In Vitro Assessment of the Satiety Effect of Non-Added Sugar Biscuit Containing Stevia, Coffee Fibre and Fructooligosaccharides

Nuria Martinez-Saez 1, Christina M. Hochkogler 2, Veronika Somoza 2 and M. Dolores del Castillo 1,*

1 Institute of Food Science Research (CIAL, UAM-CSIC). Department of Bioactivity and Food Analysis. Food Bioscience Group. C/ Nicolás Cabrera, 9. Campus de Cantoblanco. Universidad Autónoma de Madrid. 28049. Madrid, Spain; nuria.m.s@cial.uam-csic.es, mdolores.delcastillo@csic.es; 2 Department of Nutritional and Physiological Chemistry, Christian Doppler Laboratory for Bioactive Aroma Compounds, Faculty of Chemistry, University of Vienna, Vienna, Austria; veronika.somoza@univie.ac.at

* Correspondence: mdolores.delcastillo@csic.es; Tel.: +34 91 0017900 Ext. 953

Abstract: This study assessed the in vitro effects of the bioaccessible food components released during the simulated human digestion of a coffee fibre-containing biscuit (CFB) on α-glucosidase activity, antioxidant capacity and satiety hormones. This biscuit presented a significantly (p < 0.05) lower glycaemic sugar content (60.6 mg/g) and a higher antioxidant capacity (15.1 mg chlorogenic acid/g) than a sucrose-containing biscuit (SCB). The CFB significantly reduced (p < 0.05) α-glucosidase activity (IC50 = 3.3 mg/ml) compared to the SCB (IC50 = 6.2 mg/ml). Serotonin and glucagon-like peptide-1 (GLP-1) release by differentiated Caco-2 and HuTu-80 cells, respectively, were stimulated by the CFB (354.7 ± 42.7% and 277.9 ± 14.1%) to the same order of magnitude as those of the SCB. In conclusion, the CFB was demonstrated to reduce simple sugar bioaccessibility and to improve satiety.

Keywords: α-glucosidase; biscuits; coffee fibre; fructooligosaccharides; GLP-1; serotonin; stevia; non-nutritive sweeteners

1. Introduction

Consumption of energy dense foods high in fat and sugar is associated with the prevalence of obesity, type 2 diabetes, cardiovascular diseases and several cancers [1]. There is solid evidence that the risk of becoming overweight or obese is lower when the daily intake of free sugars is less than 10% of the total energy [2]. Increasing the price of unhealthy foods through sugar taxes could potentially discourage the overconsumption of sugar [1]. However, multiple strategies are needed to battle obesity and associated comorbidities, e.g. diabetes.

Consumption of foods with a low glycaemic index (GI) can help regulate blood glucose levels, improve satiety, and control body weight [3]. Recently, high-fibre and sugar-free foods containing stevia as a non-nutritive sweetener have been developed by our group.[4] Stevia glycosides can help to optimize blood sugar and insulin levels in diabetics [5]. Foods high in fibre have been demonstrated to lower the GI as, for instance, galactomannan which slows gastric emptying and inhibits diabetes-related digestive enzymes [6,7].

The α-glucosidase is a membrane-bound intestinal enzyme, essential for degrading oligosaccharides to monosaccharides [8]. Food constituents such as dietary fibres, stevia and coffee phenols have been shown to act as glucosidase inhibitors [5,7,9], and to reduce postprandial
hyperglycaemia. However, results on these compounds present in complex food matrices are missing.

Intake of satiety-inducing foods becomes a strategy to reduce sugar intake and related chronic diseases. Food ingestion activates the secretion of several gut-derived mediators [10], including the hormones localized in intestinal enterochromaffin cells, serotonin and glucagon-like peptide-1 (GLP-1). GLP-1 plays a role in the regulation of food intake and presents glucometabolic effects and serotonin is implicated in the control of satiety [10,11].

The goal of this study was to elucidate whether a replacement of sucrose by stevia, coffee fibre and fructooligosaccharides (FOS) in a sucrose-containing biscuit (SCB) improves its antioxidant capacity and outcome measures satiety in vitro. We report novel in vitro data on the potential use of a coffee fibre-containing biscuit (CFB) suitable for diabetics, people on a weight control diet and healthy people interested in maintaining a healthy body weight. This study also provides new evidence for using coffee fibre as a sustainable functional food ingredient.

2. Materials and Methods

2.1. Chemicals

Bradford reagent was provided by Bio-Rad Laboratories S.A. The following chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA): α-amylase from human saliva (type IX-A), porcine pepsin from gastric mucosa (3.200-4.500 U/mg protein), pancreatin from porcine pancreas, porcine bile extract, bovine serum albumin (BSA), chlorogenic acid (CGA) (3-CGA), 2,2’-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS)), 6-hydroxy-2,5,7,8-tetramethylethroman-2-carboxylic acid (Trolox), phenol 5% (w/v), potassium persulphate, Folin-Ciocalteu reagent, Nα-Acetyl-L-lysine, ortho-phthalaldehyde (OPA), α-glucosidase from intestinal acetone powders of rat, 4-methylumbelliferyl α-D-glucopyranoside (4-MUG), acarbose, 3-(4,5-dimethyl-2-thiazolyl) -2,5-diphenyltetrazolium bromide (MTT) and trypan blue solution. We used the D-mannose/D-fructose/D-glucose assay kit and galactomannan assay kit from Megazyme International Ireland Ldt. (Ireland), Multi Species GLP-1 Total ELISA kit (Cat-n°. EZGLPIT-36K) from EMD Millipore (Missouri, USA) and Serotonin High Sensitive ELISA kit (Cat-n°. EA630/96) from DLD Diagnostika GmbH (Hamburg, Germany). For cell culture, Dulbecco’s modified Eagle’s medium (DMEM), minimum essential medium Eagle (MEM), L-glutamine, penicillin and streptomycin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was from GIBCO Invitrogen (Karlsruhe, Germany). Water was purified using the Milli-Q and Elix system. All other chemicals and reagents were of analytical grade.

2.2. Apparatus and materials

BioTek powerWaveTM XS (BioTek Instruments, U.S.A), FP-6200 (JASCO, Easton, U.S.A) and Infinite® 200 PRO multimode (TECAN, Deutschland GmbH) microplates readers, convection oven (Romag S.A, Barcelona, Spain), UN 500 universal oven (Memmert, Germany) and Telstar Lyobeta-15 lyophilizer (Telstar, Spain) were used for analyses. For the cell assays, a Neubauer cell counting chamber (0.100 mm depth, 0.0025 mm2) (Paul Marienfeld GmbH & Co. KG, Germany), an incubator (BINDER GmbH, Germany), a Thermo Scientific™ MSC-Advantage™ class II biological safety cabinet (Thermo Fisher Scientific, USA), an autoclave (3870EA, Tuttnauer, USA), a TR400-SW TRINO microscope (VWR, Austria), CELLSTAR® multiwell culture plates and standard cell culture flasks (Greiner Bio-One GmbH, Austria) were used.
2.3. Food ingredients

Spent coffee grounds (SCG) from Robusta instant coffee were used as a source of antioxidant coffee fibre [4]. SCG were supplied by Prosol S.A (Spain) and stored at -20°C until use. FOS powder (ORAFTI®P95) was from Beneo-Orafti and stevia sweetener powder, containing 3% steviol glycosides, was purchased at a local supermarket. All other basic ingredients were purchased at specialized and certified food markets.

2.4. Food samples

Biscuit formulations can be seen in Table 1. Biscuits were prepared following Martinez-Saez et al. [4]. Briefly, the dough was prepared by mixing salt, baking powder and sucrose or stevia. Mineral water was added to the dry mixture and thoroughly blended. In a separate bowl, lecithin and oil were mixed and then added to the mixture. Finally, the flour, coffee fibre and FOS were gradually added and the dough was kneaded. The dough was allowed to rest for 30 min, and shaped into discs. The biscuits were baked at 185º C for 16 min in an air recirculation oven. Two sets of 3 biscuits were baked in duplicate (n = 6). The biscuits were placed in the centre of the tray in order to reduce process variability during baking. Biscuits were grounded to obtain a representative sample for further analyses.

<table>
<thead>
<tr>
<th>INGREDIENTS (g)</th>
<th>SCB</th>
<th>CFB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat flour</td>
<td>56.0</td>
<td>59.4</td>
</tr>
<tr>
<td>Water</td>
<td>20.0</td>
<td>21.2</td>
</tr>
<tr>
<td>Sunflower oil</td>
<td>7.75</td>
<td>8.20</td>
</tr>
<tr>
<td>Baking powder</td>
<td>0.55</td>
<td>0.58</td>
</tr>
<tr>
<td>Salt</td>
<td>0.37</td>
<td>0.39</td>
</tr>
<tr>
<td>Soy lecithin</td>
<td>0.33</td>
<td>0.35</td>
</tr>
<tr>
<td>Sucrose</td>
<td>15.0</td>
<td>-</td>
</tr>
<tr>
<td>Stevia</td>
<td>-</td>
<td>2.10</td>
</tr>
<tr>
<td>FOS</td>
<td>-</td>
<td>3.50</td>
</tr>
<tr>
<td>Coffee fibre</td>
<td>-</td>
<td>4.20</td>
</tr>
<tr>
<td><strong>TOTAL dough</strong></td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td><strong>Estimated fibre content</strong></td>
<td>1.90</td>
<td>7.50</td>
</tr>
</tbody>
</table>

*“High fibre content” (≥ 6 g fibre/100g)

Biscuits were digested in triplicate under in vitro oral gastrointestinal human digestion conditions [4]. Briefly, all three stages, salivary (pH 6.9, 10 ml, 5 min, 3.9 U α-amylase/ml, aerobic), gastric (pH 2, 13 ml, 90 min, 71.2 U pepsin/ml, aerobic), and abiotic duodenal step (pH 7, 16 ml, 150 min, 9.2 mg pancreatin and 55.2 mg bile extract/ml, aerobic) were performed in the same flask. Digests were then centrifuged and the soluble fractions containing bioaccessible compounds to be absorbed and metabolized were treated with cholestyrane resin (10% w/v) to remove the bile
acids and mimic the human bile salt reabsorption. Soluble fractions were then frozen at -20°C, lyophilized and stored at room temperature until further analysis.

Coffee fibre, stevia and FOS were also digested in vitro following the above-described procedure to gain insight into their contribution to bioactive compounds.

2.5. Cell culture: Caco-2 and HuTu-80

The human colon cancer cell line (Caco-2) was cultured in DMEM supplemented with FBS (10%), L-glutamine (2%) and penicillin/streptomyacin (1%) at 37°C and 5% CO2. Cells were grown in 12-well plates until reaching confluence, approximately after 3 days. Cell differentiation was obtained by subsequent culture for a further 21 days including medium changes every two to three days. The enterocyte-like differentiated Caco-2 cells were then used for further studies on serotonin hormone release.

The human duodenal cancer cell line (HuTu-80) was cultured in MEM supplemented with FBS (10%), L-glutamine (2%) and penicillin/streptomyacin (1%) at 37°C and 5% CO2. Cells were grown in 24-well plates until reaching confluence, approximately after 1 day. HuTu-80 cells were not differentiated with enterocyte-like properties, and 24 hours after seeding, they were ready to be used for analyses on GLP-1 hormone release.

After thawing, both cells lines were passaged 3-4 times to give cells time to recover their normal growth rate.

2.6. Bioaccessibility of food components

Digests of coffee fibre, stevia and biscuits (SCB and CFB) were characterized.

2.6.1. Total carbohydrates

Total carbohydrates were determined using the phenol–sulphuric method as described by Masuko et al. with slight modifications [12]. Samples (20 µl) were mixed with concentrated sulphuric acid (93–98%) (61 µl) and phenol solution (5%, w/v) (18 µl) in a multi-well plate. After incubation at 90°C for 5 min in a water bath, the microplate was cooled to room temperature and absorbance was measured at 490 nm. The calibration curve was constructed using glucose (0.1–0.9 mg/ml) as standard. Reagent blank and sample blank were also prepared and analysed in each set of samples. All measurements were performed in triplicate and results were expressed as mg glucose equivalents (eq.)/g digest.

2.6.2. Galactomannan

The procedure for determining galactomannan content was performed using an enzymatic kit following the manufacturer’s instructions. The method was adapted to a micromethod format. Analyses were carried out in triplicate and results were expressed as mg/g digest.

2.6.3. Free sugars

Glucose, fructose and mannose contents were determined using an enzymatic kit following the manufacturer’s instructions. The method was adapted to micromethod. The analysis was performed in triplicate. Results were expressed as mg glucose, mg fructose and mg mannose/g digest.
2.6.4. Soluble proteins and peptides

The Bio-Rad Protein Assay, based on the Bradford method in micro-method format, was used to determine proteins and peptides. Reagents were prepared according to manufacturer’s instructions. Briefly, a solution of Bradford reagent (1 : 4 reagent : milli-Q water) was prepared and filtered. Ten µl of sample and 200 µl of Bradford solution were placed in a multi-well plate. After 5 min of incubation at room temperature, absorbance was measured at 595 nm. Sample blank and reagent blank were also analysed. BSA was used as standard (0.05 – 1 mg/ml). All measurements were performed in triplicate. Results were expressed as mg BSA eq./g digest.

2.6.5. Free amino groups

The release of amino acids by proteolysis of proteins forming wheat flour and coffee fibre was measured by the OPA assay following Go et al. [13]. OPA reagent was freshly prepared by dissolving 10 mg OPA in 250 µl ethanol (95%, v/v), 9.8 ml PBS (10 mM, pH 7.4) and 20 µl β-mercaptoethanol. The reaction was carried out in a 96-well microtest plate by mixing 10 µl sample, 140µl PBS and 100 µl OPA reagent. Fluorescence was read at 360 ± 40 nm excitation and 460 ± 40 nm emission wavelengths for 15 min at 37 ºC. The calibration curve was constructed using standard solutions of Nα-acetyl-L-lysine (0.025 – 1 mM). All measurements were performed in triplicate, and data were expressed as mg Nα-acetyl-L-lysine eq./g digest.

2.6.6. Total phenolic content

Total phenolic content (TPC) was determined by the Folin–Ciocalteu method as described by Contini et al. [14], adapted to a micromethod format. Ten µl of sample and 150 µl of Folin–Ciocalteu solution were incubated at room temperature for 3 min and 50 µl of sodium bicarbonate were added. Reaction was run for 120 min at 37°C, and absorbance was read at 735 nm. Sample blank and reagent blank were also analysed in each set of samples. The CGA calibration curve (0.1-1 mg/ml) was used for quantification. Measurements were performed in triplicate and results were expressed as mg CGA eq./g digest.

2.6.7. Antioxidant capacity

The overall antioxidant capacity of the digested biscuits and coffee fibre was analysed using the indirect ABTS•+ decolourisation assay as described by Oki et al. [15]. An ABTS•+ stock solution was prepared by adding 140 mmol/l potassium persulfate (44 µl) to a 7 mmol/l ABTS•+ aqueous solution (2.5 ml), and the mixture was then allowed to stand for 16 h at room temperature. The working solution of the radical ABTS•+ was prepared by diluting the stock solution 1:7.5 (v/v) in sodium phosphate buffer (5 mmol/l, pH 7.4) to obtain an absorbance value of 0.7 ± 0.02 at 734 nm. Samples (30 µl) were added to ABTS•+ solution (270 µl) in a microplate. Absorbance was measured at 734 nm for 10 min at 30°C. CGA (0.025-0.25 mmol/l) and trolox (0.025-0.25 mmol/l) were used for quantification. All measurements were performed in triplicate and results were expressed as mg CGA eq./g digest.
2.7. Health-promoting properties of bioaccessible food components

2.7.1. Alpha-glucosidase inhibition assay

The α-glucosidase inhibitory activity of the digested biscuits, coffee fibre, stevia and FOS was analysed following the methodology described by Berthelot et al. and Geddes et al. [16,17] with slight modifications. Alpha-glucosidase enzyme was extracted previous to the assay. Briefly, 100 mg of rat intestine powder were dissolved in 3 ml NaCl (0.9%), sonicated in an ice bath for 6 min and then centrifuged at 10000 g for 30 min. The supernatant containing the enzyme was stored in the freezer. In a 96-well microplate, 100 µl of sample dissolved in PBS (100 mM, pH 6.9) were mixed with 100 µl α-glucosidase (diluted 1/10) and 100 µl 4-MUG (2 mM). Fluorescence was then monitored at an excitation wavelength of 360 nm and an emission wavelength of 460 nm for 30 min at 37 °C. Blank of sample and negative control (buffer, enzyme and 4-MUG) were included. Acarbose was used as a positive control (standard inhibitor). Curves of samples and acarbose were assayed to cover the whole range of inhibition of the enzyme (≈0.5-96%). The percentage (%) of α-glucosidase inhibition was calculated using the equation:

$$\text{α-glucosidase inhibition} \% = \frac{F_{nc} - F_s}{F_{nc}} \times 100$$

Where $F_{nc}$ is the fluorescence of the negative control (without inhibitor) and $F_s$ is the fluorescence of the sample. All measurements were performed in triplicate. Results were expressed as the concentration causing 50% inhibition (IC50 mg/ml).

2.7.2. Assays of serotonin and GLP-1 secretion

Soluble fractions recovered from the digested SCB, CFB and coffee fibre containing bioaccessible compounds for absorption and metabolism were used to stimulate the secretion of satiating hormones in cell cultures. The effect of stevia on the release of satiating hormones was also tested.

- **Cell viability:** Cytotoxic effects of test samples were excluded by performing the colorimetric MTT assay [18]. Differentiated Caco-2 cells were incubated with samples (0.05, 0.5 and 5 mg/ml) diluted in PBS containing ascorbic acid (0.1%) and after a 5-min exposure to cells, the samples were removed. HuTu-80 cells were first starved with medium free of serum, glucose and glutamine for 1 h prior to the incubation with the samples (0.01, 0.05 and 0.5 mg/ml) diluted in the starving-medium. After 90-min exposure, samples were finally removed.

  In both cellular lines, exposure was carried out at 37°C and a negative (medium) and positive control (DMSO) were also included. The MTT solution (50 mg/ml, 1:6) was left to incubate (10-15 min) and the resulting formazan diluted in DMSO was measured at 570 nm. Viability was determined relative to untreated negative control cells (100%). Three groups of different passaging and samples in duplicate were performed in each set of analysis ($n = 3$, $tr = 6$). The percentage of cell vitality was calculated as follows: vitality (%) = (dead cell number / total cell number) × 100. Results were expressed as total cell viability / control (T/C) [%].

- **Stimulation and quantification of serotonin:** Caco-2 cells were supplemented with the bioaccessible fractions (150 µl) at three different concentrations (0.5, 0.05, 0.01 mg/ml) in duplicate. Cells were washed with PBS prior to the addition of samples. The cells were then stimulated for 5 min in darkness in an orbital shaker. Supernatants were removed from the cells and frozen until...
further quantification of the serotonin hormone. Positive (cinnamaldehyde, 5 mM) and negative (buffer) controls were also tested.

The serotonin released by Caco-2 cells was quantified in darkness using a highly sensitive enzyme immunoassay kit (ELISA competitive) following the manufacturer’s instructions. Three groups of different passaging were performed in each set of analysis (n = 3, tr = 6). Results were expressed as T/C [%] compared to the control.

• Stimulation and quantification of GLP-1: HuTu-80 cells were first starved with medium free of serum, glucose and glutamine for 1 h prior to stimulation with the bioaccessible fractions (500 µl) at 0.01, 0.05 and 0.5 mg/ml in duplicate. Positive (glutamine 40 mM) and negative (medium) controls were also tested. Exposure to the cells was for 1 h 30 min at 37 ºC. After the stimulation period, supernatants were collected and frozen until further quantification of the GLP-1 hormone.

The GLP-1 hormone released from HuTu-80 cells was quantified using the sandwich ELISA kit following the manufacturer’s instructions. Three groups of different passaging were performed in each set of analysis (n = 3, tr = 6). Results were expressed T/C [%] compared to the control.

2.8. Statistical Analysis

Statistical analyses were performed using SigmaPlot 11.0 (Systat Software Inc., California, USA). Data were expressed as the mean value ± standard deviation for all analyses except for those comprising culture cells which were expressed as mean ± standard error of the mean (SEM). Differences between means were determined through analysis of variance (ANOVA), one-way ANOVA followed by Dunn’s, Fisher LSD or Holm-Sidak post-hoc tests. Differences were considered to be significant at p < 0.05.

3. Results and Discussion

3.1. Bioaccessibility of food components

Table 2 shows data on the bioaccessible food components released during the digestion process. In the case of coffee fibre, the major component of the bioaccessible fraction of the digest were carbohydrates (11.3%), whereas only trace amounts of sugars such as glucose (0.01%), fructose (0.02%) and mannose (0.03%) were detected. In contrast, a higher content in polysaccharides like galactomannan (2%) was quantitated. TPC represented 1.56% of the bioaccessible food components, and proteins (0.88%) and free amino groups (2.2%) were also present. These results suggest that SCG used as coffee fibre mainly provides complex carbohydrates to the bioaccessible fraction, which is in accordance with the literature [4,19].

In the case of the SCB, the major components of the bioaccessible fraction of the digest were also carbohydrates (64.8%). Sugars represented 18% of total carbohydrates. Fructose (52.3%) and glucose (47%) were found in the highest amounts, followed by trace amounts of mannose (0.7%). Protein and amino acid contents were lower than 1% (0.41% and 0.80%, respectively) and TPC represented 0.85%. Wheat flour starch seems to be the main contributor to the carbohydrate content of the biscuit (Table 1). According to the literature, wheat flour is composed of 65% digestible starch [20], which is hydrolysed by digestive enzymes -salivary and pancreatic α-amylases- to glucose molecules and oligosaccharides [21]. The sucrose added to the traditional formulation can also be converted into glucose and fructose, mainly by chemical reactions during the processing of cereal-based products.
[22], and the acid conditions of the stomach [23], since the intestinal sucrase enzyme was not used in this particular digestion model. These available carbohydrates may turn the traditional biscuit into a high GI food which may consequently cause fast postprandial blood glucose glycaemic responses [24].
Table 2. Bioaccessible compounds released during in vitro oral-gastrointestinal digestion of a sucrose-containing biscuit (SCB), a coffee fibre-containing biscuit (CFB), coffee fibre and stevia.

<table>
<thead>
<tr>
<th>Bioaccessible compounds</th>
<th>SCB</th>
<th>CFB</th>
<th>Coffee fibre</th>
<th>Stevia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Carbohydrates</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mg glucose eq./g digest</td>
<td>647 ± 70.0a</td>
<td>609 ± 17.2a</td>
<td>113 ± 7.57b</td>
<td>n.d.</td>
</tr>
<tr>
<td>Galactomannan</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mg/g digest ND</td>
<td>1.60 ± 0.11a</td>
<td>19.49 ± 1.19b</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>Sugars</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mg glucose/g digest</td>
<td>54.8 ± 9.94a</td>
<td>28.8 ± 0.65b</td>
<td>0.01 ± 0.0c</td>
<td>40.0 ± 0.57d</td>
</tr>
<tr>
<td>mg fructose/g digest</td>
<td>60.9 ± 3.31a</td>
<td>39.2 ± 1.48b</td>
<td>0.21 ± 0.01c</td>
<td>6.20 ± 0.46d</td>
</tr>
<tr>
<td>mg mannose/g digest</td>
<td>0.74 ± 0.11a</td>
<td>0.59 ± 0.11a</td>
<td>0.29 ± 0.01b</td>
<td>ND</td>
</tr>
<tr>
<td>Soluble proteins</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mg BSA eq./g digest</td>
<td>4.11 ± 0.09a</td>
<td>3.88 ± 0.30a</td>
<td>8.75 ± 0.28b</td>
<td>n.d.</td>
</tr>
<tr>
<td>Free amino groups</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mg Na-acetyl-Lys eq./g digest</td>
<td>7.95 ± 0.19a</td>
<td>9.16 ± 0.83a</td>
<td>24.0 ± 2.53b</td>
<td>n.d.</td>
</tr>
<tr>
<td>Total phenolic content</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mg CGA eq./g digest</td>
<td>8.86 ± 0.49a</td>
<td>8.98 ± 0.52a</td>
<td>15.56 ± 0.95b</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

ND Not Detected. n.d. not determined. Data are presented as mean ± standard deviation. Triplicate of sample preparation and triplicate of analysis (n = 9). Different letters indicate significant differences (p < 0.05) between the samples of the same row.
High GI diets are associated with decreased satiety and increased glucose intolerance, a greater risk of overweight and obesity and impaired lipid metabolism [25]. Furthermore, glucose and fructose follow different metabolic pathways after their absorption, resulting in different effects on blood glucose concentrations. Obesity is related to an elevated intake of both sugars. Type 2 diabetes is associated with high glucose diets, while the overconsumption of fructose is associated with non-alcoholic fatty liver diseases and augmented de-novo triglyceride synthesis [26].

The formulation of the CFB by replacing sucrose with stevia, coffee fibre and FOS clearly favoured the contents of ingredients with beneficial effects on GI (Table 2). The bioavailability of glycaemic sugars was significantly (p < 0.05) lower with reductions of 46.4 ± 8.8% and 35.6 ± 1.1% for glucose and fructose, respectively, compared to the SCB. The stevia sweetener did not significantly contribute to the sugar content of the CFB (Table 2), as it was hydrolysed in a small amount (4%). Stevia, in particular steviol glycosides, cannot be hydrolysed by the digestive enzymes in the small intestine, but is metabolized to steviol by the microbiota of the colon [27]. However, the stevia added to the CFB contained digestible carbohydrate –maltodextrin (97%)- as an additive. Stevia plays an important role in lowering the GI of foods. The moderate glucose levels found in CFB are a necessary primary energy source for proper cell function in the organism [28], and have a direct positive impact on the GI of the biscuit. On the other hand, galactomannan was present in the CFB unlike the SCB. The coffee fibre included in the CFB is a natural source of galactomannan (Table 2). Intake of galactomannan is associated with reduced weight gain, adiposity, liver fat and blood glucose levels [29], making coffee fibre an attractive ingredient for confectioneries. Moreover, the FOS incorporated in the CFB as soluble fibre has been shown to reduce post-prandial glycaemic responses [30]. Marangoni & Poli [25], also obtained a markedly lower glycaemic index in bread and biscuits by adding a proprietary fibre mixture to their formulations.

Regarding the antioxidant properties of the biscuits, significant differences (p < 0.05) were found between the CFB and the SCB. In the bioaccessibility of antioxidants, estimated as the overall antioxidant capacity of the food digests, the digestion of the CFB released a significantly greater amount of antioxidants (15.07 ± 1.45 mg CGA eq./g digest) than in the SCB (10.43 ± 0.90 mg CGA eq./g digest). Most of the antioxidants of the SCB may be ascribed to phenolic compounds (Table 2), in contrast, the CFB might also contain other non-phenolic antioxidants which may contribute to its overall antioxidant capacity. The bioaccessible fraction of the digested coffee fibre had a high antioxidant character (46.14 ± 3.61 mg CGA eq./g digest). Furthermore, stevia [31], FOS [32], and the gluten peptides released during the digestion process by chemical and enzymatic hydrolysis [33], may also exert an antioxidant character.

The high antioxidant properties of the CFB may play an important role in reducing the risk of obesity and diabetes. Consequently, introducing food antioxidants through the diet may be of great interest. High-antioxidant diets have been related to reduced inflammation and increased circulating antioxidants in cross-sectional and randomized intervention studies [34].

In summary, the bioaccessibility of nutrients such as total carbohydrates, proteins, amino acids and phenols were not significantly different (p > 0.05) between the two biscuits. However, the carbohydrate profile of CFB was enhanced by replacing sucrose with stevia, FOS and coffee fibre, providing the biscuits with the potential to augment satiety and reduce hyperglycaemia [24].
3.2. Health-promoting properties of foods

3.2.1. Anti-diabetic properties

IC50 values for the α-glucosidase inhibition were calculated from dose–response curves (Figure 1 (a), (b) and (c)). The IC50 for acarbose was 4.4 µg/ml. Alpha-glucosidase inhibitors were detected in the digests of the samples. However, the content of α-glucosidase inhibitors released during digestion differed significantly (p < 0.05) between samples. The CFB presented the lowest IC50 value of all the studied samples (Table 3). According to the literature, the CFB is a better inhibitor than other food products such as lemon (IC50 36.59 mg/ml), lime (10.96 mg/ml), grapefruit (62.10 mg/ml) [35], green tea (11.1 mg/ml), sardine muscle hydrolizate (48.7 mg/ml) and yogurt (519.8 mg/ml) [36].

Stevia, FOS and coffee fibre are demonstrated to contribute to the inhibitory effect of the α-glucosidase observed for the CFB, although stevia had a higher inhibitory capacity than the other two ingredients. The inhibitory effect of stevia against α-glucosidase has been previously described [9]. Phenolic compounds released during the digestion of coffee fibre may also act as α-glucosidase inhibitors, as for instance the main phenolic compound of coffee, CGA with potential benefits on type 2 diabetes [37]. Other compounds released during the digestion of the CFB such as bioactive peptides could also exhibit anti-diabetic properties [38].

These results suggest the potential use of the CFB as a diabetic-friendly biscuit with great interest for people with type 2 diabetes and/or overweight or obese, as it has the potential to delay intestinal glucose absorption and enhance postprandial hyperglycaemia levels. However, proof-of-principle human intervention studies are needed to verify this effect.

Table 3. IC50 values against α-glucosidase activity and equivalents of acarbose of the bioaccessible fractions of digested sucrose-containing biscuit (SCB), coffee fibre-containing biscuit (CFB), coffee fibre, stevia and fructooligosaccharides (FOS).

<table>
<thead>
<tr>
<th></th>
<th>IC50 (mg/ml)</th>
<th>mg acarbose eq./g digest</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acarbose</td>
<td>0.004 ± 0.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>Stevia</td>
<td>5.53 ± 0.35&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>0.79 ± 0.05</td>
</tr>
<tr>
<td>SCB</td>
<td>6.22 ± 0.33&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.70 ± 0.04</td>
</tr>
<tr>
<td>CFB</td>
<td>3.32 ± 0.12&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.32 ± 0.05</td>
</tr>
<tr>
<td>Coffee fibre</td>
<td>23.9 ± 1.31&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.18 ± 0.01</td>
</tr>
<tr>
<td>FOS</td>
<td>53.4 ± 2.22&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.08 ± 0.00</td>
</tr>
</tbody>
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Values represent mean ± standard deviation (n = 2). Differences were tested with one-way ANOVA followed by Fisher post-hoc test (p < 0.05) and marked with letters a, b, c, d and e.
Figure 1. Effect on α-glucosidase activity represented by dose-response curves of (a) acarbose (0.1 µg/ml – 30 mg/ml), (b) soluble fractions recovered from the digested sucrose-containing biscuit (SCB) (0.5-100 mg/ml), coffee fibre-containing biscuit (CFB) (0.01-100 mg/ml) and stevia (0.05-200 mg/ml), and (c) soluble fractions recovered from the digested coffee fibre (0.5-1000 mg/ml) and FOS (0.5-2000 mg/ml). Values represent mean ± standard deviation. Duplicate of sample preparation and triplicate of analysis (n = 6).
3.2.2. Release of satiety hormones

Different doses of the tested samples were not cytotoxic (Figure S1, supplementary material).

The effect of the samples on cellular secretion of serotonin is shown in Figure 2. Caco-2 cells treated with 0.5 mg/ml of the CFB digest exhibited a 4-fold increase in serotonin secretion (355 ± 42.7%). No differences in serotonin release were found (p > 0.05) between the digests of the SCB and the CFB. However, the compounds released during the digestion of the antioxidant coffee fibre had a significant effect on the secretion of serotonin, increasing serotonin release 7-fold (763 ± 81.4%) compared to the basal level. To the best of our knowledge, this is the first time that the effect of the antioxidant coffee fibre on satiety hormones has been reported. Gostner et al. found that coffee compounds such as gallic acid and caffeic acid had the potential to increase tryptophan availability, needed for the biosynthesis of serotonin, via inhibition of indoleamine 2,3-dioxygenase which is involved in tryptophan metabolism [39]. Further studies are required to elucidate which of the compounds released during the digestion of the coffee fibre is responsible for its satiating effect.

![Figure 2](image_url)

**Figure 2.** Serotonin release after stimulation of Caco-2 cells at 0.5, 0.05 and 0.01 mg/ml with soluble fractions recovered from the digested sucrose-containing biscuit (SCB), coffee fibre-containing biscuit (CFB) and antioxidant coffee fibre that contain bioaccessible compounds; as well as stevia. Results are displayed as T/C in percent compared to the control (cells with media = 100%). All measurements were expressed as mean ± SEM (n = 3, tr = 6). Significant differences between treatments were tested with one-way ANOVA followed by the Holm–Sidak posthoc test (p < 0.05) and marked with the letters a, b, c and d.

In contrast, the natural hypocaloric sweetener, stevia, did not stimulate the serotonin secretion in differentiated Caco-2 cells at the tested concentrations. This result is in concordance with that described by Ripken et al. did not found serotonin release from porcine intestinal tissue segments when incubated with Rebaudioside A, the sweetest glycoside of the stevia [40]. Moreover, Anton et al. reported in human intervention that the intake of preloads containing stevia had satiety levels
similar to those of sucrose preloads, measured by the subjective visual analogue scale [41]. Therefore, more investigations are required to clarify the role of this hypocaloric sweetener and its metabolites in serotonin secretion.

Other bioaccessible compounds may exhibit serotonin stimulation. For instance, gluten peptides from wheat flour have been shown to contribute to higher hypothalamic and cortical serotonin levels in animal models [42]. Phenolic compounds can also play an important role in appetite suppression by stimulating this important signalling molecule [43]. Furthermore, Maillard reaction products such as N(ε)-carboxymethyl lysine from heat-treated food like biscuits, have been found to contribute to satiety regulation through central/brain serotonin release in SH-SY5Y cells [44]. Therefore, biscuits made using sucrose as a sweetener might contain these advanced products of the Maillard reaction that could contribute to the stimulation of serotonin. However, replacing sucrose with stevia limits the progress of Maillard reaction in food toward advanced stages [45], and may not significantly contribute to a postprandial satiating effect of the food prepared according to the CFB. Despite the controversial effect of peripheral serotonin on satiety [46], there is evidence that peripheral serotonin unfolds similar effects on satiety than central serotonin [11,47]. Further studies are needed to identify the individual food components that contribute to the release of serotonin from the gut in the CFB and the SCB.

Regarding cellular secretion of GLP-1, significant GLP-1 values were obtained (p < 0.05) for the stimulation of HuTu-80 cells with the bioaccessible compounds released during the digestion of food formulations (Figure 3). Compared to the non-treated control cells, GLP-1 release was significantly higher (p < 0.05), i.e. three times higher (278 ± 14.1%), after incubation of HuTu-80 cells with the soluble fraction of the digested novel CFB (0.05 mg/ml). No significant differences were found (p > 0.05) between the SCB and the CFB. The same trend was observed for all concentrations of the biscuits.

The compounds released during the digestion of antioxidant coffee fibre from SCG significantly stimulated the secretion of the GLP-1 hormone. Although researchers have previously reported GLP-1 stimulation caused by coffee beverages in cells, mice and humans,[48] this is the first report of the effect of coffee fibre from SCG.

On the other hand, stevia was not effective at the three tested concentrations, which is in agreement with the results of Fujita et al. presented for an in vivo animal model [49]. FOS present in the CFB (Table 1) has been associated with appetite suppression via stimulation of GLP-1 release [50]. Other bioaccessible compounds, such as proteins and peptides, may also stimulate GLP-1 release. For instance, intact and digested wheat proteins have been found to produce satiety and have an anti-diabetic effect through GLP-1 stimulation [51]. Further studies are required to characterize the food compounds released during digestion and to determine those which contribute to the satiating effect of the biscuits.
Figure 3. Glucagon-like peptide-1 (GLP-1) release after stimulation of HuTu-80 cells at 0.5, 0.05 and 0.01 mg/ml soluble fractions recovered from the digested sucrose containing biscuit (SCB), coffee fibre containing biscuit (CFB) and antioxidant coffee fibre that contain bioaccessible compounds; as well as stevia. Results are displayed as T/C in percent compared to control (cells with media = 100%). All measurements were expressed as mean ± SEM (n = 3, tr = 6). Significant differences between treatments were tested with one-way ANOVA followed by the Holm–Sidak posthoc test (p < 0.05) and marked with the letters a, b, c and d.

4. Conclusions

To the best of our knowledge, this is the first report of anti-diabetic and satiating effects of bioaccessible compounds released during the digestion of a CFB and their ingredients, antioxidant coffee fibre, FOS and stevia. New evidence of the use of antioxidant coffee fibre as a sustainable functional food ingredient has been provided.

Supplementary Materials: The following are available online at www.mdpi.com/link, Figure S1: Cytotoxic effects of soluble fractions recovered from the digested sucrose-containing biscuit (SCB), coffee fibre-containing biscuit (CFB) and antioxidant coffee fibre that contain bioaccessible compounds; as well as, stevia, on (a) Caco-2 cells at concentrations of 5, 0.5 and 0.05 mg/ml, and on (b) HuTu-80 cells at 0.5, 0.05 and 0.01 mg/ml, compared to control (cells with media, 100% viability). All measurements were expressed as mean ± SEM (n=3, tr=6). Significant differences vs. control were determined by One-Way ANOVA followed by Dunn's posthoc test (p < 0.05) and marked as ‘*’.

Acknowledgments: This study was funded by the SUSCOFFEE (AGL2014-57239-R) project and carried out in collaboration with the University of Vienna. Martinez-Saez, N. thanks the Autonomous University of Madrid (UAM), Spain, for her FPI-predoc fellowship and the financial support for her short stay at the University of Vienna. The authors also thank Prosol for supplying spent coffee grounds and BENEO-ORAFTI for donating FOS. Moreover, the financial support by the Austrian Federal Ministry of Economy, Family and Youth and the Austrian National Foundation for Research, Technology and Development is gratefully acknowledged.

Author Contributions: “del Castillo, M.D. and Somoza, V. designed the experiments, supervised the investigation and revised the manuscript. Martinez-Saez, N. and Hochkogler, C.M. performed the experiments
and analysed the data; Martinez-Saez, N. is the principal author of the investigation since it is part of her PhD. Thesis supervised by del Castillo, PhD.”

Conflicts of Interest: Declare conflicts of interest or state “The authors declare no conflict of interest.”

References


