Title:

A natural mutation in a starch branching enzyme gene alters starch assembly in *Pisum sativum L. (pea)* and improves glucose homeostasis in humans

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Abstract: Elevated postprandial glucose (PPG) is a significant risk factor of non-communicable diseases globally. Currently there is a limited understanding of how starch structures within a carbohydrate-rich food matrix, interact with the gut luminal environment to control PPG. Here, we use pea seeds (*Pisum sativum*) and pea flour, derived from two near-identical pea genotypes (BC1/19RR and BC1/19rr) differing primarily in the type of starch accumulated, to explore the contribution of starch structure, food matrix and intestinal environment on PPG. Using stable isotope [^{13}C] labelled pea seeds, coupled with synchronous gastric, duodenal and plasma sampling *in vivo,* we demonstrate that maintenance of cell structure and changes in starch morphology are closely related to lower glucose availability in the small intestine, resulting in acutely lower PPG and promotion of changes in the gut bacterial composition associated with long term metabolic health improvements.

Introduction

Abnormal postprandial blood glucose (PPG) is a major risk factor for type 2 diabetes (T2D) and associated metabolic diseases [1]. The consumption of carbohydrate-rich foods is a determinant of PPG response [2], and the glycaemic index ranks these foods according to their impact on PPG [3]. Evidence from randomized control trials and systematic reviews shows a benefit of low glycemic index diets on long term glycaemic control in T2D [4-6]. The effect a carbohydrate-based food has on PPG is dependent on many factors, including the physical structure of food, the type of carbohydrate it contains, and neural and hormonal responses its ingestion elicits. The same food can produce different PPG and insulin responses depending purely on processing, such as in the cases of native and retrograded starch [7].

Here, we investigate the impact of food structure, carbohydrate type, and the bioaccesibility of starch in the gastrointestinal environment (gastric and duodenal digestion and colonic fermentation) on PPG and gut bacteria related to glucose metabolism. We used mature seeds of pea (*Pisum sativum L.*), as a model food in a series of experiments spanning *in vitro* laboratory to metabolic studies in humans.

There is systematic evidence that non-oil-seed pulses, of which pea is a member, have a significant impact on long term glycaemic control [8]. Additionally, the genetic variation available in pea, provides an opportunity to investigate the impact of starch assembly on digestive processes. As the physical state of food can be a significant determinant of postprandial glycaemia, using pea flour enables the study of this phenomenon in some depth.

We used two near-isogenic pea lines, which are genetically identical except that one (BC1/19rr) carries a natural mutation in the starch branching enzyme I gene (*SBEI*) [9]. In BC1/19RR, the wild-type or control line, SBEI makes a major contribution to the amylopectin fraction present in seeds, where the enzyme is active within the plastids of the cotyledonary cells (Fig. 1). The naturally occurring mutation in the *sbeI* gene (BC1/19rr) disrupts the carboxy-terminal region of the protein, affecting the structure of the starch and other seed phenotypic traits [9, 10]. In the mutant line, the majority of the starch which is synthesised has been dubbed ‘resistant starch’, reflecting its largely unbranched amylose polymers and resistance to digestion. This naturally occurring mutation is unique in *rr* peas. However, mutations in SBE exist in other species but these are mainly induced mutations, such as those in rice [11], durum wheat [12], bread wheat [13], rice [14] and potato [15].

In this work, we compared BC1/19RR wild-type and mutant BC1/19rr peas to examine the effects of genetic alterations to starch structure on digestion parameters (using *in vitro* oral/gastric and duodenal simulated digestion models) and associated health outcomes (by performing experiments *in vivo*, in human volunteers). We investigated the effects of processing and food structure by generating pea flour and producing pea-derived food products where processing had disrupted of the cell wall. The acceptability of these products and the relative effects on glucose metabolism were tested in a sample of healthy volunteers with no compromised glycaemic control.

Figure 1. Starch biosynthetic pathway in pea seeds. The contribution of different enzymes to steps in the cytosol and within the plastid and starch granule of the wild-type line, BC1/19RR, are shown in green with the metabolites in blue. The genotype of BC1/19rr carries a naturally-occurring insertion in a starch-branching enzyme gene (*sbeI-ins*, indicated by the red box, and used in this study) [9]. The mutation affects the activity of the enzyme within the structure granule of BC1/19rr, where the pathway to amylopectin is disrupted (indicated by red bar on the arrow) and starch structure is changed. Adapted from [16]**Results**

Food-structure and *rr* starch genotype attenuates postprandial glycaemia

In a randomized, controlled, double blind, cross-over trial (trial 1), 10 healthy volunteers were fed a cooked, mixed meal containing 50 g dry weight of pea seeds or flour from two pea genotypes (*RR* and *rr*; providing 31% and 26%, of the total carbohydrate content of the meal, respectively; see Methods and Supplementary Table 1). There was no significant differences in solid phase gastric emptying, assessed by the [^{13}C] octanoic acid breath test [17], between the *rr vs RR* pea seeds or flour groups (p=0.49 and p=0.59, respectively; Figure 2A, B), as determined by $T_{½}$ (half-emptying time), indicating that volunteers began digesting the different test meals in a similar time course. There was a significantly lower PPG between the *rr and RR* pea seeds and between the *rr and RR* flour groups (effect over time, p=0.02 and p=0.04, respectively; Fig. 2C, D).

In a second randomized, controlled, double blind, cross-over trial (trial 2) 12 healthy volunteers were fed 50 g dry weight of pea seeds or flour from two pea genotypes (*RR* and *rr*;) as a cooked product (rather than a mixed meal; Methods and Supplementary Table 2). For pea seeds group, plasma glucose and serum insulin concentrations were significantly lower after consumption of *rr* compared to *RR* (effect over time, p=0.02 and 0.001 respectively) (Fig. 2E, F). There was no effect on glucagon-like peptide 1 (GLP-1) (Fig. 2G). The *RR* pea seeds consumption led to a higher release of gastric inhibitory polypeptide (GIP) compared to *rr* (effect over time, p=0.01) (Fig. 2H). For flour, a lower PPG response occurred after consumption of *rr* compared to *RR* (effect over time, p=0.06) (Fig. 2I). Serum

insulin incremental area under the curve 120 min ($iAUC_{0\text{-}120min}$) was significantly reduced by 37% (p=0.04) for *rr* compared to *RR* flour (Fig. 2J). During the first 15 min post ingestion, there was a significantly higher peak in GLP-1 concentrations observed for *RR* compared to the *rr* group (p=0.001) (Fig. 2K). There was also a significantly higher $iAUC_{0\text{-}120min}$ GIP for *RR* compared to *rr* (p=0.02) (Fig. 2L).

Changes in food structure, induced through processing whole seeds to flour within genotype, showed profound effects on PPG and serum insulin. In both *RR* and *rr*, processing to flour produced a significantly larger glucose and insulin response over 180 min (Supplementary Figure 1A-D).

Together, these data demonstrate that both starch genotype and food structure have an impact on postprandial glycaemia.

Figure 2. Effects of acute consumption of 50 g dry weight *RR* and *rr* pea seeds and flour.

$t_{½}$ was determined from the modelled [^{13}C] data to describe gastric emptying rates. $t_{1/2}$ was defined as the timepoint at which 50% of exhaled $^{13}CO_2$ is recovered. (A) Gastric emptying rates for *RR* and *rr* pea seeds group during a mixed meal experiment (trial1) (n=10). (B) Gastric emptying rates for *RR* and *rr* flour group during a mixed meal experiment (trial 1) (n=10). (C, D,) Concentration of plasma glucose for *RR* and *rr* pea seeds and flour groups during a mixed meal experiment (trial 1) (n=10). (E-H) Concentration of plasma glucose, serum insulin, GLP-1 and GIP measured for 180 min for *RR* and *rr* pea seeds group (pea seeds consumed without the mixed meal, trial 2) (n=12). (I-L) Concentration of plasma glucose and corresponding serum insulin, plasma GLP-1 and GIP for *RR* and *rr* flour group (pea flour was consumed without the mixed meal, trial 2). Analysis for flour group was performed on available paired data, (n=11). Insets show the iAUC between 0 and 300/120 for the trial 1 and 180/120 min for Trial 2. The data represent mean ±SEM. Repeated Measures Anova was used to analyse time course data with pea/flour and time as within-subject factors. Fisher LSD post-hoc tests were performed between timepoints when significant pea/flour$_x$time interaction was found. Paired *t*-tests were used for iAUCs calculations and gastric emptying data analysis. Timepoints at which values differed significantly, *p<0.05, **p<0.01, ***p<0.001. Abbreviation: iAUC, incremental area under the curve

Impact of pea structure and starch assembly on starch digestion

A series of *in vitro* studies elaborated physico-chemical mechanisms of starch digestion and nutrient bio-accessibility in *RR* and *rr* pea seeds and flour.

Starch digestibility

The total starch contents of pea seeds and flour were determined at raw, post-cooking and post-simulated digestion (oral, gastric/small intestinal conditions) stages (Fig. 3A). Starch digestion in cooked pea seeds was 60% for *RR* and 24% in *rr* (Fig. 3A) indicating that the starch in *rr* was less digestible by the upper gastrointestinal enzymes versus *RR* pea seeds (p<0.0001), and corroborating Fig. 2 findings.

After cooking, the portion of analytically resistant starch (ARS) content (that is, the estimated proportion of resistant starch using standard laboratory methods), based on the AOAC 2002.02 method [18], decreased in *RR* pea seeds ($p<0.05$) but increased in the *rr* after cooking and digestion ($p<0.01$). We observed no statistical significant difference between the *RR* and *rr* flour (Fig. 3B).

^{13}C cross polarized magic angle spinning (CP-MAS) NMR was used to establish the helical structure of the starch in uncooked and cooked pea seeds and flour, a key determinant of its resistance to digestion (Fig. 3C & Supplementary Figure 2) [19, 20]. The starch in the uncooked *RR* line presented a 35% double helical structure, in both pea seeds and flour (indicating that the starch was not significantly altered during milling), whereas the *rr* line had a lower proportion of double helices (19%) (Fig. 3C). Following cooking of the flours the starches from both genotypes (*RR* and *rr*) fully gelatinized, having less than 10% double helical order. Starch from pea seeds of the *RR* genotype only partially gelatinised, with a small decrease in double helical order (from 34% to 27%) ($p<0.05$), whereas in the *rr* genotype there was an increase in double helical order observed following cooking (from 20% to 31%) ($p<0.01$). This difference in gelation behaviour suggests that the matrix structure (flour vs peas) influences how the starch granule structure rearranges upon heating, and controls the ARS. This consurs with the onserved PPG and serum insulin concentrations between the *RR* and *rr* flour compared to *RR* and *rr* pea seeds (Fig. 2) .

We hypothesized that in the spatially and water limited environment of the plant cell, the starch undergoes structural rearrangements that are different to those in the flour, leading to higher levels of ordered structures in the cooked pea seeds relative to cooked flours. These observations may be attributed to differences in the chain length distribution of the starches (Fig. 3D), where the *rr* starch has far fewer short ($R_h<4nm$) amylopectin chains and a greater proportion of longer amylose chains. A higher proportion of longer amylose chains which limits swelling of the starch (Fig. 3F) alters recrystallisation following cooling (Fig. 3C) [21] and leads to a greater proportion of starch escaping small intestinal digestion and reaching the colon to be ferment by resident gut bacteria.

Food-matrix and processing and digestion

Pea seed fragments (> 2 mm) formed following simulated oral phase ‘chewing’ survived *in vitro* digestion intact. Therefore, the particle size was measured post-gastric and intestinal phases. *rr* digesta contained more larger particles (> 700 μm) compared to *RR* (Fig 3E) The digesta from *RR*

contained a higher proportion of smaller particle (< 250 μm) than *rr* (such as individual cells and free starch), suggesting that the *RR* tissue was more friable than for *rr*. The particle size did not change significantly during simulated digestion, so the major impact on the structure was from cooking and simulated chewing.

Compression experiments to understand fracture profiles of cooked pea seeds demonstrated that force-deformation curves were higher in *rr* compared to *RR* at 1mm/s ($p<0.0001$) but were similar in both genotypes when compression rate was increased to 15mm/s (Fig. 3H). The *rr* pea seeds fractured into larger particles of tissue in contrast to *RR*, where they appeared to breakdown completely (Fig. 3H). Although the moisture content for *rr* was higher than for *RR* cooked seeds (60% vs 45%), it was noted that the physical appearance of compressed seeds was different; *rr* seeds tended to split while *RR* seeds were crushed (Fig. 3H). The greater resistance of the *rr* seeds to deformation could be attributed to known differences in cell wall properties, structural differences in the intracellular starch and/or protein and/or degree of hydration of plant tissue components [22].

Micrographs of flour and pea seed sections demonstrated the impact of cooking (Fig. 3F, G) and simulated digestion (Supplementary Figure 3) on the cellular structure and starch morphology. Micrographs of flour demonstrated the influence of cooking on pea starch following the loss of the pea matrix by milling into flour. The raw starch granules of *rr* were very different in morphology to those of the *RR* genotype (Fig. 3F), composed of a mixture of simple and compound granules due to high amylose content [23]. Starch from *RR* flour appeared to be almost fully gelatinized after cooking (Fig. 3F) and was no longer visible after digestion *in vitro* (Supplementary Figure 3). Together with the shorter chain lengths in *RR* (Fig. 3D), this may explain the loss of total starch in *RR* flour following cooking, as the shorter chains are likely to have leached out of the granules, avoiding detection by the starch assay. However, intact starch granules from *rr* flour persisted throughout cooking, showing many intact, non-gelatinised granules (Fig. 3F) and following digestion *in vitro* (Supplementary Figure 3). After simulated duodenal digestion, however, the remaining *rr* flour granules were stained pink rather than blue. After cooking, *RR* starch granules gelatinized to a greater extent than those of *rr* (Fig. 3G), where the starch granules appeared less swollen. In the *RR* peas, starch appeared to be hydrolysed in some cells and not in others, after digestion *in vitro* (Supplementary Figure 3). On the other hand, starch granules in *rr* pea cells, that were less swollen after cooking, appeared undigested within the cells (Supplementary Figure 3). Scanning electron micrographs (Supplementary Figure 4) of

uncooked and cooked pea seeds confirmed the extent of starch gelatinization within cotyledon cells seen with light microscopy and also suggested some morphological differences between *RR* and *rr.* The results show that there are at least two main factors which influence starch digestibility in the pea samples studied. Firstly, the matrix structure, where intact plant cell walls encapsulate the starch, act as enzyme barriers and also hindering gelatinization of intracellular starch by reducing access to water; and secondly the intrinsic resistance of the starch granule, with the higher ARS content of the *rr* genotype making it more resistant to digestion. Even though the *rr* starch in the flour lost much of its order, the morphology of the *rr* starch granules was affected less than *RR* starch following cooking. Furthermore, the tissue matrix affected fracture properties such that chewing produced larger particles for *rr* pea seeds and thus more intact cells acting as a barrier to digestion.

Figure 3. Impact of genotype structure and processing on starch digestibility.

Total (A) and analytically resistant starch (B) contents of uncooked (UC), cooked (C) and cooked + simulated digested (C+SD) *RR* and *rr* pea seeds and flours. Structural characteristics of starch in uncooked and cooked flour and pea seeds; helical structure (C), chain length distribution (D). Size of cooked pea seed fragments after simulated gastric and intestinal digestion (E). Micrographs of uncooked and cooked flours (F) and sections from uncooked and cooked pea seeds (G). Compression experiments (H) using hydrated/cooked *RR* and *rr* pea seeds. One-way ANOVA was used on *in vitro* digestion data. Paired Student's t-test was used for the compression experiments *p<0.05, **p<0.01, ***p<0.001. ****p<0.0001

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233 **_rr_ genotype results in reduced small intestinal glucose release in humans**

234 We investigated the impact of the *rr* mutation on duodenal glucose release in humans by intubating
235 the small intestine and stomach of 12 healthy volunteers via the nasal route (trial 2). After
236 consumption of pea seeds small intestinal glucose concentration for the *RR* group, at 30 min, was
237 3.77 ± 2.28 mmol/L which was nearly two-fold higher than for the *rr* group at the same time point (1.92
238 ± 2.21 mmol/L) (Fig 4A). We found that in the *rr* group, the area under the curve 120 min ($AUC_{0\text{-}120min}$)
239 for small intestinal glucose concentrations was significantly lower compared to the *RR* group (p=0.02;
240 Fig. 4B). We found no statistically significant differences in the small intestinal glucose responses
241 between flour groups from the two pea genotypes (Fig. 4C, D).

242 *Gastric and small intestinal metabolic profiles for pea seeds*

243 The gastric and small intestinal metabolic profiles of the aspirated samples were assessed using an
244 untargeted metabolic profiling approach by Proton Nuclear Magnetic Resonance (^{1}H-NMR)
245 spectroscopy. Significant differences were found in the metabolic profiles of gastric samples of the *RR*
246 and *rr* pea seed groups (Fig. 4E). Signals corresponding to the group of metabolites (full list
247 Supplementary Tables 3, 4), including amylopectin/maltotriose/maltose, were significantly higher for
248 the *RR* samples at 30 min post ingestion compared with those of *rr* (p=0.0004, Q=0.002).

249 We found statistically significant differences in glucose release rates, at 60 min post ingestion between
250 the two pea groups (Fig. 4F). *RR* pea seeds group resulted in higher glucose release compared with *rr*
251 group (p=0.001, Q=0.01) (Supplementary Table 5). The data suggests that leaching of
252 amylopectin/maltotriose/maltose from the *RR* seeds makes them more susceptible to early digestion
253 and release of glucose.

254 *Gastric and small intestinal metabolic profiles for pea flour*

255 ^{1}H-NMR metabolomic analysis of gastric flour samples indicated differences between the two pea
256 genotypes (Fig. 4G). Similar, to pea seeds, we found higher amylopectin/maltotriose/maltose in the
257 gastric content of the *RR* flour group at 15- and 30-min post ingestion (p=0.00004,
258 Q=0.0019/p=0.0001, Q=0.0006 respectively; Supplementary Table 6).

In the small intestinal samples, the *RR* flour group showed higher glucose concentrations at 30 min compared to *rr* ($p<0.001$, $q<0.001$) (Fig. 4H). We also observed higher release of sucrose and alanine for *rr* flour compared to *RR* (Supplementary Table 7).

α-amylase permeability in-vitro and ex vivo

Time course data from confocal microscopy showed that, within 10 min, FITC-amylase had diffused into the cell walls of both *rr* and *RR* pea seeds (Fig. 4I) but not yet passed into the intracellular space. Further ingress of the enzymes into the intracellular space was slow, as captured by the diffusion constant (given by fluorescence intensity.nm^2.min^{-1}; 6.19×10^{-10} for *rr* and 1.23×10^{-9} *RR* (summarised in histogram, Supplementary Figure 5), and there was heterogeneity in plant cells obtained from *RR* pea seeds, as seen in the time course data (Fig. 4I). For flour group, the amylase had bound to the surface of starch granules within 5 min and seemed to progressively erode the starch granules over time (Fig. 4J). These experiments indicate that the encapsulation of starch by the cell wall obstructs interaction with amylase enzyme. The diffusion of amylase across the intracellular space appeared to be slower for *rr* flour than *RR* overall.

Using the small intestinal digesta from the study *in vivo* we performed experiments *ex vivo* aiming to understand the cell wall permeability to *α*-amylase (AA). In both *RR* and *rr* peas the diffusion of AA-FITC into cells was progressive with time and the diffusion of AA in *rr* pea samples was slower than in *RR (*Fig. 4K). Uptake of AA-FITC into *RR* and *rr* pea flour was almost immediate (Fig. 4K).

Figure 4. The effect of structure and genotype of pea seeds and flour on small intestinal environment. (A) Small intestinal postprandial glucose curves for *RR* and *rr* pea seeds along with corresponding plasma glucose, where analysis was performed on available paired data, (n=8). (B) Individual responses expressed as $AUC_{0\text{-}120min}$ for small intestinal glucose for pea seeds (n=8). (C) Postprandial small intestinal glucose curves for *RR* and *rr* flour along with corresponding plasma glucose, analysis was performed on available paired data, (n=7). (D) $AUC_{0\text{-}120min}$ for small intestinal glucose for flour group (n=7). (E) RM-MCCV-PLS-DA scores plots of 1D ^{1}H-NMR gastric samples comparing volunteers at 30 min after consumption of *RR* vs *rr* pea seed groups (n=10). Model score: R^2Y 0.81, Q^2Y 0.29. Dots represent the metabolic profile of each volunteer from the study cohort; blue indicates *RR* and red indicates *rr* pea seed groups. (F) Fragment from the average 600 MHz 1D ^{1}H-NMR spectrum of the *RR* (blue) vs *rr* (red) pea seeds showing the anomeric carbon signal (5.24 (d)) of the glucose molecule. (G) RM-MCCV-PLS-DA scores plots of 1D ^{1}H-NMR gastric samples comparing volunteers at 30 min after consumption of *RR* vs *rr* flour groups. Model score: R^2Y 0.99, Q^2Y 0.85. Dots represent the metabolic profile of each volunteer from the study cohort; green corresponds to *RR* and orange corresponds to *rr* flour groups. (H) Fragment from the average 600 MHz 1D ^{1}H-NMR spectrum of the *RR* flour (green) vs *rr* flour (orange) showing the anomeric carbon signal (5.24 (d)) of the glucose molecule. (I) Diffusion of labelled α-amylase-FITC in cooked *RR* and *rr* pea seeds (green) at different timepoints. (J) Diffusion of labelled α-amylase-FITC in cooked *RR* and *rr* flour (green) at 5 and 120 min. (K) Diffusion of labelled α-amylase-FITC at 90 min in duodenal samples in *RR* and *rr* pea seeds and flour group. Data are presented as mean ±SEM. Timepoints at which values differ significantly, *$p<0.05$,

291 **p<0.01. Abbreviations: AUC (area under the curve), RM-MCCV-PLSD (Repeated measures-Monte Carlo cross validation-Partial-squares-
292 discriminant analysis), AA (α-amylase), FITC (Fluorescein isothiocyanate)

rr genotype increases SCFA production

We grew both *RR* and *rr* pea seeds in a $^{13}CO_2$ enriched environment in a hermetically sealed greenhouse, producing pea starch with an enrichment of ~0.2 atom percent ^{13}C above natural abundance and assessed labelled metabolites in plasma and urine.

Volunteers (n=10) took part in a third randomized, controlled, double blind, cross over trial (trial 3) to investigate ^{13}C glucose and ^{13}C SCFA appearance after 50 g dry weight *RR* and *rr* pea seeds or flour consumption included in a mixed meal (Supplementary Table 8). The time course and AUC for exogenous ^{13}C postprandial plasma glucose indicated significantly higher concentrations after consumption of the *RR* as opposed to the *rr* pea seeds test meal (Fig. 5A, B). There was no significant effect observed when comparing *RR* and *rr* flour for exogenous postprandial ^{13}C plasma glucose and total $AUC_{0\text{-}480min}$ (Fig. 5C, D). These observations support the PPG results observed in the Fig. 2 (trial 2).

We measured fractional recovery of ^{13}C SCFA (acetate, propionate and butyrate) in 24 h urinary collections. ^{13}C acetate excretion did not result in significant differences between *RR* and *rr* in either the seed or flour groups (p=0.65). ^{13}C propionate and ^{13}C butyrate output was significantly higher after consumption of *rr*, either seeds or flour (p=0.01, p=0.03, respectively) (Fig. 5E). This suggests that, with *rr* test meals, carbohydrate was not fully digested in the small intestine and more was delivered to the colon where it was fermented by the gut microbiota. We investigated whether or not changes in the stool gut microbiome might occur over 24 h but there was no difference in gut microbiome diversity between the pea genotypes for either test meal (Fig. 5F).

These data suggest that the main effect on glucose absorption is the structural barrier of the pea seeds which is enhanced by the *rr* genotype. However, the SCFA production highlights that the starch from the *rr* flour was not full digested in the small intestine. There was no evidence of an acute effect on stool microbiota diversity although we observed an increase in SCFA production with the *rr* genotype in both pea seeds and flour.

Figure 5. Using stable-isotope ^{13}C-enriched *RR* and *rr* pea seeds and flour to understand the digestion and fermentation process further. (A) ^{13}C plasma glucose curves for *RR* and *rr* groups after administration of 50 g dry weight ^{13}C-enriched pea seeds along with a mixed meal test (n=9). (B) Total $AUC_{0\text{-}480min}$ of exogenous ^{13}C plasma glucose concentrations for *RR* and *rr* seeds (n=9). (C) Postprandial plasma ^{13}C glucose responses for *RR* and *rr* flour and (D) corresponding $AUC_{0\text{-}480min}$ (n=8). (E) Fractional enrichment in urinary concentrations of ^{13}C acetate, ^{13}C propionate and ^{13}C butyrate after consumption of ^{13}C-enriched *RR* and *rr* seeds (n=10) and flour (n=9). (F) Gut microbiota weighted beta-diversity plots for *RR* (blue) and *rr* (red) peas and *RR* (green) and *rr* (orange) flour, where each data-point represents the microbial community of a single sample. ^{13}C plasma glucose and urine samples were analysed using gas chromatography-combustion isotope ratio mass spectrometry. Beta

diversity analysis was performed using the UniFrac metric calculated with QIIME 1.9.0 and visualized as a 3D principal coordinates analysis plot using Emperor. Data represent mean ±SEM. Repeated Measures Anova was used for testing time course data with pea/flour and time as within-subject factors. Fisher LSD post-hoc tests were performed between timepoints when significant pea/flour$_x$time interaction was found. Paired t-tests were used for AUC calculations. Time points at which values differ significantly, *p<0.05, *** p<0.001. Abbreviation: AUC, total area under the curve

Effect of *RR* versus *rr* pea-derived products consumption on glycaemic control independently of the food matrix.

To understand the effects of the pea genotype independently of the food matrix on the PPG and gut bacteria we used a randomized, double-blind, 4 phase crossover control trial (trial 4) in 25 healthy volunteers aged 40-70 years (Supplementary Table 9 , Supplementary Figure 6 for volunteer characteristics & consort diagram). Volunteers were provided with mushy-peas and pea-hummus from both the *RR* and *rr* lines to consume for 28 days in random order. The nutritional content of the products can be found in Supplementary Table 10. All measurements were performed at baseline (0 days) and follow up (28 days) after a 12 h overnight fast. We assessed the effects of repeated pea consumption exposure on stool gut bacteria, glucose metabolism, as well as GLP-1 and lipids. A full summary of all outcome variables can be found in Supplementary Table 11.

Glucose metabolism

During the experimental visits, at baseline and follow up, volunteers did not receive the interventional pea-derived products but a mixed meal tolerance test as the aim of the study was to examine the chronic effect of products consumption. No statistically significant differences in markers of plasma glucose and serum insulin within or between groups were observed (Fig. 6 A-D). We observed no differences in β-cell function and insulin sensitivity, using the Homeostatic Model Assessment 2 (HOMA 2), within or between groups. A possible explanation for this outcome might be the differences in the study design from that of the acute studies where volunteers consumed the pea seeds or flour. The rationale behind the study design here was that the chronic exposure to the intake of *rr* pea-derived food products would change the microbiota and lead to metabolic changes that would affect PPG and insulin responses, what has been termed as a ‘’second meal effect’’.

Gut microbiome 16S rRNA gene sequencing

Nonmetric multidimensional scaling plot (NMDS) indicated no statistically significant effect in the clustering within or between *RR* or *rr* interventions (within *RR*: p=0.83, within *rr*: p=0.92; between interventions: p=0.92) (Fig. 6E). Due to high inter-individual variability posed by changes in baseline gut microbiota and the habitual diets between volunteers, we decided to examine the data as paired samples per volunteer and looked specifically for gut bacteria related to insulin resistance. Within the *rr* intervention group, at genus level, there was a decrease in the relative proportion of *Bacteroides* (p=0.04) and an increase in the relative proportion of *Bifidobacterium* (p=0.007) (Fig. 6F). Within the *RR* intervention group, results indicated a statistically significant decrease in *Lachnospiraceae* and *Ruminococcaceae* (p=0.01, p=0.004 respectively), which are known as starch degraders. Between groups (*RR* and *rr* interventions), a statistically significant decrease in the relative abundance of *Collinsella* was observed after 28 days of *rr* supplementation (p=0.03). Previous studies have reported increased levels of *Collinsella* in individuals suffering from T2D [24].

Despite observing differences in gut bacteria, there was no effect on glucose and insulin responses even though controlling for adherence to the interventions by measuring trigonelline, a validated pea biomarker (Supplementary Notes and Supplementary Figure 7). We suggest that the food matrices of pea seeds have an acute impact on PPG, as seen in acute studies. The findings support the concept, that whilst dietary interventions may promote changes to gut bacterial populations, this may not automatically translate into improvements to glucose metabolism in ''free-living'' individuals.'' In an ideal situation we would have run two experiments by including the pea seeds/products and by just performing a mixed meal tolerance test (as the one we did). However, this is difficult for many possible reasons such as blood volume loss.

Figure 6. The effect of consuming products derived from the two pea genotypes for 28 days on glucose homeostasis and gut microbiota. (A) Postprandial plasma glucose for *RR* and *rr* lines (B) and serum insulin responses: *RR* (C), *rr* (D). (E) Nonmetric multidimensional scaling (NMDS) plots for *RR* and *rr* pea interventions before and after the consumption of pea derived food products. *RR* visit 1; , *RR* Visit 2 , *rr* Visit 1 , *rr* Visit 2 . NMDS represent a snapshot of the bacterial community structure, where dots close together have similar community structure while those far apart are dissimilar. Therefore, in this figure points closely located on the plot are more similar in local community composition than are more distant points. Samples were subsampled to the lowest number of reads (3926) which resulted in at least 97% coverage for each sample (Supplementary Figure 8). Analysis was performed on available paired data (n=22). Subsample read counts for Bifidobacterium and Bacteroides for both lines of peas (F)

Discussion

Utilising the mutation which results in a defective starch branching enzyme and an increase in resistant starch in *rr* compared with *RR* wild-type pea genotypes, we demonstrate that pea seeds and flour from the *rr* genotype, significantly impact glucose and insulin homeostasis compared to the *RR* wild type. This is most marked when the cellular structures remained intact during digestion suggesting that bio-accessibility of amylase to the starch structures in the cell play a major role, an effect observed previously in intact wheat endosperm with a concomitant decrease in PPG [25]. The impact of *rr* flour on post prandial blood glucose was less marked than the pea seeds. This may be due to two factors; firstly, the initial rate of amylase digestion is the same between the genotypes but decreases in the *rr* genotype over time [26]. This is due to the starch granule of the *rr* seeds having an outer layer of amorphous starch and is rapidly digested, as in the *RR* genotype, but also has an inner core of crystalline starch [27]. Secondly the transit time in the duodenal space in humans is less than two hours, which is possible not long enough of the crystalline resistant starch inner core of the *rr* genotype to make a difference to the glucose availability in the duodenal space between the two genotypes. This observation is supported by one of the unique experiments reported in this manuscript: the direct measurement of glucose in the duodenum of humans. Here we show that the availability of carbohydrate in the small intestine directly relates to plasma glucose concentrations. The more resistant starch structures in the *rr* pea seeds led to lower duodenal and lower PPG with a greater transfer of carbohydrate to the large bowel.

The higher availability of glucose in the duodenum from *RR* pea seeds is associated with an increased release of GIP which may explain the higher insulin levels. Duodenal glucose infusions in humans have shown similar findings with high concentrations and flow rates of glucose in the duodenum increasing both GLP-1 and GIP concentrations in the plasma [28]. Although we did not observe a significant difference in direct measures of duodenal postprandial glucose in the flour group, the NMR analysis suggests a higher duodenal glucose at 30 min which does coincide with the higher GIP and GLP-1 concentrations in the *RR* flour. We conclude that postprandial insulin concentrations are higher after the consumption of *RR* pea seeds and flour and this is driven through a higher availability of glucose in the small intestine and the stimulation, at least in part, of the incretin GIP. The reduction in

413 duodenal glucose and PPG in the face of lower insulin release but an increase in colonic fermentation
414 in the *rr* genotype would appear to be solely due to an increase in starch reaching the colon.
415 The experiments *in vitro* and *in vivo* performed here demonstrated the complex multifactorial nature of
416 the increased delivery of starch to the colon in the *rr* genotype. We observed that the cooked *rr* pea
417 seeds appear more resistant to fracture and during simulated gastric and duodenal digestion the size
418 of the particle population remained larger reducing the surface area for amylase activity as previously
419 shown [27]. The metabolomic profiling of the aspirated gastric and duodenal samples indicated
420 differences between the two genotypes in the amylopectin/maltotriose/maltose concentrations during
421 digestion. It is known that amylopectin is more readily digested than amylose and that amylose is a
422 poor substrate for pancreatic α-amylase [29]. By identifying higher concentrations of these metabolites
423 in the digesta from the *RR* genotype it would suggest that the greater fracturing of the food matrix in
424 the *RR* genotype leads to increases in digestible carbohydrate in the duodenum. We demonstrated
425 that the complex nature of starch digestion and the size, morphology and physical chemistry of starch
426 granules (helix ordering and chain length) are more accurate predictors of glycaemic response than
427 simply the amylose content of the pea seeds. For example, we demonstrated that cooking *rr* pea
428 seeds increased amylose double helix starch structure, creating resistant starch that is not seen in
429 *RR*. This process has previously been demonstrated to increase resistance to amylase [19]. Our data
430 also suggest that the penetration of α-amylase into *rr* cells is lower and slower than in *RR* not only in
431 the samples digested *in vitro* but in duodenal samples from humans *in vivo*, similar to observations
432 made in ileostomy volunteers using wheat flour and particles [25]. The studies *in vitro* clearly align
433 with the stable isotope experimental studies *in vivo* which demonstrate a reduced absorption of
434 carbohydrate in the small intestine with an increase in bacterial fermentation in the *rr* compared with
435 the *RR* group, as judged by fractional recovery in 24 h urinary ^{13}C SCFA propionate and butyrate
436 profiles. SCFAs, particularly butyrate, are associated with numerous health benefits [30]. There was
437 no detectable change in the stool 16S profile in the *rr* compared to the *RR* group at 24 h despite the
438 increase in SCFA production. This suggests an increase in microbial carbon flux from the labelled
439 carbohydrate in the colon from the *rr* peas without an acute change in microbial diversity compared to
440 the *RR* peas. This effect was observed in both seeds and flour from the *rr* line highlighting the
441 importance of the mutation on the starch compositional profile regardless of the food matrix,

processing and preparation. These observations highlight the multicomponent aspect leading to reduced duodenal glucose.

Observations from the 28-day supplementation study, demonstrated no effect on glucose metabolism related biomarkers despite some positive changes in the gut microbiota, with an increase in the proportion of the genus Bifidobacterium following supplementation with the *rr* genotype. Studies have shown that Bifidobacterium abundance increase with enriched carbohydrate environment and has been associated with improvements and maintenance of metabolic health [31]. However, this concept was not proven here This outcome highlights that the impact of gut microbial metabolites and/or gut microbe/host immune interactions is possibly weakly associated with glucose metabolism improvements in healthy individuals.

It is important to highlight some limitations of this work**.** Our data focuses on acute differences in glycaemic responses from an overnight fasted state and, therefore, studies are needed to confirm these results in a real-life setting. The pea-derived products were single food products, which were added to the habitual diets of volunteers without any other alterations in their diets. Given the results from the acute studies, it might have been more efficacious to have a portfolio of products with an item eaten at each meal.

Our data shows that the impact of the contrasting pea genotypes on PPG it is due to complex differences in starch structure and food matrix and their impact on cooking and digestion. Additionally, to allow comparison with a common measure of carbohydrate quality we reported the glycaemic index value. This experiment confirms that *rr* pea seeds have a lower postprandial glucose response than *RR*, garden peas and glucose control (Supplementary Figure 9).

These observations could be used to inform the production of modified food types, either through the selection of digestion resistant starch phenotypes or altered food matrices with an aim to drastically lower PPG, reducing risk of metabolic diseases at a population level.

Recent studies of SBE genetic mutations have extended to induced mutations in staple crops such as rice and wheat, adding potential and wide applicability for direct translation of our results. With modern genetic and genomic tools, the discovery or generation of *sbe* mutations across a number of seed and grain crops provides great potential for expansion of such food products to tackle major diseases, such as T2D. It is worth emphasizing that the naturally occurring mutation in pea, studied here, is one of the classical mutations studied by Mendel, on which the science of genetics is based. Accessions of

pea carrying the *sbe* mutation have been commercially cultivated for several decades as a fresh vegetable crop. Introducing this mutation into pea crops for a broader range of food uses in underway, to provide ingredients for a wide range of industries.

Materials and Methods

Food Materials

The food materials used during the experimental procedures are listed below:

1. Wild type pea seeds (BC1/19RR line), used as control group
2. Mutant pea seeds (BC1/19rr line), used as the treatment group
3. Wild type pea flour (BC1/19RR line), used as control group
4. Mutant pea flour (BC1/19rr line), used as treatment group
5. Wild type pea hummus and mushy peas (BC1/19RR line), used as control group
6. Mutant type pea hummus and mushy peas (BC1/19rr Line), used a treatment group.

The near-isogenic lines of pea (BC1/19RR, BC1/19rr) are available from the John Innes Centre Germplasm Resources Unit (JI3316 = *RR*, round-seeded, JI3317 = *rr*, wrinkled-seeded; https://www.seedstor.ac.uk). Bulked seed stocks were generated by growing plants on wire in field plots over successive seasons (March – July). The resulting seed stocks were used for studies *in vivo* and *in vitro* and supplied to the University of Glasgow for ^{13}C labelling and Campden BRI to produce the pea derived products. Campden BRI developed the two pea products for the long-term study (trial 4).

^{13}C labelling of pea seeds were sown in troughs in a glasshouse at the James Hutton Institute, Dundee. The plants developed well and were pulse labelled with $^{13}CO_2$ one week after flowering. The mature seed was collected and air dried. A sub-sample of each variety was milled to a fine flour and analysed for crude protein, C:N ratio, total ^{13}C and starch ^{13}C. The yield of the wild type was 1.24 kg at 0.242 atom % ^{13}C excess, as measured by EA-IRMS at SUERC. The yield of the *rr* mutant was 1.26 kg at 0.133 atom % ^{13}C excess.

Human Clinical Trials

Volunteers

Volunteers were provided with informed written consent forms prior to their participation in the 4 human clinical trial studies. We performed three acute studies (trial 1,2,3) and one randomized controlled trial (trial 4). In the acute studies 1 and 3 we recruited 10 volunteers per study as these were exploratory studies and the first time being conducted in humans. In the acute study 2, we recruited 12 volunteers. Although this was also an exploratory study, due to the nature and difficulty of the sample collection (intubation of gastric and duodenum of volunteers) we decided to include 12 volunteers to account for a possible higher dropout rate or difficulties in samples aspiration. This information was based on previous data by Steven Julious (2005) where he reported that a sample

size between 10-12 is enough to gain precision in the mean and variance [32]. For the final study, which was a randomized cross over, double blind clinical trial data from Te Morenga et al. (2010), were used to estimate the required sample size. Assuming a mean ±SD change in HOMA2-IR of 0.0 ±0.5 following the *RR* intervention and -0.3 ±0.5 following the *rr* intervention, a power calculation confirmed that 24 volunteers would be sufficient to detect a difference (α=0.05, power=0.80) [33]. All studies were approved by the South East Coast Surrey Research Ethics Committee (15/L0/0184) and carried out in accordance with the Declaration of Helsinki. Volunteers were recruited via a healthy volunteer's database and public advertisement. For the exploratory studies 1, 2 and 3 men and women aged 18-65 years old, with a body mass index (BMI) of 18.5-29.9 kg/m^2 were recruited. For the study 4, men and women 40 to 70 years old were recruited with same BMI as in studies 1, 2 and 3. Exclusion criteria included: weight gain or loss >3 kg in the previous 2 months, any chronic illness or gastrointestinal disorder, history of drug or alcohol abuse in the previous 2 years, use of antibiotics or medications likely to interfere with metabolic variable measured, smoking. All study visits took place at the National institute for Health Research/Wellcome Trust Imperial Clinical Research Facility, Hammersmith Hospital, London, United Kingdom and were conducted between May 2015 and December 2017. Randomization for all studies was generated by sealed envelope (Sealed Envelope Ltd, London, UK). In all human clinical studies volunteers were asked to consume the same standardized meal the evening before each study visit and avoid caffeine, alcohol consumption and strenuous exercise for 24 h before the experimental procedure. They were advised not to start any other new diets or intensive exercise regimes during the study period. Weight, height and body fat measurements were collected by using bioimpedance analysis (BC-418 Analyzer; Tanita UK).

Trial 1 - Study Day Protocol

This study was a randomized, controlled, double blind, cross-over trial. 10 volunteers were recruited for the study and attended 4 study visits (≥7days apart) after an overnight fast. The study lasted for 300 minutes. Volunteers received a standardized test meal (0 min) (details of the test meal composition, Supplementary Table 12) with 50 g dry weight of *RR* or *rr* pea seeds and flour in a random order. The test meal included 100 mg ^{13}C-octanoic acid (Sercon Ltd, Crewe, UK) which was injected in the yolk. ^{13}C-octanoic acid breath test is a non-invasive, reproducible, stable isotope method for measuring solid phase gastric emptying. By measuring the level of $^{13}CO_2$ that appears in breath samples following oxidation of the absorbed tracer, we were able to calculate how quickly the stomach empties after eating. Breath samples were taken every 15 min until the end of the study day (300 min). The breath test poses no risk to the volunteers and involves blowing through a straw into an Exetainer (Labco Co., High Wycombe, UK) until vapour condensed at the bottom of the tube. Analysis of breath $^{13}CO_2$ enrichment was by continuous flow isotope ratio MS (AP2003, UK).

Trial 2 - Study Day Protocol

Twelve healthy volunteers were recruited for this randomized, controlled, double blind, cross over study. Volunteers attended the Clinical Research Facility for 4 consecutive days (3 nights). Nasogastric and nasoduodenal feeding tubes were placed to allow aspiration of samples from the

546 stomach and small intestine. The enteral feeding tubes were placed by a doctor using the CORPAK
547 (MedSystems, Halyard UK) feeding tube model that tracks the position of the tube during placement
548 without the need for x-rays. The tubes remained in place for the duration of the 4-day visit. Each visit
549 lasted for 180 min. An intravenous cannula was inserted into one arm for blood sampling of plasma,
550 serum and gut hormones. Each morning, fasting blood samples and gastric content samples were
551 taken at –10 and 0 min. In random order, volunteers received at 0 min, a portion of 50 g dry weight *RR*
552 *or rr* pea seeds and/or flour. Postprandial blood samples were collected at 15, 30, 60, 90, 120, 180
553 min. The macronutrient profile of pea seeds can be found in the Supplementary Table 13.

554 Trial 3 - Study Day Protocol

555 Ten healthy volunteers were recruited for this randomized, controlled, double blind, cross over study
556 and attended the research facility 4 times (≥7days apart) after an overnight fast. Each study visit
557 lasted for 8 h. Volunteers received a test meal (0 min) which contained 50 g dry weight ^{13}C pea seeds
558 or flour in random order. Details of the composition of the test meal are in Supplementary Table 14.
559 Throughout the study volunteers collected urine samples and were advised to keep collecting their
560 urine samples until the following morning (24 h). The following morning, they returned to the research
561 unit with the urine sample and a stool sample.

562 Trial 4 - Study Day Protocol

563 Twenty-five volunteers were recruited for this randomized, controlled, double blind, cross over study
564 and attended the research facility for 4 visits. Volunteers had to undergo two separate 28-day
565 supplementation periods and they were provided with mushy peas and pea hummus products (*RR* or
566 *rr* line). They were advised to consume 1 can of each product per day. The products were matched for
567 dry weight. Each can contained 50 g dry weight of peas in a total amount of 210 g. Supplementary
568 Table 4 presents the macronutrient profile of *RR* and *rr* mushy peas and pea hummus analysed by
569 Campden BRI. Before and at the end of each 28-day supplementation period they attended the
570 research facility for a study visit. At time 0 volunteers received an ENSURE drink (Ensure Vanilla
571 Nutrition Shake, Abbott; 330 ml, 66.6 g carbs, 20.5 g protein and 16.2 g fat) consisting of 500 kcal.
572 Blood samples were collected throughout the time course of the study (5 h). Urine samples were
573 collected for the same time frame. Volunteers had to collect a stool sample the day before each study
574 visit. There was a 28-day washout period between the two supplementation periods.

575 ^{13}C Breath Sample Analysis

576 Breath samples analysis was performed by isotope ratio mass spectrometry (IRMS) [34]. Breath
577 samples were collected by exhalation of expired breath into an Exetainer (Labco Ltd, Lampeter,
578 Ceredigion, United Kingdom) using a straw. Volunteers were encouraged to continue to blow into the
579 Exetainer until condensate was observed in the base of the tube indicating alveolar breath collection
580 [35]. Collected breath samples were analysed by flushing a portion of breath with helium gas into the
581 IRMS where water is removed, and CO_2 separated from other gas species using gas chromatography
582 before introduction into the mass spectrometer (AP2003, GV Instruments, Manchester, UK). The

583 isotope ratio ^{13}C:^{12}C was calculated from the ion abundance of m/z 44, 45 and 46 with reference to a
584 laboratory reference CO_2 (itself calibrated against Vienne Pee Dee Belemnite (*VPDB*)) with correction
585 of the small contribution of $^{12}C^{16}O^{17}O$ at m/z 45, the Craig correction. Breath $\delta^{13}C$ enrichment (‰) over
586 baseline was calculated for each timepoint and the envelope of breath ^{13}C excretion was analysed
587 using a modified version of the curve-fitting techniques to compute gastric emptying $T_{1/2}$ times [17].

588 Biological Sample Collection and Processing

589 Ten millilitres of blood were collected at each timepoint for assay of plasma glucose (EDTA), serum
590 insulin, and plasma gut hormones (3 ml in lithium heparin tube containing 60 µl aprotinin protease
591 inhibitor; Nordic Pharma UK). All blood sample tubes were centrifuged at 2500 g for 10 min at 4^0C.
592 Samples were separated and frozen at –80^0C until the end of the study when analysis took place.

593 Biological Sample Analysis

594 Plasma glucose and luminal glucose analysis was performed using Randox Glucose (GLU/PAP) kit
595 supplied by Randox using 20 µl of plasma glucose. A human insulin radioimmunoassay kit (Millipore)
596 was used for analysis of insulin based on manufacturer's specification with 50 µl serum. GLP-1 was
597 measured with the use of previously established in-house specific and sensitive radioimmunoassay.
598 GIP was measured by using an ELIZA Human GIP (Millipore) based on manufacturer's specification
599 with the use of 20 µl serum sample.

600 Experiments *Ex Vivo*

601 FITC labelled α-amylase was added to a suspension containing pea cells. Images of the cells were
602 taken at different time points using an Olympus BX 60 Fluorescence Microscope or a Zeiss LSM 880
603 Confocal Laser Scanning Microscope.

604 Metabolomic Gastric and Duodenal Samples Analysis

605 We assessed the gastric and small intestinal metabolic profiles of the aspirated samples using the
606 metabolic profiling approach. Each metabolic profile contains hundreds of metabolites measured in an
607 untargeted manner by Proton Nuclear Magnetic Resonance (^{1}H-NMR) spectroscopy. Sample
608 extraction and analysis has been described in detailed in Supplementary Methods. NMR metabolite
609 identification strategies were used as described by Garcia-Perez et al [36].

610 Quantification of ^{13}C Plasma Glucose

611 Plasma samples were diluted 1:5 with L-fucose internal standard. The ^{13}C natural abundance of L-
612 fucose was separately calibrated against VPDB and used as a chemical and isotopic internal
613 standard. 0.5 ml of plasma was diluted with 2 ml internal standard. Samples then underwent
614 ultrafiltration using 30000 molecular weight cut-off ultrafiltration devices (Amicron Ultra 4; Millipore,
615 Watford, UK) at 3600 X g for 45 min to remove proteins and other high molecular weight compounds.
616 After this step, the samples were stored in two separate aliquots at -20°C for further analysis. Analysis
617 by liquid chromatography-IRMS (LC-IRMS) was performed as previously described. Fucose and

glucose peak areas and background-corrected isotope ratios were exported to a spreadsheet for analysis. Glucose enrichment (δ ^{13}C (‰) was calculated using an in-house routine and using a relative ratio analysis approach against the IS for each sample to report the enrichment of glucose relative to VPDB and glucose ^{13}C concentration, as the product of enrichment x concentration at each time point. Glucose concentration was calculated from the area ratio of the glucose peak area relative to fucose.

Quantification of ^{13}C SCFAs in Urine Samples

Samples were analysed as previously described [37] modified to increase sensitivity of the analysis. In brief, urine samples (7 ml) were spiked with 200 nmoles 3-methyl valerate (3mV; internal standard) and 200 µL NaOH (300 mmoles/L). A 'process blank' was prepared containing freshly deionized water and identical spikes of 3mV and NaOH. Samples and blanks for each run were dried on a vacuum concentrator (Jouan RC10 Vacuum Centrifuge, ThermoFisher, Paisley, UK) at ambient temperature. Dried samples were acidified with 100 µl HCl and SCFA extracted with 400 µl methyl-tert butyl ether. 300 µl of the MTBE phase was removed to clean vials for analysis by GC-C-IRMS as previously described [37]. The isotopic enrichment of each SCFA was calculated relative to 3mV which itself had been calibrated against laboratory standards and VPDB. Enrichment of each SCFA with time was expressed relative to the enrichment of the starting pea material ingested to derive a fractional ^{13}C enrichment curve for each SCFA.

Bacterial Composition Analysis of Stool Samples

Experimental procedures detailing DNA extractions, sequencing, and microbiome analyses can be found in Supplementary Methods section.

Digestion *In Vitro* - Study Design

Pea Preparation

Pea seeds were milled by electric coffee grinder (Krups, Berkshire, UK), and were sieved to 1 mm particles (Cole-Palmer, St. Neots, UK). All chemicals, reagents and enzymes were supplied by Sigma Aldrich (Dorset, UK). Approximately 5 g pea seeds were soaked overnight in 100 mL ultrapure water (room temperature). Flour (1 g) was weighed into 15 mL Pyrex tubes (screw cap with PTFE cap liner) and mixed with ultrapure water (4:1). Samples were hydrated, 1 h at room temperature and cooked (1 h, in a boiling water bath), cooled and further diluted (8:1). Peas were boiled for 1 h in ultrapure water, drained, and skins were removed from both uncooked and cooked peas. To mimic chewing, peas were pushed through a garlic press (Lakeland, UK) to produce chunks with particle sizes ≥ 2.5 mm.

Simulated Digestion

Triplicate digestions of flours and pea chunks were performed using a standardised static biochemical model developed by Minekus et al [38], with modifications to the composition of the simulated digestion fluids. In all cases sodium bicarbonate and ammonium bicarbonate were directly substituted with bis-tris, due to its buffering capacity which was important for maintaining pH 7.0 in the intestinal

phase. Oral phase: simulated salivary fluid (SSF) [15.1 mM KCl, 3.7 mM KH_2PO_4, 13.66 mM bis-tris, 0.15 mM $MgCl_2(H_2O)_6$, 1.5 mM $CaCl_2(H_2O)_2$] was added, 1:1 v/w, to samples immediately followed by human salivary amylase (product code A1031: type XIII-A lyophilised powder – α-amylase from human saliva) providing a final concentration of 75 U/ mL, then incubated for 2 min at 37 °C.

Gastric phase: at 2 min, the pH was adjusted to 3.0 (± 0.05) using 0.1M HCl, simulated gastric fluid (SGF) [6.9 mmol KCl, 0.9 mmol KH_2PO_4, 25.5 mmol bis-tris, 47.2 mmol NaCl, 0.1 mmol $MgCl_2(H_2O)_6$, 0.15 mmol $CaCl_2(H_2O)_2$] was added (1:1 v/v). Finally, pepsin (product code P7012: pepsin from porcine gastric mucosa) was added providing a final concentration of 2000 U/ mL. The gastric phase was incubated at 37 °C (for 1 h. The recommended time for gastric digestion is 2 h however, based on the lack of starch degrading enzymes in the gastric phase, the time for these experiments was reduced.

Intestinal phase: immediately after the gastric phase the pH was raised to 7.0 (± 0.05) using 0.1M NaOH, simulated intestinal fluid (SIF) was added [6.8 mM KCl, 0.8 mM KH_2PO_4, 85 mM bis-tris, 38.4 mM NaCl, 0.33 mM $MgCl_2(H_2O)_6$, 0.6 mM $CaCl_2(H_2O)_2$, and 10 mM bile] (1:1 v/v) and finally pancreatin (product code P7545: pancreatin from porcine pancreas) was added providing a final concentration of 100 U/ mL. The intestinal phase was incubated at 37 °C (170 rpm) for 2 h.

Flour was digested in a heated mixing vessel where samples were stirred continuously (500 rpm) at 37 °C. The pH of the intestinal phase was maintained at 7.0 by KEM AT-700 automatic titrator (Kyoto Electronics, Leeds, UK). At the end of each phase of digestion, 0.1 mL samples were taken: oral phase 2 min; gastric phase 60 min, intestinal phase 120 min.

Pea chunks were digested in disposable centrifuge tubes (Greiner Bio-One Ltd, Stonehouse, UK) at 37 °C in an orbital shaking incubator (Sartorius, Goettingen, Germany) at 170 rpm, and sample collection times were the same as for the flour.

Starch Assay

Uncooked and cooked pea chunks (100 mg ±5 mg) were digested according to protocol described in section simulated digestion. The liquid phase was removed from the samples by centrifugation (2000 g for 5 min). Additional digested samples were homogenised at 1000 rpm, using a T25 Ultra-Turrax (IKA, Oxford, England), post-intestinal digestion phase, to check that all starch in the pea chunks had been accounted for by the assay. After milling, samples were centrifuged at 10000 g for 10 min, and the pellet was retained.

Total and resistant starch contents of undigested and digested flours and pea chunks were determined using assay kits purchased from Megazyme International (Co. Wicklow, Ireland).

Total starch (assay procedure K-TSTA 07/11). Samples were heated in aqueous ethanol (80% v/v) at 80-85 °C for 5 min and centrifuged at 1800 g for 10 min. Supernatants were decanted, and excess liquid was drained from the pellets.

Resistant starch (assay procedure: KRSTAR 09/14). Samples were incubated with 4.0 mL pancreatic α-amylase (30 U/mL) and AMG (3 U/mL) for 16 h at 37 °C with continuous shaking (200 rpm), during

690 which time non-resistant starch was solubilised and hydrolysed to D-glucose. Enzymes were halted by
691 washing with 4.0 mL ethanol (99 % v/v), followed by centrifugation at 1500 g for 10 min. Supernatants
692 were decanted and pellets were re-suspended in 8.0 mL 50 % ethanol, the centrifugation step was
693 repeated, and followed by a final washing step. Supernatants were decanted, and excess liquid was
694 drained from the pellets.

695 All pellets were incubated in 2.0 mL 2 M KOH for 20 min on ice and neutralised in 8.0 mL 1.2 M
696 sodium acetate buffer (pH 3.8). Starch was hydrolysed to form maltodextrins by addition of
697 thermostable α-amylase to give a final content of 3.0 U/mL. The maltodextrins were further hydrolysed
698 by addition of AMG to give a final content of 3.3 U/mL, to form D-glucose.

699 Total starch and resistant starch contents were determined by incubating 0.1 mL of hydrolysed
700 samples with 3.0 mL GOPOD reagent [glucose oxidase plus peroxidase and 4-aminoantipyrine in
701 reagent buffer (4-hydroxybenzoic acid) at 50 °C for 20 min, where the D-glucose was oxidised to D-
702 gluconate, which was quantitively measured in a colorimetric reaction. The absorbance for each
703 sample and D-glucose controls was read at 510 nm against the reagent blank using UV tolerant
704 cuvettes (Sarstedt Limited, Leicester, UK) and a Lambda UV/Vis spectrophotometer (Perkin-Elmer,
705 Buckinghamshire, UK).

706 Starch Structural Analysis- SEC and ^{13}C CP/MAS NMR

707 SEC analysis was conducted on debranched, purified starch samples using a Waters Advanced
708 Polymer Characterisation System as described in [39].

709 Solid-state ^{13}C CP/MAS NMR experiments on all pea and flour powder samples were carried out on a
710 Bruker Avance III 300 MHz spectrometer, equipped with an HXY 4-mm probe, spun at a frequency of
711 12 kHz, at a ^{13}C frequency of 75.47 MHz, and MAS of 54.7°. Samples were manually ground using a
712 mortar and pestle and approximately 110–130 mg of each sample was packed into a 4-mm cylindrical
713 partially-stabilised zirconium oxide (PSZ) rotor with a Kel-F end cap. The ^{13}C CP-MAS NMR
714 experimental acquisition and processing parameters were 90° 1H rf pulse width of 3.50 μs and 90°
715 13C rf pulse width of 4.50 μs, contact time of 1000 μs, recycle delay of 5 s, spectral width of 22.7 kHz
716 (301.1 ppm), acquisition time of 28.16 ms, time domain points (i.e. size of FID) of 1280, line
717 broadening was set to 20, 6144 number of scans and 16 dummy scans. All experiments were
718 referenced to tetramethylsilane and hexamethylbenzene for ^{1}H and ^{13}C, respectively, and carried out
719 at approximately 26 °C.

720 Calculation of starch molecular (double helical) order was performed following the procedure
721 described by Flanagan et al [40]. In brief, following determination of the free induction decay of all
722 samples, the data was Fourier transformed, phase corrected and zero-filled to 4096 data points.
723 Chemical shift vs relative intensity data was used to obtain an estimation of the total crystallinity of
724 each sample analysed using partial least squares analysis against a reference set of 114 spectra of
725 starch with known values of molecular order obtained using spectral deconvolution and referenced
726 against x-ray diffraction data.

727 Particle Size

728 Pea chunk size (cooked) was determined after gastric and intestinal simulated digestion by dynamic
729 light scattering (DLS), using an LS13320 laser diffraction particle size analyser (Beckman-Coulter,
730 Buckinghamshire, UK), and using starch as the optical model with PIDS (Polarization Intensity
731 Differential Scattering) obscuration ≥45%. The mean particle size distribution was measured 3 times
732 over 60 second intervals.

733 Compression Test Experiments

734 An Instron machine 5540 was used to conduct the test with a 10 N load cell, model 2530-428, and
735 was connected to Bluehill3 software for the collection and analysis of the results.
736 *RR* and *rr* pea seeds were tested, using the same cooking method as for trial 2. Ten seeds from each
737 pea line using three different batches were measured for length and height using digital Vernier
738 calliper to ensure similar geometry between peas. To conduct a test, a sample seed was placed in the
739 most stable position prior to testing. A flat plate attached to Instron was used to apply load to the seed.
740 The compression test was performed at speeds of 1 mm/s and 15 mm/s. The force versus
741 deformation curves were obtained until rupture of the seed occurred.
742
743 Microscopy

744 Microscopy was used to characterise the pea seeds and flour throughout the digestion. It was
745 particularly important to image areas of damaged tissue from the action of chewing, as these areas
746 were accessible to enzymes and therefore would be susceptible to digestion. More information
747 regarding light microscopy and scan electron microscopy can be found in Supplementary Methods.

748 Statistical Analysis

749 Data were analysed using Graph Pad Prism (GraphPad Software, San Diego, CA, USA), IBM SPSS
750 (Statistics for Windows, Version 24, Armonk, NY, USA) or MatLab version R2014a, The Mathworks,
751 Inc.; Natwick, MA). Data were tested for normality using Shapiro-Wilk Test. Comparison of time series
752 data was carried out by two-way analysis of variance (ANOVA) with post hoc LSD Fisher correction.
753 Areas under the curve (AUC) were calculated using the trapezoidal rule and were compared using
754 paired Student's *t*-test. AUCs were calculated based on the time frame and parameters of each study.
755 $AUC_{0\text{-}120min}$ was calculated as this is a dynamic representation of the meal effect on postprandially
756 glycaemia which was the primary aim of this experimental study (trial 2). In trial 3, $AUC_{0\text{-}480min}$ was
757 used as ^{13}C labelled pea seeds and flour were used. The scope of this study was to understand the
758 whole-time curve and not driven by conclusion about the test meal per se. As peas and flour were
759 labelled with ^{13}C this allowed us to trace fuel metabolism and therefore a time frame to capture both
760 digestion and fermentation data was used. All results and graphs are expressed as mean ±SEM.
761 Results were considered statistically significant when $p<0.05$, two sided with the significance level
762 indicated as *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Data Availability

All presented data are tabulated and detailed din the main text and the Supplementary Information. The experimental procedures are detailed in the Methods. Quantified data are freely available from Mendeley Data Database at DOI: 10.17632/gtthhhp9wz.1

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Authors contributions

GSF oversaw the design and implementation of the *in vivo* experiments. KP managed, assist in design and performed all the experimental studies *in vivo*, samples collection, processing, and data analysis. ESC designed and applied for ethics of the human studies. RA and MK assisted in experimental human studies 2 and 3 and 4 (MK). NP was responsible for the nasogastric and nasoduodenal tubes insertion. Metabolomics analysis was performed by KP and IGP. Metabolite identification was performed by IGP and JISC. DJM and TP oversaw the stable isotope analysis and data analysis and labelled crop production (TP). LJS performed simulated digestions, starch analysis of pea seeds and particle size analysis of pea fragments; sample preparation and imaging using light microscopy and sample preparation for scanning electron microscopy. Preparation, sectioning and imaging of pea tissue sections was done by RS and KLC; KLC performed SEM. NP carried out simulated digestions

of flours and subsequent starch analysis; diffusion experiments using fluorescence microscopy were also performed by NP. RA performed the compression experiments and MC oversaw the implementation of these experiments. TK and YK carried out solid state NMR experiments. PJW, FW and CE oversaw the design and implementation of the digestions *in vitro* and microscopy studies. CD oversaw field trials of the variant pea lines and multiplication of their seeds, with quality testing for all experiments. KP, JAKM, RCS, and JMB performed 16S rRNA gene sequencing and data analysis. GF and KP led the initial drafts of the manuscript. All authors contributed to the final draft of the manuscript.

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