Analytical Methods

Assessing non-digestible compounds in apple cultivars and their potential as modulators of obese faecal microbiota in vitro

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Abstract

The health benefits of apple bioactive compounds have been extensively reported. However, only few studies have focused on bioactive compounds that are not absorbed and metabolised during gastrointestinal digestion and can induce changes in microbial populations of faeces. We have characterised Braeburn, Fuji, Gala, Golden Delicious, Granny Smith, McIntosh and Red Delicious cultivars and found significant differences for extractable phenolics (1.08–9.2 mg/g) non-extractable proanthocyanidins (3.28–5.7 mg/g), and dietary fibre (20.6–32.2%) among cultivars with Granny Smith having the highest contents. Granny Smith was used after in vitro digestion for fermentation of faeces from diet-induced obese mice. Results showed that relative abundances of Firmicutes, Bacteroidetes, Enterococcus, Enterobacteriaceae, Escherichia coli, and Bifidobacterium in apple cultured faeces tended to resemble the abundance in faeces from lean mice with increased trend in the production of butyric acid. These results suggest that apple non-digestible compounds might help to re-establish a disturbed microbiota balance in obesity.

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1. Introduction

Apples, in general, have shown to protect against human chronic diseases due to their content of fibre and phenolic compounds. These bioactive compounds have low availability and can potentially reach to colon, modulate the balance of bacterial populations in the gut, and influence the host physiology (Delzenne, Neyrinck, & Cani, 2011; Moco, Martin, & Rezzi, 2012). The apple health benefits are, in part, due to the interaction of fibre and phenolics with gut microbiota that results in changes in phenolic bioavailability and activity, and the production of short chain fatty acids (SCFAs) after fibre fermentation (Tuohy, Conterno, Gasperotti, & Viola, 2012).

Dietary fibre in apples include mainly cellulose, hemicellulose, lignin and pectin (Yan & Kerr, 2012). Previous studies have reported that apple pectin can influence the intestinal microbiota, in part, due to its bacteriostatic effects (Shinohara, Ohashi, Kawasaki, Terada, & Fujisawa, 2010). Moreover, the utilisation of apple pectin by health beneficial faecal bacteria in vitro and the effects of daily apple consumption on improving the intestinal environment due to the greater production of SCFAs and decreased ammonia and sulfide have been reported (Shinohara et al., 2010).

Extractable phenolic compounds in apples have also been extensively investigated. A survey of seven apple cultivars grown in Turkey, including Granny Smith, reported that content of phenolics vary according to the apple variety and parts (peel and flesh). The identified phenolics were flavan-3-ols ((+)-catechin, (−)-epicatechin, and procyanidins B2), dihydrochalcone (phloridzin), flavonols (quercetin glycosides (rutin + isoquercitrin) determined as quercetin), anthocyanidin (cyanidin (except for the green peel Lutz Golden and Granny Smith)), and hydroxycinnamic acids (mainly chlorogenic acid) (Karaman, Tutem, Baskan, & Apak, 2013). However, most of the studies have ignored the non-extractable phenolics in apples, which are linked to the food matrix by covalent bonds and might resist the gastrointestinal digestion to a greater extent than extractable phenolics, and therefore can reach the colon to be metabolised by intestinal bacteria (Arranz, Silvan, & Saura-Calixto, 2010). Non-extractable phenolics in apples are procyanidins mixtures, which are bound to the apple cell wall. The binding of procyanidins to the apple cell wall increases with the degree of polymerisation and inhibits the enzymatic degradation of cell walls. This strongly suggests that non-extractable phenolics are available in the large intestine for bacterial fermentation (Saura-Calixto, 2011).
In general, apples are a good source of fibre and phenolic compounds, and increasing their consumption has been shown to increase the proportion beneficial commensal bacteria (Tuohy et al., 2012). However, the content of these compounds can be significantly influenced by several factors including cultivar, soil, environment, harvest, storage conditions and processing as demonstrated by studies screening the phenolics in apple cultivars produced in Turkey (Karaman et al., 2013) and Poland (Lata, Trampczyńska, & Paczewska, 2009).

Our aim was to characterise seven apple cultivars produced in the Pacific Northwest U.S. regarding their content of non-digestible compounds and to assess how these compounds can modulate bacterial populations in faeces from obese mice as a potential underlying mechanism to help re-establish a healthy balance and protect from obesity-related disorders.

2. Materials and methods

2.1. Chemicals

Acetone, glycerol, hydrochloric acid, methanol, sodium potassium tartrate and Tris base were purchased from Fisher (NJ, USA). Acetic acid, propanol and sodium hydroxide were acquired from J.T. Baker (Center Valley, PA, USA). Bacterial growth media and supplements were obtained from BD (Franklin Lakes, NJ, USA). Primers for quantitative real time PCR were acquired from Integrated DNA Technology (San Diego, CA, USA). The remaining chemicals and reagents were obtained from Sigma–Aldrich Co. Ltd. (Saint Louis, MO, USA).

2.2. Plant material

Apple commercial cultivars Braeburn, Fuji, Gala, Golden Delicious, Granny Smith, McIntosh and Red Delicious produced in the State of Washington were purchased from a local grocery market in Pullman, WA. Apples were maintained at 4 °C to preserve chemical composition and maturity stage. Each sample was prepared out of three or more fruits, after removing the core, to study only the edible parts (flesh and peel).

2.3. Proximal analysis

Apple samples were ground with dry ice, and kept at −20 °C for moisture, and ash analysis following the AOAC standard methods (AOAC, 2006). Protein was quantified using a Perkin-Elmer 2400 Series II CHNS/O elemental analyzer (Perkin-Elmer, Waltham, MA, USA). A factor of 6.25 was used to convert the nitrogen content to crude protein content.

2.4. Colour

Colour of apple flesh was quantified by image analysis as previously reported (Medina, Skurtys, & Aguilera, 2010) with some modifications. Briefly, images were taken with a Nikon Inc COOLPIX L810 digital camera 16.1 Megapixels (Melville, NY, USA) from top view under constant distance (40 cm height) and uniform illumination of 2 bulbs (150 W each). The red (R), green (G) and blue (B) signals were obtained with the ImageJ program (http://rsb.info.nih.gov/ij/) using colour histogram plugins from 5 different regions of the picture. The colour of the apple samples were represented in the hue (H) – saturation (S) – lightness (L) model. H, S and L values were estimated as previously reported (Medina et al., 2010) using the following equation: $H = \arctan((\sqrt{3}/2(G - B))/((R - 0.5(G + B))))$, $L = (R + G + B)/3$, $S = 1 - \text{lowest value of any ratio: } R/L, G/L, \text{ or } B/L$. The RGB values were converted to CIE-Lab space using EasyRGB calculator (http://www.easyrgb.com/index.php?X=CALC#Result), to obtain L*, a* and b* values.

2.5. Soluble solids and titratable acidity

Apple samples were pressed to obtain 10 μL of juice, which was analysed with a hand refractometer (Extech model 2132, Japan) at 20 °C to determine percentage of soluble solids (°Brix).

For titratable acidity, apple samples were homogenised with an Ultra-Turrax model TR-10 (Tekman, OH, USA) (5 g/100 mL ultra-pure water), filtered through a Whatman # 1 (Whatman International Ltd, Maidstone, ME, UK) and analysed with 0.1 mol/L NaOH using phenolphthalein as an indicator. The results were expressed as g of malic acid equivalent/100 g sample wet weight (AOAC, 2006).

2.6. Total extractable phenolics

Apple samples (flesh and peel) were homogenised in an Ultra-Turrax with acidic methanol (HCl)/water solution (50:50 v/v, pH 2) (200 g/L) and left for 1 h at room temperature with constant shaking, followed by centrifugation at 2100g for 10 min at 4 °C to obtain an acid methanolic extract in the supernatant. The precipitate was extracted with acetone/water solution (70:30 v/v) (1:5 ratio, v/v) by agitation for 60 min, followed by centrifugation at 2100g for 10 min at 4 °C. The combined supernatants were analysed for total phenolic content using the Folin Ciocalteu micro-method (Abderrahim et al., 2012).

2.7. Non-extractable polymeric proanthocyanidins

Non-extractable polymeric proanthocyanidins were analysed as previously reported (Zurita, Díaz-Rubio, & Saura-Calixto, 2012) with some modifications. Briefly, 20 g of residue obtained after successive acidic methanolic and acetone extraction (as detailed in Section 2.6) was hydrolysed with 100 mL of HCl/butanol (5:95 v/v) containing 0.7 g of FeCl3·6H2O and incubated at 90 °C for 1 h. Then the sample was centrifuged at 15,000g for 3 min and the supernatant absorbance read at 555 nm and 450 nm using a µQuant microplate reader (Biotek Instrument, Rochester, VT, USA). Proanthocyanidin concentrations were taken from the sum of absorbance at 550 and 450 nm and plotted against a standard curve of polymeric proanthocyanidins (range 0–100 mg/L) and expressed as mg proanthocyanidins/g apple dry weight.

2.8. Dietary fibre and available carbohydrates

2.8.1. Dietary fibre

Apple samples were analysed by the enzymatic–gravimetric method as previously described (McCleary et al., 2012). Briefly, apple samples were blanched at 90 °C for 5 min, homogenised in a blender and freeze dried. The freeze dried samples were homogenised (1 g/20 mL) with maleate buffer (50 mmol/L, pH 6.0 plus 2 mmol/L CaCl2) containing 50 μmol/L of porcine pancreatic α-amylase and 3.4 U/mL of amyloglucosidase. This mixture was incubated at 37 °C for 16 h under agitation at 150 rpm. Thereafter, 0.75 mL Tris base (0.75 mol/L) was added to adjust pH to 8.2 and enzymes inactivated at 90 °C for 20 min. One millilitre of protease (8.8 U/mL) was added to the mixture and incubated at 60 °C for 30 min under agitation. Thereafter, acetic acid (2 mol/L) was added to the mixture to adjust pH to 4.5. Dietary fibre was precipitated by adding four volumes of absolute ethanol and 1 h incubation at room temperature followed by centrifugation at 2000g for 10 min. The pellet was filtered, dried at 105 °C and weighed to quantify dietary fibre. The supernatant was collected for quantification of available carbohydrates.
2.8.2. Available carbohydrates

Supernatants from Section 2.8.1 were assessed for available carbohydrates by quantification of reducing sugars by the 3,5-dinitrosalicylic acid (DNS) assay (King, Donnelly, Bergstrom, Walker, & Gibson, 2009). Briefly, glucose standards 0.5, 1.0, 1.5 and 2.0 mg/mL were prepared in 0.1 mol/L sodium acetate buffer pH 5.5. A 30 µL volume of the standards or diluted samples were added to 60 µL 1% DNS reagent and the mixture was heated at 95°C for 5 min before being cooled to 20°C in a T100 Thermal cycler (Bio-Rad, CA, USA). The reaction volume was transferred to 96-well plate and absorbance measured at 540 nm in a Quant microplate reader (Biotek Instrument, Rochester, VT, USA). The reducing sugars were expressed as g glucose equivalent per 100 g of freeze dried sample.

2.9. Enzymatic browning

The enzymatic browning was assessed according to the procedure described by Quintero Ruiz, Demarchi, Massolo, Rodoni, and Giner (2012) with some modifications. Briefly, a composite of 4 or more blanched apple samples were mashed with a mortar, and pictures were taken with a Nikon COOLPIX L810 digital camera 16.1 Megapixels (Melville, NY, USA) under controlled illumination, every 5 min for 55 min. The red (R), green (G) and blue (B) signals were obtained from blanched samples and from non-blanched controls (t = 0 min). The colour histogram plugin from ImageJ program (http://rsb.info.nih.gov/ij/) was used to calculate the browning index (BI) as follows: BI = R/(R + G + B). The kinetic and order of the enzymatic reaction were calculated by power law model (Quevedo, Jaramillo, Diaz, Pedreschi, & Aguiler, 2009).

2.10. Optimisation of blanching

Apple slices were immersed in distilled water (1/10, w/v) at 80°C for 10, 15 and 20 min or 90°C for 5, 10 and 15 min. The best combination time–temperature was selected as a function of available carbohydrates (as detailed in Section 2.8.2), extractable phenolics and non-extractable proanthocyanidins as detailed in Sections 2.6 and 2.7.

2.11. In vitro digestion (IVD)

Apple samples (flesh and peels) were blanched, blended, freeze dried and successively incubated with digestive enzymes to simulate salivary, gastric and duodenal digestion at 37°C in a water bath under agitation (200 strokes/min agitation using a shaking water bath (VWR, Radnor, PA, USA) (Hollebeeck, Borlon, Schneider, Larondelle, & Rogez, 2013). Briefly, 10 g of dried apples were homogenised with 20 mL of Tris-maleate buffer (0.1 mol/L, pH 6.9) and salivary digestion mimicked with 3.9 U of amylase/l for 12 h at 37°C under agitation (200 rpm). Culture experiments, an anaerobic atmosphere was generated by GasPak EZ™ Gas Systems (BD Diagnostic Laboratory, NJ, USA). A pre-culture was prepared with fresh faeces diluted with anaerobic phosphate buffer 1:10 (w/v) (1 mol/L, pH 7.2), homogenised to produce faecal slurries and used for inoculation of pre-culture (10%, v/v) in a pre-reduced sterile medium at pH 7.0 (peptone (2 g/L), yeast extract (2 g/L), NaCl (0.1 g/L), K2HPO4 (0.04 g/L), KH2PO4 (0.04 g/L), NaHCO3 (2 g/L), MgSO4·7H2O (0.01 g/L), CaCl2·2H2O (0.01 g/L), Tween 80 (2 mL/L), hemin (50 mg/L), vitamin K (10 µL/L), L-cysteine (0.5 g/L), bile salts (0.5 g/L), resazurin (1 mg/L), and distilled water) (Sanchez-Patan et al., 2012) and anaerobic incubation for 12 h at 37°C under agitation (200 rpm). Cultures were prepared with 5% (v/v) of pre-culture in modified pre-reduced medium containing the dried apples after IVD at 1% and 2% (w/v) or starch at 2% (w/v) for the obese and lean controls. After 24 h incubation at 37°C and 200 rpm, culture fermentation was left without agitation for 10 min to separate apple compounds for analysis of phenolics as detailed in Sections 2.6 and 2.7; then the culture was centrifuged at 15,000g for 3 min at 4°C to recover pellets containing faecal bacteria and supernatants containing SCFAs. Pellets and supernatants were stored at −80°C for further analysis.

2.12. In vitro faecal fermentation

Faecal samples were collected from one year old diet-induced obese mice (n = 3), body mass index (BMI) = 5.7 ± 0.7, and age matched lean mice (n = 3), BMI = 3.6 ± 0.4. Obese mice were fed a high fat diet #D12451 containing 24% protein, 41% carbohydrate, 24% fat, 5.8% cellulose and 5.2% minerals (45% kcal from fat) (Research diets, Inc., New Brunswick, NJ). Lean mice were fed a standard diet #2108 containing 18% protein, 44.2% carbohydrate, 6.2% fat, 20% fibre and 5.3% ash (18% kcal from fat) (Teklad Diets, Madison WI). Animals were housed in a temperature-controlled room with a 12 h light and 12 h darkness cycle and in compliance with the WSU Institutional Animal Care and Use Committee. Faeces from obese mice were cultured in batches from each animal donor per apple treatment (1% and 2%, w/v), i.e. no pooled faecal samples. For obese and lean controls, a composite of faeces obtained from the obese (n = 3) or lean mice (n = 3) were prepared and fermentations were carried out in duplicates. For the faecal culture experiments, an anaerobic atmosphere was generated by GasPak EZ™ Gas Systems (BD Diagnostic Laboratory, NJ, USA). A pre-culture was prepared with fresh faeces diluted with anaerobic phosphate buffer 1:10 (w/v) (1 mol/L, pH 7.2), homogenised to produce faecal slurries and used for inoculation of pre-culture (10%, v/v) in a pre-reduced sterile medium at pH 7.0 (peptone (2 g/L), yeast extract (2 g/L), NaCl (0.1 g/L), K2HPO4 (0.04 g/L), KH2PO4 (0.04 g/L), NaHCO3 (2 g/L), MgSO4·7H2O (0.01 g/L), CaCl2·2H2O (0.01 g/L), Tween 80 (2 mL/L), hemin (50 mg/L), vitamin K (10 µL/L), L-cysteine (0.5 g/L), bile salts (0.5 g/L), resazurin (1 mg/L), and distilled water) (Sanchez-Patan et al., 2012) and anaerobic incubation for 12 h at 37°C under agitation (200 rpm). Cultures were prepared with 5% (v/v) of pre-culture in modified pre-reduced medium containing the dried apples after IVD at 1% and 2% (w/v) or starch at 2% (w/v) for the obese and lean controls. After 24 h incubation at 37°C and 200 rpm, culture fermentation was left without agitation for 10 min to separate apple compounds for analysis of phenolics as detailed in Sections 2.6 and 2.7; then the culture was centrifuged at 15,000g for 3 min at 4°C to recover pellets containing faecal bacteria and supernatants containing SCFAs. Pellets and supernatants were stored at −80°C for further analysis.

2.13. Analysis of bacterial populations

Relative abundances of bacterial populations were analysed by quantitative real-time PCR (qPCR). Briefly, DNA was extracted from pellets recovered after faecal fermentation using QIAamp DNA Stool Mini Kit (QIAGEN Inc, CA, USA) according to the manufacturer’s protocol. The concentrations of DNA and absorbance ratio 260/280 nm as measure of purity were assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific, NC, USA). PCR reaction mixtures (total of 10 µL) contained 5 µL of SsoAdvanced™ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA), 0.4 µL of primer mix (forward and reverse, final concentration 200 nmol/L), 2 µL of adjusted (2 ng/µL) template DNA or non-template control, and 2.6 µL nuclease-free water (Qiagen GmbH, Hilden, Germany). PCR conditions were as follows: 95°C for 30 s and 40 cycles at 95°C for 5 s and 55°C for 30 s. A melt curve analysis was performed to verify the specificity of the primers using the following conditions: 65–95°C at 0.5°C increments. Relative bacterial population was quantified by the Livak Method (2-∆∆CT) using, as a reference, the universal primer CT values. Data was normalised to obese controls fermented with starch (2% w/v) as a carbon source. Primer sequences were obtained from Bergstrom et al. (2012). The pairs of forward and reverse primers were purchased from Integrated DNA Technologies, Inc. (San Diego, CA, USA) and sequences were:
Millennium software for HPLC data analysis. A sample of 20 tdiode array detector (999 PAD) (Waters, Milford, MA, USA) and a 1–3 guard column (Bio-Rad, Hercules, CA, USA) in a Waters 600S by HPLC using the Aminex HPX-87H column (8% cross-linked resin UV–visible spectral data, and pick areas to known standards. were identified and quantified by comparing retention times, between these values. In general, these values are in agreement with RGB was converted to CIE-Lab space, the values obtained for Golden Delicious and Granny Smith cultivars (Drogoudi et al., 2008; Hoehn et al., 2003; Vieira et al., 2009). The content of protein in fruits is, in general, low and apple has around 0.26% dry weight. Results from protein analysis ranged from 0.53 ± 0.23% to 1.85 ± 0.01% (dry basis) for Red Delicious and McIntosh, respectively.

Results from colour analysis are presented in Fig. 1 and in Supplementary material Table 1. Values of H were from 1.9 ± 0.6 to 21.4 ± 2.5 for the red apple cultivars (Braeburn, Fuji, Gala, McIntosh and Red Delicious), and from 47.7 ± 0.9 to 60.7 ± 0.6 for Golden Delicious and Granny Smith, respectively. The L and S of Fuji were substantially different from the other red cultivars (Fig. 1A and B). When RGB was converted to CIE-Lab space, the values obtained for Golden Delicious (L* = 55, a* = −18 and b* = 30) and Granny Smith (L* = 62, a* = −12 and b* = 28) were comparable to those previously reported for the same cultivars (Drogoudi et al., 2008).

3.2. Bioactive compounds in apple cultivars

Previous studies have reported that apples are a good source of dietary fibre, and phenolic compounds are variable among cultivars (Karaman et al., 2013; Tsao, Yang, Christopher, Zhu, & Zhu, 2003). To our knowledge, this is the first study assessing the variability in the content of bioactive compounds in apple cultivars produced in the U.S. Pacific Northwest. Our results showed that, among the seven apple cultivars analysed, Granny Smith had the highest content of dietary fibre and phenolics (extractable and non-extractable) (Table 2). Red Delicious and Granny Smith represented the lowest and highest extremes regarding the contents of extractable phenolics (1.08 ± 0.04 to 9.2 ± 1.8 mg GAE/g dry weight, respectively). Results were consistent with values of phenolics reported for Fuji, Gala, Golden Delicious, Granny Smith, and McIntosh (Lata et al., 2009). However, in another study of apples grown in Ontario, Canada, the content of phenolics in peel and flesh in Red delicious was higher than in Golden delicious and McIntosh (Tsao et al., 2003). Discrepancies with our findings might be due the influence of maturity stage and environmental conditions.

The content of non-extractable phenolics ranged from 3.28 ± 0.02 to 5.70 ± 0.9 mg proanthocyanidins/g dry weights for Golden Delicious and Granny Smith, respectively. The other cultivars were between these values. Likewise, content of fibre ranged from 20.6% to 32.2% dry weight for Fuji and Granny Smith, respectively.

<table>
<thead>
<tr>
<th>Apple cultivars</th>
<th>Moisture (%)</th>
<th>Titratable acidity (g malic acid/100 g wet basis)</th>
<th>Soluble solids ('Brix)</th>
<th>Protein (% dry basis)</th>
<th>Ash (% dry basis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Braeburn</td>
<td>86.6 ± 0.4a</td>
<td>0.18 ± 0.01a</td>
<td>11.6 ± 0.3a</td>
<td>1.70 ± 0.46a</td>
<td>1.2 ± 0.1a</td>
</tr>
<tr>
<td>Fuji</td>
<td>85.4 ± 0.3a</td>
<td>0.16 ± 0.01a</td>
<td>12.0 ± 0.0a</td>
<td>1.28 ± 0.02a</td>
<td>1.7 ± 0.4a</td>
</tr>
<tr>
<td>Gala</td>
<td>89.1 ± 0.1b</td>
<td>0.59 ± 0.04b</td>
<td>9.1 ± 0.1b</td>
<td>1.79 ± 0.03a</td>
<td>2.5 ± 1.5a</td>
</tr>
<tr>
<td>Golden Delicious</td>
<td>84.7 ± 1.3a</td>
<td>0.18 ± 0.01a</td>
<td>10.1 ± 0.2b</td>
<td>1.52 ± 0.47a</td>
<td>2.1 ± 0.5a</td>
</tr>
<tr>
<td>Granny Smith</td>
<td>86.9 ± 0.2a</td>
<td>0.25 ± 0.01c</td>
<td>13.1 ± 0.1c</td>
<td>1.31 ± 0.23a</td>
<td>3.1 ± 0.5a</td>
</tr>
<tr>
<td>McIntosh</td>
<td>86.8 ± 0.2a</td>
<td>0.34 ± 0.03c</td>
<td>14.3 ± 0.6d</td>
<td>1.85 ± 0.01a</td>
<td>2.0 ± 0.3a</td>
</tr>
<tr>
<td>Red delicious</td>
<td>84.9 ± 0.3c</td>
<td>0.30 ± 0.02c</td>
<td>14.5 ± 0.1d</td>
<td>0.53 ± 0.23b</td>
<td>2.9 ± 0.7a</td>
</tr>
</tbody>
</table>

Table 1
Physicochemical characteristic of apple cultivars. Values are mean of three or more samples ± SD, different letters within same column indicate statistical significance (p < 0.05).
respectively, with other cultivars between these values. Fruits and vegetables supply a major proportion of the non-extractable polyphenols to the human diet (Arranz et al., 2010; Saura-Calixto, 2011). Results showed that non-extractable proanthocyanidins ranged from 3.28 ± 0.02 to 5.7 ± 0.9 mg of proanthocyanidins/g dry weight for Golden Delicious and Granny Smith, respectively (Table 2). These values were comparable to those previously reported for Golden Delicious (3.2 ± 0.07 mg of proanthocyanidins/g dry weight) (Arranz et al., 2010; Zurita et al., 2012). In general, non-extractable proanthocyanidins were similar among the apple cultivars and variability in the ratio of extractable phenolics to non-extractable proanthocyanidins were determined by the contents of extractable phenolics.

Dietary fibre has been consistently found to be inversely associated with chronic human diseases such as cancer, obesity and obesity-related diseases (Saura-Calixto, 2011). Recently, dietary prebiotics have been defined as an ingredient that is selectively fermented, resulting in specific changes in composition and/or activity of gastrointestinal microbiota, thus conferring benefits to the host health (Tuohy et al., 2012). Results of dietary fibre contents ranged from 20.6 ± 2.4% to 32.2 ± 1.3% in dry weight, with Granny Smith being the highest, whereas no statistical difference was found in the contents of available carbohydrates (Table 2). Previous studies have reported values of 12.4% and 48% in dry weight for dietary fibre of freeze dried apple flesh and pomace, respectively (Yan & Kerr, 2012), and effects of apple pectin on improving the faecal environment have been reported (Shinohara et al., 2010).

Finally, a principal component analysis of extractable phenolic, non-extractable proanthocyanidins, dietary fibre, available carbohydrate, and titratable acidity allowed us to position apple

![Color parameters](image)

**Table 2**

<table>
<thead>
<tr>
<th>Apple cultivars</th>
<th>Total extractable phenolics (mg GAE/g of dry weight)</th>
<th>Non-extractable proanthocyanidins (mg proanthocyanidins/g dry weight)</th>
<th>Dietary fibre (% dry weight)</th>
<th>Availability carbohydrate (g glucose/100 g lyophilised)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Braeburn</td>
<td>5.91 ± 0.13a</td>
<td>5.23 ± 0.96a</td>
<td>24.9 ± 1.4a</td>
<td>7.3 ± 0.7a</td>
</tr>
<tr>
<td>Fuji</td>
<td>5.78 ± 0.83a</td>
<td>5.30 ± 1.19a</td>
<td>20.6 ± 2.4a</td>
<td>7.1 ± 0.7a</td>
</tr>
<tr>
<td>Gala</td>
<td>8.04 ± 0.27a</td>
<td>4.47 ± 1.30a</td>
<td>26.7 ± 1.4a</td>
<td>8.1 ± 1.1a</td>
</tr>
<tr>
<td>Golden Delicious</td>
<td>3.93 ± 0.24a</td>
<td>3.28 ± 0.02a</td>
<td>23.1 ± 2.1a</td>
<td>7.3 ± 0.6a</td>
</tr>
<tr>
<td>Granny Smith</td>
<td>9.18 ± 1.79b</td>
<td>5.70 ± 0.90a</td>
<td>32.2 ± 1.3b</td>
<td>7.3 ± 2.3a</td>
</tr>
<tr>
<td>McIntosh</td>
<td>7.23 ± 0.39a</td>
<td>5.65 ± 0.87a</td>
<td>21.5 ± 2.1a</td>
<td>7.5 ± 1.3a</td>
</tr>
<tr>
<td>Red delicious</td>
<td>1.08 ± 0.04c</td>
<td>4.15 ± 0.25a</td>
<td>24.3 ± 3.3a</td>
<td>6.6 ± 0.9a</td>
</tr>
</tbody>
</table>

**Fig. 1.** Colour parameters lightness, saturation (%) and hue (°) of apple cultivars. Values are mean ± SD (n = 3).
cultivars based on their similarity and identify those with enhanced content of dietary fibre and low content of available carbohydrates (Fig. 2). In general, we identified Granny Smith as a cultivar with enhanced content of bioactive compounds (dietary fibre, extractable phenolics, and low in available carbohydrates) and based on these results, Granny Smith was selected for future studies.

3.3. Optimisation of processing to stabilise bioactive compounds in the selected apple cultivar

Blanching optimisation aimed to protect apples from enzymatic browning and oxidation of phenolic compounds while decreasing the amount of reducing sugars. Granny Smith apples were blanched at 80 °C for 10–20 min or 90 °C for 5–15 min and results showed that inactivation of enzymatic browning was similar for all blanching conditions tested with not statistical difference on kinetic of enzymatic browning (0.0011 < K < 0.0139), while K for the non-blanced control was 0.15 ± 0.02 with a reaction order (n) of 0.3 ± 0.1 (Supplementary material, Table 2). Simultaneously, the blanching conditions required to significantly decrease reducing sugars (approximately 40% of control) were 80 °C for 20 min or 90 °C for 5 min (Supplementary material, Fig. 1) while none of the blanching temperature–time combinations decreased extractable phenolics (data not shown). Based on these results, blanching at 90 °C for 5 min was used for further experiments due to lowest content of available carbohydrates.

3.4. Modulation of faecal bacteria and production of SCFAs by non-digestible compounds in Granny Smith

The beneficial effects of apple consumption and apple pectin in promoting the growth of Bifidobacterium and Lactobacillus and...
improving intestinal and faecal environment has been reported (Shinohara et al., 2010). However, to our knowledge, this is the first study assessing the effects of apple non-digestible compounds containing dietary fibre and phenolic compounds on faecal bacterial changes in faeces from obese mice. Extractable phenolics were reduced in apple after IVD to ~48% of initial content (from 6.3 ± 0.3 to 3.05 ± 0.23 mg GAE/g dry weight). Similar losses in extractable phenolics have been previously reported for Golden Delicious and Mutzu apples (from 36.3–51.4 mg/100 g to 18.0–27.4 mg/100 g) (Bouayed, Deußer, Hoffmann, & Bohn, 2012). Proanthocyanidins, instead, resisted the IVD almost completely (Supplementary material, Fig. 2), due to their covalent link to dietary fibre, which makes possible for them to reach the colon and to be metabolised by faecal bacteria (Arranz et al., 2010).

Altered proportions of the two dominant bacteria phylum Bacteroidetes and Firmicutes in the human gut have been reported in obese state (Lyra, Lahtinen, Tiihonen, & Ouwehand, 2010). Our results, although not statistically significant (p = 0.06), showed a trend to increase the relative DNA abundance of Firmicutes in lean controls compared to obese controls (from 1.00 ± 0.03-fold to 1.91 ± 0.11-fold), and this trend increased in faeces from obese mice cultured with 1–2% apple compounds (to 2.3 ± 0.6 and 2.6 ± 0.2 of obese controls) (Fig. 3A). The relative abundance of Bacteroidetes DNA, instead, decreased significantly (p < 0.05) in lean controls to 0.35 ± 0.01-fold of obese controls. When faeces from obese mice were cultured with 1–2% apple compounds, Bacteroidetes DNA abundance also decreased significantly (p < 0.05), to 0.22 ± 0.04– and 0.23 ± 0.1-fold of obese controls, respectively (Fig. 3A). Previous studies have reported that relative abundance of the two phyla Firmicutes and Bacteroidetes differ among lean and obese mice, with obese mice having a higher proportion of Firmicutes to Bacteroidetes and similar results were observed in obese humans compared to lean subjects (Kallus & Brandt, 2012). However, our results showed an inverse trend between the proportions of Firmicutes and Bacteroidetes in faeces from obese and lean mice and apple compounds, which tended to increase Firmicutes (p = 0.06), and decrease Bacteroidetes significantly (p < 0.05). This was consistent with a previous study showing that consumption of apple pectin increased the population of Firmicutes and decreased Bacteroidetes in the rat gut (Licht et al., 2010). The discrepancies regarding the association of bacterial groups with obesity might be due to the complexity of the microbial ecosystem with high subject specificity (Lyra et al., 2010).

Similarly, relative DNA levels of Enterococcus, which belong to the phylum of Firmicutes were assessed and results showed that Enterococcus DNA in lean controls tended to be higher (p = 0.07), compared to the obese controls (1.84 ± 0.84-fold). Enterococcus DNA levels also tended to increase in faeces from obese mice cultured with 1–2% apple compounds (3.55 ± 0.37– and 1.84 ± 0.84-fold of obese control, respectively) (Fig. 3B). We also found that relative DNA proportions of Enterobacteriaeae, which belong to the phylum of Proteobacteria, were higher in faeces from lean controls (5.82 ± 0.16-fold of obese controls). Similarly, Enterobacteriaeae DNA levels tended to increase in faeces from obese mice when cultured with 1–2% apple compounds (to 2.4 ± 0.65– and 2.29 ± 0.36-fold of obese control, respectively) (p = 0.1025) (Fig. 3C). Escherichia coli belongs to the family of Enterobacteriaeae and, accordingly, the relative DNA abundances of E. coli were consistent with the trend found for Enterobacteriaeae, with lean controls having 2.15 ± 0.95-fold of obese controls, and an increasing trend for rising levels in faeces from obese mice when cultured with 1–2% apple compounds (4.04 ± 0.94 and 2.77 ± 0.65-fold of obese controls, respectively) (p = 0.1025) (Fig. 3C). Most of E. coli strains are harmless and part of the normal flora of the gut and can benefit the host by preventing pathogenic bacteria from establishing within the intestine. A recent study has reported a negative correlation between E. coli and BMI (Million et al., 2013).

Finally, relative abundances of Bifidobacterium spp., which belong to the Actinobacteria phylum, tended to increase in faeces from lean controls (2.22 ± 0.99-fold of obese control) (p = 0.58); this trend was also observed when faeces from obese mice were cultured with 1–2% apple compounds (1.84 ± 0.56– and 1.6 ± 0.33-fold of obese controls) (Fig. 3D). Increases in the numbers of Bifidobacterium and Enterococcus have been found in caecal contents and faeces of mice fed with high fat diet containing fermentable carbohydrates, which prevented body weight gain (Tuohy et al., 2012). Moreover, Bifidobacterium is a genus known as probiotic beneficial bacteria inversely associated with BMI (Million et al., 2013).

Shinohara et al. (2010), demonstrated that Bifidobacterium and Enterococcus increased in faeces cultured with apple pectin and in faeces from healthy adults after apple consumption. Our results are consistent with this study, which attributed major changes in gut microbiota to pectin. However, we found that phenolics are also being metabolised by faecal bacteria since the concentrations of extractable phenolics and non-extractable proanthocyanidins decreased after faecal fermentation by 74% and 38%, respectively. This is in agreement with studies reporting the effects of phenolic compounds on the intestinal environment due to modulation of the intestinal bacterial populations and implications in the maintenance of gastrointestinal health (Mico et al., 2012). Overall, the non-extractable proanthocyanidins resisted the IVD and were further metabolised by the faecal bacteria (Supplementary material, Fig. 2). Therefore, they might have contributed to changes in faecal bacterial populations. This is consistent with studies reporting that part of the health benefits of non-extractable phenolics are related to their effects on modulation of intestinal bacteria population (Saura-Calixto, 2011). Finally, principal component analysis of relative microbiota populations showed that faeces cultured with Granny Smith non-digestible compounds shifted the profile of bacterial DNA abundances of obese faecal material towards the profile of faeces from lean mice (Fig. 4).

![Fig. 4. Principal component analysis. Relative DNA abundances of bacterial populations in faeces from obese mice were positioned closer to the lean controls when fermented with apple compounds. Data was analysed by SPSS using the dimension reduction.](image-url)
Results from SCFAs analysis showed that butyric acid in culture supernatants were 90.15 ± 54 μmol/g starch in obese + stanch control, 141.8 ± 78 μmol/g, and 154.28 ± 77 μmol/g apple in obese + digested apple (1% and 2%, respectively). Butyric acid in faeces from obese mice cultured with 1–2% apple compounds was 1.57 ± 0.86- and 1.71 ± 0.85-fold of stanch control, respectively (Supplementary material, Fig. 3). These values show that apple compounds promote the production of butyric acid, even though the increase was not statistically significant due to the high variability (p = 0.53). The SCFAs, particularly butyric acid, are beneficial for the host colonic cells because these nutrients help increase colon barrier integrity and protect against pathogens and blood endotoxins, the presence of endotoxins in the blood (Cani & Delzenne, 2011). Levels of acetic acid instead, were lower compared to butyric acid levels (from 2.3 to 11 μmol/g), and apple compounds decreased acetic acid concentrations (Supplementary material, Fig. 3). Levels of propionic acid were below the limits of detection.

4. Conclusions

Granny Smith apples were identified as a cultivar with enhanced content of bioactive compounds including dietary fibre, extractable and non-extractable phenolics; these compounds can potentially reach the colon. After faecal fermentation of Granny Smith non-digestible compounds, we demonstrated that relative abundances of bacterial populations in faeces from obese mice tended to be similar to the lean controls. These results suggest that dietary fibre and phenolic compounds remaining in apple after IVD might help to prevent metabolic disorders driven by an altered microbiota in obesity, and potentially protect from an obesity-disturbed balance of microbiota.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.03.122.

References


