The solution properties of galactomannan after simulated digestion of guar fortified bread predict the extent of postprandial insulin reduction in healthy adult overweight subjects

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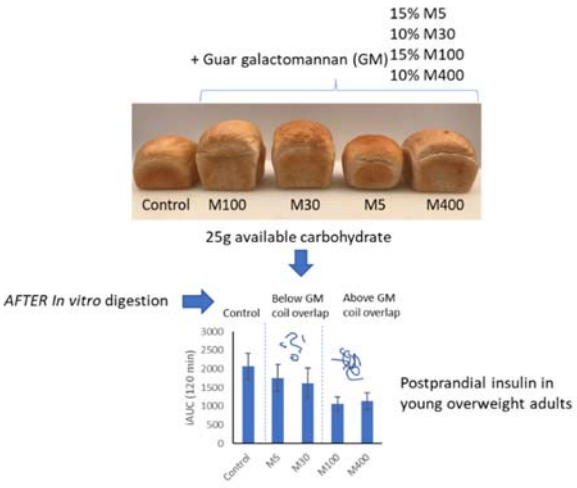
GRAPHICAL ABSTRACT



The concept of coil overlap can explain the postprandial blood insulin lowering effect of guar galactomannan in overweight adults when it is eaten as an ingredient in bread

ABSTRACT

Coil overlap occurs when random coil polysaccharides such as cereal beta-glucan or galactomannan in solution are abundant enough and large enough to entangle with one another to form networks. It was recently shown that this concept applied to *in vitro* digested cereal-based foods could predict the efficacy of the food to reduce postprandial glycaemia. In the current study we further investigate the role of coil overlap for prediction of glycaemic and insulinaemic responses using four guar fortified breads (10-15% wheat flour replacement level) with galactomannans of different weight-average molecular weight (M_w). The breads, including a wheat flour control, were tested in a randomised crossover study in 12 overweight adults. Addition of guar reduced postprandial serum insulin, but not glucose responses. The extent of postprandial insulin reduction correlated with the solution properties of galactomannan after *in vitro* digestion. A significantly greater reduction in insulin response was observed for two of the breads where the galactomannan M_w and concentration in solution after *in vitro* digestion was above coil overlap, in contrast to two other breads, which resulted in digests containing galactomannan below coil overlap and a significantly lower reduction of postprandial insulin. Further *in vitro* digestion experiments focusing on amylolysis of starch with kinetic modelling showed a greater proportion of slowly digested starch in breads with galactomannan above coil overlap than below. A combination of the molecular weight of dietary fibre in a food and its soluble concentration are key parameters explaining its physiological efficiency in the upper gastrointestinal tract.

KEYWORDS: starch, alpha-amylase, digestion modelling, viscosity, dietary fibre, blood glucose, serum insulin

INTRODUCTION

White bread is a staple food in many countries. However, due to its high content of readily digestible starch it has a high glycaemic index (GI) [1]. Diets rich in high-GI foods are associated with increased risk of development of impaired fasting glucose and subsequent type-2 diabetes [2]. Reducing the GI of white bread could therefore have important beneficial health implications.

Guar is a viscous dietary fibre preparation dominated by galactomannan. It is industrially extracted from the endosperm of the cluster bean *Cyamopsis tetragonoloba* (L.) Taub. When incorporated into foods or drinks rich in starch/and or glucose it has been shown to blunt the postprandial rise in blood glucose and insulin levels in both healthy and diabetic subjects [3-6]. Several studies have shown postprandial glucose and/or insulin attenuating effects to varying degrees for white bread meals providing 5 to 16 g guar and 50 to 106 g available carbohydrate [5, 7-10]. When hydrolysed non-viscous galactomannan was used in a glucose drink, this effect was abolished and has therefore been ascribed to the highly viscous properties of galactomannan [11].

While the ability of galactomannan, and several other soluble dietary fibres with potential to decrease postprandial glycaemia is linked to their viscous properties, the exact molecular mechanisms by which they exert their effects in the body are not entirely clear. Increased viscosity of the digesta resulting in delayed gastric emptying and slower glucose uptake across the small intestine is believed to play a role [12]. Interaction of soluble dietary fibres with the intestinal mucus layer resulting in increased intestinal barrier function and delayed glucose uptake is another explanation [13, 14], as is reduced starch digestion through interference of enzymatic accessibility of starch and changes of gastrointestinal hormone regulation [12, 15].

It is well-known that the viscous properties of galactomannan and other water soluble/semi-soluble random coil type polysaccharides/dietary fibres are a function of their space occupancy in solution which is described by the product of its hydrated concentration and its molecular weight or intrinsic viscosity [16-18]. Yet in clinical studies with white bread where different molecular weight grades of galactomannan 'ingredient Mw' have been evaluated in subjects for their effect on postprandial glycemia and insulinemia (e.g. [9, 19]), no information is provided on the weight-average molecular weight of galactomannan in the actual test breads. There was a general assumption that the baking process had no effect on the average molecular weight of galactomannan. However, we have recently shown that galactomannan is partially depolymerised during dough proofing [20] most likely by the activation of endogenous endo-β-mannanase activity in wheat flour. Thus, previous conclusions on the effects of different galactomannan molecular weights on postprandial glycemia [9, 19] should be treated with caution, even though it was shown that at the 10% addition level galactomannans with low molecular weights were still physiologically active. There is a clear need to undertake new clinical studies with knowledge of galactomannan molecular weight and concentration in white breads as 'ready to eat'.

In a recent study with cereal beta-glucan, a dietary fibre with similar solution properties, and postprandial benefits on glycaemia, as galactomannan, we showed that an understanding of the viscous solution properties following *in vitro* digestion could predict their potential health benefits including effects on postprandial glycemia [21]. Through an assessment of previous clinical studies with beta-glucan it was revealed that the concept of coil overlap could possibly be used to predict the efficacy of the food on reducing postprandial glycemia [21]. The concept of coil overlap comes from the field of rheology and describes the fundamental relationship of viscosity of soluble random coil polysaccharides in aqueous solutions as a function of their concentration and the volume each coil occupies[16]. Above the critical overlap parameter (C*) random coil polysaccharides are either long enough and/or abundant enough to entangle with one another and other polymers to form non-covalently cross-linked networks.[22] Galactomannan has an almost identical dilute, semi-dilute solution behaviour to beta-glucan [18, 23].

In this work we have prepared four different guar fortified breads. Two breads contained galactomannan of such amounts and chain length that following simulated *in vitro* digestion of the bread the galactomannans are forecast to lie below the critical overlap parameter while the other two were prepared to contain galactomannan that would result in digests above C*[16]. We used a dough preparation process that sought to optimise the quality, palatability, and metabolic properties of these guar breads [20]. The main aim of our study was therefore to evaluate if the solution properties of galactomannan after simulated digestion of guar fortified bread could predict the extent of postprandial glycaemic and insulinemic responses in adult overweight subjects. This population sub-group was selected because it was expected to see higher glycaemic responses than in healthy weight subjects (BMI <25) [24].

MATERIALS AND METHODS

Raw materials

Commercial wheat flour of high protein strength was obtained from Lantmännen (Lantmännen Cerealia, Oslo, Norway). Four different guar flour samples extracted from the endosperm of *Cyamopsis tetragonoloba* (L.) Taub. were a kind gift from Dr. Graham Sworn, DuPont Nutrition & Health, Paris, France. The flours were labelled M5, M30, M100 and M400 each with different respective weight average molecular weights (M5: ~ 40 kDa, M30: ~ 300 kDa, M100: ~ 1350 kDa and M400: ~ 2200 kDa).

Experimental foods

Five doughs were prepared as previously described [20], up-scaled to a basis of 2000 g wheat flour. Guar gum of different grades were added, one grade to each of the four doughs, to constitute 15 % M5, 10 % M30, 15 % M100 and 10 % M400 of total solid ingredients. The M5 and M30 doughs were designed so that following their simulated *in vitro* digestion the galactomannan would be below C* whilst the M100 and M400 doughs contained guar amounts and grades to give digests above C*. One dough served as control (no added guar gum). Water at 14.4 ± 0.6°C was added to each dough according to the water absorptions determined previously [20] and the doughs were mixed in a Diosna Table Spiralmixer SP12 (Diosna Dierks & Söhne GmbH, Osnabrück, Germany) until an accumulated specific mixing energy of 12 Wh Kg^{-1}, which resulted in dough temperatures of 25.8 ± 0.6 °C after mixing. The control dough and the M5 and M30 doughs were fermented for 1 h, while the M100 dough was fermented for 2.5 h at 27°C and 70 % RH in a fermentation cabinet (Lillnord A/S, Odder, Denmark). The M400 dough was prepared in a two-step procedure to limit the degradation of galactomannan as described previously [20]. First, a dough without guar was prepared and fermented as described for the control dough above. Guar gum was pre-hydrated by mixing with 1482 mL water in a Bear Varimixer Teddy (Bear Varimixer, Brøndby, Denmark) equipped with a whisk for 5 min at speed 4 and left to hydrate for 20 min. Following fermentation of the wheat flour dough, the pre-hydrated guar and the dough were mixed for 3 min at speed 3 using the dough hook attachment. The differences in dough preparation were chosen to try to maximise the differences in galactomannan weight-average molecular weights in the resulting breads.

After fermentation, the doughs were divided into pieces of 65 g (control), 76 g (M5), 83 g (M30), 94 g (M100) and 99 g (M400), corresponding to 25 g of available carbohydrate in each piece. The pieces were moulded by hand (M30 only due to stickiness) or in a Dough Rounder R10 (FriulCo Srl, Maniago (PN), Italy) and placed in 8 x 8 cm greased pans, before proving and baking as described previously [20]. Upon removal from the oven the breads were de-paned and cooled at room temperature for 1 hour before they were snap frozen in a bakery shock freezer for 1 hour at -35°C (Lillnord A/S Odder, Denmark). All bread was then transferred to the freezer and stored at -18 °C. Frozen bread for the clinical study were subsequently packed in styrofoam boxes and stored at -18 °C prior to frozen overnight shipment to Bergen. Upon arrival all bread was stored frozen at -18 °C. The day before the study visit the bread was thawed on a plate, covered with plastic foil, and marked with participant ID number and bread number. Bread for the *in vitro* experiments was thawed at room temperature before use while bread for compositional analysis was freeze-dried.

Compositional analyses of bread

The weight-average molecular weight (M_w), weight-average intrinsic viscosity ($[\eta_w]$), proximate composition (water, ash, starch, lipids, proteins and non-starch polysaccharides (NSP), including

galactomannan) of the breads were analysed as described previously [20]. Energy content of the breads was calculated according to EU directive 1169/2011 Annex XIV.

In vitro digestion to assess galactomannan solution properties

Breads were subjected to a simulated static *in vitro* digestion as described by Brodtkorb et al. [25]. A total amount of 2 g breadcrumbs per tube were digested in triplicate as previously described [21]. After digestion, enzymes were inactivated in a boiling water bath for 10 min. Tubes were cooled and centrifuged at 4000 × *g* for 10 min. The supernatants were aliquoted for determination of M_w,, $[\eta_w]$ and galactomannan concentration which was then measured as described previously [20]. Galactomannan solubility was defined as the galactomannan content in the *in vitro* digesta relative to the theoretical galactomannan content calculated from the galactomannan content of the corresponding freeze-dried guar breads. Apparent viscosity in simulated *in vitro* digests was measured at 37 °C using a Physica MCR 301 rheometer (Anton Paar GmbH, Graz, Austria) equipped with double gap geometry (DG26.7) as described previously [21]. The viscosity measurements were performed using 10 mL of the supernatants from the *in vitro* digestions. Viscous samples were analysed over a shear rate range of 0.1 – 1000 s^{-1}, ramping both forwards and backwards. There were seven measurement points per decade and the duration of the measurement points was 20 – 0.1 s during the forward ramp and 0.1 – 20 s during the backward ramp. Zero shear viscosity was calculated by fitting the Cross equation [26] to the raw data using Anton Paar RheoCompass software. Low viscosity samples displaying a constant viscosity independent of shear rate (no shear thinning) were analysed at a fixed shear rate of 10 s^{-1} with 10 measurement points. Prior to all measurements there was a temperature equilibration period of 60 s. Zero shear specific viscosity (η_{sp0}) was calculated as the difference of viscosity (at zero shear or at 10s^{-1}) between the *in vitro* digesta of the guar breads and the control bread (equation 2):

$$\eta_{sp.0} = (\eta_{guar\ bread} - \eta_{control\ bread}) / \eta_{control\ bread} \quad (2)$$

The solution properties were evaluated by a power law model defining two linear regions with slopes of ≈ 1 (below) or ≈ 3.5-4 (above random coil overlap) [17]. The transition from one region to the other is the critical overlap parameter (C*). Based on previous studies with guar galactomannan the region below C* is taken as $c[\eta]<2.5$, $\eta_{sp.0}<10$ mPas, and above C* $c[\eta]>2.5$, $\eta_{sp.0}>10$ mPas [18].

In vitro simulated starch digestion and subsequent kinetic analysis

Breads were subjected to a simulated static *in vitro* digestion [25, 27]. For each bread, breadcrumbs corresponding to an amount containing 200 mg starch, were made to 1 g by addition of water. 1 mL of simulated saliva fluid (SSF) and 2 mL simulated gastric fluid (SGF) containing 17.4 mg pepsin and 1 µL 0.3 M $CaCl_2.2H_2O$ was added and pH was adjusted to 3. The samples were then incubated horizontally for 1h at 37°C and 175 rpm in a shaking incubator (Innova 40R Incubator Shaker Series, New Brunswick Scientific, Edison, NJ, USA). After incubation, 4 mL of 0.1 M Na-maleate buffer (pH:6) containing 0.02 % NaN_3, 1 mM $CaCl_2$ and 2.5 mg mL^{-1} pancreatin (Sigma P1750 with 4x USP units) was added and pH was adjusted to 6. Thereafter, 165 U amyloglucosidase (Megazyme Ltd., Bray, Ireland) was added and the samples were placed in the shaking incubator at 37°C and 175 rpm. Samples were removed in triplicate after 0, 5, 10, 15, 20, 40, 60, 120 and 180 min and the enzymatic reaction was stopped with addition of 32 mL 96 % w/v ethanol. Finally, the samples were centrifuged at 4000 *g* for 10 min and the supernatants were collected.

Aliquots of 100 µL supernatants were mixed with 500 µL amyloglucosidase (33 U mL^{-1} in 0.2 M Na-acetate buffer, pH: 5.2) and the mixtures were incubated for 20 min at 50°C. Then, 3 mL glucose oxidase peroxidase (GOPOD) reagent (Megazyme Ltd., Bray, Ireland) was added, and the mixtures were incubated for another 20 min at 50°C. Absorbances were read at 510 nm and compared against a blank sample and a 1 mg mL^{-1} glucose standard.

Starch amylolysis data of the fraction of starch digestion as a function of time were first visually analysed to check for multiple digestion reactions by making a logarithm of slope (LOS) plot as previously described [28] and recommended by [29]. Secondly in SigmaPlot 14.5 a least squares non-linear regression fit was made to mean values with a double 4-parameter first-order rate function $C_t = C_f(1\text{-}e^{-k1t}) + C_s(1\text{-}e^{-k2t})$ to describe two parallel digestion processes [30] were C_f is the fraction of rapidly formed product and C_s is the fraction of slowly formed product with respective rate constants k^1 and k^2. C_t is the fraction of digested starch at any given time (t).

Clinical study

A clinical study based on international recommendations for glycaemic index testing [31] was performed at the Research Unit for Health Surveys (RHUS) at the University of Bergen, Norway. The study was conducted in agreement with the ethical principles of the Declaration of Helsinki and the study design was approved by the Regional Committees for Medical and Health Research Ethics (REK Vest) in August 2019 (Reference number: 2019/912-1). The study protocol was subject to minor changes which were approved January 2020 (ID 11367) and the study is registered at ClinicalTrials.gov with identifier: NCT04289545. All participants provided written informed consent. All experiments were performed in compliance with relevant laws and institutional guidelines. The study was a double-blind randomized block design with crossover where all participants consumed all experimental breads, with at least a 72 h wash out period between each test food.

Twelve healthy adults meeting the inclusion and exclusion criteria (age 18 – 60 years, body mass index: BMI 25-30 kg/m2, no history of diabetes) were recruited. The number of subjects was based on recommendations of at least 10 subjects for glycaemic index testing to obtain a reasonable degree of power and precision[31]. Based on a within subject standard deviation of 25, twelve participants provide 80% power to detect a 30% difference in iAUC blood glucose after 120 min (two-tailed t-test, α set at 0.05). During a screening visit, the subjects were asked to fill in a questionnaire on self-reported health. In addition, anthropometric measurements (height, weight, and waist circumference) were obtained, and glycated haemoglobin (HbA1c) was analysed to identify undiagnosed diabetes mellitus.

Prior to each test day, the participants were instructed to fast for at least 12 h, after consuming a self-selected and prepared carbohydrate-based evening meal of approximately the same composition before each test day. The participants were instructed to avoid smoking, alcohol consumption and any strenuous exercise the day before a study visit.

On the study day, a cannula was inserted in the medial cubital vein in the upper arm. After resting for 10 minutes, baseline venous blood samples for determination of plasma glucose and serum insulin and a finger-prick capillary blood sample for glucose analysis were collected (time 0, fasting sample). Then, the subjects consumed the test bread at a comfortable pace within 15 minutes along with 250 ml of water. Blood sampling was then performed 15, 30, 45, 60, 90 and 120 min after the participants had fully consumed the test bread (without any added toppings or spreads), and the participants were instructed to remain seated and were not permitted to eat during this period.

Blood samples were collected into one serum tube containing an acryl-based gel with silica particles as clot activator, and one plasma tube containing Na2-EDTA, NaF, and sodium citrate-citric acid. Serum samples were used for insulin analysis, and plasma samples for glucose analysis. After blood draw serum tubes were left undisturbed at room temperature for 30 min. Blood samples for plasma were centrifuged immediately. Blood samples were centrifuged (serum samples: Hettich 320 centrifuge at 2200 g for 10 minutes at 20°C, plasma samples: Eppendorf Centrifuge 5702 R at 1800 g for 10 min at 4°C) on-site and then aliquoted into test tubes and either stored at room temperature (one plasma sample) or placed at -80°C. Plasma samples were delivered daily to the central laboratory at Haukeland University Hospital for glucose analyses (Cobas 8000 series c702 module by Roche Diagnostics AS, Mannheim, Germany). Frozen serum samples were used for insulin analysis in the same laboratory, using a Siemens Immulite 2000 XPi chemiluminescence immunoassay (Siemens Healthineers AG, Erlangen, Germany). The assay has 6 % analytical variation at 45 mIE/L. Remaining samples were destroyed after obtaining the respective results. Capillary blood samples were collected by finger-prick, simultaneous as the venous blood samples and analysed on-site using an Accu-chek blood glucose meter (Roche Diagnostics Norge AS, Oslo, Norway).

Incremental peak and iAUC calculation

All collected raw data were used for subsequent calculations. In the cases where raw data points were missing these were estimated [32]. Incremental peak response was calculated by subtracting the fasting value for each subject from their peak recorded value for each test bread. The incremental area under the glucose and insulin response curves (iAUC120min) above fasting baseline between 0-120 min was calculated for each subject and for each bread type based on the standard trapezoid geometric method [33]. A custom R-script (iAUC.R) was employed to automate the calculation using R-Studio version 0.99.491.

Statistical analysis of clinical raw data

Statistical analyses of clinical data were performed using Minitab 19 (Minitab Inc., PA, USA). Statistical differences between peak incremental response and $iAUC_{120min}$ for glucose and insulin responses for the different guar breads (fixed factor) were assessed for subjects (random factor) using a mixed-effect model. Where raw data was not normally distributed a Johnson Transformation[34] was conducted prior to further analysis. The criterion for significance was a two-tailed confidence interval where $P < 0.05$. Differences between bread type were assessed with the *post-hoc* Tukey pairwise comparison test at a confidence interval of 95%.

Results

Study bread

Serving sizes ranged from 53.7-82.3 g to provide a similar (ca. 25 g) dose of available carbohydrate (Table 1). Protein, lipid and ash (minerals) amounts were also very similar per type of test bread per serving (Table 1). Differences in total dietary fibre content between breads reflected the galactomannan addition levels (Table 1). The calculated energy content per serving was consequently similar between 128-140 kcal (Table 1) and the variation in serving size was due to differences in water content, which ranged from 18.3 g – 42.4 g water and was a function of the different water absorption capacity of the added guar gums. As observed in a previous study some of the galactomannans with the largest M_w (M100 and M400) underwent a significant reduction in M_w during dough fermentation [20]. The M_w of galactomannan in these guar fortified breads was therefore much lower than when initially added as an ingredient (Table 2).

Solution properties of galactomannan extracted from *in vitro* digested bread

In vitro simulated digestion of the different bread types had no further impact on galactomannan M_w (Table 3). Similarly, $[\eta]_w$ was also not much affected (Table 3). Following *in vitro* digestion virtually all galactomannan was soluble. Calculated solubilities ranged from 96-107%. Solubility was independent on the M_w of guar and the amount added. Zero shear specific viscosities, on the other hand, were very low for M5 and M30 breads <5 mPas while for the breads containing M100 and M400 they were higher with 26 and 55 mPas respectively (Table 3). To be certain that galactomannan was the sole cause of the measured specific viscosity, the digested samples were treated with β-mannanase. This caused the viscosity to drop to close to that of the solvent (ca. 1 mPas) and thus proved that soluble galactomannan was the sole cause of the viscosity in the digests. The product of $[\eta]_w$ and concentration of galactomannan (reduced concentration with units of reciprocal concentration) further confirmed that two of the breads 15% M5 and 10% M30 contained galactomannan after *in vitro* digestion that was below (c*)' ($c[\eta]<2.5$, $\eta_{sp.0}<10$)' while the breads containing 15% M100 and 10% M400 resulted in digests with galactomannan's above C* ($c[\eta]>2.5$, $\eta_{sp.0}>10$) (Table 3).

Clinical study

Among the recruited volunteers, 24 subjects agreed to come for a screening visit to assess if they were eligible to participate in the study. Five people did not fulfil the inclusion criteria. Three subjects subsequently dropped out before their first test day. Another four subjects dropped out during study visits. All data from these was excluded from the study. In total, 12 subjects (8 males and 4 females) completed all five visits and were included in the analysed dataset. A CONSORT flow diagram for the study is presented in Supplementary Figure 2. The mean (± SD) age of subjects was 30 ± 8 years (range 22-48 years), weight was 87.8 ± 8.5 kg, waist circumference was 91 ± 9 cm with a BMI of 27.6 ± 2.5 Kg m^{-2} and HbA1c of 32 ± 3 mmol mol^{-1}.

Fasting capillary whole blood glucose levels during the 5 study visits ranged from 4.2-6.5 mmol L^{-1} with a mean and SEM of 5.3 ± 0.08 mmol L^{-1}(Table 4A, Figure 1A). Out of 12 overweight subjects five displayed fasting capillary whole blood glucose levels ≥5.6 mmol L^{-1} and two subjects ≥6.1 mmol L^{-1} on more than one occasion. According to the respective ADA and WHO definitions these subjects could be considered to have an impaired fasting glucose[35] . Postprandial incremental rises in capillary whole blood glucose for all test meals, including the white bread control, did not differ

significantly. (Figure 1A) with mean and SEM maximal increments between 0.78 mmol L^{-1} ± 0.2 and 1.24 mmol L^{-1} ± 0.22 (Table 4A). On two occasions, two of the subjects presented no postprandial excursion from baseline capillary fasting glucose level including one of the subjects for the control bread. All other subjects displayed positive increments for all breads. Similarly, for capillary whole blood glucose for $iAUC_{120}$ values were generally small and not significantly different. The largest mean value was observed for the white bread control (60.75 mmol min L^{-1}) and the lowest for the bread containing 15% M100 guar (Table 4A). However, the inter-subject variation (SEM) was large, especially for the control bread, at 21.62 mmol min L^{-1} (Table 4A).

Venous plasma glucose measurements mirrored very closely those seen for the capillary whole blood glucose measurements and no significant difference was observed between the two (Figure 1B). Fasting baseline values ranged from 4.4 -6.5 mmol L^{-1} with a mean of 5.47 ± 0.06 mol L^{-1} (Figure 1B, Table 4B). Except for one subject, those who had fasting capillary whole blood glucose levels above 5.6 mmol L^{-1} on more than one occasion in the study, also displayed the same elevated values for venous plasma glucose. Likewise, postprandial rises in venous plasma glucose were even smaller than for capillary blood glucose and spanned a narrow range with means of 0.66 to 0.93 mmol L^{-1} with quite large inter-subject variation (SEM of 0.16-0.23 mmol L^{-1}). there was no significant difference in mean incremental venous plasma glucose peaks for the test breads (Table 4B). There was also no significant difference in the corresponding $iAUC_{120}$ values for venous plasma glucose between the different test breads (Figure 1B, Table 4B). These values were generally lower, and over a narrower range, than those for capillary whole blood glucose. Means ranging from 28.4-47.9 mmol min L^{-1} were observed with SEMs between 8.1 and 13.0 mmol min L^{-1} (Table 4B).

Fasting serum insulin levels ranged from 2-20.9 mIU L^{-1} with a mean and SEM of 7.5 ± 0.58 mIU L^{-1} . These levels are within the normal range for healthy subjects [36]. One subject repeatedly displayed no change in plasma insulin response following ingestion of all the different guar breads and only a minor response (peak serum insulin response 7.8 mIU L^{-1} and $iAUC_{120}$ 188 mIU min L^{-1}) to consumption of the control bread. Mean incremental insulin peak and corresponding mean $iAUC_{120}$ values for breads fortified with guar were significantly lower ($p<0.05$) than the control bread containing no guar (Table 4C). Further the breads prepared with guars M100 and M400, which resulted in *in vitro* digests containing galactomannan above the critical overlap parameter (C*) (Table 3), had a significantly lower ($p<0.001$) mean incremental peak insulin and $iAUC_{120}$ than the breads prepared with M5 and M30. In contrast, the simulated *in vitro* digests of M5 and M30 contained galactomannan below C* (Figure 1C, Table 4C). However, there was no significant difference in mean incremental peak and $iAUC_{120}$ for insulin values between M5 and M30 nor M100 and M400 (Table 4C, Figure 1C). This shows there is a clear difference in insulin response for breads with *in vitro* digests containing galactomannans above and below C*.

Kinetics of *in vitro* starch digestion

Figure 2 shows the *in vitro* starch digestion profile for each bread type. The curves follow similar pseudo-first order kinetics as is typical for the amylase catalysed hydrolysis of starch. Application of the logarithm of the slope (LOS) approach [28], however, revealed discontinuity (Supplementary Figure 2) perhaps dominated by gelatinised and retrograded wheat starch respectively. Assuming parallel digestion kinetics [30] a double exponential least squares function was fitted to the digestion curves with a correlation coefficient of R_2 = 0.99. Two rate constants and the proportion of rapidly and

slowly digested starch could be obtained. For all breads the 95% confidence limits of k^1 and k^2 did not overlap further indicating that there were two different parallel reactions taking place [30]. The rate of the rapid reaction (k^1 = 0.18-0,51 min^{-1}) was an order of magnitude greater than the rate for the slower reaction (k^2 = 0.02-0.04 min^{-1}) (Table 5). In general, k^1 for all samples were similar, with the exception for bread M5, which had almost double the rate of reaction than other test breads. On the other hand, k^2 was similar for all test breads. The amount of starch digested in the rapid phase was markedly greater, while the slowly digested starch fraction was smaller, for the control bread compared to the breads containing guar (Table 5) with a ratio of C^f:C^s of 2.17. A smaller, but similar, C^f:C^s ratio was observed for the M5 and M30 test breads, while an even smaller but almost identical C^f:C^s ratio was noted for the M100 and M400 test breads (Table 5).

DISCUSSION

The addition of guar galactomannan to white wheat bread reduced the postprandial insulin response, but not the corresponding glucose response, in overweight adult subjects. Indeed, significant reductions in post prandial plasma insulin responses, but not blood glucose responses, to breads made with 5-15% guar (wheat flour replacement level) and served as a meal containing 50-75 g available carbohydrates have been observed in healthy nondiabetic human subjects before [8, 19]. Similar significant reductions of postprandial insulin, but not blood glucose responses have also been observed with guar ingested as a drink with 50 g glucose in solution [37] or as a mixture of 50 g glucose/α-linked glucose polymers [6].

Several possible explanations for these discrepancies have been proposed. Discrete post prandial measurements of blood glucose concentration in the peripheral circulation are suggested to be a poor index of the rate of glucose absorption in the small intestine [19]. Although not ethically possible in humans, a more precise method would be to measure the glucose in blood sampled from the portal vein. This has been demonstrated in pigs fed guar where a reduction in the rate of blood glucose absorption was seen[38]. Blood glucose levels are tightly regulated and thus, unlike their main coordinator insulin, do not fluctuate by several orders of magnitude[39]. The blood glucose responses to all breads, including the wheat control, observed in the current study, was low but within the normal range[40]. Due to our crossover design, we could not determine within subject variation in postprandial blood glucose response, and for our subject group (overweight but healthy) no data for this variation was available. We cannot exclude that our study was underpowered with too few subjects especially since our subject group might be expected to have a larger within subjects' variation in blood glucose response than healthy and normal weight subjects used in our power calculation.

The low blood glucose response to all breads was also noted in the above cited human studies[6, 8, 19, 37] with bread and glucose solutions and might be another reason for the lack of significant difference in blood glucose responses to the different breads, while significant differences in postprandial insulin responses were observed. In the current study a serving size of 25 g available carbohydrate was chosen to ensure subject compliance as an earlier study [41] revealed difficulties for subjects to eat a portion of bread with 50 g available carbohydrate and a large amount of fibre in the allocated 15 minutes. This relatively low amount of available carbohydrate per serving might also have contributed to the low blood glucose rise observed in the current study. In our previous study with 25g available carbohydrate per serving of mixed-linkage beta-glucan fortified bread glucose responses were in the upper normal range[40] and thus much higher than in the current study. While capillary glucose was measured in the plasma of healthy normal weight subjects in the beta-glucan bread study, whole blood capillary glucose was measured in overweight subjects in the current study. The glucose concentration in whole blood is higher than plasma because the water content of whole blood is lower than in red blood cells [33], but this factor is much smaller and cannot explain the differences in blood glucose response. Opposed to what is expected the blood glucose response in overweight subjects was lower. One aspect might be the younger age of the subjects in the current study[42]. However, these differences could simply be a consequence of natural variation in blood glucose responses.

Another main finding of the study was that the postprandial insulin response could be predicted by the solution properties of galactomannan following *in vitro* digestion of the guar fortified breads. The extent of the insulin sparing effect achieved with our guar breads may be related to whether galactomannan after *in vitro* digestion resulted in solutions above or below the onset of coil overlap. However, all guar fortified breads performed better than the control bread. If the amount of guar in the bread is high enough, in our current study 5 g per 25 g available carbohydrate, then even low-M_w galactomannan of 37 x 10^3 g mol^{-1} can reduce postprandial insulin responses. Bread containing 7.6 g guar galactomannan per serving, which provided 75 g available carbohydrates and had an ingredient galactomannan M_w of 520 x 10^3 g mol^{-1}, was also found to significantly lower insulin responses [19]. Yet addition of the same amount of a higher M_w-grade guar (2.36 x 10^6 g mol^{-1}) in the same study resulted in no further statistically significant reduction in insulin response. In a recent study [20], we found evidence for endogenous endo-β-mannanase catalysed partial depolymerisation of galactomannan during the bread baking process. High-M_w galactomannan, ca. 1.5-2 x 10^6 g mol^{-1} could be reduced to 0.5-0.7 x 10^6 g mol^{-1} during dough mixing, fermentation, and proofing. Therefore, the galactomannan M_w as eaten could, in earlier studies, have been much smaller than assumed. Thus, initial conclusions [19] about the relative effectiveness of galactomannans of different M_w on their ability to lower insulin responses may be incorrect. Apart from M_w the amount of added guar is also an important factor to consider.

A better way to assess the physiological effectiveness of guar galactomannan to lower postprandial glycaemic or insulinemic responses might be to assess the solution properties of galactomannan after *in vitro* digestion which are an effect of both M_w and soluble concentration. Indeed, we show that insulin reduction is significantly greater for bread with galactomannan above the onset of coil overlap than below. Breads with galactomannan above the onset of coil overlap after *in vitro* digestion also had the largest proportion of slowly digestible starch. While those with galactomannan below the critical overlap parameter after *in vitro* digestion had smaller amounts of slowly digestible starch, but a lot more than bread with no galactomannan. An explanation for this effect is probably linked to the greater propensity of galactomannan chains above coil overlap to interact with one another and with other macromolecules such as starch in the bread. For example, non-competitive inhibition of alpha-amylase by galactomannan was observed in an *in vitro* kinetic assay [43]. In another *in vitro* study galactomannan incorporated into wheat bread was closely associated with starch and it could thus act as a barrier to starch digestion [44]. The result of our *in vitro* starch digestion study and subsequent kinetic modelling could be explained by one or both mechanisms. These results fit very well with those observed *in vivo* for the insulin lowering effect and may be linked to retarded starch digestion throughout the upper gastrointestinal tract. How much direct galactomannan-substrate-enzyme interactions contribute to the insulin sparing effect *in vivo* is, however, unknown.

An increase in viscosity and subsequent reduced mixing and free water availability in the small intestine could also minimise interactions between α-amylase and starch. Similarly, it could also reduce diffusion of starch degradation products to the brush boarder either through intestinal content viscosity or through interactions of galactomannan with small intestinal mucus that could increase barrier function [45]. Another factor that we did not investigate is gastric emptying. However, following consumption of galactomannan-rich locust bean preparations of different viscosity, only a minimal delay in gastric emptying was observed [46]. However, in a follow up study with added nutrients the effect of viscosity on delayed gastric emptying was found to be additive [47]. Although the serving size of the control bread (no guar added) delivering 25 g available carbohydrate was half

that of the bread containing M400, the energy content of the different breads per serving was similar. Since calorie intake is the main determinant of gastric emptying rate [48] no large difference in this parameter between test meals is expected. Coupled with the fact that the breads were consumed with 250 ml water then the difference in total meal volume was less than 10%. Nevertheless, we have no information on the length of the lag-time (if any) before gastric emptying starts and the emptying half-time for the guar breads or whether the water consumed with the guar breads mixed with the chyme of the meal in the stomach or emptied fast via the Magenstrasse [49] . Whether gastric emptying is affected by galactomannan solution properties related to the concept of coil-overlap is currently unknown.

All these different mechanisms may contribute to the lower postprandial insulin responses from guar fortified breads. It seems that most of the mentioned mechanisms are connected to the solution properties of galactomannan in the upper digestion tract. A comprehensive understanding of the solution properties of galactomannan under simulated upper gastrointestinal tract conditions seems to be a key predictor of their potential physiological efficiency. In vitro digestion models thus are important tools to extract information on soluble dietary fibres that correlates with in *vivo* measures such as postprandial insulin responses. In the current case for galactomannan this information includes its M_w, viscosity, concentration in solution, occurrence of coil overlap, and the kinetic modelling of its effects on starch digestion.

Conclusion

There is evidence from this and other studies, that guar galactomannan can significantly reduce postprandial insulin responses. In this study we have shown that the extent of the effect can be predicted from solution properties of galactomannan following *in vitro* digestion through the concept of coil overlap. This *in vivo* - *in vitro* correlation provides a potentially useful approach to understanding the various mechanisms responsible for the health effects of different soluble dietary fibres. It may also provide a valuable tool to develop soluble dietary fibres fortified food products with optimal physiological effects.

Future studies should focus on expanding the scope of the current study e.g by studying guar galactomannan, or other soluble dietary fibres in different food matrixes, using a similar in vivo -in vitro approach. A study on gastric emptying with these same breads would also be informative. Future work is also warranted on further *in vivo* correlation of *in vitro* starch digestion models especially by applying the most recent advances in kinetic starch digestion modelling. Finally, future work may also seek to shed further light on why postprandial blood glucose responses from wheat bread are sometimes very low even at higher available carbohydrate loads (e.g., 50g) than the current study.

TABLES AND FIGURES

Table 1. Serving size, macronutrient content, calculated energy content of the breads (n = 3). Values are reported as mean ± SD and in g per serving unless otherwise stated.

	Serving Size (g)	Available carbohydrates*	Dietary fibre	Galactomannan	Protein**	Fat	Ash (minerals)	Water	Calculated energy (kcal)
Control	53.7 ± 0.6	26.5 ± 0.5	1.05 ± 0.05	0	4.33 ± 0.02	0.25± 0.01	0.78± 0.07	18.3 ± 0.01	128.9
15 % M5	67.0 ± 1.0	26.8 ± 0.8	6.28 ± 0.04	4.98 ± 0.07	4.60 ± 0.03	0.20± 0.01	1.14± 0.03	24.8 ± 0.01	140.9
10 % M30	71.0 ± 1.0	27.3 ± 1.2	4.67 ± 0.08	3.43 ± 0.06	4.61 ± 0.04	0.30± 0.05	1.01± 0.19	30.6 ± 0.02	140.1
15 % M100	76.7 ± 1.5	25.1 ± 0.2	6.72 ± 0.08	5.39 ± 0.08	4.64 ± 0.04	0.26± 0.01	0.92± 0.01	36.3 ± 0.03	135.7
10 % M400	82.3 ± 0.6	26.6 ± 1.0	4.53 ± 0.07	3.30 ± 0.04	4.62 ± 0.02	0.27± 0.01	0.83 ± 0.02	42.4 ± 0.04	137.3

* Available carbohydrate was calculated from the measured amount of available starch using a conversion factor of 1.1 [31]

** Protein calculated from total nitrogen content. conversion factor 5.4 [50]

Table 2. Weight-average molecular weight and weight-average intrinsic viscosity of galactomannan in the test breads and original guar flour.

	BREAD		ORIGINAL GUAR FLOUR	
Guar addition	Weight-average molecular weight (g mol^{-1} x 10^3)	Weight-average intrinsic viscosity (mL g^{-1})	Weight-average molecular weight (g mol^{-1} x 10^3)	Weight-average intrinsic viscosity (mL g^{-1})
15 % M5	37.1 ± 0.6	95.5 ± 1.5	39.3 ± 0.2	101.3 ± 1.1
10% M30	194.5 ± 1.6	346.9 ± 11.1	303.3 ± 13.9	468.6 ± 7.0
15% M100	511.1 ± 4.6	686.1 ± 14.7	1347 ± 2.4	1381.6 ± 10.6
10% M400	720.1 ± 13.4	859.7 ± 11.3	2194 ± 44	1939.6 ± 28.9

522 Table 3. Solution properties of galactomannan extracted from *in vitro* digested bread

	Guar addition			
	15 % M5	10 % M30	15 % M100	10 % M400
Galactomannan concentration [g 100 ml^{-1}]	0.95 ± 0.04	0.69 ± 0.03	0.87 ± 0.04	0.49 ± 0.02
Solubility of galactomannan [%]	95.83 ± 4.85	107.4 ± 1.24	96.14 ± 2.82	96.05 ± 2.14
Weight-average molecular weight (g mol^{-1} x 10^3)	37.2 ± 0.4	200.8 ± 1.7	505.6 ± 9.7	737.0 ± 9.3
Weight-average intrinsic viscosity $[\eta]_w$ (mL g^{-1})	94.0 ± 0.3	342.6 ± 3.5	672.2 ± 14.7	864.0 ± 10.6
Zero shear specific viscosity ($\eta_{sp.0}$) (mPas)	1.24 ± 0.05	4.38 ± 0.36	55.06 ± 3.43	26.02 ± 4.81
Reduced concentration (extent of space occupancy) (c[η])	0.89 ± 0.04	2.36 ± 0.11	5.81 ± 0.17	4.19 ± 0.21
[A]Coil overlap (C*)	BELOW	BELOW	ABOVE	ABOVE

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524 [A]Below C* = (c[η]<2.5, $\eta_{sp.0}$ <10 mPas); Above C* (c[η]>2.5, $\eta_{sp.0}$ >10 mPas)

Table 4. Postprandial capillary whole blood (**A**) and venous blood plasma (**B**) glucose response, and serum insulin response (**C**). Values are mean ± SEM from 12 healthy overweight participants after consumption of bread providing 25 g available carbohydrate.

A	Bread	PBGR [mmol L^{-1}]	$iAUC_{120}$ [mmol min L^{-1}]
	Control	1.07 ± 0.30^{A}	60.75 ± 21.62^{A}
	15 % M5	1.24 ± 0.22^{A}	52.74 ± 10.77^{A}
	10 % M30	1.08 ± 0.18^{A}	49.91 ± 10.99^{A}
	15 % M100	0.78 ± 0.22^{A}	34.46 ± 11.05^{A}
	10 % M400	1.13 ± 0.20^{A}	42.73 ± 7.36^{A}

B	Bread	PBGR [mmol L^{-1}]	$iAUC_{120}$ [mmol min L^{-1}]
	Control	0.87 ± 0.18^{A}	34.05 ± 10.15^{A}
	15 % M5	0.93 ± 0.21^{A}	47.85 ± 12.98^{A}
	10 % M30	0.90 ± 0.17^{A}	41.96 ± 10.85^{A}
	15 % M100	0.66 ± 0.16^{A}	28.36 ± 11.04^{A}
	10 % M400	0.79 ± 0.23^{A}	31.54 ± 8.19^{A}

C	Bread	Peak insulin response [mIU L^{-1}]	$iAUC_{120}$ [mIU min L^{-1}]
	Control	28.6 ± 5.0^{A}	1414.7 ± 250.8^{A}
	15 % M5	15.8 ± 4.8^{B}	828.0 ± 215.6^{B}
	10 % M30	14.3 ± 3.9^{B}	863.8 ± 263.8^{B}
	15 % M100	$4.7 \pm 1.0B^{C}$	272.0 ± 66.2^{C}
	10 % M400	8.0 ± 2.6^{C}	436.6 ± 147.4^{C}

$iAUC_{120}$ is the integrated area under the curve above fasting baseline, PBGR is the peak incremental blood glucose/insulin response

Different letters indicated significant differences ($P<0.05$).

Table 5. Mean values for *In vitro* digestion of starch in the bread fitted to a 4 parameter first-order kinetic model

	K^1 min^{-1}	C^f	K^2 min^{-1}	C^s	Ratio C^f:C^s
Control	0.25	0.63	0.02	0.29	2.17
15 % M5	0.51	0.51	0.03	0.42	1.21
10 % M30	0.25	0.57	0.02	0.46	1.23
15 % M100	0.27	0.42	0.04	0.49	0.86
10 % M400	0.18	0.45	0.03	0.52	0.87

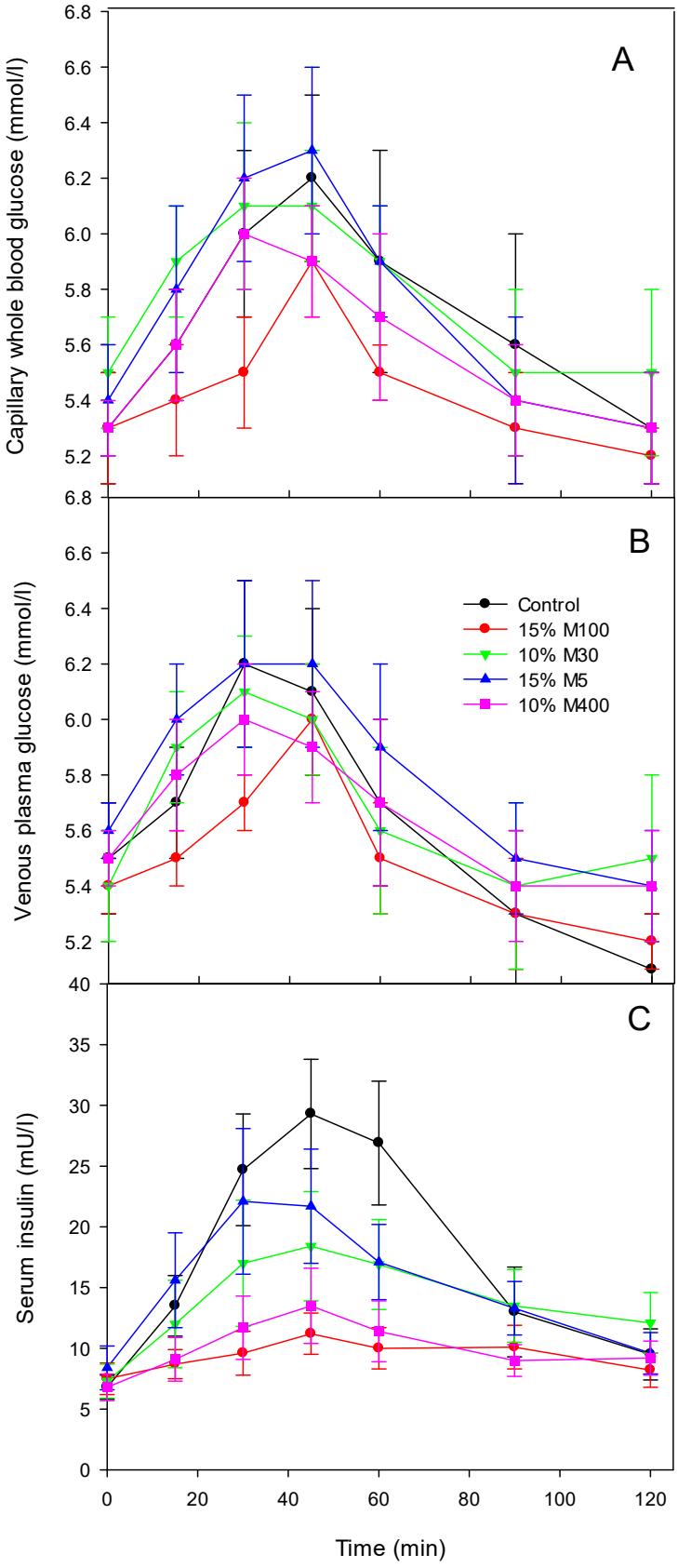

A
B
C
Capillary whole blood glucose (mmol/l)
Venous plasma glucose (mmol/l)
Serum insulin (mU/l)
Time (min)
Control
15% M100
10% M30
15% M5
10% M400

Figure 1

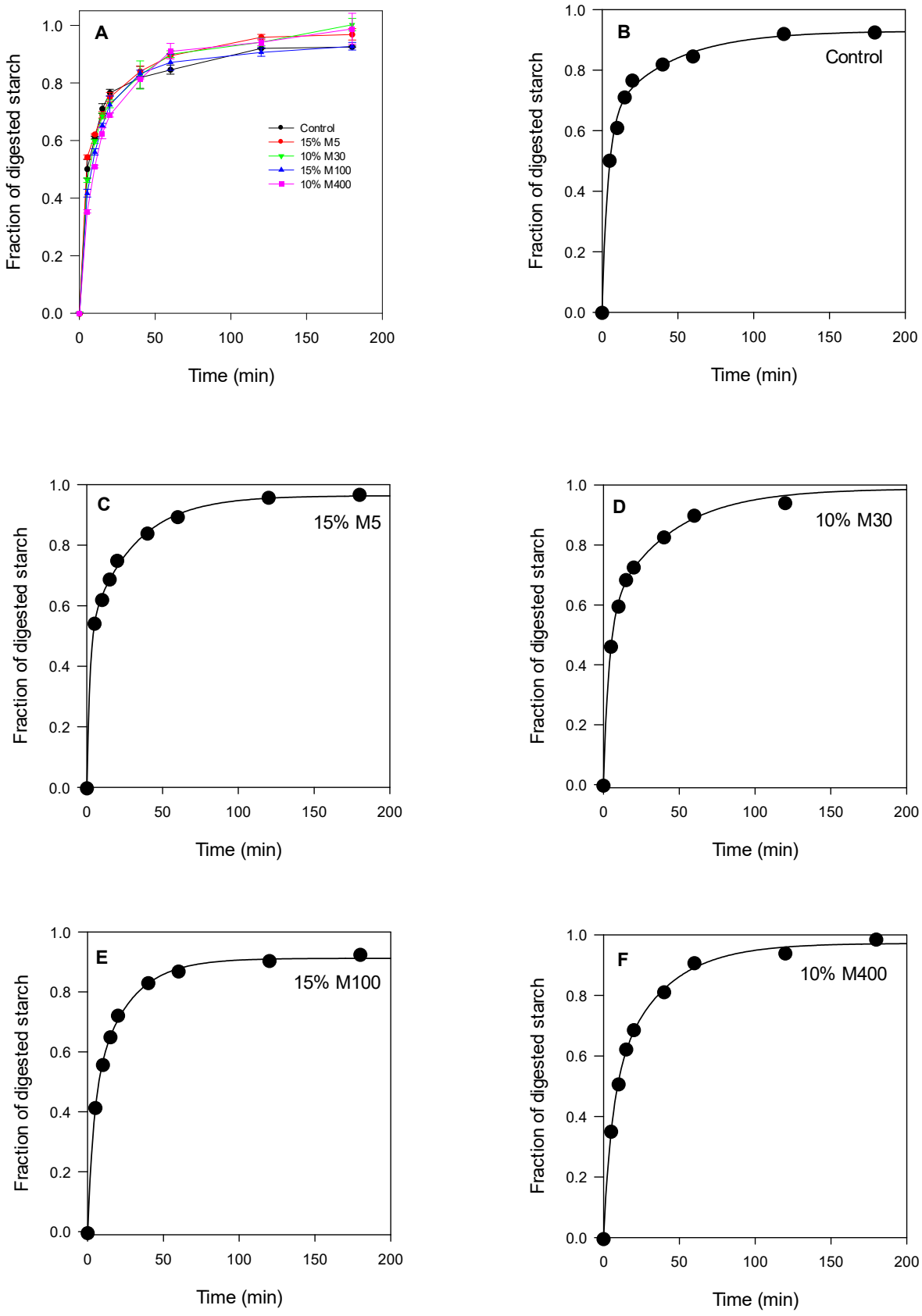
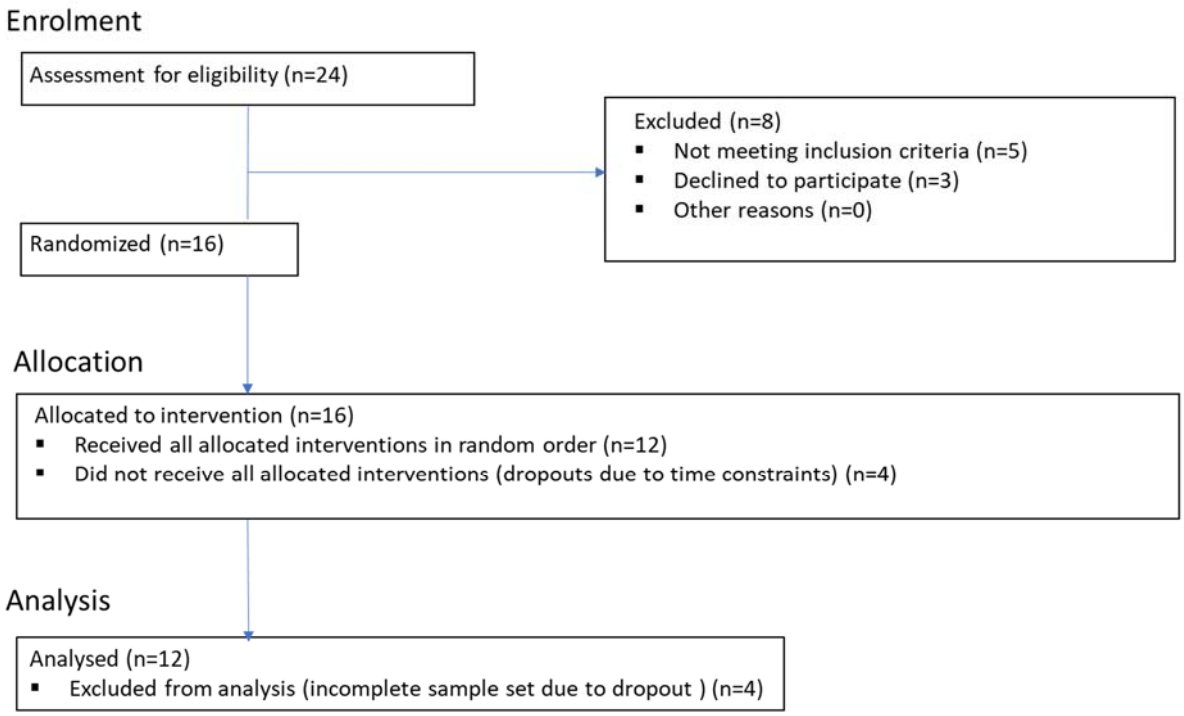

A
Control
15% M5
10% M30
15% M100
10% M400
Fraction of digested starch
Time (min)
B
Control
C
15% M5
D
10% M30
E
15% M100
F
10% M400

Figure 2

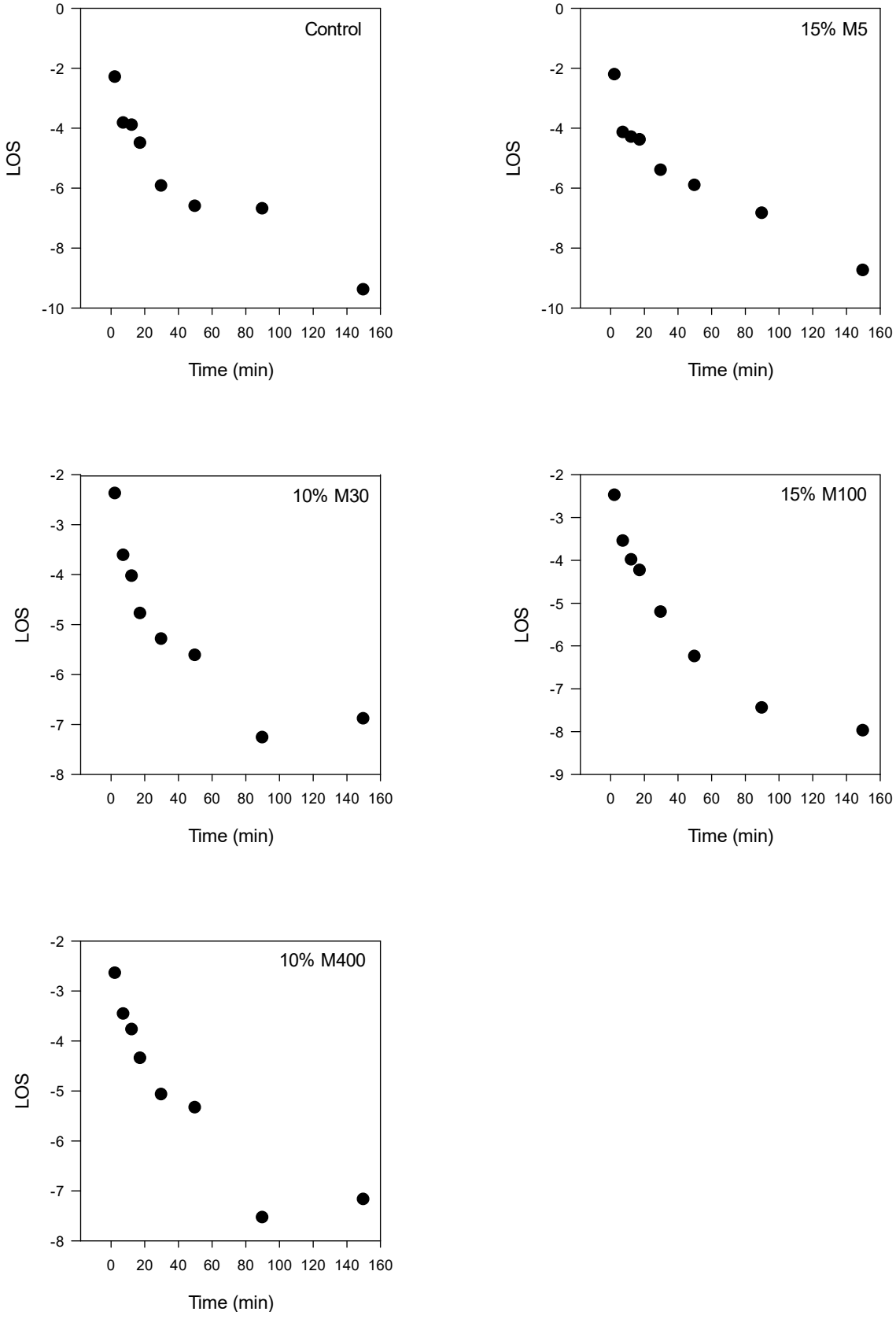
FIGURE LEGENDS

Figure 1. Changes in whole blood capillary (**A**) and venous plasma (**B**) glucose and serum insulin (**C**) with time within 2 h postprandial. Values are mean ± SEM from 12 adult overweight subjects after consumption of control and guar fortified breads providing 25 g available carbohydrate.

Figure 2*. In vitro* starch digestion curves for the control and guar fortified bread. Values are mean n=3 ± SEM (**A**) and plotted means n=3 for each test bread (**B**-**F**) fitted to a 4 parameter first-order kinetic model $C_t = C_f(1\text{-}e^{-k1t}) + C_s(1\text{-}e^{-k2t})$. Text labels in each figure represent the wheat flour replacement level by the different guar grades.



Supplementary Figure 1 Consolidated Standards of Reporting Trials (CONSORT) flow diagram



Supplementary Figure 2. Logarithm of slope (LOS) plots of *in vitro* digestion of starch in the guar fortified test breads. Text labels in each figure represent the wheat flour replacement level by the different guar grades.

AUTHOR CONTRIBUTIONS

Simon Ballance: Conceptualization, Methodology, Validation, Formal Analysis, Visualisation, Writing – original draft, Writing – review and editing, Project administration, Supervision, Funding acquisition. **Hanne Mæhre**: Investigation, Validation, Formal Analysis, Data Curation, Writing – review and editing. **Anne Rieder**: Conceptualization, Validation, Formal Analysis, Visualisation, Writing – original draft, Writing – review and editing, Supervision. **Gülen Arslan Lied**: Writing – review and editing. **Espen K. Hindar Tvedt:** Investigation, Data Curation. **Jutta Dierkes:** Conceptualization, Validation, Visualisation, Writing – review and editing, Project administration, Supervision, Funding acquisition.

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CONFLICT OF INTEREST

There are no conflicts of interest to declare

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