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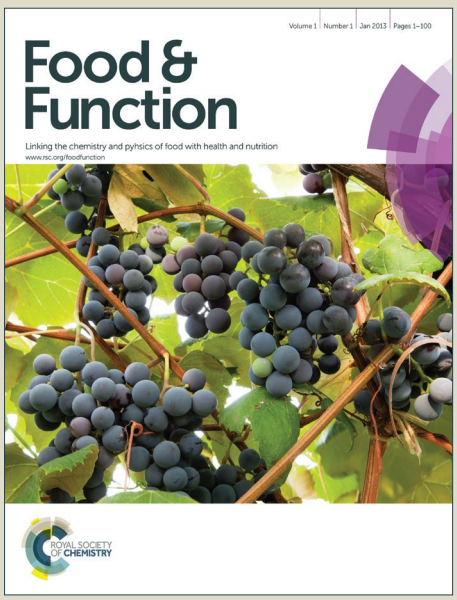

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The structure of wheat bread influences the postprandial metabolic response in healthy men[1-3]

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Abbreviations used: BA, bile acid, CA, cholic acid, CB, control bread, CCK, cholecystokinin, CDCA, chenodeoxycholic acid, dAUC, decremental area under the curve, DCA, deoxycholic

acid, EGP, endogenous glucose production, FB, flat bread, GC/C/IRMS, gas chromatography combustion isotope ratio mass spectrometry, GCR, glucose clearance rate, GI, glycemic index, GIP, glucose-dependent insulinotropic polypeptide, GLP-1, glucagon-like peptide-1, G_{RA}, rapidly available glucose, G_{SA}, slowly available glucose, G_T, total glucose, G_{TA}, total available glucose, iAUC, incremental area under the curve, LCA, lithocholic acid, LC/MS, liquid chromatography mass spectrometry, LOQ, limit of quantitation, PA, pasta, RaE, rate of appearance of exogenous glucose, RaT, rate of appearance of total glucose, RS, resistant starch, UDCA, ursodeoxycholic acid, VAS, visual analogue scale, XRT, X-ray microtomography

RUNNING TITLE: Postprandial metabolic effects of wheat bread structure

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This trial was registered at trialregister.nl as NTR3020.

ABSTRACT

Postprandial high glucose and insulin responses after starchy food consumption, associated with an increased risk of developing several metabolic diseases, could possibly be improved by altering food structure. We investigated the influence of a compact food structure; different wheat products with a similar composition were created using different processing conditions. The postprandial glucose kinetics and metabolic response to bread with a compact structure (flat bread, FB) was compared to bread with a porous structure (control bread, CB) in a randomized, crossover study with ten healthy male volunteers. Pasta (PA), with a very compact structure, was used as control. The rate of appearance of exogenous glucose (RaE), endogenous glucose production, and glucose clearance rate (GCR) was calculated using stable isotopes. Furthermore, postprandial plasma concentrations of glucose, insulin, several intestinal hormones and bile acids were analyzed.

The structure of FB was considerably more compact compared to CB, as confirmed by microscopy, XRT analysis (porosity) and density measurements. Consumption of FB resulted in lower peak glucose, insulin and glucose-dependent insulinotropic polypeptide (ns) responses and a slower initial RaE compared to CB. These variables were similar to the PA response, except for RaE which remained slower over a longer period after PA consumption. Interestingly, the GCR after FB was higher than expected based on the insulin response, indicating increased insulin sensitivity or insulin-independent glucose disposal. These results demonstrate that the structure of wheat bread can influence the postprandial metabolic response, with a more compact structure being more beneficial for health. Bread-making technology should be further explored to create healthier products.

INTRODUCTION

Frequent consumption of starchy foods that result in a high postprandial blood glucose response may contribute to an increased risk of developing type 2 diabetes.[1] In addition, high postprandial insulin concentrations could play a role in developing obesity[2] and insulin resistance.[3] Therefore, efforts to modulate the postprandial glucose and insulin responses after consumption of starchy foods are highly relevant.

The glycemic response after starchy foods is determined by three glucose fluxes: glucose which is derived from digested starch entering the circulation (RaE: rate of appearance of exogenous glucose), glucose produced in the liver (EGP: endogenous glucose production) and glucose uptake into tissues (GCR: glucose clearance rate). The postprandial glucose-induced rise in insulin concentrations, facilitating glucose uptake into tissue, can be potentiated by up to 70% by the incretin hormones; glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1).[4] They are released postprandial from intestinal K- and L-cells, respectively, and have several other physiological functions. GLP-1 is involved in the regulation of gastric emptying[5] and satiety[6] and, in rodents, found to preserve β-cell function.[7] GIP may be implicated, at least in animals, with increased nutrient uptake into adipocytes and fat accumulation,[4, 8] and inhibition of GIP signaling in mice prevented obesity.[9] It has been shown that GIP plasma concentrations are closely correlated with the RaE.[10, 11] Therefore, slow starch digestion may also exert beneficial effects by reducing GIP release. The relationship of GLP-1 and RaE is weaker [10] and in contrary to GIP, GLP-1 would need to be elevated to exert its beneficial effects.

One way to influence postprandial glucose kinetics is by applying different food processing techniques and by consequence changing food structure. A slower RaE, reflecting decreased starch digestibility, was observed after ingestion of breakfast biscuits, which were produced from the same ingredients, but underwent different processing conditions than breakfast

cereals.[12] Extrusion rendered starch granules in breakfast cereals more accessible to starch digestion, whereas the integrity of starch granules was preserved by preventing complete gelatinization of starch by the biscuit making process (heating with low water content). Previously, we compared postprandial glucose kinetics of fiber-rich fresh pasta and wheat bread which were composed of similar ingredients, but were processed differently.[11] The RaE was slower and the insulin response lower after pasta than after bread, but unexpectedly, total blood glucose did not differ. The same discrepancy between the glucose and insulin response was observed in several studies comparing different types of wheat[13] and rye breads.[14, 15] A common characteristic of the products exerting this effect was their relatively compact food structure - likely resulting in slow starch digestion - which was in agreement with the observation for pasta in our previous study.[11] After consumption of starchy products with a compact food structure, the food particles leaving the stomach can still be relatively compact, and accordingly the accessibility to α-amylase is reduced,[16] resulting in a slower RaE. As a consequence GIP is lower and results in a lower insulin response.[11] This causes a slower GCR and therefore total blood glucose is not necessarily decreased. Products with slowly digestible starch could thus exert beneficial effects by reducing insulin and GIP concentrations even without lowering total postprandial blood glucose.

Bread is widely consumed worldwide, but many types result in undesirable high glucose and insulin responses,[17] whereas the glucose kinetics and GIP response are mostly unknown. We aimed to produce bread with a compact structure, flat bread, by changing processing conditions, and compared this to a porous control bread and compact wheat pasta. All high-fiber products were manufactured from the same ingredients, except for the addition of yeast to the control bread, thus only differed in structure. We investigated whether consumption of this flat bread could result in a more beneficial postprandial ‘pasta-type’ response compared

72 to the control bread. For characterizing the metabolic response, we measured glucose kinetics,

73 insulin, GIP and GLP-1, various bile acids (BA) and cholecystokinin (CCK).

SUBJECTS AND METHODS

Subjects

Ten healthy men [age 24 ± 0.6 y, BMI 22 ± 0.2 kg/m^2 (mean ± SEM)] were recruited (Sept-Oct 2011). The main criteria for exclusion were use of medication, blood donation or use of antibiotics in the past 3 mo, gastrointestinal surgery or dysfunction, inflammatory diseases, and diabetes mellitus. Approval was obtained from the Medical Ethics Committee of the 'Beoordeling Ethiek Biomedisch Onderzoek' foundation, Assen, The Netherlands. Each subject gave written informed consent for the study. This trial was registered at trialregister.nl as NTR3020.

Test meals

Three wheat-based test products were prepared (TNO, Zeist, The Netherlands) that had a similar composition, but differed in food structure due to different processing conditions: control bread (CB), flat bread (FB), and pasta (PA).

By conventional roller-milling, refined white flour and wheat bran were obtained from unlabeled (1.085 Atom % ^{13}C) wheat [T. *aestivum* var *Capo*, grown in Austria]. The products were ^{13}C-enriched by using 12% whole meal flour from ^{13}C-labeled wheat [*T. aestivum* var *Paragon* (1.359 Atom % ^{13}C)] cultured in a $^{13}CO_2$ enriched atmosphere.

The control bread (CB) was prepared with 1446 g unlabeled white wheat flour, 240 g ^{13}C-labeled whole meal wheat flour, 314 g wheat bran, 1300 g water, 33.4 g yeast, 36 g salt, 3 g malt and 70 ppm ascorbic acid. After kneading, the dough was left to rise for 30 min, moulded, and left to rise for 60 minutes. Subsequently, the bread was baked for 30 min at 240 °C.

Flat bread (FB) was prepared with 1450 g unlabeled white wheat flour, 240 g ^{13}C-labeled whole meal wheat flour, 310 g wheat bran, 1300 g water, 36 g salt, and 3 g malt. After

kneading, the dough was left to rest for 30 min, sheeted to 1.5 mm thickness and disks with a diameter of 14 cm were cut. The thin dough pieces were baked on a hot stone plate in an oven at 350 °C for 30 s at each side to provide flat breads with a brown colored dry crust and a soft moist crumb inside. Bread portions were stored at -20 °C until use.

Pasta (PA) was prepared with 763 g unlabeled white wheat flour, 120 g ^{13}C-labeled whole meal wheat flour, 117 g wheat bran, 390 g water and 20 g salt. Dough was mixed in a z-blade mixer and spaghetti was prepared using a sheeting method, creating an elastic dough sheet after multiple rolling and folding steps. This dough sheet was cut in spaghetti strings which were about 2*2 mm and 25 cm long. Portions of pasta dough were stored at -20 °C until use and cooked for 3 min in 2 L water before consumption.

All test meals provided 50 g available carbohydrates; portion sizes were 138 g for CB, 119 g for FB, and 127 g for PA (uncooked weight). The breads and pasta were consumed together with 10 g margarine light (4 g fat), 2 slices lean ham (5 g fat, 6 g protein) and 250 mL tap water within 20 min.

Experimental design

The total registered study, addressing two different research questions, consisted of 4 test products. To increase clarity and be able to focus on the results of one of these questions, three meals are described in this paper and a part is described elsewhere.[18] The study was performed in a randomized, crossover manner, with at least 1 wk between each study day. The subjects were asked to refrain from consuming ^{13}C-enriched foods, such as cane sugar, corn products and pineapple, for 3 d preceding the experiments and from alcohol consumption and strenuous exercise for 24 h before each study day. Food intake on the day before each experiment was individually standardized using a diary. A standard evening meal was provided at the commercial research facility (QPS Netherlands B.V.), where the men stayed

overnight. In the evening, a venous catheter was inserted in each forearm for blood collection and for infusion of D-[6,6-$^2H_2$]glucose (98% ^2H atom percent excess) (Isotec). Subjects fasted overnight, but were allowed to drink water. In the morning (t = -122 min), 26.7 mL D-[6,6-$^2H_2$]glucose solution (80 × 0.07 mg/kg body weight) was infused, and a continuous infusion of 0.07 mg/kg body weight D-[6,6-$^2H_2$]glucose per min was started (t = -120 min) and maintained for 8 h (until t = 360 min). Two hours after the start of the infusion, the test meal was ingested (t = 0 min). Water (150 mL) was provided hourly, starting at t = 120 min. Physical activity was limited during the day.

Test meal characterization

Starch, dietary fiber and moisture contents were determined [Starch, enzymatic method (W55110); TDF (AOAC), Gravimetric method (W5586); Moisture 102 °C, Gravimetric method (W5549)] at Eurofins Analytico Food, The Netherlands. To quantify starch fractions (G_T, G_{RA}, G_{SA}, G_{TA} and RS) of the test meals in vitro, an adapted version of the Englyst method[19] was used.[20]

Bread density (g/mL) was calculated by the bread weight (g) divided by volume (mL), which was determined by rapeseed displacement method in duplicate. The density of cooked PA was measured in 10-fold (10 replicates of 1 pasta string) by paraffin oil displacement.

Stereomicroscopy was performed without sample preparation using an Olympus SZX-9 microscope connected with a DP-50 digital camera (settings: Red 1.22; Green 1.22 and Blue 1.22). Light microscopy was performed with an Olympus BH-2 light microscope and the same digital camera type and settings. Sections of 10 μm thickness were cut in a cryostat-microtome at -26°C and applied on gelatin/glycerol coated slides. Sections were stained for 1 minute with Lugol (KI-I2) to color the starch (blue) and for 10 minutes with 0.05 % Ponceau

2R solution in 50% glycerol in water to color the protein (red). The excess of stain was rinsed with a minimum of water and the sections were covered with glycerol.

To determine test meal porosity, samples were scanned using a desktop X-ray microtomography (XRT) system (Model 1172, SkyScan, Aartselaar, Belgium) consisting of an X-ray tube, an X-ray detector and a CCD camera. The X-ray tube was operated at a voltage of 40 kV/250 µA to obtain optimum contrast between void (air cells) and matter (cell walls) according to a modified method. A 12-bit cooled CCD camera (512 x 1024 pixels) was used to collect the X-ray data. Bread samples were sealed in small bags and rotated by a total of 180° during the scanning process with a pixel size of 24.31 µm to obtain optimum resolution, which gave a total scanning time of 18 min. Pasta samples were cooked in boiling water as described above and then sealed in microcentrifuge tubes with water to avoid drying out during the scanning period. Pasta samples were rotated by a total of 180^{o} with a pixel size of 4.86 µm and a total scanning time of 30 min. The initial X-ray radiographs or raw images were obtained at every 0.7° of rotation. Samples were scanned in triplicate. After scanning, radiographs were loaded into NRecon reconstruction software (v. 1.6.6). The software combines the images graphically into a 3-D object from which 2-D cross sectional images can be taken. Before the reconstruction, the CS rotation feature was used to rotate the sample cross sections, making them parallel to the view window. Beam hardening correction was set to 40% in order to reduce the number of artefacts. Cell walls of the solid matrix appear grey, whereas air cells appear black. The reconstructed 2-D slices were then loaded into CTAn software (v. 1.12, Skyscan, Belgium) to obtain the parameters of porosity, air cell wall thickness, and air cell diameter.

Sample collection

Blood was collected into 2 mL fluoride tubes (NaF), 3 mL EDTA tubes [+ 30 µL DPP-4 inhibitor (Millipore)], and 3 mL Lithium/Heparin tubes (BD Diagnostics). Three basal blood samples were collected (t = -60, t = -30, t = -15 min) and postprandial samples were drawn every 15 min for 2 h, every 30 min for an additional 3 h, and once after 6 h. To obtain heparin plasma, blood was collected at t = -60, t = -15 min and then every 30 min for the first 3 hours, and hourly for the last 3 hours. After centrifugation (1300 × g for 10 min at 4°C), plasma aliquots were stored at –20°C (NaF and EDTA plasma) or –80°C (heparin plasma) until analysis.

Breath samples were collected by breathing through a straw into 10 mL Exetainer® vials (Labco Limited). Two basal breath samples were collected (t = -30, t = -5 min) and after the test meal a sample was taken every 30 min until t = 360 min.

Subjects were asked to rate their feeling of hunger using a visual analogue scale (VAS) 15 min before and hourly after the test meal. At the same time points, their feeling and extent of discomfort (abdominal pain, flatulence, other complaints) were recorded (0 = no complaints, 3 = severe complaints). The liking of the test meal was rated using a VAS (0 = not tasty, 100 = very tasty) half an hour after the start of test meal consumption (t = 30 min).

Measurement of plasma glucose, insulin, glucagon, incretin and CCK concentrations

Plasma glucose concentrations were measured on a Roche/Hitachi Modular automatic analyzer (Roche Diagnostics, Hitachi) using a glucose hexokinase method. The within- and between-run CV were ≤ 2 %. The ARCHITECT® insulin assay (Abbott Laboratories) was used to determine insulin concentrations in plasma. The total CV of this chemiluminescent microparticle immunoassay was ≤ 7%. The glucagon assay was directed against the C-terminal of the glucagon molecule (antibody code no. 4305) and therefore measures glucagon of mainly pancreatic origin.[21] Total GIP was measured using the C-terminally directed

antiserum (code no. 80867),[22] which reacts fully with intact GIP (1-42) and the N-terminally truncated metabolite GIP (3–42). Total GLP-1 concentrations were determined as previously described,[23] using a radioimmunoassay (antiserum no. 89390) specific for the C-terminal of the GLP-1 molecule and reacting equally with intact GLP-1 and the primary (N-terminally truncated) metabolite. The glucagon and incretin assays have detection limits of < 2 pmol/L and an intra-assay CV of approximately 6%. CCK was measured using antiserum no. 92128, which binds the bioactive forms of CCK with equal potency without cross-reactivity with any gastrin.[24] The detection limit of the CCK assay is 0.1 pmol/L, and the intra-assay CV approximately 5%.

Measurement of plasma bile acids

Fasting and postprandial concentrations of 15 individual BAs were determined using LC/MS; the primary BAs [cholic acid (CA) and chenodeoxycholic acid (CDCA)], the secondary BAs [deoxycholic acid (DCA), ursodeoxycholic acid (UDCA) and lithocholic acid (LCA)], as well as their glycine (G, glyco-) and taurine (T, tauro-) conjugates. Concentrations of 7 of the BAs were below the lower limit of quantitation (LOQ) of 0.05 µM. The intra- and inter-assay CV ranged from 1.6% to 11.3% and 4.4% to 13.3%, respectively.

For sample preparation, 250 µl of internal standard solution was mixed with 25 µl plasma, and centrifuged at 15.900 x g for 10 min. The supernatant was transferred into a new vial, evaporated under nitrogen at 40 °C, and reconstituted in 100 µl of 50% methanol. The solution was filtered with a 0.2 µm centrifugal filter at 2000 x g for 10 min. After this step, the samples (injection volume 10 µl) were ready for analysis, using two different LC-MS systems. A detailed description of the systems and settings can be found in the Electronic Supplementary Information.

Analysis of isotopic enrichment in breath and plasma samples

Analysis of ^{13}C abundance in breath CO_2 was performed using GC/IRMS (Delta Plus XL; Thermo Fisher Scientific) measuring the $^{13}C/^{12}C$ ratio versus the international standard Pee Dee Belemnite ($\delta^{13}C_{PDB,}$ in ‰).

Plasma sample preparation required for analysis of isotopic enrichment by GC (derivatization) is described in detail elsewhere.[10, 25] ^{2}H enrichment was measured by GC/MS as previously described[25] and $^{13}C/^{12}C$ isotope ratio was measured using GC/C/IRMS as previously described[26], both with some modifications.[27]

Calculation of glucose kinetics

The molar percentage enrichment of [6,6-$^{2}H_2$]glucose and the ^{13}C atom% were calculated as previously described,[25] except that data were not smoothed this time. The rate of appearance of total glucose [RaT, glucose from exogenous (meal) and endogenous (hepatic) sources] was calculated from total plasma glucose concentrations and ^{2}H-enrichment data by using the non-steady-state equation of Steele et al.[28] as modified by De Bodo et al.[29] It was assumed that labeled and unlabeled glucose molecules showed identical metabolic behavior. The effective volume of distribution was assumed to be 200 mL/kg and the pool fraction to be 0.75.[30] The systemic RaE was calculated from the RaT and ^{13}C-enrichment data, as described by Tissot et al.[30] The EGP was calculated by subtracting RaE from RaT.[30] The GCR, which reflects the tissue glucose uptake, was calculated as described by Schenk et al.[31]

Incremental areas under the curve

To determine differences in glucose kinetics and plasma glucose, insulin, glucagon, incretin, CCK and bile acid concentrations, the 0–2 and 0-6 h iAUC were calculated using the trapezoidal rule.[32] The averages of fasting measurements were used as baseline values, and

areas below baseline were not included. For the iAUC calculations of RaT, RaE and GCR, the values were multiplied by body weight. The iAUC of RaE was expressed as a percentage of the administered dose of glucose equivalents (cumulative dose %). Because EGP and unconjugated BA were suppressed after the test meals, the area beneath baseline (dAUC) was calculated.

Insulin sensitivity

Post hoc analyses of possible differences in insulin sensitivity were performed according to a previously reported adapted method[33, 34] using GCR and insulin data. The time periods 0-2 and 0-6 h, but also the time period 0-3 h were analyzed; the latter because this time period included the postprandial insulin response until it returned back to baseline.

Statistics

Data are presented as means ± SEM (n=10), unless indicated otherwise. Analyses were based on the total registered study, so including data from 4 test meals (see experimental design). For parameters that result in individual points of a time curve, we fitted a model that accounts for the differences in the means of the 40 subject/occasion combinations, the main effects of treatment and time and the time x treatment interaction. Residual plots based on such a model were used to assess approximate normality and homogeneity of variances. Parameters that were not compatible with these assumptions were transformed before definitive analysis. We performed F tests on time × treatment interaction effects to assess whether the test meals gave rise to curves of a different shape. If the test resulted in a P value < 0.05, we tested differences between the meals for each time point. If there were no statistically significant differences in shapes, we tested overall differences between the meals based on the above model without the time x treatment interaction.

To assess summary measures such as iAUC, we fitted a model accounting for the differences in the means of the 10 subjects and the four occasions, and the main effects of treatment. Transformed data were used whenever residual plots showed incompatibility with normality and homogeneity assumptions. We performed an F test to see whether there were differences between the meals. If this test resulted in a P value < 0.05, pairwise comparisons among the meals were conducted. A Benjamini-Hochberg correction was applied on P values from all partial tests to correct for multiple comparisons, resulting in a set of differences in which at most 5% were falsely selected (P < 0.0052 for partial tests was considered significant). The analyses were performed with the software package GenStat, release 13.

The within-subject relationship (correlation) between variables was tested by regression analysis according to the method of Bland and Altman.[35] Test meal differences (e.g. density, porosity) were assessed using a Student's t test; a P value < 0.05 was considered significant. These analyses were performed using SPSS 20.0 for Windows (SPSS Inc., Chicago).

RESULTS

Characterization of test meals

Results from *in vitro* quantification of starch fractions suggested that CB and FB were most similar, whereas the PA contained approximately 10% less G_{RA} and 10% more G_{SA} (**Table 1**). The products (CB vs FB vs PA (cooked)) varied in amount of starch (36.2, 42.1 and 20.1%), dietary fiber (7.6, 7.5 and 3.5 %) and moisture content (38.6, 32.6 and 69.5%). Based on the portion size (50g available carbohydrate) the products contained 10.5, 8.9 and 8.7g dietary fiber, respectively.

Product density varied substantially between the meals (**Figure 1** A). CB had a density of 0.29 g/mL ± 0.01 compared to 0.47 g/mL ± 0.00 for FB and 1.15 g/mL ± 0.01 for PA (cooked). The difference in density was clearly visible in the product structure (Figure 1 B). Where CB had large air cells and thin cell walls due to yeast leavening, FB had a more compact structure with fewer air cells in absence of yeast leavening. Only small air cells were visible in FB originating from air incorporation during the mixing process, as well as a larger cavity in the middle of the product as a result of puffing of the dough during baking. PA had a compact structure without any air cells. This was reflected by measures of porosity using XRT (**Supplemental Table 1**). Porosity was highest for CB (83 %). FB, which had a more closed structure, had a porosity of 47%. The 3% porosity obtained for PA was mainly related to the structural disintegrates (e.g. internal cracks) coming from the added bran (**Figure 2**). Average cell wall thickness of CB was significantly smaller than of FB and the average air cell diameter was larger in CB due to the processing differences described above.

The porosity and average air cell diameter obtained from image analysis are closely related (r = 1.00) and inversely related to the product density as determined by physical measurement (r = -0.97 and -0.95 respectively).

The products' microstructure (Figure 1 C) showed that FB appeared to have a protein continuous phase (protein stained red) in which the starch granules are dispersed, whereas the microstructure of PA and even more of CB appeared to be dominated by swollen starch granules (starch stained blue-purple), with patches of protein. This may be explained by the very short baking process of FB, resulting in less time for complete swelling and gelatinization of the starch. For PA, a clear gradient from the center to the surface of the structure can be observed. In the center (left top corner) the starch granules were dark and compact, whereas more and more swelling of the granules was observed towards the outside, with lighter color of the granules and some colored areas in between the starch granules due to amylose leakage.

Postprandial glucose and insulin response

Postprandial glucose concentrations did not differ after the consumption of CB, FB and PA based on comparisons (all meals) of iAUC and time points (**Figure 3** A**, Table 2**). However, significant differences in glucose peak value between CB-FB (8.3 ± 0.3 vs 7.5 ± 0.2, $P < 0.002$) and CB-PA (8.3 ± 0.3 vs 7.6 ± 0.3, $P < 0.003$) were found (Table 2).

The insulin response was lower after PA consumption compared to CB at several time points (t = 45, 60, 105 min, $P < 0.005$) (Figure 3 B), which resulted in a smaller 0-6 h iAUC compared with CB consumption ($P < 0.005$, Table 2). FB and PA showed similar low insulin responses, which were lower compared to CB based on the 0-2 h iAUC ($P < 0.005$). A lower average peak value was observed after FB and PA consumption compared to CB ($P < 0.005$) as well. At t = 210 min ($P < 0.005$), insulin was higher after PA compared to CB and FB.

Glucose kinetics

FB intake resulted in a slower RaE compared to CB at t = 30 min ($P < 0.005$), but was similar to CB in the later postprandial phase. The RaE was slower after PA compared with CB intake at several time points (**Figure 4** A), resulting in a lower 0-2 h iAUC ($P < 0.005$) (Table 2). In the later phase, the RaE after PA was higher compared to CB (t = 210 and 270 min, $P < 0.005$). The average peak values of RaE from FB and PA were also lower compared to CB (Table 2). The EGP was not significantly different after consumption of the meals (**Supplemental Figure 1**). The GCR after FB and CB showed a remarkable similarity, whereas after PA consumption, glucose was cleared from the circulation at a slower rate (Figure 4 B). The GCR after PA and FB consumption was different at t = 150 min (FB higher) and at t = 240 min (PA higher) ($P < 0.005$). No partial tests for iAUC were performed because of a lack of difference based on the F test.

Postprandial glucagon, incretin and CCK response

The postprandial glucagon responses were not significantly different between the test meals (**Supplemental Figure 2**). The average postprandial GIP response after FB consumption tended to be lower compared to CB and was similar compared to the response after PA intake (**Figure 5** A). However, no partial tests could be performed on iAUC data (Table 2; F-test, $P = 0.16$). Looking at time points, a significant difference in GIP response after CB and PA consumption was found at t = 120 min ($P < 0.005$).

The GLP-1 response showed a different pattern after PA compared to CB and FB consumption (Figure 5 B), especially around t = 30 min, where PA consumption did not evoke the sharp peak in GLP-1 concentrations observed after FB and CB consumption. However, time x treatment interaction failed to reach significance ($P = 0.0733$). The GLP-1 response to FB was similar compared to CB in the first hour, but tended to drop thereafter. The GLP-1 response after PA increased gradually, and reached a similar average increase

from baseline compared to CB (t = 150 min). The iAUCs of GLP-1 responses were similar between meals (Table 2). The average postprandial CCK response was lower after PA consumption compared to CB between t = 0 and 180 min (0-2 and 0-6 h iAUC, $P < 0.005$), whereas the response to FB was intermediate (**Figure 6**).

Postprandial bile acid response

The postprandial pattern of each individual (glycine- and taurine-) conjugated BA was identical, with the highest concentrations for GCDCA, followed by either GDCA or GCA. The unconjugated BAs (both primary and secondary) also responded in the same way within each person per test period. Therefore, by summing up concentrations, individual BAs were grouped as conjugated and unconjugated BAs, and together they formed the total BA response (**Figure 7** A). Except for the high peak at t = 30 min after FB, conjugated BAs after FB and PA showed a similar pattern, whereas CB consumption showed a biphasic pattern with a second peak around t = 120 min (Figure 7 C). Due to the great inter-individual differences in postprandial responses, F tests did not result in significant *P*-values.

$^{13}CO_2$ excretion in breath

$^{13}CO_2$ excretion in breath, reflecting the rate of oxidation of the ^{13}C-labeled substrate, was higher after CB compared to PA (t = 90 – 210 min, and 0-2 h iAUC 120, $P < 0.005$) and to FB (t = 120 – 180 min, and 0-2 and 0-6 h iAUC, $P < 0.005$) (Table 2, **Figure 8**). The $^{13}CO_2$ responses were similar after PA and FB consumption.

Rated hunger, discomfort and liking of test meal

The subjective sensation of hunger, as determined hourly using a VAS, did not differ after consumption of CB, FB, and PA (**Supplemental Figure 3**), which might be due to a lack of

power. Occasional mild complaints of flatulence were not meal type related. The VAS scores for liking (CB = 57, PA = 62, FB = 62) indicated that all meals were appreciated similarly by the subjects.

Correlations

Correlations between several variables (all time points, 0-6 h) were determined. RaE and GIP correlated very well (CB r = 0.84; FB r = 0.81, PA r = 0.75), whereas RaE and GLP-1 correlated moderately for CB (r = 0.70) and FB (r = 0.69), but less for PA (r = 0.47). The correlation between GLP-1 and conjugated BA was moderate for CB (r = 0.60), but was lower for FB (r = 0.40), and PA (r = 0.28).

Insulin sensitivity

Post hoc analysis of possible difference in insulin sensitivity revealed that for the time period 0-3 h, there was a difference between PA and FB (t-test, $P < 0.05$) **(Supplemental Table 2)**. A higher relative value indicates increased insulin sensitivity.

DISCUSSION

Because food structure may be important in determining starch digestibility and the metabolic response to a food product, we hypothesized that fiber-rich wheat bread with a compact structure would result in a more beneficial response compared to normal fiber-rich bread, and show similarities to fiber-rich wheat pasta with respect to postprandial glucose kinetics and associated processes. Therefore, we made compact flat breads (FB) without the use of a leavening agent and compared this to a yeast-leavened porous wheat bread (CB, control bread with open structure) and to pasta (PA, control wheat product with compact structure). All products had the same composition, but different food structures due to the processing conditions.

As confirmed by microscopy, XRT analysis (porosity) and density measurements, the structure of FB was indeed considerably more compact compared to CB (porosity CB: 83% vs FB: 47%, density CB 0.29 g/mL vs FB: 0.47 g/mL). However, although no leavening agent was used in these breads, some air bubbles were present because of the mixing and baking procedure, resulting in a porosity of 47%. FB was, therefore, not as compact as pasta (density cooked PA 1.15 g/mL), which did not contain any air cells. Our pasta did show some porosity (3%), which was due to the addition of fiber resulting in small cracks in the pasta. These cracks, making the pasta structure less dense, might also (partly) explain why this pasta did not result in a low glucose response (neither in the present nor the previous study[11]) as most often is the case for pasta as described in previous literature.[36] From microscopy data FB appeared to contain less swollen starch granules. This can be explained by the very short baking process of FB, which leaves little time for complete swelling and gelatinization of the starch and is likely to decrease starch digestibility as well.

The in vivo digestibility of the products is reflected by the RaE. After FB consumption the RaE was lower compared to CB in the first hour postprandial (t = 30 and peak value), and

comparable to the low PA response. However, although not significantly different, the RaE between 45 and 150 min postprandial was intermediate for FB compared to PA and CB, indicating that starch digestibility and/or oral processing, gastric emptying, etc was different between the meals. The *in vitro* digestibility data (Table 1) suggested that FB was most similar to CB, but contained some more resistant starch.

Despite the differences in RaE, the resulting total glucose response after consumption of the test meals was similar based on the iAUC. This corresponds to the findings in our previous study comparing CB and PA,[11] although this time the peak value of glucose was somewhat lower for PA and also for FB compared to CB. The insulin response was, however, clearly lower after FB compared to CB, and was similar to the low insulin response after PA consumption, as observed previously.[11]

GIP, one of the incretin hormones involved in insulin potentiation, responded very similarly after FB and PA consumption, whereas the increase was less pronounced than after CB. In previous studies, we showed that the RaE (influx rate of glucose from the meal) and the GIP response are in good agreement,[10, 11] which can be explained by the importance of the intestinal glucose transporter SGLT-1 in GIP secretion.[37] The relation between glucose absorption and GIP release (dose-response) was also shown in several duodenal perfusion studies.[38, 39] In the present study, this relationship between RaE and GIP was again demonstrated by the good correlation coefficients (CB r = 0.84, FB r = 0.81, PA r = 0.75). However, the obviously faster RaE from t = 45 min after FB as compared to PA (ns) was not reflected in the GIP response. It might be that the initial glucose influx rate is the most important factor in determining the extent of the GIP response, as the RaE at 30 min was the same after FB and PA and significantly lower than after CB in this early postprandial phase (t = 30 min FB, t = 45 min PA). In support of this, the initial rate of delivery of glucose to the duodenum was found to be important in determining the pattern of the incretin response.[40] An

initial rapid infusion rate (3 kcal/min) for 15 min and subsequent slower infusion rate of 0.71 kcal/min until t = 120 resulted in a high peak in plasma GIP and GLP-1 responses around 30 min, whereas the same amount of calories infused at a constant rate of 1 kcal/min resulted in a low response.[40]

The initial high influx rate of glucose in our study, however, cannot explain the difference in GLP-1 response. We observed a sharp GLP-1 peak at t = 30 min after FB, which was very similar to that after CB, but absent after PA consumption. As the RaE indicated that the rate of glucose absorption was similar between PA and FB at around 30 min postprandially, the 'difference' in the initial GLP-1 peak between FB and PA cannot be explained by this mechanism. Although SGLT-1 plays a role in stimulating GLP-1 release to some extent,[37, 41] other factors are capable of stimulating GLP-1 secretion from L-cells as well. For instance, GLP-1 release, but not GIP release, was found to be influenced also by BAs via the BA receptor TGR5 in the small intestine.[42] Interestingly, we observed a similar pattern when comparing GLP-1 and plasma BA concentrations after FB and PA, mainly with respect to the first postprandial hour. Conjugated BA peaked after FB around 30 min postprandial, whereas PA consumption resulted only in a modest increase in BAs. It could thus be speculated that the high conjugated BA peak after FB had an additional effect on GLP-1 secretion, thereby explaining the initial high GLP-1 peak. The reason for this 'difference' in plasma BA peak is not evident. CCK is known to stimulate gall bladder contraction and thus BA release,[43] but the CCK response in our study did not correspond well with the BA concentrations.

Insulin is an important factor in glucose uptake into insulin sensitive tissues (via GLUT4 translocation), and therefore a low insulin response would be expected to result in a slow GCR. This was indeed observed in previous studies.[11, 31] Interestingly, despite the lower insulin response (peak value and 2 h iAUC) to FB as compared to CB, the GCR after CB and FB consumption was almost identical. In addition, although insulin was very similar after PA

and FB, the GCR was higher after FB compared to PA intake. Taken together, data from this study indicate either increased peripheral insulin sensitivity or increased insulin-independent glucose absorption after FB leading to augmented glucose disposal. Only speculations can be offered about the underlying mechanism.

One factor involved in increasing insulin sensitivity and glucose disposal could be BAs. After FB consumption, the conjugated BA concentrations rose steeply (peak at t = 30 min), whereas no pronounced peak was observed after PA consumption. There are indications from rodent studies that BAs, via activation of their receptor FXR, could be involved in improving insulin sensitivity and increased glycogen storage.[44-46] In addition, treatment of obese and diabetic mice with TUDCA resulted in normalization of hyperglycemia (within 10 days), restoration of systemic insulin sensitivity, and enhancement of insulin action in liver, muscle, and adipose tissue.[47] Treatment of obese human subjects with TUDCA for 4 weeks improved muscle and hepatic insulin sensitivity.[48] Although these are mainly longer term effects, it indicates the potency of BAs in regulating insulin sensitivity.

Previously we observed that a slow GCR (together with a slow RaE) resulted in a relatively 'high' glucose response after pasta consumption.[11] Therefore, with an increased GCR after FB compared to PA, total glucose concentrations after FB would be expected to be lower. However, the similar glucose responses after FB and PA may be explained by the somewhat higher RaE, and a slightly less suppressed EGP after FB consumption.

To summarize, consumption of a compact flat bread resulted in a more moderate postprandial response compared to CB intake based on glucose, insulin and GIP responses, resembling that of pasta, although RaE was only lower in the first hour. Interestingly, the GCR after FB was higher than expected based on the insulin response and seems to be due to insulin-independent glucose disposal or increased insulin sensitivity. The role (and regulation) of BA

and possibly other factors stimulating GLP-1 release and affecting insulin sensitivity after ingestion of starchy food products needs further study.

Conclusion: The results of this study clearly show that the structure of bread can influence the postprandial metabolic response. A more compact bread structure, due to reduced porosity and/or air cell diameter, may be advantageous because of the lower peak glucose, insulin and GIP (ns) response, via a slower initial RaE after FB consumption. Therefore, the effect of bread processing on postprandial metabolism and related health aspects deserves further study.

ACKNOWLEDGEMENTS

We thank Theo Boer for GC/MS and GC/C/IRMS analyses, Marianne Schepers and Gerlof Reckman for technical support/laboratory procedures, and Tom Preston for providing ^{13}C enriched wheat kernels. From TNO, Zeist, The Netherlands we thank Carina de Jong for statistical analysis using GenStat and Thérèse Maarschalkerweerd for making microscopic images. All authors read and approved the final manuscript and have no conflicts of interest. This study was funded by the Dutch Top Institute Food and Nutrition (TIFN).

Table 1 *In vitro* analysis of starch fractions in the test meals[1,2]

Starch fraction	Test meal/portion		
	CB Total	FB Total	PA Total
		%	
G_T	100	100	100
G_{RA}	78.8	77.9	69.4
G_{SA}	11.8	9.1	20.3
G_{TA}	90.6	87.0	89.7
RS	8.4	11.7	9.3

[1]Analyses were performed in duplicate.

[2]CB, control bread; FB, flat bread; G_{RA}, rapidly available glucose (20 min); G_{SA}, slowly available glucose (20-120 min); G_T, total glucose (indicated as 100%); G_{TA}, total available glucose (120 min); PA, pasta; RS, resistant starch.

Table 2 Indices reflecting the metabolic response after ingestion of 138 g ^{13}C-enriched control bread, 119 g ^{13}C-enriched flat bread and 127 g ^{13}C-enriched pasta (uncooked weight) in healthy men[1]

	Basal concentrations	Peak concentrations	Time to peak (min)	iAUC (0-2 h)	iAUC (0-6 h)
Glucose (mmol/L)					
CBread	5.1 ± 0.1	8.3 ± 0.3	40.5 ± 3.2	163.2 ± 20.5	197.0 ± 23.4
FBread	5.0 ± 0.1	7.5 ± 0.2*	36.8 ± 2.4	130.1 ± 15.6	169.5 ± 17.0
Pasta	5.0 ± 0.1	7.6 ± 0.3*	34.5 ± 2.3	134.5 ± 22.6	191.1 ± 30.9
Insulin (µU/mL)					
CBread	4.9 ± 0.5	69.9 ± 10.5	43.5 ± 3.5	3290.9 ± 528.8	4193.0 ± 936.6
FBread	4.9 ± 0.5	44.5 ± 5.3*	43.5 ± 3.5	2168.9 ± 238.8*	2714.4 ± 329.0
Pasta	4.6 ± 0.6	36.0 ± 5.6*	37.5 ± 4.0	1689.0 ± 186.3*	2334.3 ± 248.3
Glucagon (pmol/L)					
CBread	8.3 ± 1.0	15.8 ± 1.7	175.5 ± 33.4	272.9 ± 84.6	980.5 ± 163.4
FBread	8.9 ± 0.8	13.7 ± 1.4	101.3 ± 31.9	226.5 ± 52.1	587.2 ± 143.3
Pasta	9.6 ± 1.1	14.6 ± 1.1	147.0 ± 31.4	139.3 ± 36.9	442.3 ± 127.4
GIP (pmol/L)					
CBread	9.2 ± 1.8	84.9 ± 10.5	93.0 ± 13.6	4904.6 ± 426.7	9089.2 ± 113[illegible]
FBread	10.6 ± 2.1	68.4 ± 6.1	84.0 ± 13.5	3882.3 ± 296.0	7471.2 ± 615.3
Pasta	10.9 ± 0.9	63.8 ± 4.6	90.0 ± 14.8	3796.5 ± 396.7	7746.5 ± 837.9
GLP-1 (pmol/L)					
CBread	15.5 ± 1.4	30.9 ± 2.1	86.3 ± 15.7	949.9 ± 95.0	2019.9 ± 183.0
FBread	16.9 ± 1.7	30.7 ± 3.6	82.5 ± 14.2	765.9 ± 111.7	1489.7 ± 219.9
Pasta	17.1 ± 1.5	30.5 ± 3.2	121.5 ± 21.7	686.5 ± 94.5	1747.6 ± 256.3
RaT (mg/kg·min)					
CBread	2.0 ± 0.1	6.5 ± 0.3	57.0 ± 14.5	39.1 ± 1.4	88.1 ± 1.6
FBread	2.0 ± 0.0	5.7 ± 0.3	55.5 ± 13.0	37.0 ± 1.6	87.9 ± 2.4
Pasta	2.0 ± 0.1	6.0 ± 0.5	34.5 ± 3.2	33.7 ± 2.1*	84.1 ± 1.8
RaE (mg/kg·min)					
CBread	0 ± 0	4.9 ± 0.2	66.0 ± 14.9	49.7 ± 1.6	95.9 ± 4.7
FBread	0 ± 0	4.0 ± 0.2*	61.5 ± 13.7	43.5 ± 2.2	91.0 ± 3.3
Pasta	0 ± 0	3.9 ± 0.3*	48.0 10.9	37.5 ± 2.9*	88.6 ± 2.6
EGP (mg/kg·min)[2]					
CBread	2.0 ± 0.1	0.9 ± 0.2	111.0 ± 12.1	43.4 ± 9.3	157.8 ± 26.7
FBread	2.0 ± 0.0	1.1 ± 0.2	103.5 ± 22.4	34.7 ± 8.7	133.6 ± 19.8

Pasta	2.0 ± 0.1	0.9 ± 0.1	100.5 ± 18.7	42.0 ± 11.1	171.6 ± 24.8
GCR (mL/kg·min)					
CBread	2.5 ± 0.1	6.4 ± 0.2	100.5 ± 12.5	15.6 ± 1.5	32.7 ± 1.7
FBread	2.5 ± 0.1	6.2 ± 0.3	99.0 ± 11.7	15.0 ± 1.7	33.4 ± 2.1
Pasta	2.5 ± 0.1	5.7 ± 0.4	111.0 ± 17.2	11.8 ± 2.0	29.1 ± 2.0
$^{13}CO_2$ (%dose/h)					
CBread	0 ± 0	7.7 ± 0.2	228.0 ± 9.2	4.3 ± 0.3	31.7 ± 0.7
FBread	0 ± 0	7.4 ± 0.2	243.0 ± 10.4	3.6 ± 0.3*	29.2 ± 1.0*
Pasta	0 ± 0	7.6 ± 0.2	267.0 ± 14.5	3.5 ± 0.3*	29.6 ± 0.8
CCK (pmol/L)					
CBread	0.5 ± 0.1	2.6 ± 0.3	115.5 ± 10.5	142.4 ± 5.7	295.0 ± 26.4
FBread	0.7 ± 0.1	2.5 ± 0.2	126.0 ± 10.8	122.3 ± 14.6	242.9 ± 30.8
Pasta	0.6 ± 0.1	2.1 ± 0.2	103.5 ± 15.2	104.1 ± 12.8*	231.9 ± 28.9*
Total BA (µmol/L)					
CBread	2.1 ± 0.4	4.3 ± 0.7	61.5 ± 19.7	93.0 ± 23.3	171.2 ± 40.9
FBread	2.4 ± 0.7	5.4 ± 1.1	64.5 ± 24.4	86.0 ± 19.2	162.0 ± 40.7
Pasta	2.0 ± 0.5	4.1 ± 0.9	87.0 ± 30.5	80.8 ± 19.9	147.4 ± 46.1
Conjugated BA (µmol/L)					
CBread	1.2 ± 0.3	3.6 ± 0.6	63.0 ± 19.3	110.8 ± 21.3	216.2 ± 43.4
FBread	1.4 ± 0.5	4.5 ± 0.9	90.0 ± 25.8	100.1 ± 16.9	213.5 ± 34.9
Pasta	1.3 ± 0.3	3.1 ± 0.5	85.5 ± 30.6	67.3 ± 18.0	133.8 ± 46.1
Unconjugated BA (µmol/L)[2]					
CBread	0.9 ± 0.3	0.2 ± 0.1	211.5 ± 38.0	47.7 ± 26.9	185.2 ± 96.9
FBread	1.0 ± 0.3	0.2 ± 0.1	271.5 ± 32.7	46.3 ± 18.1	220.1 ± 80.2
Pasta	0.6 ± 0.2	0.3 ± 0.2	282.0 ± 36.7	8.3 ± 3.2	49.0 ± 15.9

[1]Values are means ± SEM, n=10. * Significantly different from control bread. BA, bile acid; CB, control bread; CCK, cholecystokinin, EGP, endogenous glucose production; FB, flat bread; GCR, glucose clearance rate; GIP, glucose-dependent insulinotropic polypeptide; GLP-1, glucagon-like peptide-1; iAUC, incremental area under the curve; PA, pasta; RaE, rate of appearance of exogenous glucose; RaT, rate of appearance of total glucose.

[2] Because EGP and unconjugated BA were suppressed after the test meals, the nadir concentrations and time to nadir are presented. Also, the area beneath baseline (dAUC) was calculated using mirrored data.

FIGURE LEGENDS

FIGURE 1 Pictures of ^{13}C-enriched control bread (CB), flat bread (FB) and pasta (PA): A, overview; B, stereo microscopy; C, light microscopy with starch (lugol; blue) and protein (Ponceau 2R; red) staining.

FIGURE 2 Representative 2D XRT images of control bread (CB), flat bread (FB) and cooked pasta (PA).

FIGURE 3 Mean (± SEM) changes from baseline in (A) plasma glucose concentrations and (B) plasma insulin concentrations, after ingestion of 138 g ^{13}C-enriched control bread (●), 119 g ^{13}C-enriched flat bread (▪), and 127 g ^{13}C-enriched pasta (Δ) in healthy men (n=10). There was no significant time × treatment interaction for glucose (P = 0.1773), but there was for insulin (P < 0.0001). *Significantly different between CB and PA per time point (after Benjamini Hochberg correction). $^{\#}$Significantly different between PA and FB per time point (after Benjamini Hochberg correction).

FIGURE 4 Mean (± SEM) of (A) RaE and (B) changes from baseline in GCR, after ingestion of 138 g ^{13}C-enriched control bread (●), 119 g ^{13}C-enriched flat bread (▪), and 127 g ^{13}C-enriched pasta (Δ) in healthy men (n=10). There was a significant time × treatment interaction for RaE (P < 0.0001) and GCR (P = 0.0002). *Significantly different between CB and PA per time point (after Benjamini Hochberg correction). °Significantly different between CB and FB per time point (after Benjamini Hochberg correction). $^{\#}$Significantly different between PA and FB per time point (after Benjamini Hochberg correction). GCR, glucose clearance rate, RaE, rate of appearance of exogenous glucose.

FIGURE 5 Mean (± SEM) changes from baseline in (A) plasma GIP concentrations and (B) plasma GLP-1 concentrations, after ingestion of 138 g ^{13}C-enriched control bread (●), 119 g ^{13}C-enriched flat bread (▪), and 127 g ^{13}C-enriched pasta (Δ) in healthy men (n=10). There was a significant time × treatment interaction for GIP (P = 0.0355), but not for GLP-1 (P = 0.0733). *Significantly different between CB and PA per time point (after Benjamini Hochberg correction). GIP, glucose-dependent insulinotropic polypeptide, GLP-1, glucagon-like peptide-1.

FIGURE 6 Mean (± SEM) changes from baseline in plasma CCK concentrations after ingestion of 138 g ^{13}C-enriched control bread (●), 119 g ^{13}C-enriched flat bread (▪), and 127 g ^{13}C-enriched pasta (Δ) in healthy men (n=10). There was no significant time × treatment interaction (P = 0.4559). CCK, cholecystokinin.

FIGURE 7 Mean (± SEM) changes from baseline in (A) total plasma BA concentrations, (B) unconjugated BA concentrations and (C) conjugated BA concentrations, after ingestion of 138 g ^{13}C-enriched control bread (●), 119 g ^{13}C-enriched flat bread (▪), and 127 g ^{13}C-enriched pasta (Δ) in healthy men (n=10). There were no significant time × treatment interactions. BA, Bile acids.

FIGURE 8 Postprandial breath $^{13}CO_2$ response after ingestion of 138 g ^{13}C-enriched control bread (●), 119 g ^{13}C-enriched flat bread (▪), and 127 g ^{13}C-enriched pasta (Δ) in healthy men (n=10). There was a significant time × treatment interaction for breath $^{13}CO_2$ (P < 0.0001) *Significantly different between CB and PA per time point (after Benjamini Hochberg correction). °Significantly different between CB and FB per time point (after Benjamini Hochberg correction).

Figure 1

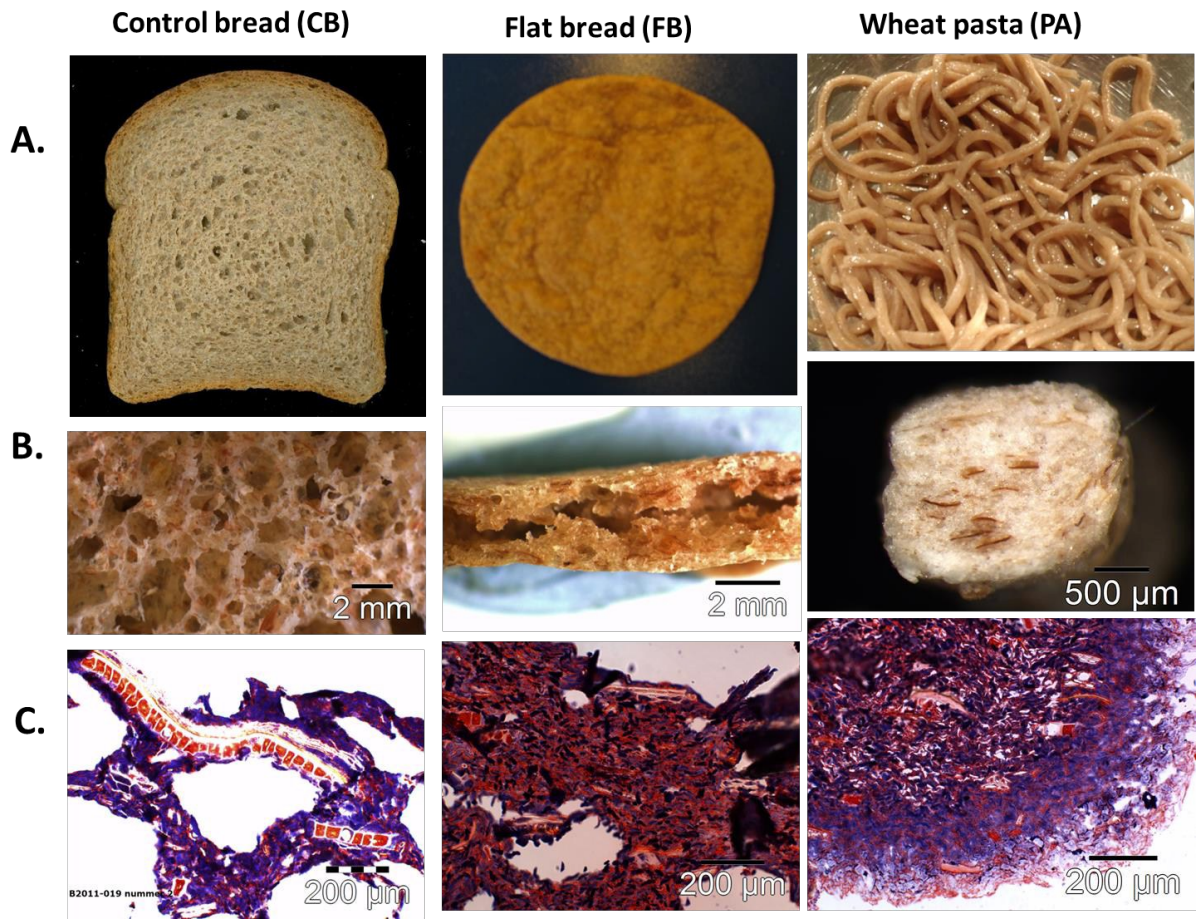

Figure 2

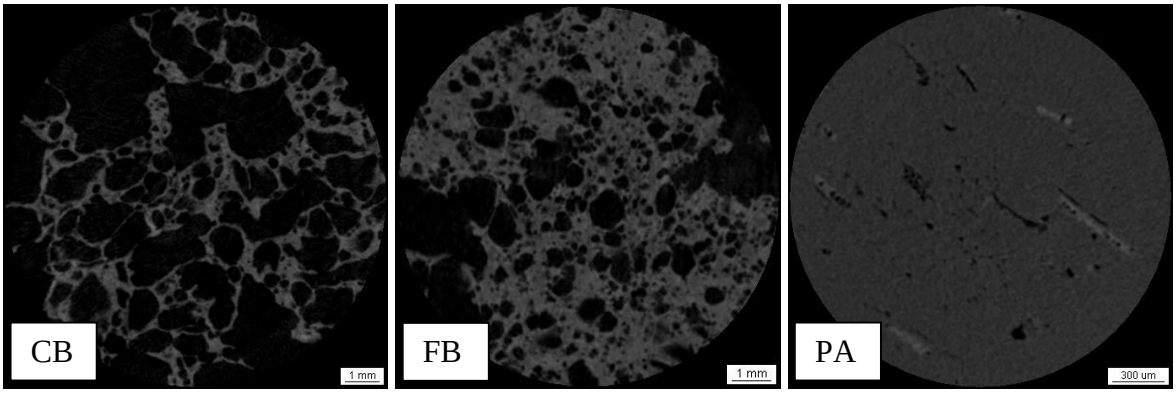

Figure 3

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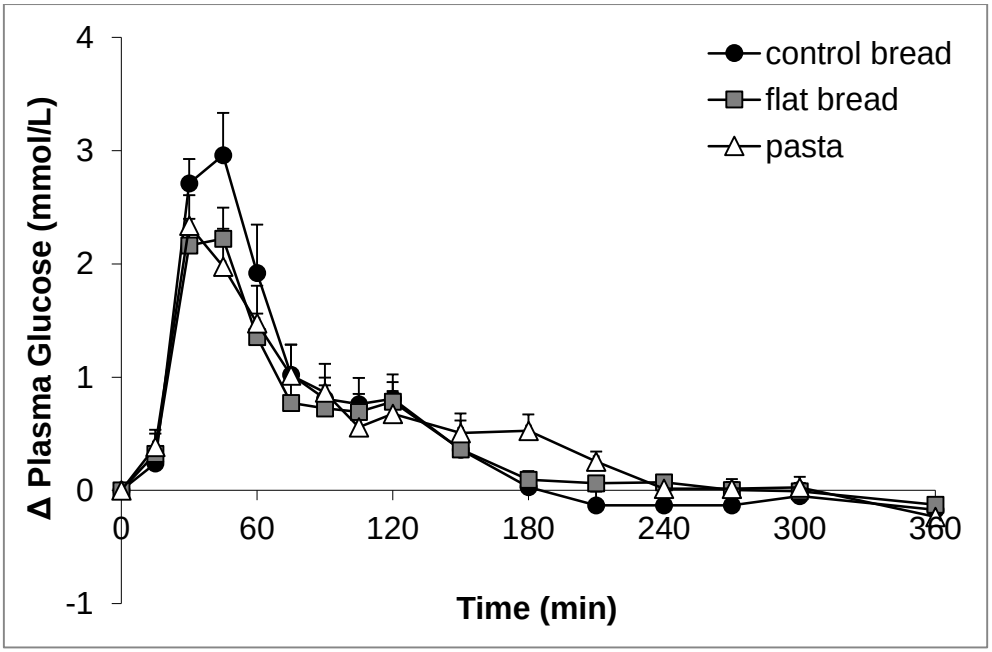

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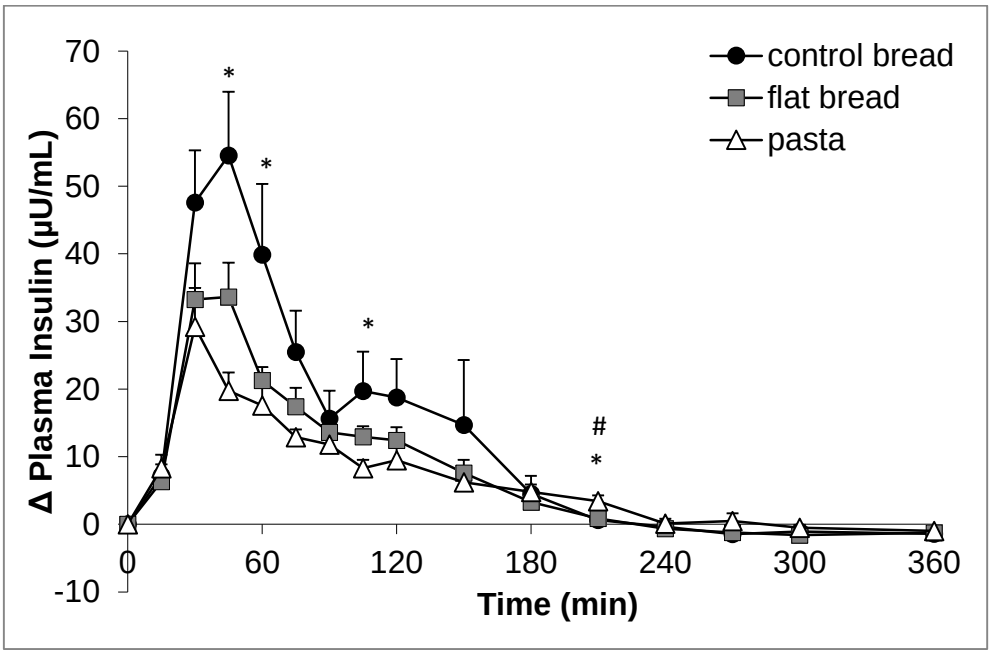

Figure 4

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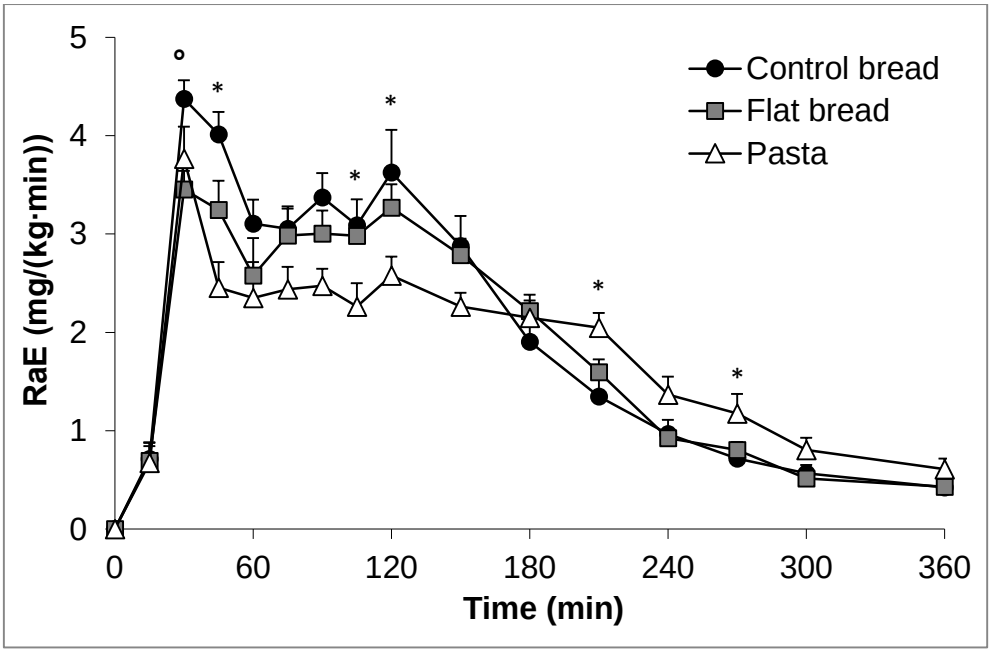

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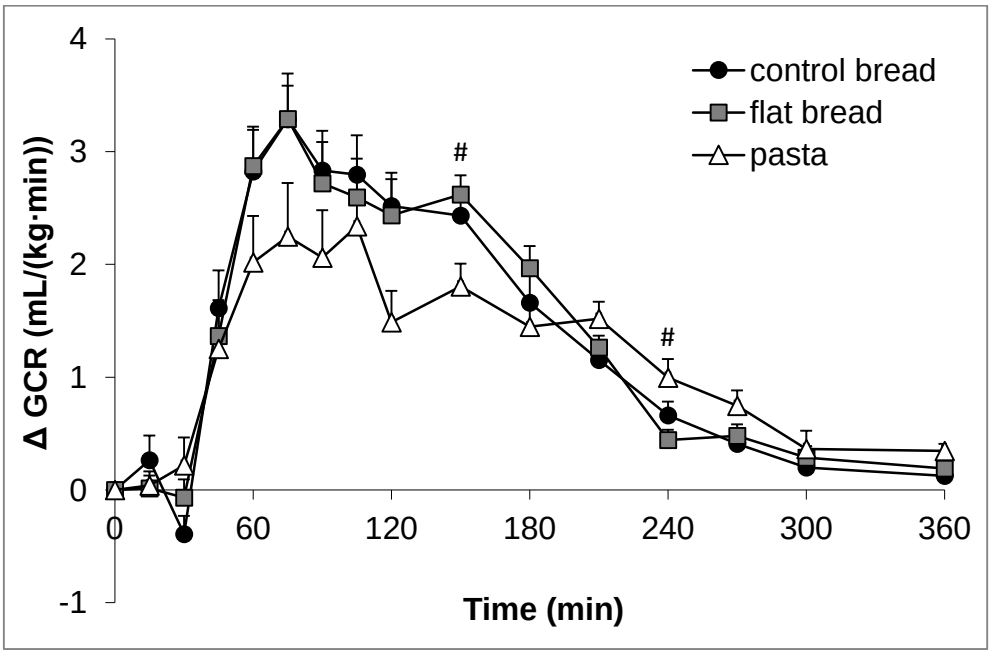

Figure 5

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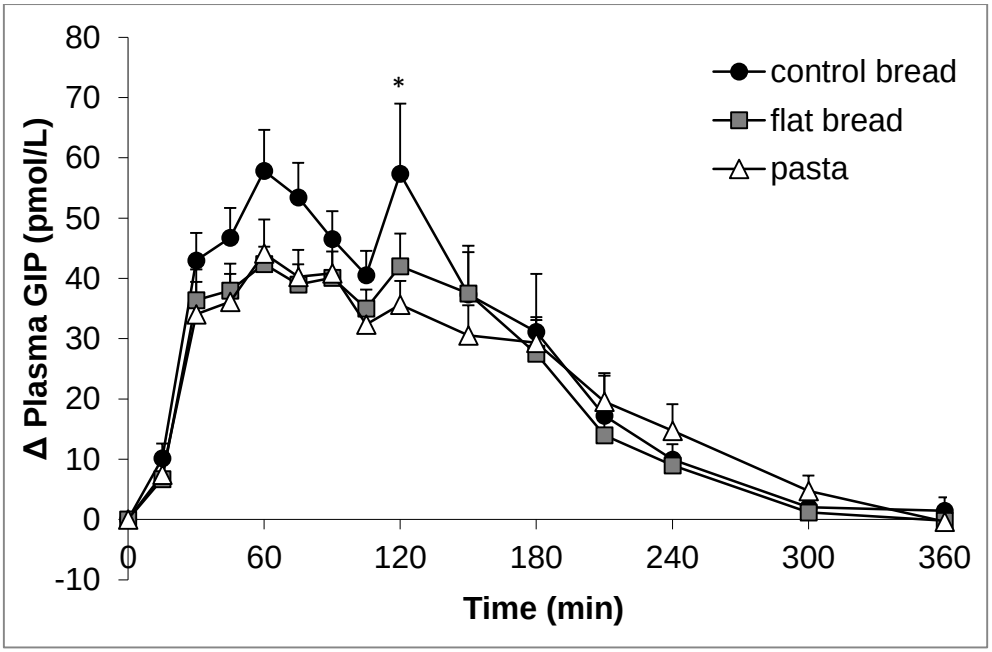

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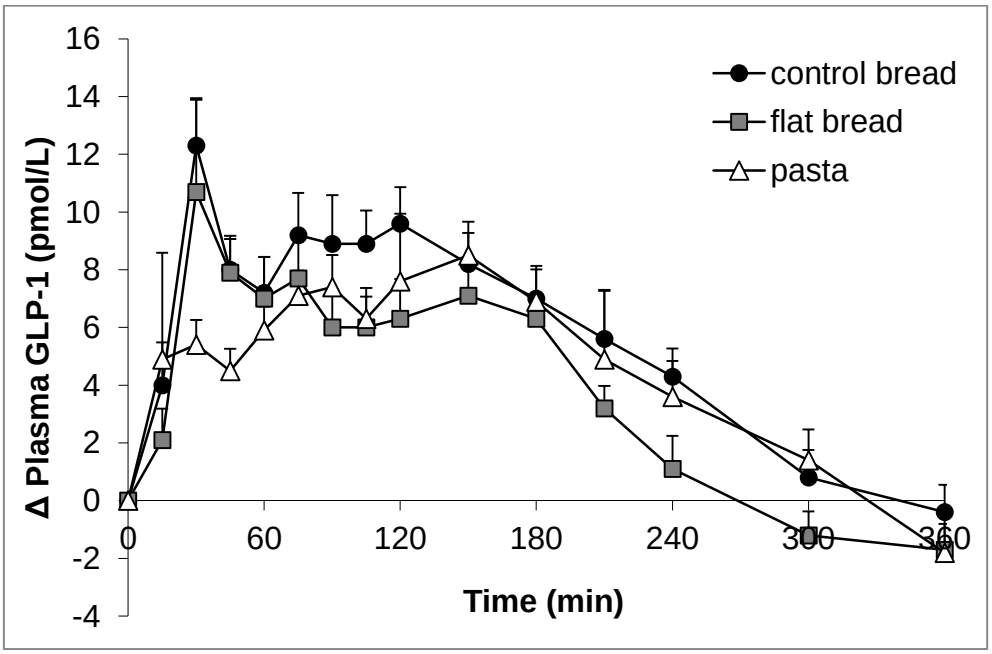


Figure 6

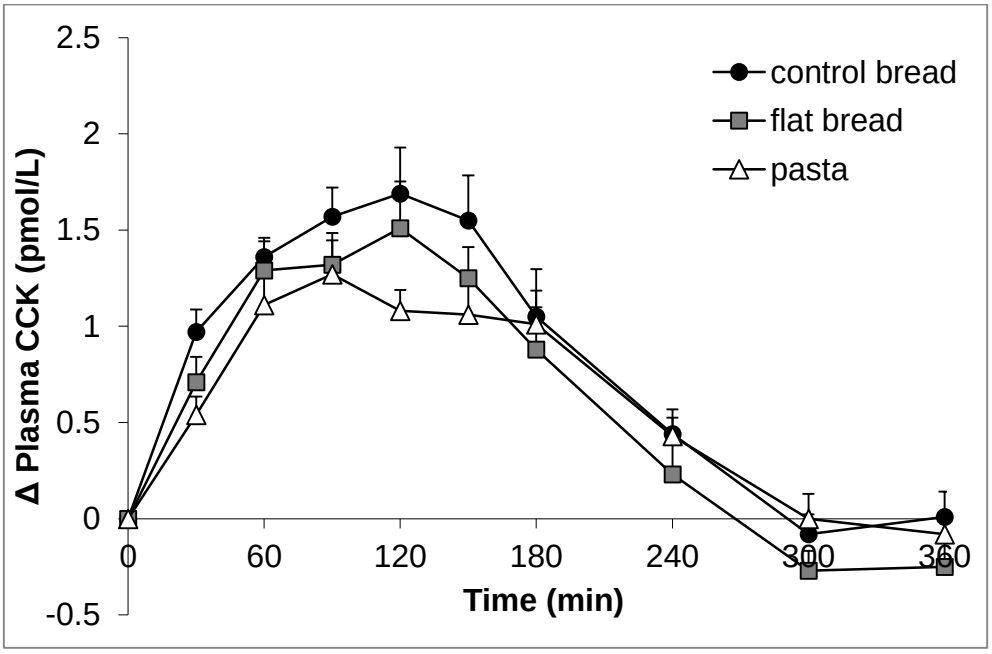
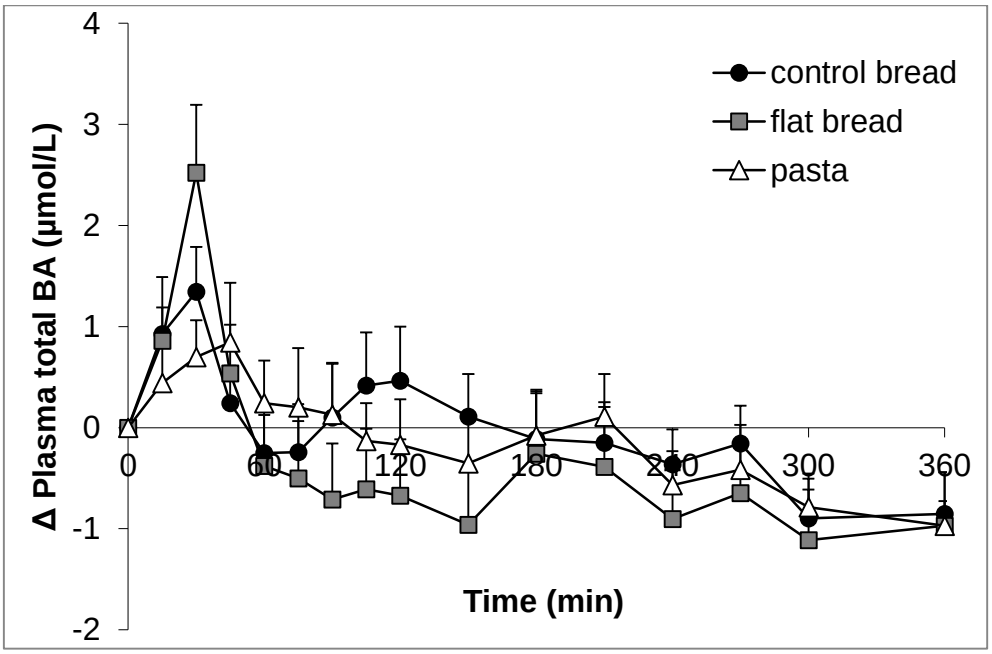


Figure 7

A



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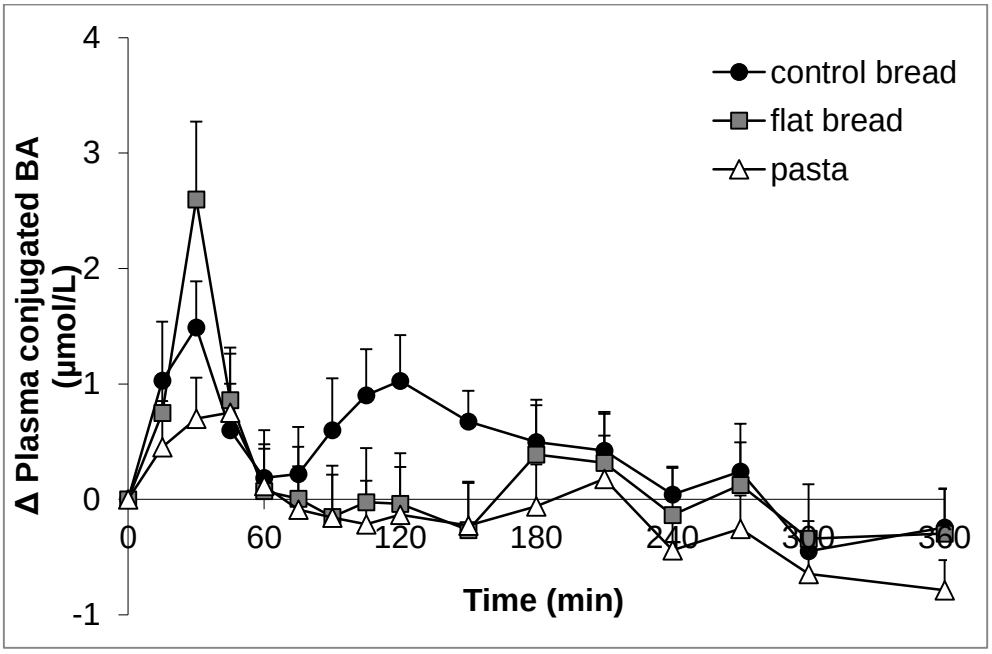


C

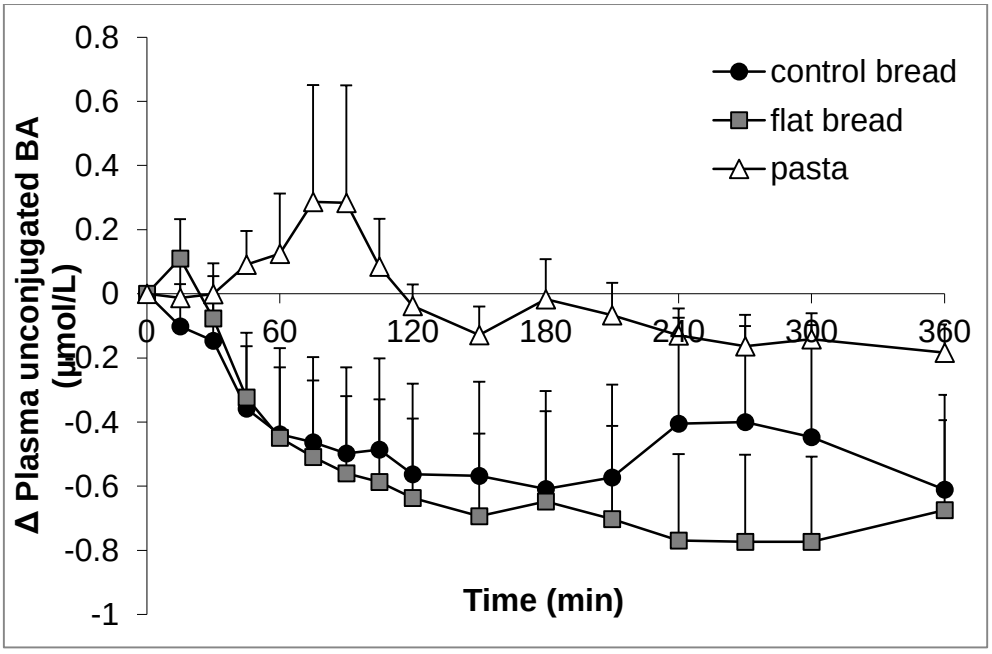
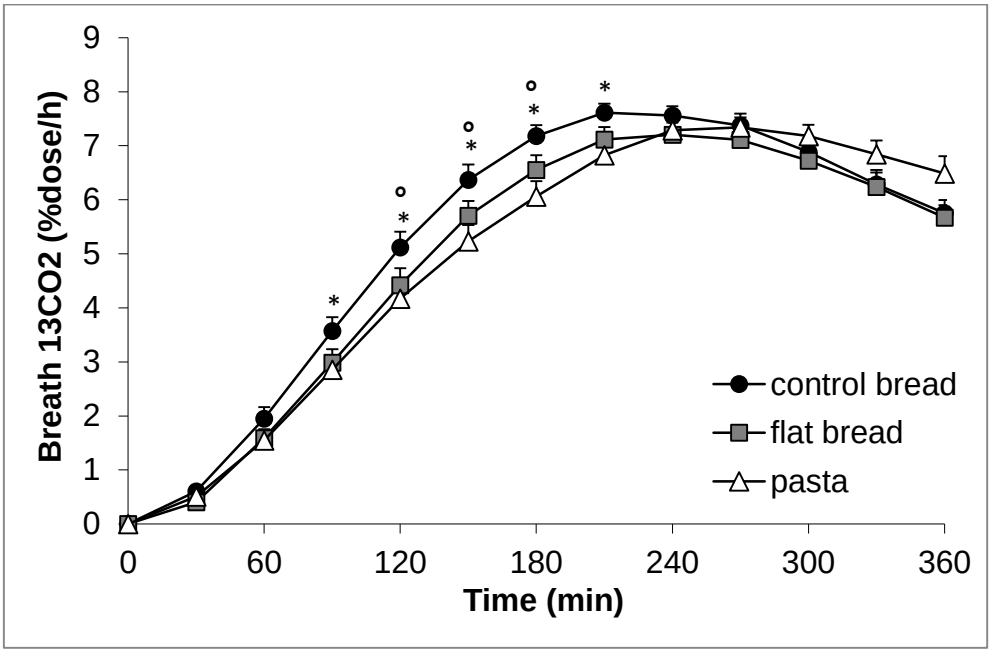


Figure 8



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