Release of phenolic compounds with antioxidant activity by human colonic microbiota after *in vitro* fermentation of traditional white and blue maize tortillas

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Abstract

The attention gained by cereals and derived products, such as tortillas, is due to their richness in phenolic and anthocyanin compounds. Although white maize tortillas have been a staple of the Mexican diet for centuries, blue maize has been adopted as a healthier alternative because of its important natural antioxidant source. The aim of the present work was to evaluate the involvement of colonic microbiota in the release of phenolic compounds with antioxidant activity present in traditional tortillas made from commercial white (WMT) and blue (BMT) maize flours. Nutritional composition in WMT and BMT exhibited no differences in protein (9.10 and 9.20%), ash (1.33 and 1.39%), energy (384.30 and 384.70 kcal), or phenolic consumption (323.44 and 437.33 mg/day) among tortillas. The highest anthocyanin (6.61 CGE/100 g), total phenolic contents (235.76 mg GAE/100 g), and antioxidant activity (5,992.14 and 1,651.64 µmol TE/100 g in ORAC and ABTS, respectively) were observed in BMT. Through microbiota fermentation, phenolic released content (13.4 mg GAE/g), ORAC (804.6 and 880.7 µmol TE/g), ABTS (27.4 and 30.7 µmol TE/g), and bioaccessibility (> 80%) displayed the highest values at 5 h. The present work demonstrated that colonic microbiota improved bioaccessibility of insoluble phenolics present in tortillas, favouring an antioxidant environment that positively impacts colonic health.

Introduction

In recent years, the increasing consumer awareness of the relation between diet and health has encouraged the research of bioactive compounds with biological and functional activities that can be used as food ingredients or dietary supplements. Cereals, in particular maize and their products, have drawn more attention from a nutraceutical point of view owing to consumer knowledge of their association to several health benefits (Bello *et al*., 2015; Herrera *et al*., 2020). In Mexico, white maize (*Zea mays* L.) is the most frequently produced cereal, and the main ingredient in commercial and traditional tortillas (Colín *et al*., 2020). Each year, an average of 68 kg of tortillas are consumed by Mexicans; this corresponds to 410 kcal (around 20% of the daily calorie ingestion) of the total energy daily consumption (Serna and Chuck, 2016). To be consumed, maize is subjected to an alkaline cooking process known as nixtamalization, where maize kernels are cooked in a lime solution, followed by steeping and washing. Finally, the nixtamal, a composition of endosperm, germ, and pericarp in minor proportion (mostly composed of insoluble-bound phenolics) is dried and milled until flour is obtained, from which tortillas are made (Mora *et al*., 2010).

Nowadays, other maize genotypes have been used in the food industry, such as blue maize, which is used to create healthier alternatives for tortillas, and enhance the nutritional features of other food products (Bello *et al*., 2015; Mora *et al*., 2016; Colín *et al.,* 2020; Herrera *et al*., 2020). The pigmentation in blue maize is provided mainly by anthocyanins (Mora *et al*., 2016), which, together with other polyphenols (present in free, soluble conjugated, and

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insoluble bound forms), dietary fibers (soluble and insoluble fractions), and vitamins and minerals provide antioxidant properties (Singh *et al*., 2011; Bello *et al*., 2015; Shahidi and Yeo, 2016) that are associated with the prevention of intestinal and cardiovascular diseases, diabetes, obesity, and some types of cancer (Shahidi and Yeo, 2016; Herrera *et al*., 2017; 2020).

Even though polyphenols are abundantly found in maize, most of them, which are found in insoluble form covalently bound to indigestible matrices such as polysaccharides, may be metabolized and released in the colon by the microbiota; a process known as colonic fermentation (Dall´Asta *et al*., 2012; Shahidi and Yeo, 2016). The diverse microbial ecosystem carries out chemical reactions to transform complex polyphenolic structures to lower molecular weight phenols that can be absorbed more easily by the organism to exert their health benefits (Dall´Asta *et al*., 2012; Lafiandra *et al*., 2014).

In vitro fermentation models are usually performed with human or animal faeces as inoculums to study polyphenol catabolism. However, these models do not reflect the *in vivo* conditions, but are still useful for understanding the metabolic fate of bioactive compounds in the colon (Mosele *et al*., 2014). Unfortunately, there is little information related to the colonic microbiota capability to metabolized phenolic compounds in tortillas made from blue and white maize flours. Hence, the objective of the present work was to evaluate the release of phenolic compounds, along with their antioxidant activity present in traditional tortillas made from commercial white and blue maize flours, by the human faecal microbiota.

Materials and methods

Tortilla processing

The white (WMT) and blue (BMT) maize tortillas used in the present work were made from commercial nixtamalized flours purchased from a local market to accomplish the following experiments. To elaborate both types of tortillas, we followed the protocol described by Mora *et al*. (2016). In brief, 400 g of commercial flour (white and blue maize, separately) and 400 mL of water were mixed until an appropriate mass consistency was attained. Small portions of mass or dough (30 g) were pressed and shaped into flat disks (15 cm) using a manual press (Casa Herrera, México DF, México).

The disks were cooked on a hot griddle ($270 \pm 10°C$) for 15 s on one side, followed by 30 s on the other side. Finally, the first side was cooked again until a puffing tortilla was observed. Fresh tortillas were dehydrated and milled (UD Cyclone Sample Mill, UD Corp. Boulder, CO, USA) until the flour passed through an 80-US mesh (0.180 mm) sieve. The resulting flours were packed and stored in plastic bags at -20°C until their use.

Nutritional composition

The chemical composition of WMT and BMT was determined following the standards prescribed by the Association of Official Analytical Chemists (AOAC, 2005). All experiments were conducted in triplicate. Moisture was determined by drying the samples at 105°C for 24 h. Protein was determined by the micro-Kjeldahl method ($N \times 6.25$). Lipid was determined by a Soxhlet apparatus with petroleum ether. Ash was determined by incineration at 550°C. Caloric content (Kcal) was calculated using Eq. 1:

$$Kcal = (4\ Kcal \times 1\ g\ carbohydrates) + (4\ Kcal \times 1\ g\ proteins) + (9\ Kcal \times 1\ g\ fat)$$

$$(Eq.\ 1)$$

Determination of anthocyanin content

Anthocyanins were determined according to Abdel and Huel (1999), by measuring the absorbance of methanolic supernatants at 535 nm and corrected for background absorbance at 700 nm using a Microplate Reader (Synergy HT, Bio-Tek Instrument, Inc., Winooski VT, USA). The molar extinction coefficients ($\in$) of 25,965 Abs/M $\times$ cm, and a molecular weight (MW) of 449.2 g/mol were used to calculate the total anthocyanin content, and results were expressed as mg of cyanidin 3-glucoside equivalent (CGE) per 100 g of dry weight (DW), using Eq. 2:

$$C = [(A_{535nm} - A_{700nm})/\in] \times mL\ extract \times MW \times \frac{1}{sample\ weight}$$

$$(Eq.\ 2)$$

Extraction and quantification of soluble and insoluble phenolic compounds

Soluble and insoluble phenolics extractions were performed according to Mora *et al*. (2010). Briefly, 1 g of sample flour was added into 10 mL of chilled ethanol-water (80:20, v/v), and shaken for 10 min. Samples were centrifuged at 2,500 *g* for 10 min.

Supernatants were reduced to 2 mL using a rotary evaporator at 45°C, and the extracts obtained were kept at -20°C until their use.

Insoluble phenolic fractions were extracted from the residue from the previous extraction also according to Mora *et al*. (2010). The extraction residue was digested for 1 h with 10 mL of 2 mol/L NaOH in a water bath at 95°C. Later, it was agitated for another hour at room temperature. The mixture was acidified with HCl, and hexane was used to remove the lipids. The final residue was extracted five times with ethyl acetate. Finally, the ethyl acetate was pooled and evaporated at 45°C. Insoluble phenolic extracts were reconstituted with 2 mL of methanol 50%, and kept at -20°C until their use.

The total phenolic content of soluble and insoluble extracts was spectrophotometrically quantified by Folin-Ciocalteu method. The results were expressed as milligrams of gallic acid equivalents (GAE) per 100 g of dry weight (DW). The average daily intake of total phenolics present in tortillas (mg GAE/day) was calculated based on per capita consumption of tortilla.

Antioxidant capacity determination

The antioxidant activity was determined by ORAC and ABTS assays as described Re *et al*. (1999) and Mora *et al*. (2010). In brief, peroxyl radicals were generated by AAPH to achieve the ORAC assay, and fluorescence loss was measured using a microplate reader (Synergy HT Multi-Detection Microplate Reader; BioTek Instruments, Inc., Winooski, VT, USA). The absorbances of excitation and emission were set at 485 and 538 nm, respectively. ABTS radical cation (ABTS$^{\bullet+}$) was generated by oxidation of 2 mM ABTS with 2.45 mM potassium persulfate ($K_2S_2O_8$) solution for 12 h. The absorbance measurements of all samples with the ABTS$^{\bullet+}$ were read at 734 nm using a microplate reader (Synergy HT Multi-Detection Microplate Reader, BioTek Instruments, Inc., Winooski, VT, USA) 6 min after the initial mixing. A standard Trolox curve was used as control in both assays, and the antioxidant capacity was expressed as micromoles of Trolox equivalent (TE) per 100 g of dry weight (DW). All samples were analyzed in triplicate.

In vitro fermentation by human microbiota

Fermentation experiments for both types of tortilla flours were done according to Campos *et al*. (2009) with slight modifications. Most of the soluble phenolic compounds present in samples of WMT and BMT were removed by subjecting them to a washing treatment with chilled ethanol-water (80:20, v/v). Fresh faecal samples were donated by four healthy individuals (marked with letters from A to D, where donors B and C were women, and A and D were men) between 18 - 28 years old, who did not have reports of previous intestinal disease, and were not treated with antibiotics at least for the past three months. Sterile containers were used to keep the faecal samples within a maximum of 2 h from recollection. Next, 2 g of each sample were homogenised with 18 mL of 0.1 mol/L sodium phosphate buffer, pH 7.0. This faecal slurry was used as the fermentation starter. Sterile tubes (15 mL capacity) were filled with 9 mL of sterile basal culture medium. Tubes were sealed and maintained under a headspace containing H_2-CO_2-N_2 (10:10:80, by volume), O_2-free for 24 h. The tubes containing basal culture medium were inoculated with 1 mL of faecal slurry and 0.1 g of tortilla flour, except for blanks. The samples were shaken in vortex for 30 s, and placed in water bath at 37°C. In parallel, two different controls were conducted under the following conditions: (i) the flour was incubated in buffer solution without faeces to determine the possible release of phenolics, and (ii) the faecal suspension was incubated without flour as a negative control. The samples and controls were collected at 0, 1, and 5 h. Fermentation was terminated by placing the tubes in a freezer at -70°C. All experiments were carried out in triplicate. Fermentation-derived phenolic compounds and antioxidant activity were analyzed by the assays previously described.

Bioaccessibility percentage

The percentage of phenolic compounds (PC) that could become available for absorption after *in vitro* fermentation was calculated using Eq. 3 (Blancas *et al*., 2018):

Bioaccessibility percentage (%B) =

$$\frac{(PC\ RCF) - (SPC)}{(PC\ RCF) + (IPC)} \times 100 \qquad \text{(Eq. 3)}$$

where, the PC released on the colonic fermentation = PC RCF, the PC associated with the soluble fraction = SPC, and the PC of the insoluble fraction = IPC. The quantification of each phenolic fraction was calculated following the methodology earlier described.

Release kinetics analysis

Release kinetics parameters resulting from the *in vitro* fermentation were calculated according to Blancas *et al.* (2018), using Eq. 4:

$$Vf = \Sigma \left(\frac{\Delta C}{\Delta t} \right) \qquad \text{(Eq. 4)}$$

where, ΔC = concentration difference between the final and initial phenolic compounds, Δt = time difference between a specific time and the initial time, and Vf = final rate of phenolic compounds release during the *in vitro* fermentation, and expressed in mg GAE/h.

Statistical analysis

All experiments were carried out in triplicate, and data were reported as mean ± standard error. Statistical analysis and comparisons among means were performed using the statistical package MINITAB version 19. ANOVA procedures were used, and differences among tortilla flours were determined using Tukey's Test. Differences were considered significant at $p \leq 0.05$.

Results and discussion

Nutritional composition of tortillas

The nutritional analysis of WMT and BMT showed differences ($p \leq 0.05$) in some properties. BMT revealed higher moisture percentage (51.2%) than WMT (48.0%). Moisture content in tortillas has been reported to be between 35 - 50%, thus becoming an issue during tortilla storage. Such values could be related to the maize variety and the maize kernel components preserved after nixtamalization (Bello *et al.*, 2015; Colín *et al.*, 2020). No differences between results were observed in protein content (9.10 and 9.20%), ash (1.33 and 1.39%), and energy (384.3 and 384.7 Kcal), but lipid (1.76 and 2.20%) and carbohydrate (83.01 and 82.01%) contents showed differences among tortillas ($p \leq 0.05$) (Table 1).

These agree with Colín *et al.* (2020) who reported similar protein (9.63 and 9.45%), lipid (1.78 and 1.87%), and ash (1.80 and 1.85%) values in white and blue maize tortillas, respectively. Differences in nutritional composition content in tortillas are associated with maize varieties used in the production (Bello *et al.*, 2015; Colín *et al.*, 2020).

Table 1. Nutritional and bioactive compositions of traditional tortillas made from commercial flours.

Parameter	WMT	BMT
Nutritional composition[1]		
Moisture	48.0 ± 0.0[b]	51.2 ± 0.0[a]
Protein	9.10 ± 0.1[a]	9.20 ± 0.0[a]
Lipid	1.76 ± 0.1[b]	2.20 ± 0.2[a]
Carbohydrate	83.01 ± 0.1[a]	82.01 ± 0.1[b]
Ash	1.33 ± 0.08[a]	1.39 ± 0.1[a]
Energy[2]	384.30 ± 0.6[a]	384.70 ± 1.63[a]
Phenolic composition		
Anthocyanin[3]	0.15 ± 0.0[b]	6.61 ± 0.0[a]
Total phenolic[4]	173.61 ± 8.4[b]	235.76 ± 10.9[a]
Soluble	17.11 ± 0.6[b] (10)	30.64 ± 1.8[a] (13)
Insoluble	156.50 ± 8.3[b] (90)	205.10 ± 11.8[a] (87)
Phenol intake[5]	323.44 ± 14.4[b]	437.33 ± 8.0[a]

Values are means of three replicates ± standard error. Means in a row followed by different lowercase superscripts are significantly different ($p < 0.05$). [1]Expressed in percentage; [2]Expressed in Kcal; [3]mg cyanidin 3-glucoside equivalent (CGE)/100 g DW (dry weight); [4]mg gallic acid equivalents (GAE)/100 g DW; [5]mg/day. Values in parenthesis indicate percentage contribution of this fraction corresponding to the total phenolics. WMT = white maize tortilla; BMT = blue maize tortilla.

Bioactive compounds of tortillas

The highest value of anthocyanin content was observed in BMT (6.61 mg CGE/100 g) ($p \leq 0.05$) showing around six-fold higher anthocyanin content as compared to the one observed in WMT (Table 1). Similar results were previously reported in nixtamalized tortillas (2.1 to 15.5 mg CGE/100 g) produced with 15 different blue maize genotypes (Mora *et al.*, 2016). Also, different authors (Herrera *et al.*, 2017; Colín *et al.*, 2020) observed higher amounts of anthocyanin (21.8 - 27.8 mg CGE/100 g) in blue maize tortillas.

The potential health benefits of anthocyanin are well known, highlighting their protective role against oxidative stress by acting as radical scavengers (Mora *et al.*, 2016; Gaxiola *et al.*, 2017; Salinas *et al.*, 2017). However, during nixtamalization, blue maize kernels suffer a significant loss of bioactive compounds (> 50%). This occurs due to the alkaline pH (approximately 10) and high-temperature conditions used which affect the anthocyanins directly (Mora *et al.*, 2016).

Soluble, insoluble, and total phenolic contents of traditional tortillas made from commercial flours are presented in Table 1. BMT contained the highest ($p \leq 0.05$) amount (235.76 mg GAE/100 g) of total phenolics as compared to WMT (173.61 mg GAE/100 g). In contrast, other reports have observed higher phenolic contents in nixtamalized white maize tortillas. This difference could be attributed to the genetic background, grain physical characteristics, and the relative ratio of the structural parts of the grain since phenolic compounds are the most abundant in pericarp and endosperm structure (Mora *et al.*, 2016).

The results obtained in the present work confirmed that most phenolics occurred in an insoluble form (> 85%) in BMT and WMT, while soluble phenolics contributed only to < 15%. These results agree with those of other authors who reported that most phenolic compounds in maize are found in an insoluble form representing approximately 85% of

the total phenolic content (Mora *et al.*, 2010; Gaxiola *et al.*, 2017).

In addition, phenolic intake (mg/day) was also calculated based on the per capita consumption of tortilla (Table 1). The results revealed that even an average consumption of WMT and BMT contribute to a higher amount of natural antioxidants (323.44 and 437.33 mg phenols/day) than the daily recommended intake of vitamins A, E, and C (84.75 mg/day), which are the most commonly consumed antioxidants naturally present in foods (FAO, 2021). Therefore, the results of the present work could be considered to promote the consumption of white and blue maize products as alternative antioxidant sources.

Antioxidant activity of tortillas

In the present work, free and bound phenolic extracts of traditional tortillas made from commercial flours were analyzed by two *in vitro* antioxidant assays. In ORAC and ABTS assays, insoluble-bound phenolics showed the highest values for BMT (3,999.64 and 1,118.96 µmol TE/100 g) and WMT (3,530.57 and 861.10 µmol TE/100 g), respectively (Figure 1). These results agree with other authors who observed that insoluble phenolics are the main antioxidant contributors with approximately 85% of the total antioxidant content in maize (Gaxiola *et al.*, 2017).
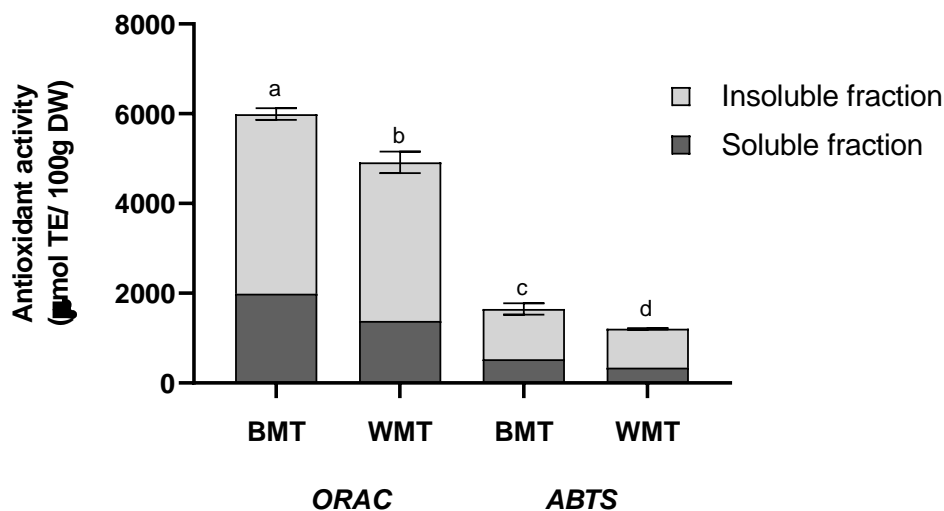

Figure 1. Antioxidant activity of soluble, insoluble, and total phenolic fractions in white and blue maize tortillas. Results are the average of at least three independent experiments. Results are expressed as µmol Trolox equivalents (TE)/g DW (dry weight). Error bars correspond to the total antioxidant activity. Bars with different lowercase letters are significantly different ($p \leq 0.05$). WMT = white maize tortilla; BMT = blue maize tortilla.

BMT showed the highest values in both assays. However, significant differences were only observed in ORAC ($p = 0.003$). This is in accordance with Colín *et al.* (2020) who reported that tortillas produced from blue maize contained a higher antioxidant activity than their counterparts produced from white maize. On the other hand, it is well known that blue maize is a great source of phenolics (Mora *et al.*, 2010; Gaxiola *et al.*, 2017). It is crucial to keep in mind that the insoluble fraction in cereals is covalently conjugated to the food matrix, and not bioaccessible during intestinal digestion (around 90% of total phenolics). Therefore, to exert its health benefits, this fraction must undergo a fermentation process in the colon, where different microbial species are involved. As a result, lower molecular weight phenolics are obtained, becoming more absorbable. Also, some of their functional groups are exposed, thus helping with the scavenging of free radicals and other oxidant molecules, and contributing to the colonic environment by decreasing pH and inhibiting cancer development (Lafiandra *et al.*, 2014; Shahidi and Yeo, 2016).

Phenolic release during in vitro fermentation

With its symbiotic microbiota, the colon is an active site where complex phenolic compounds are hydrolysed into smaller molecular weight phenolics, thus allowing them to be more bioaccessible and absorbable (Shahidi and Yeo, 2016).

During *in vitro* fermentation with human faecal suspensions, an important microbial metabolic or catabolic activity was detected, which resulted in the degradation of most of the naïve phenolic compounds within 5 h of incubation.

The release of phenolic compounds during *in vitro* fermentation was donor-dependent. From 0 to 5 h, the amounts of phenolics released showed values from 0.01 to 13.4 mg GAE/g (Table 2). It is also important to mention that the results obtained in the present work demonstrated that the phenolics trapped in the cell-matrix were released in higher proportions by colon microbiota than by chemical methods. This is in agreement with Babbar *et al.* (2014) who mentioned that the polarity of organic solvents together with the phenolic solubility properties can generate interferences during the phenolic release process.

At the initial incubation period (0 h), some degree of biotransformation, which may have been initiated during the first contact with the faecal inoculum, was revealed. After 1 h of fermentation, significant differences ($p \leq 0.05$) were observed between individuals, where donor A showed the highest released in WMT (3.83 mg GAE/g).

In contrast, the rest of the donors (B, C, and D) showed higher ($p \leq 0.05$) phenolic contents in BMT. These differences may be associated with the characteristics of the phenolics present in tortillas, such as types and proportions, and whether they occur in soluble or conjugated forms. Previous studies also pointed out that one of the most significant limitations to study phenolic metabolism in humans is the high interindividual microbiota variations (Faraldo *et al.*, 2019).

Interestingly, at 5 h of fermentation, we noticed significant differences ($p \leq 0.05$) in the amount of phenolics released between genders, where female donors (B and C) showed the lowest values in WMT and BMT (Table 2). Gender differences in gut microbiota composition remain unclear. While some authors suggested that gender has no direct effect on microbiota variations, other authors have reported inconclusive evidence to support how sex hormones may influence the commensal microbial community (Org *et al.*, 2016).

Overall, these results suggested that individual variations in gut microbiota composition influenced the formation of phenolic metabolites. Although diet is one of the strongest factors that modulate gut microbiota, it is important to emphasise that genetics and the environment also influence the microbiome. Therefore, metabolite concentrations might differ among subjects with the same diet. In fact, this could probably explain the contrasting results from different sources that evaluate the colonic fermentation process using human faeces (Willson and Situ, 2017).

Bioaccessibility of phenolic compound after in vitro fermentation

Despite all health benefits phenolics have to offer, their simple quantification is insufficient to demonstrate their functionality. In this context, bioaccessibility assessment is critical to estimate if a compound is available for absorption and health benefit exertion after passing though different digestion stages (Shahidi and Yeo, 2016; Blancas *et al.*, 2018). Table 2 shows the bioaccessibility of WMT and BMT after *in vitro* fermentation. The data

Table 2. Release of phenolic compounds and percentage of bioaccessibility from different maize tortillas flour after colonic fermentation *in vitro*.

Individual	WMT						BMT					
	0 h	(%)	1 h	(%)	5 h	(%)	0 h	(%)	1 h	(%)	5 h	(%)
A	0.01 ± 0.0[azA]	ND	3.83 ± 0.0[ayA]	67.8	13.4 ± 0.0[axA]	88.4	0.01 ± 0.0[bzA]	ND	3.17 ± 0.0[ayB]	54.8	13.4 ± 0.4[axA]	84.7
B	0.02 ± 0.0[azA]	ND	1.54 ± 0.0[bcyA]	44.0	6.12 ± 0.2[dxA]	77.3	0.02 ± 0.0[bzA]	ND	1.72 ± 0.1[cyA]	37.5	6.49 ± 0.1[cxA]	72.3
C	0.05 ± 0.0[azA]	ND	1.64 ± 0.0[byB]	45.8	7.67 ± 0.0[cxB]	81.2	0.05 ± 0.0[abzA]	ND	1.89 ± 0.0[cyA]	40.2	11.6 ± 0.1[bxA]	82.7
D	0.06 ± 0.0[azA]	ND	1.41 ± 0.0[cyB]	41.7	9.14 ± 0.1[bxB]	83.7	0.08 ± 0.0[azA]	ND	2.93 ± 0.1[byA]	52.6	12.9 ± 0.4[axA]	84.2

Means values ± standard error at 0,1 and 5 hours (h) post incubation. Bioaccessibility (%) values are expressed in percentages. Results are the average of at least three independent experiments. Phenolic compounds are expressed as milligram gallic acid equivalent (GAE)/g DW (dry weight). A-D = faecal sample donors, WMT = white maize tortilla, BMT = blue maize tortilla. ND = no detected. [abc] Means in the same column for each individual at the same time for the same tortilla with different low case letters are different ($p < 0.05$). [xyz] Means in the same line at each time for the same tortilla with different low case letters are different ($p < 0.05$). [ABC] Means for each time comparing both tortillas with different upper-case letter are different ($p < 0.05$).

showed that after 1 h of fermentation, up to 35% of phenolics were bioaccessible. However, the highest bioaccessibility was observed after 5 h (up to 70%) in both tortillas.

These results are lower than those reported by other authors, with bioaccessibility values greater than 100% from maize and bean chips (Luzardo *et al.*, 2017). These differences could be related to the various techniques of the transformation of maize products, and their phenolic profiles.

Unfortunately, documentation related to phenolics released by human microbiota during *in vitro* fermentation of white and blue maize tortillas is almost non-existent, which becomes a limitation that should be considered when discussing the results obtained in the present work. However, a study by Kroon *et al.* (1997) reported that over 95% of ferulic acid, a phenol present in most cereals, was released during colonic fermentation in wheat, while only 2.6% was released in gastric and small intestinal digestion, thus highlighting the impact of colonic microbiota in the release of this particular phenolic acid. It is pertinent to mention that further experiments are required to determine the levels and profiles of phenolic acids released during the simulated fermentation. Nevertheless, previous reports have determined the presence of six major phenolic acids in raw maize and tortillas: ferulic, *p*-coumaric, *p*-hydroxybenzoic, vanillic, syringic, and sinapic acids (Shahidi and Yeo, 2016; Gaxiola *et al.*, 2017; Luzardo *et al.*, 2017).

Kinetics of release of phenolic compounds

Phenolic compound release kinetics were considered to complement the study of *in vitro* colonic fermentation by human microbiota on insoluble phenolics present in WMT and BMT (Figure 2). No significant differences ($p \leq 0.05$) in the release of phenolics from either WMT or BMT were observed. In both types of tortillas, a constant release of phenolics during the digestion process was observed. The total release rates of phenolics in WMT and BMT were 1.8 and 2.2 mg GAE/h, respectively. It is important to mention that this assessment, along with bioaccessibility, estimates the expected length of time in which the insoluble phenolics are released from the food matrix once they interact with the colonic microbiota, becoming absorbable, and being able to circulate in the bloodstream (Blancas *et al.*, 2018).
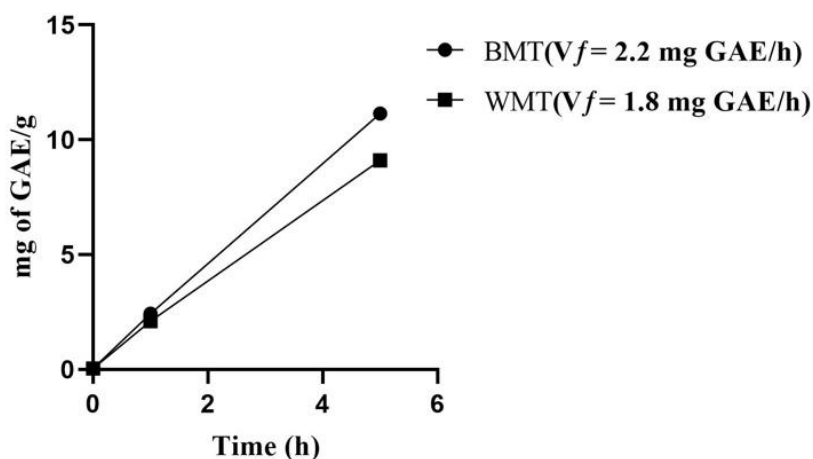

Figure 2. Phenolic compound release kinetic during colonic fermentation *in vitro*. Means values ± standard error at 0, 1, and 5 hours (h) post-incubation. Results are the average of at least three independent experiments. Phenolic compounds are expressed as milligram gallic acid equivalent (GAE)/g DW (dry weight). WMT = white maize tortilla; BMT = blue maize tortilla.

Antioxidant activity during in vitro fermentation

Phenolics constitute one of the most important bioactive compounds with antioxidant and chemopreventive activity. Therefore, fermented metabolites with antioxidant properties may protect the colon against oxidative damage (Shahidi and Yeo, 2016).

In the present work, the antioxidant capacity of the release of the phenolic compounds by human microbiota was determined by ORAC and ABTS assays. As shown in Table 3, the antioxidant activity varied based on the assay type performed. As previously mentioned, the results were donor-

Table 3. Antioxidant activity by ORAC and ABTS assays from different maize tortillas flour after colonic fermentation *in vitro*.

Individual	WMT						BMT					
	0 h		1 h		5 h		0 h		1 h		5 h	
	ORAC	ABTS	ORAC	ABTS	ORAC	ABTS	ORAC	ABTS	ORAC	ABTS	ORAC	ABTS
A	0.7 ± 0.0^{uzB}	0.03 ± 0.0^{uzA}	119.0 ± 7.6^{byA}	27.8 ± 0.1^{axA}	426.7 ± 24.0^{bxB}	27.4 ± 0.0^{ayB}	0.9 ± 0.0^{czA}	0.03 ± 0.0^{czA}	121.5 ± 6.8^{byA}	26.3 ± 0.2^{ayB}	527.6 ± 30.2^{cxA}	28.9 ± 0.2^{bxA}
B	0.6 ± 0.0^{bzB}	0.03 ± 0.0^{uzA}	156.4 ± 13.2^{ayB}	19.2 ± 0.1^{cyB}	804.6 ± 31.2^{axB}	24.4 ± 0.2^{cxA}	0.8 ± 0.0^{bzB}	0.03 ± 0.0^{bzB}	197.6 ± 6.7^{ayA}	22.1 ± 0.1^{cyA}	880.7 ± 34.9^{axA}	24.4 ± 0.1^{cxA}
C	0.7 ± 0.0^{uzB}	0.02 ± 0.0^{bzB}	66.8 ± 4.4^{cyA}	12.7 ± 0.1^{dyB}	243.5 ± 19.8^{dxA}	16.8 ± 0.1^{dxA}	0.8 ± 0.0^{bzA}	0.03 ± 0.0^{uzA}	59.1 ± 0.9^{cyA}	13.4 ± 0.0^{dyA}	208.3 ± 5.4^{dxB}	13.9 ± 0.3^{dxB}
D	0.7 ± 0.0^{uzA}	0.03 ± 0.0^{uzB}	47.2 ± 4.9^{dyB}	24.1 ± 0.2^{byB}	246.6 ± 15.2^{cxB}	25.5 ± 0.3^{bxD}	0.9 ± 0.0^{uzA}	0.03 ± 0.0^{bzA}	141.1 ± 12.7^{byA}	24.6 ± 0.2^{bxA}	735.7 ± 35.4^{bxA}	30.7 ± 0.0^{axA}

Means values $\pm$ standard error at 0, 1 and 5 hours (h) post incubation. Means in a column for each component with different letters are significantly different ($p < 0.05$). Results are the average of at least three independent experiments. Values are expressed as μmol Trolox equivalent (TE)/g DW (Dry weight). A-D = faecal sample donors, WMT = white maize tortilla. BMT = blue maize tortilla. [abc] Means in the same column for each individual in the same antioxidant assay with different low case letters are different ($p < 0.05$). [xyz] Means in the same line at each time and same assay for same tortilla with different low case letters are different ($p < 0.05$). [ABC] Means for each antioxidant assay and time comparing both tortillas with different upper-case letter are different ($p < 0.05$).

dependent. Throughout the fermentation, the antioxidant activity was significantly increasing ($p \leq$ 0.05) in ORAC (0.6 to 804.6 and 0.8 to 880.7 TE/g) and ABTS (0.02 to 27.4 and 0.03 to 30.7 TE/g), in WMT and BMT, respectively.

Similar results were reported by Burgos *et al.* (2018) who studied Chilean currants, and reported an increase in antioxidant activity at 8 h post-fermentation. In contrast, we observed only a slight increase at 5 h in WMT and BMT during ABTS assay with no significant differences. These results agree with Wootton *et al.* (2011) who observed an increase in ABTS values from 23 commercial vegetable juices after an *in vitro* digestion model. Likewise, Chandrasekara and Shahidi (2012) showed higher ABTS values after an *in vitro* gastric and intestinal digestion using millet grains.

The differences in the antioxidant activity can be partly explained by the method principles. Each of these assays is based on one feature of the antioxidant activity. ORAC assay measures the radical chain-breaking ability of antioxidants by monitoring the inhibition of peroxyl radical-induced oxidation. Meanwhile, ABTS measures the ability of antioxidant to scavenge free radicals. Therefore, it is almost impossible to evaluate the antioxidant properties of food material using a single method, which provides the most basic information about the antioxidant properties; but a combination of methods can define them in detail (Číž *et al.*, 2010; Salinas *et al.*, 2017).

Conclusion

In general, traditional tortillas made from commercial white and blue maize flours showed considerable differences in nutritional compositions. Blue maize tortilla noticeably exhibited higher anthocyanin, total phenolic content, phenols intake, and antioxidant activity. On the other hand, phenolic release, bioaccessibility, and antioxidant activity resulting from the *in vitro* colonic fermentation were donor-dependent, and increased during the fermentation. This is the first study showing the effect of diverse human microbiota on the release of phenolic compounds and their antioxidant activity in traditional tortillas made from commercial white and blue maize flours, thus suggesting that their consumption could positively affect colonic health. This evidence highlights the importance of colonic microbiota in improving the bioaccessibility of insoluble phenolics present in tortillas. Further studies must determine phenolic acid profiles and their concentration released during simulated fermentation using human microbiota.

Acknowledgement

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