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Effect of cooking, 24 h cold storage, microwave reheating, and particle size on *in vitro* starch digestibility of dry and fresh pasta

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The aim of this study was to investigate the effect of preparation method and particle size on digestion of starch in fresh and dry pasta types. Pasta samples were boiled, refrigerated and re-heated, with samples collected after each stage, and then prepared as small (2 mm) and large (5 mm) particles for subsequent starch digestibility testing and logarithm of slope analyses. There were significant main effects of particle size ($F_{1,24}$ = 568.895, p < 0.001, η_p^2 = 0.960) and processing treatment ($F_{2,24}$ = 19.897, p < 0.001 η_p^2 = 0.624) on starch digestibility overall, however the main effect of pasta type was not significant at the p < 0.05 level ($F_{1,24}$ = 2.978, p = 0.097, η_p^2 = 0.110). Particle size had the largest effect on digestibility, and the extent of starch digestion was at least 50% lower in samples prepared as large particles compared with small particles. The most digestible sample was the boiled fresh-type pasta prepared as small particles (C_∞ = 57.9%) and cold storage alone and/or with subsequent re-heating significantly reduced the extent of digestion in this sample to ~40.6%. In the dry pasta type, processing treatment had no significant effects on starch digestibility. The rate constant, k, was not significantly altered by processing treatment or pasta type (k = 0.0275 min^{-1}, mean of all samples). These findings suggest that cold-storage and re-heating treatments have limited potential to impact on glycaemic responses and highlight the importance of masticated particle size as a potential rate-limiting factor in digestibility studies.

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Introduction

Starch-rich foods that evoke a high glycaemic response are modifiable risk factors for obesity, type 2 diabetes and cardiovascular disease and there is a need to promote staple foods that attenuate postprandial blood glucose responses.[1–3] Pasta is a widely consumed food product typically prepared from extrusion of wheat-based dough and is available either as a 'fresh type' (*pasta fresca*) where egg is included in the dough, or as a 'dry-type' (*pasta secca*), in which extruded pasta shapes are dried at high temperature. The glycaemic index (GI) of most pasta types falls within the 'low to medium' range (*i.e.* GI < 70),[4] and its inclusion as part of a low GI diet seems to support cardiometabolic health.[5] However, there is large variation in GI between pasta types and the mechanisms that underpin these differences are not well understood.

The susceptibility of starch to amylolysis during digestion regulates the availability of glucose for absorption and is known to have a major impact on the postprandial glycaemic response to carbohydrate foods. Starch digestibility has been shown to occur more slowly in pasta than in other high glycaemic carbohydrate foods,[6,7] but it is unclear if the low digestibility is due to intrinsic properties of the starch or the properties of the surrounding food matrix. Boiled pasta has a compact microstructure in which partially swollen starch granules are entrapped within a continuous protein matrix. The gluten protein network has been shown to delay the activity of α-amylase on starch during *in vitro* digestion,[7–9] and the type and amount of protein may therefore impact on texture and glycaemic responses. Additionally, amylose and amylopectin, the α-glucan polymers of starch, are known to undergo structural changes when exposed to different processing conditions, with implications for its susceptibility to amylolysis.[10–13] For instance, refrigeration of gelatinised starch is known to promote the formation of retrograded starch, in which the re-association between α-glucan chains of amylose and eventually amylopectin reduces its susceptibility to amylolysis.[11,12] On a macroscopic level, the surface area to volume ratio of food macroparticles that are swallowed are also recognised to impact on metabolic responses.[14,15] Mastication

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studies have shown that spaghetti (*i.e.* pasta in the shape of cylindrical strands) is reduced to shorter cylindrical particles with a size range of 2.5 to 30 mm during chewing.[16–18] Studies in ileostomy patients have demonstrated that 2 mm particles of cooked durum wheat endosperm can remain intact through upper-gastrointestinal transit to the end of the small intestine.[14] Particle size is inversely related to the rate of starch amylolysis,[19,20] and the low surface area per volume may be a rate-limiting factor when pasta types such as spaghetti are swallowed as large particles.

Although the mechanisms of starch gelatinisation and retrogradation are well known to occur in isolated starches it remains unclear if these starch structural changes drive the glycaemic response to pasta. Furthermore, under domestic cooking, boiled pasta may be refrigerated and either eaten cold or subsequently re-heated in a microwave prior to consumption, yet there is a very limited understanding of how refrigeration and re-heating affects starch structures and indeed digestibility. One human study did not find any significant differences between postprandial glycaemic responses to freshly cooked pasta compared with pasta that was cooked, chilled and then reheated,[21] whereas another study recently reported that re-heated pasta evoked somewhat lower postprandial glycaemic responses compared with freshly boiled pasta, but observed no difference between freshly boiled and refrigerated meals.[22] Furthermore, the mechanistic basis for these observations has yet to be established.

We have previously demonstrated the application of *in vitro* starch digestibility assays and Logarithm of Slope analyses to examine mechanisms affecting starch amylolysis in foods.[6,19,23,24] In the present study we use these established methods to compare the effect of different preparation methods (boiling, storage, reheating) on starch digestion kinetics of dry and fresh pasta types and compare two different particle size fractions to elucidate the relative importance of masticated particle size. The results obtained with this *in vitro* method are highly relevant to understanding the *in vivo* glycaemic response to pasta.[6]

Materials and methods

Food materials

Fresh pasta (Tagliatelle made in Italy, Tesco Stores Ltd, UK) made with durum wheat and fresh egg, and dry pasta (Spaghetti, Napolitana Ltd, Liverpool, U.K.) made from 100% durum wheat semolina, were purchased from a local Tesco supermarket. The nutrient composition of both pasta types was measured by ALS Laboratories (UK) Ltd, Chatteris, UK using standard UKAS accredited methods and is shown in Table 1. In summary, total fat was determined by NMR (ALS method no. AM/C/1015), protein by Dumas technique using Nx6.25 (ALS method no. AM/C/224), total dietary fibre by AOAC method no. 985.29 [25] (ALS method AM/C/309), total sugars determined by HPLC (ALS method no. AM/C/1014), starch measured by polarimetry (ALS method no. AM/C/401, based on EC Directive 79/1999/EC); moisture was determined by loss on drying (ALS method no. AM/C/1015), ash determined as per BS 4401-0126 [26] (ALS method no. AM/C/803) and energy was calculated using standard conversion factors (EC 2008/100 and 90/496).

Table 1 Nutrient composition (per 100 g uncooked) of fresh tagliatelle and dry spaghetti pasta[a]

	Fresh tagliatelle Per 100 g uncooked	Dry spaghetti Per 100 g uncooked
Energy (kJ)	1201	1526
Energy (kcal)	284	360
Fat (g)	2.4	0.8
Sugars (g)	1.1	3.9
Starch (g)	48.5	66.5
Dietary fibre (g)	2.4	2.8
Protein (g)	10.5	12.1
Ash (g)	0.8	0.8
Moisture (g)	32.5	11.7

[a] Values obtained from proximate analyses performed by ALS Laboratories (UK) Ltd, and obtained by UKAS accredited methods: energy was calculated using standard conversion factors, total fat determined by NMR; total sugars determined by HPLC; starch by polarimetry; total dietary fibre by AOAC official method no. 985.29, protein by Dumas method using Nx6.25, and moisture was determined by loss on drying.

Sample preparation

To prepare the 'boiled' pasta samples, the fresh (50 g) and dry pasta (100 g) were each boiled for 3 min or 10 min respectively, in a saucepan using 1 L of water per 100 g of pasta and stirred occasionally. These samples were prepared in accordance with the cooking instructions provided by the manufacturer. The pasta was drained in a colander immediately after boiling and the cooking water was discarded. To prepare the 'refrigerated' samples, the fresh and dry pasta types were boiled and drained as described above and then transferred to a bowl and covered with cling film then immediately placed into a refrigerator at 4 °C and stored there for 24 hours. To prepare the 're-heated' pasta samples, the fresh and dry pasta were boiled, drained and refrigerated as described above, and then reheated in a microwave at 1000 W for 1 min and 30 seconds. After each stage, boiled pasta samples were taken and reduced into two different particle sizes ('large' and 'small'), and evaluated immediately for starch digestibility. The particles were obtained either by cutting boiled pasta to a length of 5 mm ('large particles') or by passing the boiled pasta through a garlic press with spherical aperture of 2 mm diameter ('small particles'). We chose these particle size fractions based on previous literature which states that masticated pasta particle size could be between 0.5 to 30 mm (ref. 16–18) and our earlier observations that intact durum wheat endosperm can remain intact through gastro-ileal transit.[14] The sample moisture content was determined after each stage.

In vitro starch digestion assay and determination of reducing sugars

Starch digestibility was determined according to the amylolysis assay procedure described previously.[6] In brief, pasta samples

(n = 3 per treatment) were weighed into 15 mL Corning tubes and suspended in 10 mL PBS (pH 7.4). Porcine pancreatic α-amylase (EC 3.2.1.1, A6255, Sigma-Aldrich Co. Ltd) was added so that each tube contained 10 U amylase (1 U liberates 1 mg maltose from starch in 3 minutes at pH 6.9, 37 °C) and 46 mg starch in the 10 mL digestion mixture. Samples were incubated on a rotary mixer (30 rpm, Stuart rotator SB3) at 37 °C. Aliquots were taken before addition of amylase, and after 5, 10, 15, 30, 45, 60 and 90 min and immediately diluted 1 : 2 in 0.3 M Na_2CO_3 and centrifuged at 13 000g for 5 min (Heraeus Fresco 17 Centrifuge; Thermo Electron Corporation) to obtain supernatants for further analysis of starch amylolysis products (reducing sugars) by PAHBAH assay, as described previously,[6,23] except that sample absorbance (λ = 405 nm) was measured in a microplate reader (Bio-Rad Benchmark Plus, Waukegan, Illinois, USA).

Data processing and logarithm of slope analyses

Logarithm of Slope (LOS) analysis was applied to the first-order starch amylolysis curves to obtain kinetic parameters as described previously[19,27] In brief, this analysis enables the value of rate constant, k, and the maximum extent of digestion at the endpoint, C_∞, to be obtained from the slope and y-intercept of linear LOS plots of digestibility data (*i.e.* maltose concentration measured at each time point). For ease of interpretation, data obtained in units of maltose equivalent concentrations were converted to show the corresponding percentage of starch digested by α-amylase (eqn (1))

$$(\text{Starch amylolysis }\%)_t = \frac{[\text{maltose}]_t}{[\text{maltose}]_s} \times 100 \quad (1)$$

in which $[\text{maltose}]_t$ is the maltose equivalent concentration (after baseline correction) measured in the liquid phase of the reaction mixture at a time point t, and $[\text{maltose}]_s$ is the theoretical maltose equivalent concentration that would be present at the start of the reaction, assuming that all starch within the food sample can be converted to maltose.

Optical microscopy

Images were captured using an Olympus BX60 Microscope equipped with a Jenoptik ProgRes camera. A small amount of sample previously treated with an iodine/potassium iodide solution (I_2/KI, 0.2% iodine in 2% potassium iodide) to stain starch, or with a 1% w/v toluidine blue solution to stain cell wall and protein, was placed in a glass slide with a drop of water and covered with a cover glass before image capture.

Statistical analyses

All data are presented as mean ± standard deviation (SD) or standard error (SE) of triplicates (n = 3) unless otherwise specified. Statistical analyses of the experimental results were performed using the statistical programs IBM SPSS Statistics 23.0 (©IBM Corp. 2011) and curve-fitting was performed using SigmaPlot 12.0 (©Systat software 2011) statistical and graphical software. Repeated measured analysis of variance (ANOVA) was used to compare starch digestibility curves, with time as the 'within-sample' factor, and type, treatment and size as 'between-sample' factors, and using Bonferroni correction for multiple comparison procedures and Greenhouse-Geisser correction for sphericity. ANOVA results are reported as an F-statistic with factor and error term degrees of freedom shown as subscript letters, and together with the significance level and partial eta-squared (η_p^2) value as a measure of effect size. Tukey's *post hoc* test was carried out to identify homogenous subsets. Statistically significant differences were accepted at $p < 0.05$.

Results

The typical size and shape of particles obtained from boiled fresh and dry pasta is shown in Fig. 1. The garlic press produced smaller cylindrical particles ~2 mm from both fresh and dry pasta type. Larger sections cut to a length of ~5 mm from dry pasta were also cylindrical, whereas the larger sections cut from fresh pasta had a somewhat flattened appearance.

Starch digestibility curves obtained for fresh and dry pasta cut into large or small particles prepared from boiled, refrigerated and reheated samples are shown in Fig. 2. The measured amount of starch digestion products increased over time for all samples and the resulting starch digestibility curves obtained from large particles were essentially linear over the 90 min time-course, whereas curves obtained from small particles were first-order. There were significant main effects of particle size ($F_{1,24}$ = 568.895, $p < 0.001$, η_p^2 = 0.960) and processing treatment ($F_{2,24}$ = 19.897, $p < 0.001$ η_p^2 = 0.624) on starch digestibility overall, however the main effect of pasta type was not significant at the $p < 0.05$ level ($F_{1,24}$ = 2.978, p = 0.097, η_p^2 = 0.110).

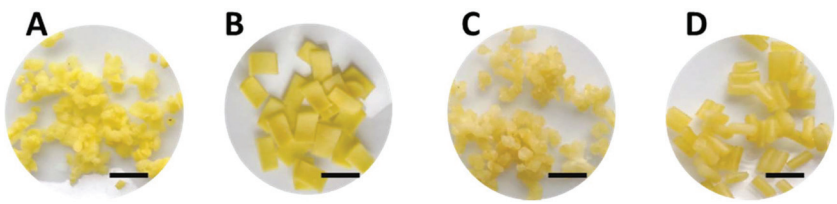

Fig. 1 Photograph of boiled pasta samples prepared to small and large particle size. Fresh (AB) and dry-type pasta (CD) were boiled and either passed through a garlic press with 2 mm aperture (AC) or cut to a length of 5 mm to obtain small and larger particle sizes, respectively. Scalebar = 1 cm.

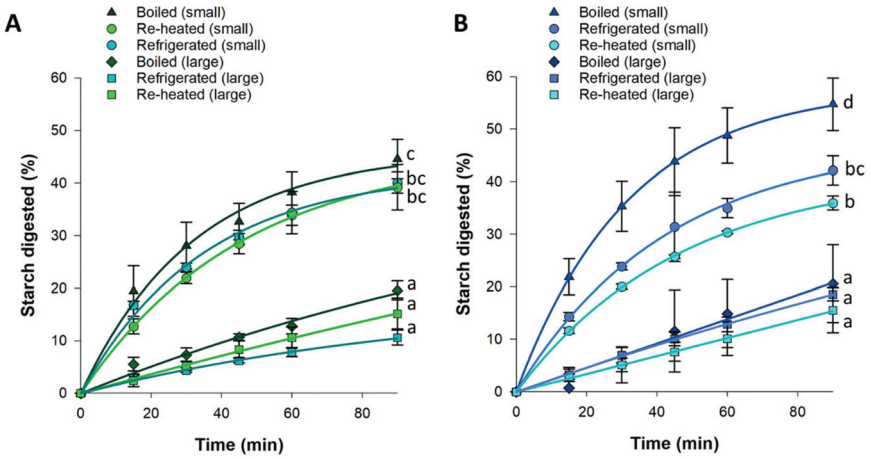

Fig. 2 Digestibility curves obtained for dry (A) and fresh (B) pasta types processed in different ways. Each pasta type was 'boiled' in water and drained, 'refrigerated' at 4 °C for 24 and 're-heated' in a microwave with samples collected after each processing stage. Collected samples were then reduced to a 'small' (~2 mm) or 'large' (~5 mm) particle size prior to incubation with porcine pancreatic α-amylase. Legend applies to both panels. Data points are mean values with error bars as SD (n = 3). Letters indicate statistical differences and apply to both panel A and B. Curves with different letters are significantly different ($p < 0.05$, ANOVA with Tukey's b *post-hoc* analyses).

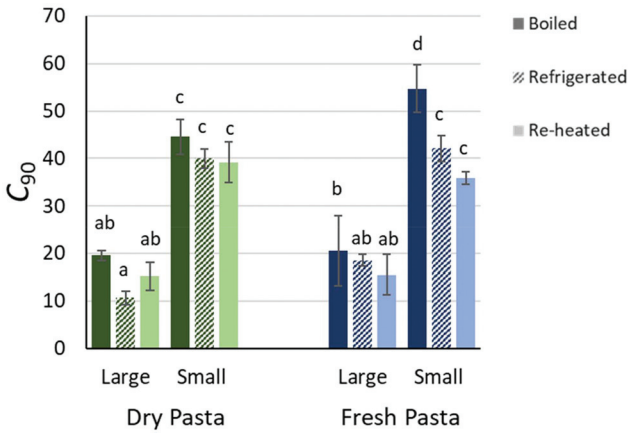

Fig. 3 Effect of processing on the quantity of starch digested after 90 min in small and large particles of dry and fresh pasta types. C_{90} is the proportion of starch digested after 90 min amylolysis. Values are mean with error bars as SD (n = 3). Data points annotated with different letters are significantly different (Tukey's B, $p < 0.05$).

An overview of the C_{90} values (*i.e.* the extent of starch digested after 90 min) is provided for all samples in Fig. 3. Statistical analysis by ANOVA revealed a significant main effect of processing treatment on C_{90} ($F_{2,12} = 19.587$, $p < 0.001$, $\eta_p^2 = 0.766$). *Post-hoc* analyses on C_{90} values showed that significantly less starch was digested ($p < 0.05$, Tukey's b) when samples were prepared as large particles (group mean C_{90} with SD = 16.7 ± 3.7%) compared with small particles (group mean C_{90} with SD = 42.8 ± 6.5%). For samples of the dry pasta type, there were no significant differences between boiled, refrigerated and re-heated samples within the same particle size group ($p < 0.05$). In the small particle size of the fresh pasta type, however, the boiled samples (C_{90} = 54.7%) had a significantly higher starch digestibility than the refrigerated (C_{90} = 42.1%) and re-heated (C_{90} = 35.9%) samples of the same particle size, with no significant difference between the latter treatments (Tukey's b, $p < 0.05$).

LOS analysis was applied to starch digestibility curves and used to obtain kinetic parameters for the small particles of fresh and dry pasta type (Table 2). It was not however applied to the digestibility curves obtained for large particles, as the linear nature of these curves meant that they were unsuitable for LOS analysis. For the small particle size fractions, the LOS plots were linear and consisted of a single phase of digestion (see Fig. 4) and the high R^2 values (≥0.98) indicate that the value of C_∞ and k obtained from LOS analyses provided a good fit to experimental data (see Fig. 4B). There was a significant main effect of processing treatment on C_∞ ($F_{2,12} = 18.122$, $p < 0.001$, $\eta_p^2 = 0.751$), but not on k ($F_{2,12} = 3.135$, $p = 0.080$, $\eta_p^2 = 0.343$). *Post-hoc* comparison revealed that C_∞ values of boiled fresh-type pasta (C_∞ = 57.9%) was significantly higher than all other samples. Although the mean k value of the re-heated samples was somewhat lower than the k value obtained for the boiled and refrigerated samples, this was not found to be statistically significant.

Micrographs (Fig. 5) showed that both dry and fresh type pasta consisted of large and small wheat starch granules surrounded by a protein matrix. The large granules were partially swollen and elongated before boiling treatment was applied (Fig. 5A and B). Starch granules that leached into the cooking water (Fig. 5C and D) had a swollen more rounded appearance.

Discussion

Starch digestibility analyses of freshly boiled, refrigerated and-reheated pasta of two types (fresh and dry), prepared as large

Table 2 Kinetic parameters obtained from LOS analyses of *in vitro* digestibility curves[a]

Type	Treatment	k (min^{-1})	C_∞ (%)	R^2
Dry pasta (small)	Boiled	0.029 ± 0.004	44.6 ± 2.2	0.995 ± 0.002
	Refrigerated	0.031 ± 0.006	40.8 ± 3.1	0.994 ± 0.002
	Re-heated[b]	0.022 ± 0.001	44.4 ± 0.6	0.999 ± 0.000
Fresh pasta (small)	Boiled	0.034 ± 0.006	57.9* ± 2.1	0.997 ± 0.003
	Refrigerated	0.027 ± 0.003	39.1 ± 1.5	0.985 ± 0.005
	Re-heated	0.021 ± 0.001	42.2 ± 1.3	0.999 ± 0.001

[a] Values are mean of triplicate analyses with error bars as SE. [b] Data in this row are from duplicate LOS analyses due to outlier exclusion. Kinetic parameter k is the rate constant, C_∞ is the proportion of starch digested at the reaction endpoint, and R^2 describes the correlation between experimentally obtained data and values calculated for matched time points using C_∞ and k from LOS plot analyses of the experimental data. Differences between k-values were not statistically significant (Tukey's b, $p < 0.05$). *Value is significantly different (Tukey's b, $p < 0.05$) from all other values within the same column.

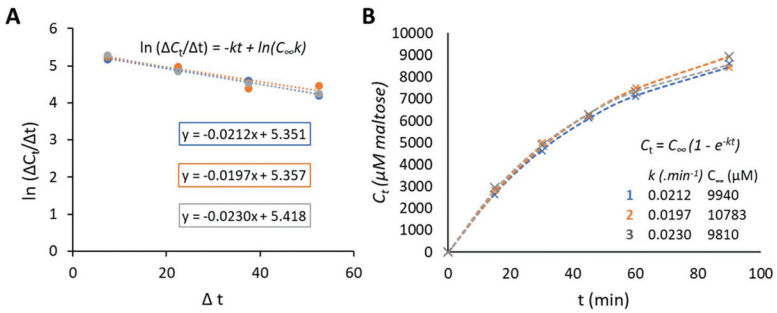

Fig. 4 LOS plot analyses of digestibility data from re-heated fresh pasta sample at a small particle size. LOS plots (A) plotted from experimentally obtained digestibility data (C_t = maltose equivalent concentration, μM) measured at different time points, (t, min) were used to obtain C_∞ and k. A linear, single-phase plot was obtained (linear correlation co-efficient $r^2 \geq 0.86$). Amylolysis progress curves (B) show product formation C_t from starch amylolysis over time, t. The experimentally obtained data points are marked with X symbols, whereas the dashed lines are the calculated fits obtained using values of C_∞ and k from the LOS plots in the first order equation. Table insert in (B) shows the value of kinetic parameters obtained from LOS analyses used to obtain curves in B. The correlation between experimentally obtained and calculated data points given was very good, with $R^2 \geq 0.998$ for all replicates. Data shown in this example are from triplicate analyses of fresh pasta, at a small particle size, that has been reheated prior to amylolysis.

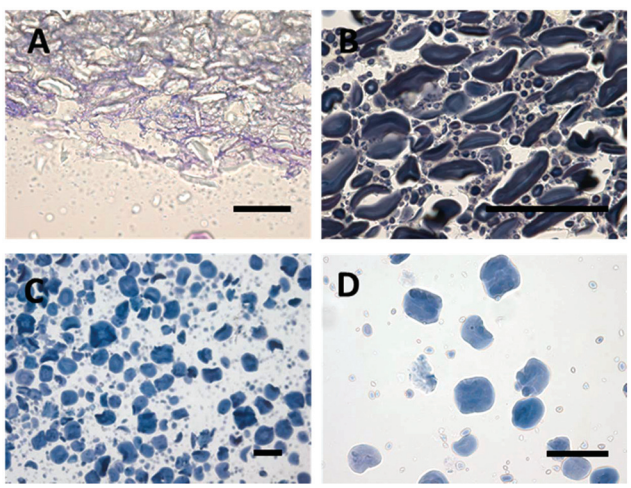

Fig. 5 Optical micrographs of starch granules in fresh and dry pasta types before (AB) and after boiling (CD). Sections cut from the pasta solids before cooking (AB) reveal a protein matrix, stained purple with toluidine blue (A) which surrounds distorted shape pre-gelatinised starch granules, stained blue with iodine/potassium iodide (B). Some starch granules (stained blue with iodine) leached out from fresh-type pasta (C) and dry-type pasta (D) into the boiling liquor and appeared swollen and rounded compared with in the dry solids. Scalebar = 50 μm.

and small particles, was performed to gain new insight into mechanisms by which food processing methods influence starch digestibility. This study provides an improved understanding and perspective of how different food preparation methods influence starch digestibility and thereby acute postprandial glycaemic responses of relevance to health.

Out of all the factors examined, particle size was found to have the greatest impact on starch digestibility, with at least twice as much starch digested from small particles (2 mm, obtained with a garlic press) compared with large particles (5 mm), irrespective of pasta type and cooking-cooling treatment. Particle size has been shown to influence amylase ingress and starch digestion kinetics in cereal products,[19,20,23] with direct implications for the glycaemic and insulinaemic response to food.[9,14] The particle sizes prepared in this study are within the physiological range that was reported by Hoebler *et al.*, who demonstrated that *in vivo* mastication of pasta produced particles up to 3 cm in length for both spaghetti or tortoglioni.[17] Thus, even the 'large' 5 mm particles included within the present study are still considerably smaller than many of the particles that may be expected to arise *in vivo.* Furthermore, the 'small' particle size of samples

obtained with the garlic press may not be representative of the main type of particle to occurs in the duodenum. Representative particle size selection is a particularly important consideration when preparing samples for *in vitro* digestibility analysis. A previous study reported that pasta samples prepared for *in vitro* digestibility analysis by maceration were more digestible than *in vivo* masticated samples.[28] The preparation of pasta samples by maceration or garlic press prior to *in vitro* digestion may therefore overestimate starch digestibility and mask the potentially rate-limiting effect of food macrostructure.

Cooking and cooling treatments were found to have relatively subtle effects on starch digestibility in both pasta types, but significant differences were evident within the small size fractions of the fresh pasta type. Within the fresh pasta type, refrigeration (for 24 h) of boiled pasta reduced the final amount of starch that could be digested (C_∞) from small particles without affecting the rate constant. The enzyme kinetic parameters obtained in the present study accord with previous studies performed on isolated starch.[29] It is likely that during cold storage of pasta, some α-glucan chains within the starch may have re-associated ('retrograded') and become inaccessible to α-amylase action. The finding that there was no statistically significant difference between refrigerated and re-heated pasta indicates that mechanisms hindering the extent of starch amylolysis in refrigerated samples are not reversed by microwave cooking. Moreover, our finding that cold-storage had larger effects on starch digestibility in fresh pasta compared with the dry pasta type suggests that other characteristics (such as the protein component or hydration characteristics) of these samples could influence starch susceptibility to amylolysis and/or the behaviour of starch during cooling and reheating treatments. While the protein component is also susceptible to processing conditions and has been implicated in amylolysis rates,[8,30] it does not seem to influence starch retrogradation behaviour.[31] The present study was limited to studies of amylolysis kinetics, however the potential role of protein in influencing starch accessibility and properties have not been fully explored and requires further investigation.

Although the formation of retrograded starch upon cold storage has been widely reported, there are relatively few studies of re-heated retrograded starch, and current understanding of the starch structural changes and mechanisms that occur under re-heating conditions is limited. A recent human study reported a slightly lower area under postprandial glucose curves following ingestion of reheated pasta compared with freshly cooked pasta,[22] however contrary to what would be expected from the literature on cooling and resistant starch,[32,33] this study found no significant difference between hot and cold pasta.[22] Studies performed on other carbohydrate foods types suggest that refrigeration prior to re-heating reduces starch digestibility and in some case, glycaemic responses. For instance, in a study on parboiled rice, there was a tendency for glycaemic responses to be lower when boiled rice had been refrigerated prior to re-heating, however the difference in the postprandial glycaemic response between freshly cooked and re-heated rice was not statistically significant.[32] Another study reported that cooled potato had a lower starch digestibility than freshly boiled potato, and that some starch digestibility was re-gained when the cooled potato was re-heated.[33] Our finding that refrigeration causes a subtle reduction in starch digestibility does fit with several studies in the literature, however there are some inconsistencies between *in vitro* and *in vivo* findings, and the mechanistic basis for different physiological effects observed remain uncertain. There is a need to combine further mechanistic studies of digestibility and structural changes that occur within realistic foods with well-designed human studies to improve understanding of the physiological responses.

Starch-rich foods that evoke low glycaemic responses are desirable to support the prevention and management of obesity and type 2 diabetes.[1] Refrigeration of carbohydrate foods could be incorporated into domestic cooking and industrial processing to promote starch retrogradation, with the aim of attenuating postprandial glycaemic responses to starch. However, in the present study, refrigeration of boiled dry-type spaghetti for 24 h did not have a signficant effect on starch amylolysis. In comparison, the particle size had a far greater effect on both the rate and extent of starch amylolysis, and would be more likely to influence postprandial glycaemic responses. An interesting industrial application for further work would be the development of pasta types or shapes that naturally and safely break down into large particle sizes during mastication.

Conclusions

Overall, our study demonstrated that effects of particle size on starch digestibility are greater than subtle changes resulting from refrigeration and re-heating, irrespective of the type of pasta. The effects of refrigeration were consistent with expectations of retrograded starch formation; however, our finding suggest that protein and other non-starch components may play a role in influencing starch susceptibility to amylolysis after cold-storage and re-heating. The subtle reductions in starch amylolysis obtained by refrigeration in this study would likely have relatively minor implications for lowering of glycaemic response.

Author contributions

CE conceived and designed the analyses; SC collected the data; SC, NP and CE performed the analyses; SC and CE wrote the paper; CE had responsibility for the final content. All authors read and approved the final manuscript.

Conflicts of interest

There are no conflicts of interest to declare.

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