CHAPTER 6

Effect of fibre additions to flatbread flour mixes on glucose kinetics: A randomised controlled trial

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ABSTRACT
We previously found that guar gum (GG) and chickpea flour (CPF) added to flatbread wheat flour lowered postprandial blood glucose (PPG) and insulin responses dose-dependently. However, rates of glucose influx cannot be determined from PPG, which integrates rates of influx, tissue disposal and hepatic glucose production. The objective was to quantify rates of glucose influx and related fluxes, as contributors to changes in PPG with GG and CPF additions to wheat-based flatbreads. In a randomized cross-over design, 12 healthy males consumed each of three different 13C-enriched meals: control flatbreads (C), or C incorporating 15% CPF with either 2% (GG2) or 4% (GG4) guar gum. A dual isotope technique was used to determine the time to reach 50% absorption of exogenous glucose (T50%abs, primary objective), rate of appearance of exogenous glucose (RaE), rate of appearance of total glucose (RaT), endogenous glucose production (EGP) and rate of disappearance of total glucose (RdT). Additional exploratory outcomes included PPG, insulin, GIP and GLP-1 were additionally measured over 4h. Compared to C, GG2 and GG4 had no significant effect on T50%abs. However, GG4 significantly reduced 4h area-under-the-curve (AUC) values for RaE, RaT, RdT and EGP, by 11%, 14%, 14% and 64%, respectively, whereas GG2 showed minor effects. Effect sizes over 2h and 4h were similar except for significantly greater reduction in EGP for GG4 at 2h. In conclusion, a soluble fibre mix added to flatbreads only slightly reduced rates of glucose influx, but more substantially affected rates of postprandial disposal and hepatic glucose production.

This trial is registered at ClinicalTrials.gov with identifier NCT 01734590.
INTRODUCTION
The worldwide prevalence of metabolic diseases such as diabetes mellitus type 2 (T2DM) is rapidly increasing, especially in China and South-East Asia (1). Consequently, there is considerable public health and consumer interest in taking steps to reduce risk of these diseases. Repeated exposures to high post-prandial glucose (PPG) and insulin (PPI) responses are implicated in pre-diabetes and T2DM (2). There is evidence that reducing PPG reduces progression from pre-diabetes to T2DM and risk of cardiovascular disease, as is shown in studies with the alpha-glucosidase inhibitor Acarbose or low Glycaemic index /Glycaemic load diets (3; 4; 5; 6).

Carbohydrate-rich staple foods are interesting candidates for reducing PPG and PPI exposures, because of their widespread and frequent consumption (7). The two most common carbohydrate-rich staple foods in South Asia are rice and wheat-based flatbreads (8), the latter generally prepared at home from commercially-manufactured whole-wheat flour mixes (“atta”). Therefore, cost-effective feasible approaches to further reduce the PPG and PPI response to these staple foods are of interest.

Soluble fibres, especially soluble viscous fibres, can lower PPG (9). An increased viscosity delays gastric emptying (10) and inhibits the propulsive and mixing effects of intestinal contractions (11; 12) resulting in a lower PPG (13). In addition, legume flours, such as chickpea flour (CPF), are known to give a lower PPG response than wheat flours (14). We observed that viscous guar gum added to flatbread flour in combination with CPF dose-dependently lowered PPG and PPI responses (15; 16). It is assumed that viscous gums mainly affect PPG by reducing rates of glucose absorption (17; 18; 19; 20), but testing this hypothesis would require a determination of glucose fluxes. The PPG response profile is the net result of the rate of appearance of glucose from food in the peripheral circulation (rate of appearance of exogenous glucose (RaE); tissue disposal (rate of disappearance of total glucose (RdT); disposal of all glucose to the tissues) and hepatic glucose production (endogenous glucose production (EGP); rate of glucose production by the liver) (21). RaE is a surrogate for glucose absorption in the gut, though slightly different because it does not take account of liver glucose uptake on first pass metabolism and metabolism of glucose in enterocytes. The dual stable isotope technique is often applied to distinguish RaE from other glucose flux parameters (22). For example, this technique has previously demonstrated that differences in the glycaemic responses to some starch-rich foods reflect changes in post-absorptive events more than (as might be assumed) differences in digestibility and absorption (23).

The present research was therefore carried out in the context of the general question of how (soluble) fibres in foods influence glucose fluxes. More specifically, we wished to establish this for a commercially feasible combination of soluble viscous fibre and legume flour in a popular southeast Asian staple food. The primary objective of this
study was to determine the effect of incorporation of GG and CPF in flatbreads on the rate of exogenous glucose uptake, expressed as the time to reach 50% absorption of exogenous glucose (T50%abs). Other objectives, which contribute to getting an overall picture of glucose metabolism, were to 1) estimate the main kinetic parameters, viz. RaE, rate of appearance of total glucose (RaT), RdT and EGP, and 2) assess the possible involvement of incretins (GLP-1 and GIP) as contributors directly to the observed PPI and indirectly to the PPG responses (because of their potential effects on PPI and gastric emptying). In addition, the glucose clearance rate (GCR), the rate of plasma volume being cleared of glucose, was estimated.

SUBJECTS AND METHODS

Subjects
Fifteen healthy men were recruited locally for screening from an existing database of potential participants of Quality Performance Service (QPS) Netherlands B.V. (Groningen, The Netherlands), a clinical research organization (CRO), where the study was executed. Twelve subjects were planned to be randomized and 3 subjects were available in case a subject became ill or did not eat at least 95% of the weight of the test meal in the first test period. Subjects who met all the inclusion criteria and had none of the exclusion criteria were considered for participation (see Supplemental Table 1). The study was conducted according to the principles of Good Clinical Practice, the Declaration of Helsinki (2008) and according to applicable local laws and regulations concerning studies conducted on human subjects. Ethical approval was obtained from the Medical Ethics Committee of the “Beoordeling Ethiek Biomedisch Onderzoek” Foundation (Assen, The Netherlands). Each participant provided written informed consent for the study. The trial was registered at clinicaltrials.gov.com as NCT01734590.

Experimental design
This study used a double-blind, randomized, controlled, full cross-over (within-subject) design. Treatment orders were balanced according to a Williams-type design, and a randomized schedule for allocation to treatment orders was generated with SAS software (version 9.4, SAS Institute Inc., Cary, NC, USA) by a statistician not involved with subject contact or subsequent data analyses. A representative of Unilever who was not involved in the analyses, randomly assigned a product (flour mix) to a product code (A/B/C) and randomly assigned a product code sequence to subject number (001 to 012). The CRO randomly assigned a subject to a subject number using a computer-generated sequence. All persons involved in the study were blinded as to the nature of the test products until after the blind data review. One password-protected memory
stick with the personal code-treatment combination was prepared and provided to the study coordinator of the CRO, to be accessed only if de-blinding of the study was necessary. The code was not broken during the study.

Subjects attended the initial screening day followed by 3 test days, at least 1 week apart. Participants were instructed to minimize changes in their habitual diet and activity during the study period. The subjects were asked to refrain from consuming $^{13}$C-enriched foods such as corn, pineapple, quorn, cane sugar, millet, purslane, tequila and some fishes (trout, haddock, tuna, whiting), for 3 days preceding the experiments and from exercise and alcohol consumption the day prior to each test day. A standardized evening meal consisting of pasta with vegetables, sauce and meat, a dessert (yogurt, fruit yogurt or custard) (3912 kJ (935 kcal), 24 en% protein, 43 en% carbohydrate, 33 en% fat and 13.5g dietary fibre) and mineral water was provided at the research facility, where the subjects stayed overnight. All participants fasted overnight (from 19.30 until consumption of the test product) but were allowed to drink water ad libitum.

In the evening a venous catheter was inserted in each subject’s forearm for blood collection and for infusion of D-[6,6-$^2$H$_2$] glucose (98% $^2$H atom percent excess) (Isotec, Miamisburg, OH, USA). In the morning ($t$=-120 min), a priming dose of deuterated glucose (80 x 0.07 mg/kg bw) was administered as a bolus in 2 min in 26.7 ml of water followed by a continuous infusion of 0.07 mg/kg bw/min in 0.33 ml/min for 8 hours. Two hours after the start of the infusion ($t$=0) each morning of each test day, subjects consumed three freshly made flatbreads (105 g flour total) with 250 ml mineral water as breakfast, and completed this within a 15 min period at every visit at the same time and day of the week. A 5% deviation in consumption of the standard test product quantity (measured in weight) was allowed. Subjects were allowed to drink up to 150 ml water every subsequent hour, to be consumed after venous blood drawings. The volume of water consumed was recorded on the first test day and the same volume of water was consumed on subsequent test days.

**Test product and preparation**

The composition of the test products is given in Table 1. For the flour mix with 2% guar gum (GG2) a small amount of barley flour (BF) was added to improve the sensory quality as a prototype of a commercially-acceptable formulation. Based on our previous results (15; 16) the flour mix with 4% guar gum (GG4) was used as a positive control and therefore no BF was added. For the control product (C) a refined wheat flour was used, because this is the widely used market standard product in India. The coarse flour was chosen for the experimental product because it also has a higher dietary fibre content. This was intended to be a test of an optimised, realistic potential ‘healthier’ commercial product relative to the current market standard, hence the use of this coarse flour and also barley flour in the experimental product (along with
chickpea flour and guar gum). Our previous research showed PPG responses to the ‘standard’ and higher fibre atta bases did not differ (16), and it seems unlikely the small amount of barley flour would have had much impact. Nevertheless, results are reflecting the total product formulation and cannot be definitively assigned to any single component.

Table 1. Composition of Test flatbreads + all components in weight (grams) with the exception of water in w/w% and APE (%)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>13C-wheat (%)</th>
<th>APE (%)</th>
<th>Wheat¹ flour</th>
<th>Chickpea Flour²</th>
<th>Guar gum³</th>
<th>Barley flour⁴</th>
<th>Total carbs</th>
<th>Dietary fibre</th>
<th>Water content (%)</th>
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<tbody>
<tr>
<td>Control</td>
<td>2.05</td>
<td>2.17</td>
<td>103.0¹</td>
<td>69.5</td>
<td>10.1</td>
<td>23.4</td>
<td></td>
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<td></td>
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<tr>
<td>GG2$</td>
<td>1.84</td>
<td>1.97</td>
<td>83.2²</td>
<td>15</td>
<td>2</td>
<td>3</td>
<td>62.5</td>
<td>10.6</td>
<td>24.8</td>
</tr>
<tr>
<td>GG4$</td>
<td>1.80</td>
<td>1.93</td>
<td>84.2²</td>
<td>15</td>
<td>4</td>
<td></td>
<td>61.4</td>
<td>11.6</td>
<td>28.4</td>
</tr>
</tbody>
</table>

Note: To prepare the flatbreads 2.5 g of wheat flour was initially needed to avoid sticking. The amount of 13C labelled wheat flour is based on this extra 2.5 g of wheat flour. However, in practice it was found that 5g flour was needed. Although the amount of 13C wheat flour in the flatbread was not corrected for this change in the amount of extra wheat flour.

$ GG = guar gum
¹ Flour mix Annapurna Atta (100% refined wheat flour, Hindustan Unilever Ltd, India)
² Flour mix Annapurna Atta with traditional coarse whole wheat flour, Hindustan Unilever Ltd., India.
³ Chickpea flour (Cardin Healthcare Pvt. Ltd.; Gujarat, India; 99% passing through 420 micron)
⁴ Guar gum (P.D. Bros, Bangalore, India; creamish white powder, 93% passing through 200 mesh)
⁵ Barley flour (dehulled) (Cardin Healthcare Pvt. Ltd., Gujarat, India; 99% passing through 420 micron)

Viable wheat seeds (*Triticum aestivum* L. cv. ‘Sharbati C306’) were obtained from the Centre for Genetic Resources (Wageningen, The Netherlands). After germination, the plants were grown in IsoLife’s labelling facility (Wageningen, The Netherlands) and continuously labelled for 15 weeks until maturity in an atmosphere containing 13CO2 (>97 atom % 13C). After harvesting, the uniformly 13C-labelled seeds (97.1 atom % 13C) were milled (Meneba, Rotterdam, The Netherlands) according to the same specifications (particle size, ash content and starch damage) as the non-enriched Sharbati whole wheat flour from India, obtained from the same cultivar as the 13C-labelled wheat. After mixing the enriched with non-enriched flour, all test products obtained a final 13C-enrichment of approximately 2% atom percent excess (APE; values for GG2, GG4 and C were 1.97 %, 1.93 % and 2.17 %, respectively).

All test flour mixes were formulated in the kitchen of the Consumer Centre of Unilever R&D (Vlaardingen, The Netherlands), and flatbreads were prepared fresh at the test site in a tortilla roti maker (Jaipan Jumbo Roti Maker, Jaipan Kitchen Appliances, Mumbai, India). For each single test serving, 100 g flour (+ 5 g for kneading) was kneaded to a soft and uniform consistency with the addition of ~77 ml water and allowed to rest for 30 min, then divided into 3 equal balls of each 53 g and rolled. More water was added and absorbed when fibres or legume flour was incorporated (see Table 1). Flatbreads were subsequently baked for 15 minutes and kept warm until
consumption within 10 min after preparation. The $^{13}$C abundance of the $^{13}$C-labelled wheat flour was verified at 97.05 atom% with isotope ratio mass spectrometry and this was used for the calculation of the kinetic parameters.

**Sample collection**

Blood samples were collected according to the scheme in Supplemental Table 2. At each time point blood was collected in two different blood collection tubes (BD Vacutainer): NaF-tubes (0.9 ml plasma) and K2-EDTA-tubes (1.35 ml plasma), the latter containing dipeptidyl peptidase-IV inhibitor for GLP-1 and GIP preservation (BD Diagnostics). After blood collection, tubes were directly mixed by inversion (8-10 times) and placed on ice. Within 30 min the tubes were centrifuged at 1300 x g for 10 min at 4°C. The resulting plasma was frozen in 2 ml aliquots at -20°C until analysis.

**Isotopic analysis of plasma glucose**

To ensure proper calculation of the kinetic parameters (RaE, RaT, GCR, EGP, and RdT), fractional enrichments of orally and intravenously administered D-[U-$^{13}$C] glucose and D-[6,6-$^{2}$H$_2$] glucose tracers were determined in the plasma samples at the time points indicated in Supplemental Table 2. Samples were deproteinized by adding 400 μl ice-cold ethanol to 40 μl of plasma and placing on ice for 30 minutes. This mixture was centrifuged for 10 minutes and the supernatant collected for further analysis. 200 μl of the supernatant was transferred to a Teflon-capped reaction vial and dried at 60°C under a nitrogen stream. To convert glucose to its pentaacetate derivative, 100 μl of pyridine and 200 μl of acetic anhydride were added to the residue and this mixture was incubated for 30 min at 60°C. The solution was evaporated at 60°C under a stream of nitrogen and the residue redissolved in 200 μl of ethyl acetate. The solutions were transferred into injection vials for analysis by gas chromatography-mass spectrometry. The derivatives were separated on AT-1701 30 m x 0.25 mm ID (0.25 μm film thickness) capillary column. Mass spectrometric analyses were performed using positive chemical ionization with ammonia, with ions monitored for mass-to-charge ratio (m/z) ranging from m/z 331-337 (m$_0$-m$_6$).

**Calculation of glucose kinetics**

The first step in data analysis was the adjustment of the fractional distribution of glucose isotopologues as measured by GC/MS (m$_0$–m$_6$) for the natural abundance of $^{13}$C atoms (m$_0$–m$_6$), using the method of Lee et al. (24). Calculations were performed using the non-steady-state equations of Steele et al. (25) as modified by Debodo et al. (26). We used an approach suggested by Radziuk et al. (27), including the assumption that the clearance rates of all glucose isotopologues, i.e., tracers and tracee, are identical. Furthermore, the volume of glucose distribution was considered to be 200...
ml/kg and the pool fraction 0.75 \(^{(28)}\). The non-steady-state elimination rate of the infused tracer was initially calculated and used to determine the GCR. Next, from the GCR and glucose concentrations the disposal rates of all glucose isotopologues (RdT), as well as the disposal rates of glucose that was absorbed from the meals (RdE) can be calculated. Using the non-steady-state equations, the rates of appearance (RaT and RaE) can be calculated from these disposal rates. Finally, the difference between RaT and RaE reflects the endogenous glucose production rate (EGP).

The primary outcome measure \(T_{50\%\text{abs}}\) was calculated using the Wagner-Nelson deconvolution method \(^{(29)}\). The percentage of \(^{13}\)C-glucose absorbed at time \(t\) (\(F[t]\)) was calculated as: \(F[t] = \frac{(\text{AUC}(0-t) + \text{glucose}[t]/\text{elim.rate})}{\text{AUC}(0-\text{inf})}\). AUC\((0-\text{inf})\) was the sum of the actually measured AUC\((0-360)\) and the extrapolated AUC\((360-\text{inf})\) based on the elimination rate of \(^{13}\)C-glucose in the terminal phase. AUC\((360-\text{inf})\) was calculated by \(^{13}\)C-glucose predicted at \(t=360\) min divided by the elimination rate.

**Measurement of plasma glucose, insulin, GLP-1 and GIP**

Plasma glucose concentrations were measured on a Roche/Hitachi Modular automatic analyser (Roche Diagnostics, Hitachi, Rotkreuz, Switzerland) using a glucose hexokinase method. Insulin was measured by a chemiluminescent microparticle immunoassay (The ARCHITECT® insulin assay, Abbott Laboratories, Abbott Park, USA). GIP was determined by a radioimmunoassay \(^{(30)}\), based on an antibody which fully reacts with the primary metabolite GIP3-42. The plasma concentration of GLP-1 was also analysed by radioimmunoassay \(^{(31)}\), which measured the sum of the intact GLP-1 and its primary metabolite GLP-1 9-36amide.

**Statistical Methods**

All statistical analyses were performed with SAS version 9.4 (SAS Institute Inc., Cary, NC, USA).

The time to reach 50% absorption of exogenous glucose (\(T_{50\%\text{abs}}\)) was the primary outcome. A power calculation indicated that a minimum of 12 subjects would be needed for 80% power to detect a mean change in \(T_{50\%\text{abs}}\) of 20 min at two-sided significance level of 0.05. This estimation was based on research from Eelderink et al. \(^{(21)}\) who used similar techniques to compare wheat bread with pasta, and observed a 38 min difference (SD= 11 min) in \(T_{50\%\text{abs}}\). We proposed about half that effect size as a reasonable basis for power in the current study (20 min).

The responses were summarized as areas under the curve (AUC \((t0 – t120\ min)) over a period of 120 minutes. The AUC was calculated using the trapezoidal rule \(^{(32; 33)}\).
Values obtained before consumption of the meal (T= -60, -30 and -5 min) were averaged and used as the baseline. For glucose the positive incremental AUC (+iAUC \( t_0 \rightarrow t_{120} \text{ min} \)) was calculated by subtracting 120 * baseline glucose value from the AUC 2h. For RaE, RdT and GCR, +iAUC was calculated by subtracting 120 * baseline values from the AUC \( t_0 \rightarrow t_{120} \text{ min} \). For EGP a decremental AUC (dAUC \( t_0 \rightarrow t_{120} \text{ min} \)) was calculated by subtracting the AUC 2h from 120 * baseline EGP. Similar calculations were also used to derive the data values over a period of 240 minutes.

The cross-over design aspect of the study was taken into account when statistically assessing the difference between the meals for log transformed AUC, +iAUC or dAUC using a linear mixed model with subjects as random effect. The model included as fixed effects, the meal, baseline characteristics and visit number.

The results based on least squares (LS) means were expressed as a percentage change and its 95% confidence interval (CI) via back transformation using the control meal as a reference.

Only the primary objective (T 50%abs) underwent pre-planned statistical hypothesis testing with \( p<0.05 \) as the criterion for statistical significance. A Dunnett adjustment was made to correct for the proposed multiple comparisons using control as a reference. For other secondary and exploratory objectives there was no pre-planned hypothesis testing and the data are described by the mean and 95% confidence intervals. For ease of interpretation we have however used the convention of describing these results as “significant” where the 95% CI does not include 0.

RESULTS

Subjects
From 24 male subjects screened for participation, six were not eligible for the study. Eighteen subjects were eligible for the study and 16 subjects were selected by lot as potential participants, one of whom cancelled his participation for personal reasons before the first intervention. In total 15 subjects including three reserve subjects were available, of whom 12 started and completed the study, with no dropouts or missing visits (Figure 1). One subject arrived ill at the first intervention day and was replaced by a reserve subject. The baseline characteristics of participants were: mean (sd) age 23.0 (2.0) years, height 186.1 (8.1) cm, body weight 78.7 (8.8) kg, BMI 22.6 (1.0) kg/m², fasting plasma glucose 5.0 (0.4) mmol/l and HbA1c 30.7 (3.7) mmol/mol. As blind review identified only trivial deviations from protocol, only the results of the Per Protocol analysis are shown and discussed here.
Postprandial glucose and insulin response

PPG and PPI response curves are shown in Figures 2a and 2b, respectively, and absolute values and percent differences between treatments are summarized in Table 2. Compared to C, GG2 reduced mean $+\text{iAUC}_{(t0 \rightarrow t120\text{min})}$ glucose by 14% and insulin by 16%, while GG4 reduced these by 26% and 23%, respectively.
Figure 2: Effects of flatbread consumption with different amounts of guar gum and legume flour on plasma glucose concentration (a) and plasma insulin concentration (b) (mean ± SEM). Control = closed circle, GG2 = square and GG4 = triangle
Glucose kinetics
The T50%abs values did not differ significantly between treatments (Table 2).

Glucose kinetics curves are shown for RaE, RaT, EGP and RdT in Figures 3a, 3b, 3c and 3d respectively, and for GCR in Supplemental Figure 1. The absolute values and percent difference for GG2 and GG4 vs C are summarized in Table 2. For all parameters, effects for GG4 were generally larger and more sustained than for GG2.

As can be seen in Figure 3a, from ~60-150 min, RaE was generally lower for GG2 and GG4 when compared to C, reflected in a reduction in RaE AUC(t0 – t240 min) (Table 2) for both GG treatments vs C, which was significant only for GG4.

After consumption of GG2 and GG4 EGP was more suppressed compared to C (Figure 3c), reflected by a significantly lower AUC(t0 – t120 min) for GG2 and GG4, and a significantly lower AUC(t0 – t240 min) for GG4.

RdT was similar for all treatments up to ~60 min, and from then up to ~210 min was lower for GG2 and GG4 relative to C (Figure 3d). This effect was somewhat more pronounced in GG4 than in GG2, resulting in significant differences from control for both GG2 and GG4 in AUC(t0 – t120 min) and AUC(t0 – t240 min) only for GG4 (Table 2).

The curves for GCR (Supplemental Figure 1) for all meals were similar to those for RdT. For these curves only the AUC(t0 – t240 min) for GG4 was significantly different from C (Table 2).

Data for the cumulative exogenous glucose appearance and disappearance can be found in Figure 4 and Supplemental Table 3. The cumulative amount of glucose appearing from exogenous (RaE) and endogenous sources (EGP) for GG4 compared to C was decreased by 2.4 and 2.9 g, respectively, over 2h, and by 4.3 and 6.4 g, respectively, over 4h. The RdT over the 2 h period was decreased by 5.0 g for GG4, and by and 11.1 g for GG4 at 4 h, compared to C.
Fig. 3a

Fig. 3b
Figure 3: Effects of flatbread consumption with different amounts of guar gum and legume flour on RaE (a), RaT (b), EGP (c) and RdT (d) (mean ± SEM). Control = closed circle, GG2 = square and GG4 = triangle
Figure 4: Cumulative appearance of total and exogenous glucose, and glucose from the liver in the peripheral circulation, and cumulative disappearance of glucose from the peripheral circulation.

Incretin response
GLP-1 and GIP response curves are shown in Figures 5a and 5b, respectively, and the absolute values and percent difference between treatments are summarized in Table 2. While GLP-1 did not differ among the treatments, the mean AUC\(_{(t_0 - t_{240} \text{ min})}\) for GIP was somewhat lower after GG4 vs C.
Figure 5: Effects of flatbread consumption with different amounts of guar gum and legume flour on GLP-1 (a) and GIP (b) (mean ± SEM). Control = closed circle, GG2 = square and GG4 = triangle.
Table 2: Overview of results (mean and %difference (%Differ.) and 95% CI vs. control (Contr)) for kinetic parameters, glucose, insulin and incretin responses

<table>
<thead>
<tr>
<th></th>
<th>Contr</th>
<th>GG2</th>
<th>GG4</th>
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<tr>
<td></td>
<td>Mean</td>
<td>%Differ.</td>
<td>CI †</td>
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<tr>
<td>T50abs#</td>
<td>91.4</td>
<td>95.0</td>
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<tr>
<td>RaE AUC0-120</td>
<td>269.3</td>
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<td>RaE AUC0-240</td>
<td>534.12</td>
<td>515.8</td>
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<tr>
<td>RaT AUC0-120</td>
<td>512.7</td>
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<td>RaT AUC0-240</td>
<td>970.5</td>
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<td>GCR AUC0-120</td>
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<td>3057.6</td>
<td>-2.5</td>
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</table>

† Difference= difference vs. control and expressed in % as 100× (GG2 – control) / control (similar for GG4), except for T50abs expressed in min and % change from control.
CI= 95% confidence interval; data in cells shown in grey to highlight outcome where 0 is not contained within the CI.
* Because EGP was suppressed after the test meals, the area beneath baseline was calculated as decremental area under the curve (dAUC).
# Units of RaE, RaT, EGP and RdT are in mg/kg·min of GCR in ml/kg·min. Unit of glucose is mmol/l and insulin is μU/ml. Units of GLP-1 and GIP are pmol/l.

**DISCUSSION**

Using the dual isotope technique, we found that the lower glucose response to flatbreads incorporating soluble fibre mixes was not the result of a reduced absorption rate only, as might be expected for these ingredients, but reflected a greater contribution from post-absorptive effects (RdT and EGP). The data suggest that small initial changes in RaE are part of a wider cascade of metabolic effects including somewhat reduced RdT and substantial reduction in EGP. These data confirm previous observations that changes in the rate of intestinal glucose release from carbohydrate-rich foods and its contribution to the PPG response cannot be assumed from the response profile itself (21).
For this study $T_{50\% \text{abs}}$ was chosen \textit{a priori} as the primary objective and RaE as the secondary objective. However, it appears that $T_{50\% \text{abs}}$ was less sensitive than RaE as a measure of change in the rate of release of glucose from the test foods, probably because the change in $T_{50\% \text{abs}}$ is based on the difference of two cumulative curves, while RaE is measured as percentage change.

An explanation of the modest reductions in RaE observed here may lie in the lower peak values of RaE in C as compared to previous studies. RaE for all treatments was around 3.1 mg/kg/min (range 2.3-3.4 mg/kg/min), whereas Eelderink et al. \citep{23} reported peak values of about ~3.4 mg/kg/min and ~4.3 mg/kg/min for pasta and bread (50 g available carbohydrates), respectively \citep{23}. In other research, RaE reached values of about 4.3 mg/kg·min after wheat bread \citep{34} (50g available carbohydrates) and of about 7.2 mg/kg·min after a large meal of either polished or parboiled rice (5g dry mass per kg body weight) \citep{35}. Interestingly in another study with a flatbread RaE reached peak values of 4.0 mg/kg·min \citep{36}. The low rate of glucose influx from all flatbreads (including C) in the present study may be attributed to the denser and drier structure of the product \citep{37} whereby soluble fibres and legume flours may make only a modest additional contribution toward further reducing the influx rate.

Most studies assessing glucose kinetics to foods have compared different foods (e.g. pasta versus wheatbread) \citep{21}, rather than a change in a defined ingredient within the same food format. An exception is the study by Nazare et al. \citep{38} in which adding 5g gram of beta-glucan to a polenta meal reduced RaE by 18% during the first two hours, after which this phenomena was reversed. Eelderink et al \citep{21} observed that RaE over 2h was about 30% lower after pasta as compared to wheat bread.

In addition to getting insight into how the combination of guar gum and legume flour in flatbread could influence the influx of glucose into the circulation, this study was designed to understand the extent to which absorptive processes and metabolic handling play a role in total blood glucose and insulin response. The post-meal glucose and insulin responses in the current study were in line with our previous results \citep{15; 16}, which were powered for PPG as a primary outcome. Other studies \citep{21; 39} have shown similar PPG responses for different treatments, yet a difference in the RaE, or vice-versa. Eelderink et al. \citep{21} found that the glycaemic response did not differ between pasta and bread, although the RaE was 30% lower for pasta compared to bread and this was compensated by a lower RdT \citep{23}. In contrast, Schenk et al.\citep{39} observed a pronounced difference in PPG response to two breakfasts with a similar RaE. In that study the difference was explained by a difference in RdT. In the current study the differences in PPG, especially between GG4 and control, were not only due to a lower RaE, but also to concurrent, larger reductions in EGP and RdT. Nazare et al. \citep{38} also found that beta-glucan added to polenta not only lowered PPG and RaE, but also inhibited EGP to a greater extent. Similarly, Péronnet et al. \citep{40} found that the exchange
of extruded cereals (low slowly digestible starch (SDS) content) for biscuits (high SDS content) slowed down the availability of glucose and RaE, and also reduced RdT, while the reduction of EGP was lower (40). This shows that both absorptive processes (reflected in RaE) and perhaps even more prominently metabolic handling (reflected in RdT and EGP) can all contribute to the effect of changing carbohydrate type on PPG response. It underscores that the observation of a lower glycemic responses (glycemic index) cannot be interpreted as indicative of or attributable to significantly reduced rates of release from the food matrix, without additional evidence.

The postprandial increase in RdT was generally reduced by GG2 and GG4 in the current study, which would tend to dampen effects on PPG from their lower RaE. Indeed, it has been shown that a reduced RaE leads to a decreased direct glucose stimulation of the beta-cells and to a low GIP response, both contributing to a lower insulin response and resulting in a lower RdT (41). The quantitative cumulative reductions in glucose influx were largely matched by reductions in glucose disappearance (Figure 3a), resulting in little net effect on the overall PPG response. Therefore, the reduction in PPG responses for GG2 and GG4 reflect the additional suppression of EGP. Given that suppression of EGP is an important action of insulin, it is of interest to note that the suppression of EGP by GG4 in particular occurred over a period when insulin levels were also relatively reduced. This apparent paradox was also seen in previous studies by Eelderink et al. (23) and Priebe et al. (34), in which EGP was more suppressed together with reduced PPI after both pasta compared to wheat bread (23) and wheat bread compared to glucose (34). In addition, Nazare et al. (38) found that the addition of beta-glucan to a polenta meal resulted in no differences in plasma insulin levels for the first hour compared to the polenta meal without beta-glucan, together with an enhanced inhibition of EGP. Other mechanisms for suppression of EGP could be involved, such as inhibition of glucagon secretion, decrease in release of non-esterified fatty acids and glycerol from adipose tissue or to a lesser extent gluconeogenic amino acids from skeletal muscles (42), however all these mechanisms are also influenced by insulin. There might also be a direct effect of plasma glucose concentration suppressing glucose efflux from the liver via the hepatic glucose-sensing system (43). There might also be a contribution from production of SCFA by small intestinal fermentation of fibres (44), stimulating hepatic AMP-activated protein kinase, which controls liver glucose homeostasis mainly through the inhibition of gluconeogenic gene expression and hepatic glucose production (45). Den Besten et al. (46) indeed showed in mice that the SCFA uptake fluxes inversely correlated with genes involved in gluconeogenesis. However, studies concerning the relationship between SCFA and liver glucose homeostasis in humans are lacking (47).

To obtain more information about possible underlying mechanisms we also measured the hormones GIP and GLP-1. These hormones have been shown to affect insulin production and hepatic glucose production (via glucagon) and could therefore
indirectly influence glucose kinetics (48; 49). In addition, GLP-1 has been shown to delay gastric emptying which also influences PPG response (50; 51). We did not see any effect on GLP-1 in this study, and a small effect on GIP only for GG4. The negligible effect on GLP-1 suggests that delivery of glucose to the GLP-1 producing cells (L-cells) in the distal part of the small intestine or colon was similar for all treatments. A recent review has concluded that fibres in general do not increase GLP-1 concentrations compared to control in the acute intake situation (52) and possibly longer-term consumption of particular fermentable fibres (e.g., fructooligosaccharide) is needed to increase GLP-1 secretion (53). The lower GIP is likely explained by the slower digestion rate of the flatbreads with the fibre/flour mix, as reflected by the lower RaE, and as such slower delivery to GIP producing K-cells in the duodenum and jejunum (54; 55; 56). In other studies comparing slowly and rapidly absorbed carbohydrates there was a strong correlation shown between GIP and RaE (23; 36; 40; 57).

Lowering insulin and GIP are generally seen as a beneficial physiological effect. In the longer-term, regular consumption of diets with a low PPI response is supposed to improve pancreatic β-cell function due to the lower strain on the β-cells especially in individuals with impaired first-phase insulin secretion (58). A lower GIP response may prevent an unhealthy fat distribution independent of insulin (59).

A limitation of the present study is that the amount of carbohydrates differed slightly between treatments; however, these small differences would not realistically explain the differences in postprandial glucose, insulin and the different fluxes (60). Furthermore, the amount of carbohydrates was very similar for GG2 and GG4, yet GG4 was much more effective. Another limitation is the number of subjects, which is underpowered for statistical comparison of PPG, but considered sufficient for estimating flux parameters (60). While the dual tracer method is suitable for measuring the different glucose fluxes, it is suggested that a triple tracer methodology can provide a more accurate assessment of the EGP, RaE and glucose disposal following ingestion of a carbohydrate-containing meal (61). A study assessing the accuracy of both techniques conformed that the triple tracer technique tends to slightly outperform the dual tracer technique, but the latter benefits from reduced experimental and computational complexity (62).

The main conclusion of this work is that incorporating GG and CPF in flatbread only slightly reduced the influx of glucose, but more substantially affected postprandial disposal as well as hepatic glucose production in healthy subjects. Future research could test other putative ‘slow-release’ carbohydrates for their effects on RaE and other flux parameters. At present, these studies are also quite resource-intensive, especially if they require growing 13C-labelled substrates, and the future development of alternative methods which do not require this would be advantageous. Another important research question is how these flux parameters differ in individuals with (pre-
diabetes, and also whether effects on the different flux parameters contribute to explaining the associations of different dietary patterns with disease risk. Lastly, glucagon should also be measured in future flux studies, because the ratio between levels of insulin and glucagon determines EGP and RdT (63).

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Conflict of interest
HMB, HH, ARH, DJM and HPFP are employees of Unilever, which manufactures and markets consumer food products, including the flour used for the flatbreads in this study.

Authorship
HMB, MGP and HPFP designed the research; MGP and ARH facilitated execution of the study; THvD executed the calculation of the glucose kinetics. HH performed statistical analysis. HMB wrote the manuscript with significant contributions from DJM, MGP, THvD. HH, HPFP, ARH and RJV. HMB, MGP, RJV and DJM had primary responsibility for final content. All authors read and approved the final manuscript.
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36. Schenk S, Davidson CJ, Zderic TW et al. (2003) Different glycemic indexes of breakfast cereals are not due to glucose entry into blood but to glucose removal by tissue. Am J Clin Nutr 78, 742-748.


Supplemental Table 1: Inclusion and exclusion criteria

<table>
<thead>
<tr>
<th>Inclusion criteria</th>
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<tbody>
<tr>
<td>- Healthy male subjects between ≥ 18 and ≤ 50 years of age</td>
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<td>- BMI of ≥ 20.0 and ≤ 28.0 kg/m²</td>
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<tr>
<td>- Apparently healthy: no medical conditions which might affect study measurements, absorption, metabolism and distribution (including diabetes type 1 and type 2, gastrointestinal dysfunction, gastrointestinal surgery and inflammatory diseases)</td>
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<tr>
<td>- Fasting blood glucose value of volunteer is ≥ 3.4 and ≤ 6.1 mmol/l (i.e. 62-110 mg/dl) at screening</td>
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<td>- Having a general practitioner</td>
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<td>- Agreeing to be informed about medically relevant personal test-results by study physician</td>
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<tr>
<td>- Informed consent signed</td>
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<td>- Willing to comply to study protocol during study</td>
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<td>- Accessible veins on arms as determined by examination at screening</td>
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<td>- Use of antibiotics within 3 months before first day of intervention (day 01); use of any other medication within 14 days before day 01</td>
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<td>- Blood donation in the past 3 months</td>
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<tr>
<td>- Reported participation in another nutritional or biomedical trial 3 months before the pre-study examination or during the study</td>
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<tr>
<td>- Reported participation in night shift work two weeks prior to pre-study investigation or during the study. Night work is defined as working between midnight and 6.00 AM.</td>
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<td>- Reported intense sporting activities ≥ 10 h/w</td>
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<td>- Consumption of ≥ 21 alcoholic drinks in a typical week</td>
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<td>- Not being used to eat breakfast</td>
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<td>- Reported use of any nicotine containing products in the six months preceding the study and during the study itself</td>
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<tr>
<td>- Reported use of medically prescribed diet or slimming diet</td>
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<td>- Not used to eat 3 meals a day</td>
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<td>- Vegetarian</td>
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<tr>
<td>- Reported weight loss / gain ≥10% of body weight in the six months before screening</td>
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<td>- Positive drug screen or alcohol breath test during the screening and /or at the day before the first intervention day.</td>
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<tr>
<td>- Being an employee of Unilever, QPS or UMCG</td>
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<tr>
<td>- Allergy or intolerance to food products and aversion to food products provided during the study</td>
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Supplemental Table 2: Sampling scheme (in min.)

<table>
<thead>
<tr>
<th>-60</th>
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<th>15</th>
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<th>75</th>
<th>90</th>
<th>105</th>
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<th>150</th>
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</table>

a = total blood glucose, fractional enrichment of D-[U-13C] glucose, and D-[6,6-2H2] glucose; b = insulin; c = GLP-1 and GIP
Supplemental Table 3: Cumulative appearance of exogenous glucose and total glucose, and glucose from the liver in the peripheral circulation, and cumulative disappearance of glucose from the peripheral circulation (g) (mean and 95% CIs).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>GG2</th>
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<th>GG4</th>
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<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Confidence interval</td>
<td>Mean</td>
<td>Confidence interval</td>
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<td>Cum RaE&lt;sub&gt;0-120&lt;/sub&gt;</td>
<td>22.3</td>
<td>18.2; 25.8</td>
<td>19.9</td>
<td>17.0; 22.8</td>
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<tr>
<td>Cum RaE&lt;sub&gt;0-240&lt;/sub&gt;</td>
<td>42.9</td>
<td>37.5; 45.9</td>
<td>38.6</td>
<td>35.1; 42.0</td>
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<tr>
<td>Cum RaT&lt;sub&gt;0-120&lt;/sub&gt;</td>
<td>40.4</td>
<td>33.9; 42.8</td>
<td>35.2</td>
<td>32.0; 38.4</td>
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<tr>
<td>Cum RaT&lt;sub&gt;0-240&lt;/sub&gt;</td>
<td>75.3</td>
<td>65.5; 76.2</td>
<td>64.7</td>
<td>60.7; 68.8</td>
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<tr>
<td>Cum EGP&lt;sub&gt;0-120&lt;/sub&gt;</td>
<td>18.2</td>
<td>15.4; 17.5</td>
<td>15.3</td>
<td>14.3; 16.3</td>
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<tr>
<td>Cum EGP&lt;sub&gt;0-240&lt;/sub&gt;</td>
<td>32.6</td>
<td>27.6; 31.1</td>
<td>26.2</td>
<td>24.6; 27.9</td>
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<tr>
<td>Cum RdT&lt;sub&gt;0-120&lt;/sub&gt;</td>
<td>38.4</td>
<td>32.1; 41.3</td>
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<td>75.1</td>
<td>65.1; 76.4</td>
<td>64.0</td>
<td>59.9; 68.1</td>
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CI= 95% confidence interval; data in cells shown in grey to highlight outcome where 0 is not contained within the CI

Supplemental Figure 1: Effects of flatbread consumption with different amounts of guar gum and legume flour on GCR (mean ± SEM). Control = closed circle, GG2 = square and GG4 = triangle