylopectin (α-1,4-linked chains with α-1,6-
60 linked branches). In their native state, starch polymers assemble to form partially crystalline granules,
61 which are difficult for humans to digest, thus starch-based foods are typically cooked prior to
62 consumption. Heating starch in the presence of water leads to starch gelatinization making starch
63 polymers more accessible to digestive enzymes (Wang and Copeland, 2013, Chung et al., 2006, Parada
64 and Aguilera, 2009). Subsequent cooling leads to retrogradation in which starch polymers form
65 crystalline structures that are less digestible (Wang et al., 2015, Corrado et al., 2020). Other intrinsic
66 properties of starch, like its molecular structure can also influence starch digestibility, including the
67 length of its glucan chains and amylose-to-amylopectin ratio (Hazard et al., 2015, Schönhofen et al.,
68 2016, Anugerah et al., 2022).

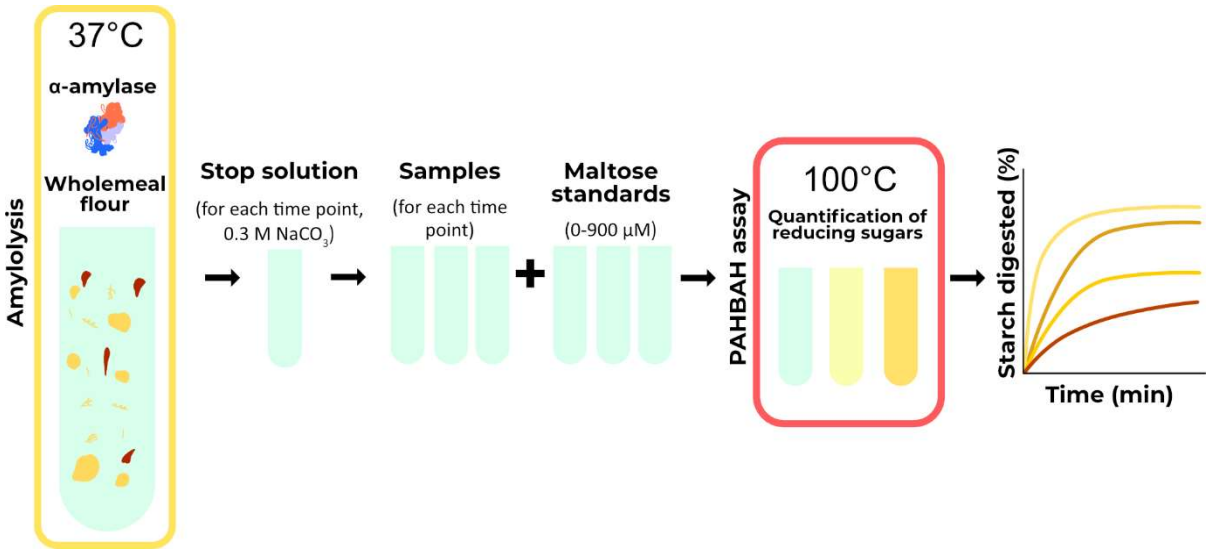
69 Reducing the rate and extent of starch digestibility in wheat-based foods can help to maintain healthy
70 blood glucose levels, which is important for the prevention and management of obesity and chronic
71 diseases like type II diabetes (Blaak et al., 2012). Furthermore, there is substantial evidence that
72 consumption of resistant starch, that is the starch that escapes digestion in the small intestine and
73 reaches the colon, can reduce blood glucose levels and help to maintain a healthy gut (Corrado et al.,
74 2022, Belobrajdic et al., 2019, Hughes et al., 2021). Thus, breeding for wheat with less digestible starch
75 is an important strategy to develop healthier staple foods. So far, reverse genetic studies in wheat
76 have demonstrated potential for increasing resistant starch levels using induced mutations in starch

biosynthesis genes (Botticella et al., 2018, Schonhofen et al., 2017, Hazard et al., 2015, Fahy et al., 2022). However, initial analyses of some mutants have shown detrimental effects on yield and on pasta and bread-making quality, thus identifying additional sources of genetic variation for starch digestibility could support the development of improved traits for commercial breeding applications (Schonhofen et al., 2017, Hazard et al., 2015).

Wheat landraces, locally adapted lines that have not been modified through modern breeding techniques, present reservoirs of genetic diversity that can be introduced into modern cultivars. Of special note is the A.E. Watkins bread wheat landrace collection (Wingen et al., 2014) encompassing 826 bread wheat landraces collected in the 1920s and 1930s from a wide geographic distribution. This collection contains useful variation for a number of agronomic traits and has been used to identify resistance genes for a variety of diseases (Dyck and Jedel, 1989, Bansal et al., 2011, Bansal et al., 2013, Burt et al., 2014). The Watkins lines have been purified by single-seed descent from which many genomic and genetic resources have been developed; a core set of 118 accessions was used to generate nested association mapping populations, all of which were genotyped, have genetic maps available, and are free to access (http://wisplandracepillar.jic.ac.uk/) (Wingen et al., 2017).

Despite the availability of diverse wheat germplasm resources like the Watkins collection, forward screening approaches for starch digestibility have been limited due to the lack of informative, accurate, and efficient phenotyping methods. Screening based on amylose content, which has a positive association with resistant starch content, can identify lines with high levels of resistant starch but cannot identify factors beyond amylose content that may cause resistance to digestion (Chen et al., 2017, Mishra et al., 2016). Only a few studies have developed methods to screen large populations for starch digestibility and they have focused on analyzing purified starch (Wang et al., 2022). However, other components of the wheat flour matrix could potentially impact starch digestibility (Edwards et al., 2014, Sissons et al., 2021, Qi et al., 2022). To our knowledge no previous studies have screened flour samples of germplasm collections.

102 Recently, a new *in vitro* method for measuring starch digestibility was published which produces
103 results that are well correlated with the glycaemic response to foods (Edwards et al., 2019) (Figure 1).
104 This method has proven useful for measuring starch digestibility in mechanistic studies (Edwards et
105 al., 2019) and testing early-stage food products, but modifications and improvements were required
106 to accurately and efficiently use it for screening large wheat germplasm collections. Here we describe
107 the development of a high-throughput starch digestibility assay based on the Edwards et al. (2019)
108 protocol, which can be used for the simultaneous analysis of 96 samples. Using standard samples of
109 wheat and maize starch, we validated the assay by comparing starch digestibility profiles to those
110 produced by the Edwards et al. (2019) protocol. The high-throughput assay was then used to screen
111 processed flour samples of the core Watkins (c.Watkins) collection (Wingen et al., 2014) and
112 commercial wheat lines (for bread, biscuit, and animal feed) which revealed natural phenotypic
113 variation for starch digestibility.



114

115 **Figure 1.** *Principle of the in vitro starch digestibility method. For amylolysis, a known enzyme-substrate*
116 *ratio is used, and starch is hydrolysed by porcine pancreatic α-amylase to produce reducing sugars.*
117 *During amylolysis, aliquots are transferred to a 'stop solution' at predetermined time points to*
118 *inactivate amylase activity. The reducing sugar concentration is quantified using a colorimetric p-*
119 *hydroxybenzoic acid hydrazide (PAHBAH) assay and maltose standards (Lever, 1972). The portion of*

120 *starch digested for each timepoint is calculated based on reducing sugars and is then displayed against*
121 *time.*

122 Materials and Methods

123 Chemicals

124 Chemicals used in this study: Percoll™ (17-0891-01, GE Healthcare), 4-Hydroxybenzhydrazide
125 (PAHBAH) (5351-23-5), TRIS (77-86-1), EDTA (60-00-4), SDS (151-21-3), DTT (3483-12-3), Phosphate
126 buffered saline (PBS) (P4417-100), Sodium Carbonate (497-19-8), DMSO (67-68-5), maltose (6363-53-
127 7), sodium hydroxide (1310-73-2) and α-amylase (DFP Treated, Type I-A, saline suspension, 647-015-
128 00-4) were purchased from Sigma-Aldrich Company Ltd., Poole, UK.

129 High-throughput starch digestibility assay

130 Starch digestion assays were carried out on samples that were gelatinized and allowed to retrograde
131 following a protocol by Edwards et al. (2019), modified for screening a large number of samples.
132 Wholemeal flour samples were weighed (6 mg) and transferred into a deep well plate (96/1000 µL,
133 Eppendorf). Phosphate buffered saline (600 µL, PBS, pH 7.4) was added to each sample with a 1 mm
134 glass ball to improve mixing. The deep well plate was sealed and secured with a Cap-mat (96-well, 7
135 mm, Round Plug, Silicone/PTFE) and added to a preheated thermal mixer (80°C) with a 96
136 SmartBlock™ DWP 1000n attachment (Thermomixer C, Eppendorf Ltd., Stevenage, UK) for 15 min at
137 1500 rpm to gelatinize the starch, then cooled at 4°C for 21 h to accelerate retrogradation. The plate
138 was then briefly spun (100*g* for 1 min) to collect condensed liquid on the Cap-mat and placed on the
139 thermal mixer for 30 min at 37°C, 1600 rpm. Time zero samples (50 µL) were collected using a 12-
140 multichannel pipette and transferred into a 2 mL deep well plate containing 1.95 mL of stop solution
141 (17 mM $NaCO_3$). Digestion was started by adding pancreatic α-amylase suspended in PBS targeting
142 2U/mL activity into the samples. Enzyme activity was determined by applying the starch digestibility
143 assay on gelatinized potato starch and obtaining the linear rate of maltose release every 3 minutes
144 (mg/mL). Aliquots (50 µL) were then taken after 6, 12, 18, 24, 40, and 90 minutes from the onset of
145 digestion and transferred to the stop solution.

146 The stopped reactions were then centrifuged at 1500*g* for 5 minutes to avoid transferring any starch
147 remnants, and 50 µL of the supernatant was transferred to a new deep well plate. The PAHBAH
148 reducing end assay was used to quantify the reducing ends released (Lever, 1972). Briefly, 0.5 mL of
149 freshly prepared reagent (2 g of p-hydroxybenzoic acid hydrazide dissolved in 38 mL of 0.5 M HCl and
150 360 mL of 0.5 M NaOH) was added to each sample. The deep-well plate was held at 100°C for 7 min
151 and then placed in an ice bath for 10 min. Samples were then transferred to a microplate, and
152 absorbance was measured at 405 nm using a microplate reader (Bio-Rad Benchmark Plus, Waukegan,
153 Illinois, USA). Reducing sugars were expressed as maltose equivalents, using a standard curve of
154 maltose standards (5 - 1000 µM). Starch digestibility (%) was expressed according to Edwards et al.
155 (2019); each timepoint's maltose equivalents were corrected by subtracting the baseline maltose
156 (time zero) and then divided by the maltose equivalent of total starch. Four technical replicates were
157 used, each carried out on a different day.

158 **Validation of the high-throughput starch digestibility assay**

159 Starch digestion profiles from the high-throughput assay and the Edwards et al. (2019) protocol were
160 compared using standards of purified starch from standard maize, waxy maize, and high-amylose
161 maize (purchased from Merck, formerly Sigma-Aldrich, Darmstadt, Germany) and previously
162 characterized by Koev et al. (2020)), and standard wheat starch (purchased from Merck, formerly
163 Sigma-Aldrich, Darmstadt, Germany), and two high-amylose starches: *sbeII* and *ssIIIa*, previously
164 characterized respectively by Corrado et al. (2022), (Fahy et al., 2022). Starch samples were aliquoted
165 to make 5.4mg of starch/mL of PBS, and α-amylase activity was adjusted at 2U/mL. The thermal
166 treatment procedure was followed as described above for the high-throughput starch digestibility
167 assay. Three runs were obtained for both protocols over different days. For each run, six replicates
168 per starch sample were placed randomly in the 96-well plate for the high-throughput assay, and two
169 replicates were used for the standard protocol. For each run, starch samples and enzyme solution
170 were prepared in stock and aliquoted for use in both the high-throughput and standard method.

171

172 **Field trial design**

173 Grains for the c.Watkins lines were ordered from the Germplasm Resources Unit (John Innes Centre
174 in Norwich, UK) using the publicly accessible SeedStor system https://www.seedstor.ac.uk/;
175 permission to use the materials for research purposes was obtained. The 118 c.Watkins lines were
176 grown in Autumn 2018 in 1 m^2 plots (one plot per line, except for the low yield lines) at Church Farm,
177 Norfolk UK (52°37'49.2" N 1°10'40.2" E) using standard agronomic practices (Supplemental Figure 1).
178 Based on yield data from previous years, lines with a lower yield performance were grown in duplicate
179 or triplicate (to ensure production of sufficient grains), and grains were pooled for analysis
180 (Supplemental Figure 2). Seeds from elite varieties of bread, biscuit, and animal feed commercial
181 groups of the UK Agriculture and Horticulture Development Board Recommended List (Cougar,
182 Crusoe, Dickens, Diego, Myriad, Paragon, Santiago, Skyfall) were kindly provided by Brendan Fahy
183 (Fahy et al., 2018) (ahdb.org.uk). Elite varieties were grown in 2013 in plots (one per genotype) of
184 1.5m^2 at Morley Farm, Norfolk, UK (52°33'15.57" N 1°10'58.956" E).

185 **Milling and sieving**

186 Grains from the c.Watkins and elite varieties were coarsely milled in a cyclone mill fitted with a 0.5
187 mm screen (UDY Corporation). Milled samples were passed through a 0.3 mm sieve to produce
188 'wholemeal' flour samples, and selected lines were passed through a 0.053 mm sieve to produce
189 'sieved' flour samples. The flour samples were kept in a vacuum desiccator for five days before
190 analysis.

191 **Starch isolation**

192 Starch was isolated using an adapted method reported in Hawkins et al. (2021). Wheat flour samples
193 were resuspended with water and filtered through a 100 µm cell strainer (BD Falcon #352360).
194 Samples were then centrifuged at 3000*g* for 5 min, and the pellets were resuspended in 2 mL of water.
195 The starch suspensions were then overlayed into a Percoll solution (90% v/v) and centrifuged at 2500*g*
196 for 15 minutes to remove cell walls and proteins. The recovered starch pellets were washed with 1 mL
197 buffer (50 mM Tris-HCl, pH 6.8; 10 mM EDTA; 4% SDS; and 10 mM DTT), transferred into a 2 mL tube

198 and left to incubate for 5 minutes. The starch suspension was then centrifuged at 4000*g* for 1 min.
199 The pellets were recovered, and the washing procedure was repeated once more. The pellets were
200 then washed three times with 1 mL of water, then once with 100% ethanol. Samples were then kept
201 one day in the fume hood, followed by five days in a desiccator containing silicon dioxide prior to
202 analysis.

203 **Total starch assay**

204 Wholemeal flour samples were weighed (~8 mg) and transferred into a deep well plate (96/1000 µL,
205 Eppendorf), each well containing 20 µL of DMSO and a 3 mm glass ball to improve mixing. The plate
206 was mixed for 5 min at 1600 rpm to disperse the samples before adding 500 µL of a thermostable α-
207 amylase to each sample. The thermostable α-amylase was solubilized at 1:30 (v/v) in 100 mM sodium
208 acetate buffer, pH 5.0 (Total Starch hexokinase kit, AOAC Method 996.1 1; Megazyme, Bray, IE). The
209 deep well plate was sealed and secured with a Cap-mat (96-well, 7 mm, Round Plug, Silicone/PTFE).
210 Samples were heated at 90°C for 10 minutes at 1600 rpm using a thermal mixer with a 96 SmartBlock™
211 DWP 1000n attachment (Thermomixer C, Eppendorf Ltd., Stevenage, UK). Total starch content was
212 determined using a Total Starch HK kit (Total Starch hexokinase kit, AOAC Method 996.1 1; Megazyme,
213 Bray, IE) following manufacturer instructions, except volumes of reagents were scaled down by a
214 factor of 10.

215 **Tools**

216 During optimization of the high-throughput assay, a low-cost 3D-printed pipetting tool was developed,
217 which allowed for manageable weighing and transferring of samples into 96-sample deep well plates
218 and improved speed and control of pipette aspiration (Plate Z). The PLZ 3D design is available to
219 download and print for free (https://www.hackster.io/386082/high-throughput-pipetting-plate-z-
220 bde2c7).

221 **Data analysis**

222 Statistical analyses and graphs were obtained with R (R version 4.2.1). Datasets of the validation of
223 the high-throughput *in vitro* starch digestibility assay were analysed using the packages lme4 (v 1.1-

224 30) and lmerTest (v 3.1-3) for a mixed model fit (Kunzetsova et al., 2017, Bates et al., 2015, Pinheiro
225 et al., 2022). Plots were made using the ggplot2 package (v 3.3.6) (Wickham, 2016).
226 For validation of the high-throughput assay, the methods were compared by plotting the estimated
227 starch digestion profiles and by comparing the estimated starch digested at 90 minutes. The bias
228 (difference in estimates between methods) and variation in starch digested at 90 minutes measured
229 by the high-throughput assay was estimated using linear mixed models, including the type of starch
230 as a fixed effect and sample batch as a random effect. The variance was estimated using separate
231 models for each method, while the bias was estimated using a single joint model including all data
232 points, with 'method' corresponding to an additional fixed effect.
233 A linear mixed model including line as a fixed effect and experimental run as a random effect was used
234 for analysis of *in vitro* starch digestibility of the c.Watkins and elite lines, and one-way ANOVA for
235 selected low- and high-digestibility lines as wholemeal flour, sieved flour, purified starch and total
236 starch. The correlation between starch digestibility and total starch was estimated using a linear
237 regression model. All values reported represent the mean, and the number of replicates and variance
238 metrics are specified in the description of the corresponding figure and supplementary datasets. Data
239 sets of starch digestibility and total starch and all statistical analysis code are included as
240 supplementary material.

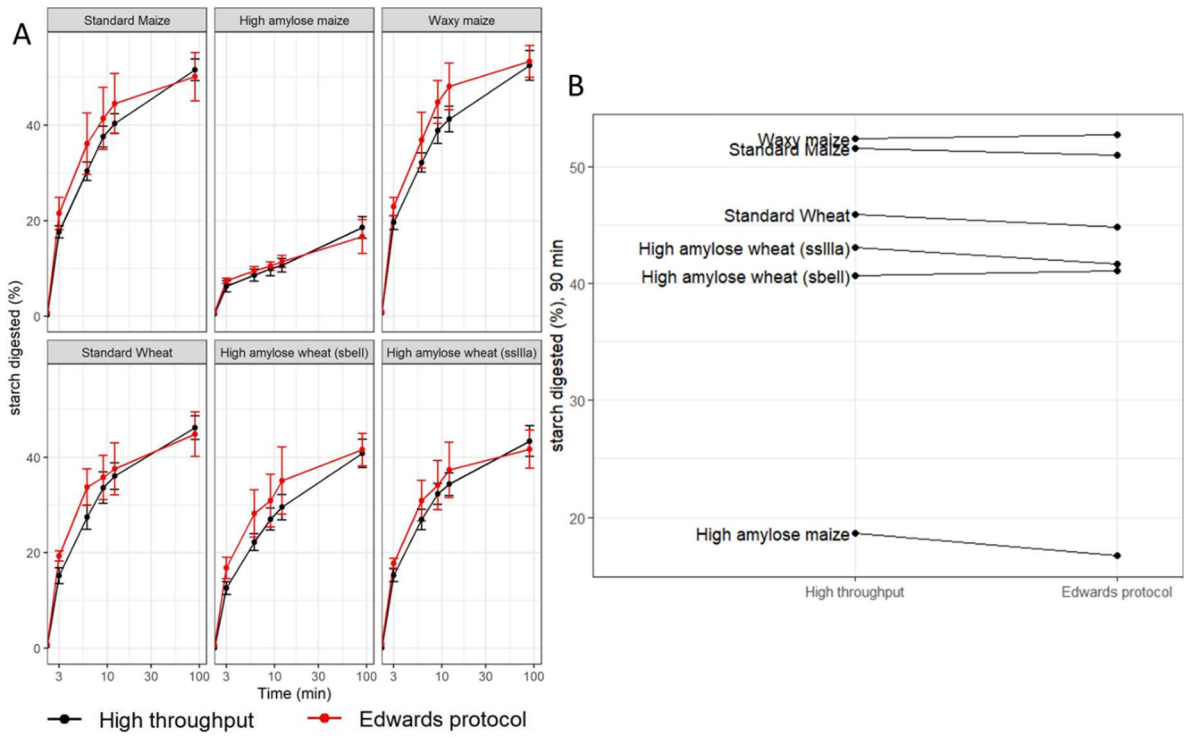
241 Results and Discussion

242 Establishment and validation of a high-throughput starch digestibility assay

243 A key outcome of our study was the development of a high-throughput assay to directly measure
244 starch digestibility of hydrothermally treated flour samples (cooked and cooled wholemeal wheat
245 flour, a processed matrix), based on the Edwards et al. (2019) protocol (referred to as 'Edwards
246 protocol' throughout). The high-throughput assay (detailed in the methods section) was scaled from
247 a 6-tube format to a 96-well plate format which required optimizations for maintaining even heat
248 distribution during starch digestions and PAHBAH assays, for the mixing ability of samples, and for

recovering adequate sample volumes for analysis. We also developed new tools to allow faster sample handling for sample preparation and pipetting (Plate Z, available free to download from hackster.io).

Digestion profiles of maize and wheat starch standards were comparable to profiles generated using the Edwards protocol (Figure 2), particularly with respect to the variance. For example, the difference in the average estimates at 90 mins from the two methods was -0.72 percentage points, and the variation observed at 90 min within the runs was 2.1 percentage points in the high-throughput assay and 2.2 percentage points in the Edwards protocol. There were no significant differences in the percent of starch digested at all the time points measured between the Edwards protocol and the high-throughput assay. Thus, the high-throughput assay provided comparisons between samples that were accurate and reproducible, and the reliability of the assay was deemed sufficient for use as a screening tool to aid in the selection of low- and high-digestibility samples. For future work, it will be important to consider improving the efficiency of upstream steps, including milling, sieving, and weighing samples which present additional bottlenecks.



263 ***Figure 2.*** *Comparison of the high-throughput starch digestibility assay to the Edwards protocol* ***A.***
264 *digestibility profiles of purified maize and wheat starch produced by the high-throughput assay (black)*
265 *and the Edwards protocol (red).* ***B.*** *Starch digested (%) at 90 min, ranking comparison of the high-*
266 *throughput assay (left) to the Edwards protocol (right). Values represent the mean ± SD of n = 18*
267 *replicates for the high-throughput assay and n = 6 for the Edwards protocol.*
268
269 Recent studies have reported improved methods to increase the throughput of starch digestibility
270 assays. Wang et al. (2022) developed a 96-sample format assay to screen starch from a wheat MAGIC
271 population and used a multifactorial analysis to identify the most influential factors for starch
272 digestibility (starch granule size distribution, amylopectin chain length distribution, amylose content
273 and endogenous α-amylase activity). Other studies have also aimed to increase the throughput of
274 starch characterization techniques however, the starch properties analyzed may or may not have a
275 direct impact on starch digestibility (Kaufman et al., 2015, Perez-Moral et al., 2018). It is important to
276 consider that other components of wheat flour and factors like processing are likely to have a major
277 impact on starch digestibility thus, determining starch digestibility on processed flour samples may
278 better represent what people may consume in wheat-based foods (Edwards et al., 2014, Edwards et
279 al., 2019, Toutounji et al., 2019, Wang et al., 2022). Some progress has been made for other crops like
280 rice; Toutounji et al. (2019) developed a 15-sample format assay for screening starch digestibility of
281 cooked rice grains with potential to allow analysis of 60 samples per day (if 15 samples are prepared
282 every two hours). In this method, samples were handled individually with a single pipette, which was
283 a limiting factor for the number of samples that could be processed simultaneously. Furthermore, the
284 assay was performed using a set of 8 samples and this assay still needs to be validated on a larger
285 sample set. The high-throughtput assay described here addresses some of these key limitations
286 presented by prior assays namely the type of sample analyzed (hydrothermally processed flour) and
287 sample size (96-sample format).
288 **Screening of c.Watkins landraces and elite varieties**

289 ***In vitro* starch digestibility**

290 Results of the high-throughput assay revealed a wide range of variation in starch digestibility among
291 c.Watkins landraces; starch digestibility profiles formed a gradient of low to high digestibility rather
292 than two distinct groups, which is expected from complex traits. This variation was evident at all time
293 points (Figure 3). For the c.Watkins lines, the levels of starch digested ranged between 9.7 - 31.6% (at
294 6 min), 13.2-35% (at 12 min), 14.8-37.2% (at 18 minutes), 16.2 - 36.1% (at 24 min), 19 - 37.8% (at 40
295 min), and 23.5 - 39.9% (at 90 min). The residual variance from the mixed effect model, measuring the
296 variability between technical replicates from the same sample in the same run, was 3.7 percentage
297 points. The variance using wholemeal flour was higher than the variance using starch standards in the
298 validation experiment, and this could be due to the higher variability in wholemeal flour composition
299 compared to purified starch samples. The number of technical replicates needed to estimate starch
300 digestibility to a given precision can be calculated from residual variance.

301 There was no evidence for a relationship between the plot location in the field and the starch
302 digestibility at 90 min, as no trend was observed between field rows and columns (Supplemental
303 Figure 3). Elite UK varieties showed less variation and greater starch digestibility compared to most
304 c.Watkins lines, although a direct comparison is confounded by the different growing conditions
305 between the two groups. For example, the levels of starch digested for the elite varieties ranged
306 between 26.8 – 36.4% (at 6 min), 29.4-40.4% (at 12 min), 30.6 – 43.8% (at 18 min), 30 – 43.4% (at 24
307 min), 31.3 – 43.4% (at 40 min) and 31.2 – 43.5% (at 90 min).

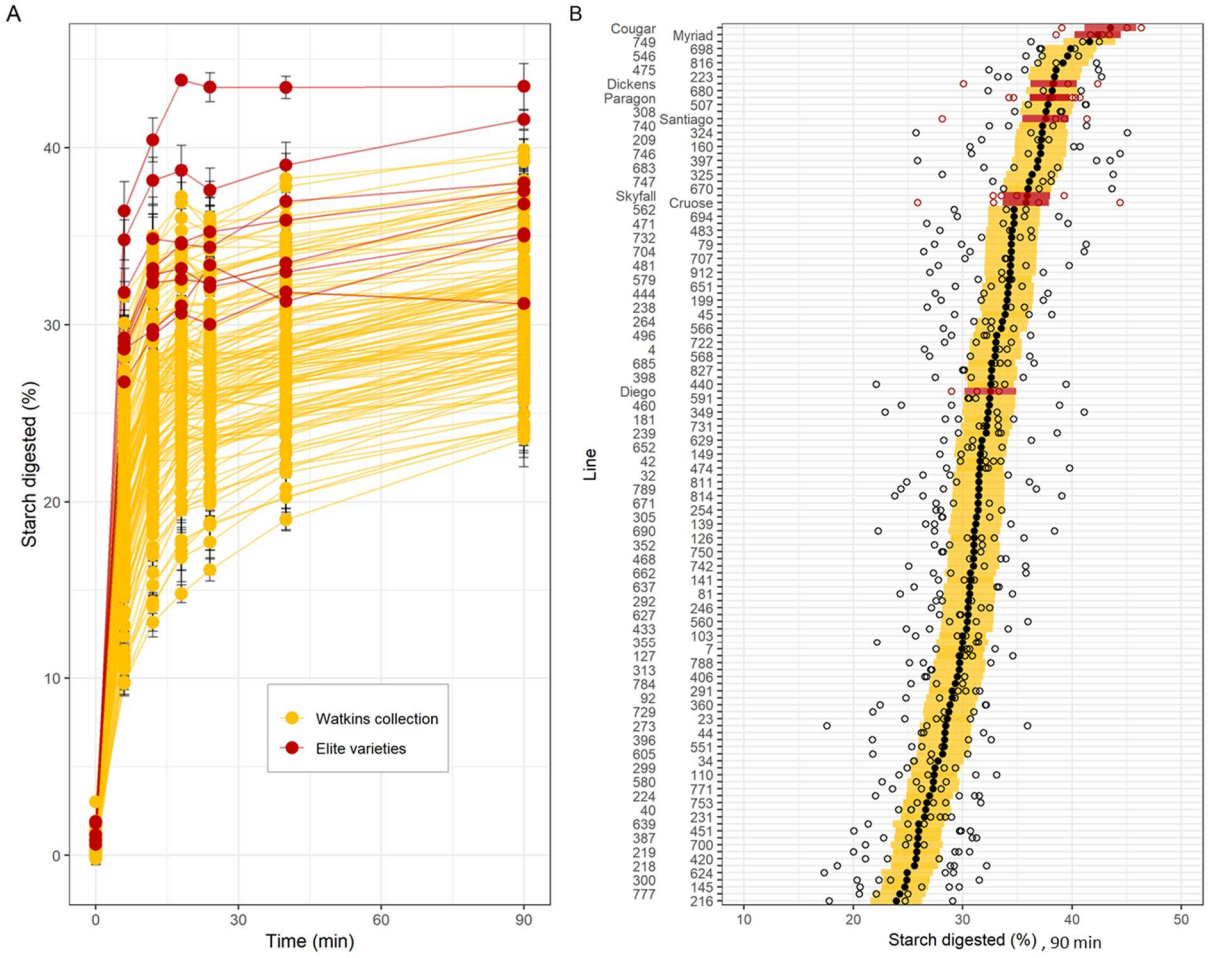


Figure 3. *Starch digestibility and total starch of c.Watkins landraces (yellow) and elite varieties (red) of wholemeal flour.* ***A.*** *Starch digested (%) for c.Watkins (yellow) and elite varieties (red). Points and lines represent the mean and standard error from between 3 and 6 technical replicates per line.* ***B.*** *Starch digested (%) at 90 min. Individual data points from technical replicates are shown in white dots, and marginal mean values from a mixed effects model with line as a fixed effect and experimental run as a random effect are shown in black (c.Watkins) and red dots (elite). The standard error estimated from the model is displayed as yellow (c.Watkins) and red (elite) bars.* ***Values represent mean ± SE of*** *n ≥ 3 replicates.*

Our results demonstrate that natural phenotypic variation for starch digestibility exists in the c.Watkins collection (representing the genetic diversity of the entire Watkins collection, 826 lines) as well as in elite varieties representing commercial groups of bread, biscuit and animal feed groups, thus

321 there is potential for identifying underlying genetic diversity. The levels of variation observed in the
322 c.Watkins were greater than the elite varieties screened, which is consistent with the higher levels of
323 genetic diversity previously reported for the c.Watkins collection compared to modern European
324 bread wheat varieties, as well as increased phenotypic variation for agronomically desirable traits,
325 such as stripe, leaf, and stem rust resistance, as well as grain surface area and grain width (Wingen et
326 al., 2014, Bansal et al., 2011, Bansal et al., 2013, Randhawa et al., 2015, Toor et al., 2013). It is
327 important to note that a limitation of our study was that the grain samples of c.Watkins lines and elite
328 varieties were not produced in the same field trial, and each used a plot per line, so it is possible that
329 environmental conditions between and within fields could influence the average starch digestibility
330 levels and the variation observed; this will be important to consider in future trials. Furthermore, with
331 the availability of structured germplasm panels (nested association mapping populations) and
332 genotype data (Wingen et al., 2017), there is now potential for use of the high-throughput assay to
333 investigate the genetic factors underlying the variation in starch digestibility observed through
334 approaches like QTL analysis which will aid in identification of underlying candidate genes and
335 development of tools for marker assisted-selection.

336 **Total Starch**

337 Total starch (TS) content of wholemeal flour varied significantly for c.Watkins landraces (43 ± 3.3 g/100
338 flour to 61 ± 2.4 g/100 flour, mean ± SE, $p \leq 0.001$), and the elite varieties (46 ± 3.6 g/100 flour to 61
339 ± 0.9 g/100 flour, mean ± SE, $p \leq 0.05$) (Supplemental data). The elite variety Diego had the highest TS
340 content (61 ± 0.9 g/100 flour, mean ± SE), and the c.Watkins line 651 had the lowest (43 ± 3.3 g/100
341 flour, mean ± SE). Most of the samples had a TS content between 47 to 57 g/100 flour. Linear
342 regression analysis showed that total starch content only weakly correlated ($R^2 = 0.0108$) with starch
343 digested at 90 min which suggests that the differences in total starch content cannot explain the
344 variation observed for starch digestibility (Supplemental Figure 4).

345 **Analysis of low- and high-digestibility lines as wholemeal flour, sieved flour, and purified starch**

346 Three low-digestibility lines: 777 (WATDE0111), 216 (WATDE0025), and 639 (WATDE0083); and five
347 high-digestibility lines: including two c.Watkins landraces 816 (WATDE0117), 308 (WATDE0042) and
348 the three UK elite varieties Myriad, Dickens, and Paragon were selected for further analysis. The
349 selection was based on the high-throughput starch digestibility assay results, specifically the ranking
350 (low to high), to represent the variation observed in the screen.
351 Starch digestibility profiles differed considerably for wholemeal flour, sieved flour and purified starch,
352 suggesting that other flour components besides starch likely contributed to the variation in starch
353 digestibility observed (Figure 4). For wholemeal flour (<0.3 mm), starch digestibility profiles of selected
354 'low-' and 'high-digestibility' lines grouped separately where the high-digestibility samples varied
355 between 37.6 - 41.6% and low-digestibility samples varied between 23.5 - 24.3% (at 90 min) (Figure
356 4A). For sieved flour (< 0.05 mm), the digestibility of two low-lines (216 and 639) increased significantly
357 ($p < 0.05$) compared to wholemeal samples, and the third low-line (777) remained low (Figure 4B). In
358 sieved flour, the high-digestibility samples varied between 38.1 - 42.7%, whereas low-digestibility
359 samples varied between 26 - 35.4% (at 90 min). Purified starch samples also showed a greater extent
360 of starch digested at 90 minutes compared to wholemeal and sieved flour fractions ($p < 0.001$) (Figure
361 4B) but there were no differences between the high- and low-digestibility groups in purified starch
362 samples. For example, high-digestibility lines differed between 46.9 - 52.3% and low-digestibility lines
363 varied between 47.6 - 49.2% (at 90 min); the variation observed at 90 min within the runs was 3 SD
364 (95% CI= 2.3 to 4.2%).

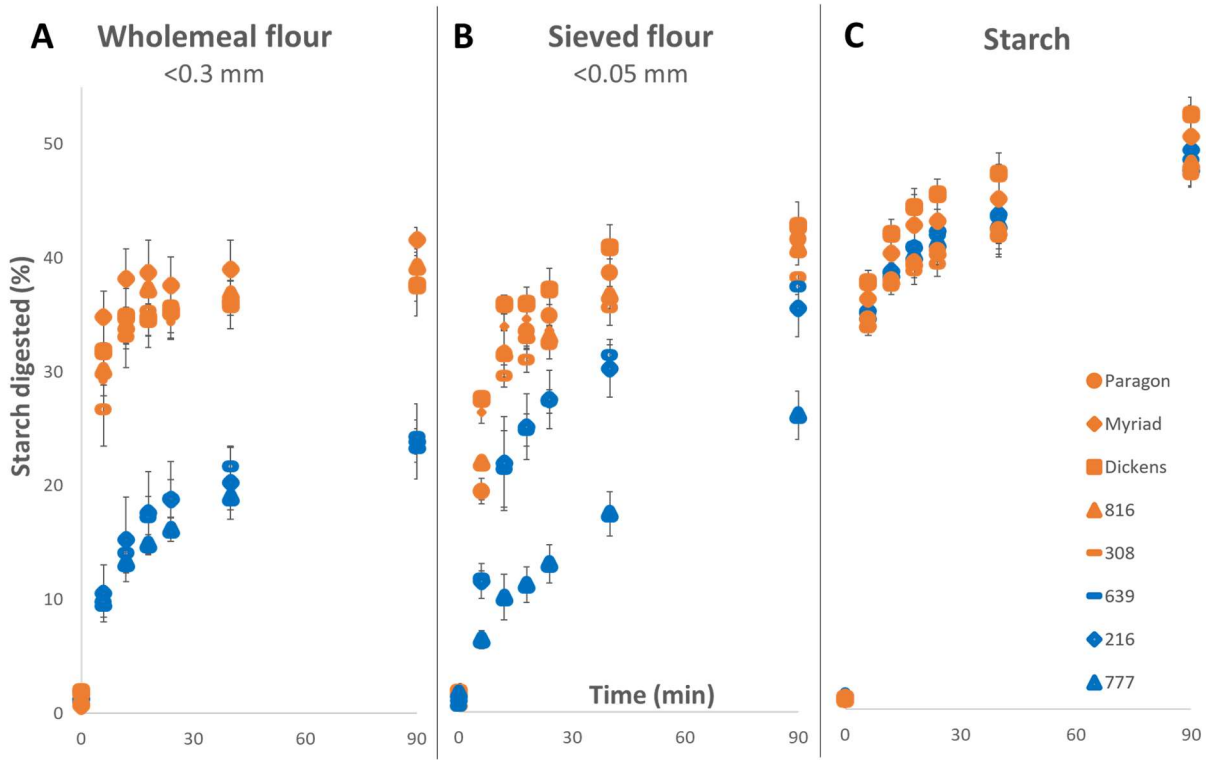


Figure 4. *Starch digestibility of selected low- and high-digestibility lines measured in wholemeal flour (A), sieved flour (B) and purified starch (C) using the high-throughput assay. Samples in blue represent the low-digestibility lines, and samples in orange represent the high-digestibility lines. Values represent mean ± SE of n = 3 replicates.*

The varying digestibility profiles observed across purification steps suggest that multiple mechanisms in the selected lines could be affecting starch digestibility and that different factors in each line could have a distinct effect. This is consistent with prior studies which have shown effects of flour characteristics and starch structure on starch digestibility in cooked wheat samples including particle size of wholemeal flour, protein content, and chain-length distribution of starch polymers (Obadi et al., 2020, Lin et al., 2020, Sissons et al., 2021). In our study, selected lines were also analyzed for flour properties (flour particle size, endogenous α-amylase and protein content) and starch structural properties (starch granule size distribution and chain-length distribution), however, none of the factors strongly correlated with starch digestibility, which could be due to the high phenotypic variation of the Watkins collection (Supplemental Table 1 and Supplemental Figures 5,6,7). An

important limitation of this aspect of the study was the small number of lines selected for this analysis; prior work by Wang et al. (2022) identified starch properties influencing starch digestibility in a screen of 224 wheat starches. Nevertheless, our study suggests that a combination and interaction of many factors are required to achieve low starch digestibility profiles in flour, so using a high-throughput starch digestibility assay for screening or selection approaches may be more efficient and informative than selection based on underlying factors such as starch molecular structure.

Abbreviations:

c.Watkins: core Watkins

PAHBAH: p-hydroxybenzoic acid hydrazide

TS Total: starch

Authors contributions:

Petros Zafeiriou: Conceptualization, investigation, methodology, project administration, data curation, and formal analysis, writing – original draft preparation, writing – review & editing, George M. Savva: methodology, data curation, visualization, formal analysis, writing – review & editing, Jennifer Ahn-Jarvis: investigation, methodology, writing – review & editing, Frederick J. Warren: conceptualization, methodology, supervision, writing – review & editing, Marianna Pasquariello: investigation, methodology, writing – review & editing, Simon Griffiths: conceptualization, methodology, supervision, writing – review & editing, David Seung: conceptualization, investigation, methodology, supervision, writing – review & editing, Brittany A. Hazard: conceptualization, funding acquisition, methodology, supervision, writing – review & editing.

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