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ORIGINAL ARTICLE

Release of health-related compounds during in vitro gastro-intestinal digestion of okara and okara fermented with *Lactobacillus plantarum*

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Abstract Okara is a highly perishable by-product remaining after filtration of the smashed soybeans seeds in the production of soymilk. Due to its nutritional value, different approaches have been developed to use it as functional ingredient. Fermentation of okara appears as an interesting strategy to preclude spoilage, providing a more stable matrix to be incorporated in the formulation of functional foods. Okara has antioxidant compounds but the effect of fermentation, and their bioaccessibility still need to be investigated. To achieve this aim, the phenolic compounds (as determined by TPC and TFC assays) and the antioxidant properties (as determined by $ABTS^{\cdot+}$, $DPPH^{\cdot}$, $O_2^{\cdot-}$ assays) of okara and okara fermented with *Lactobacillus plantarum* CIDCA 83114 were assessed both before and after exposure to simulated gastro-intestinal conditions. Before digestion, okara showed higher values of TPC and TFC than the fermented counterpart. Although a decrease of TPC and TFC was observed after exposing okara to gastric conditions, no significant differences between okara and fermented okara were detected. No further decrease of TPC were observed in intestinal conditions. Okara showed higher antioxidant activity than fermented okara. There was a considerable decrease in the antioxidant activity for both samples when exposed to gastric and intestinal conditions. A good correlation between TFC and antioxidant activities was detected, suggesting that flavonoids play an important role as antioxidants. As a whole, this work provides a solid support for the stability of phytochemicals along the digestive process of both okara and fermented okara.

Keywords Okara · Fermentation · *Lactobacillus plantarum* · Antioxidants · Polyphenols · In vitro digestion

Abbreviations

TPC	Total phenolic content
TFC	Total flavonoid content
GAE	Gallic acid equivalents
RUE	Rutin equivalent
Trolox	6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
TE	Trolox equivalents
$ABTS^{\cdot+}$	2,2′-Azinobis-(3-ethylbenzthiazoline-6-sulfonic acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl
$O_2^{\cdot-}$	Superoxide anion

Introduction

Okara is a white-yellowish residue produced in large quantities, after filtration of the mashed soybeans in the production of tofu or soymilk. It is generally treated as an industrial waste with little market value because of its high perishability. However, the okara residue still contains (in dry basis) 21% proteins of high nutritional value, 55% whole fiber, 1.5% ash and 13–14% fats and oils (polyunsaturated fatty acids) (Quintana et al. 2017; Isanga and Zhang 2008). The presence of health beneficial compounds, mainly represented by phenolics, converts okara in an interesting source of bioactive substances. As phenolics

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have been associated with different biochemical and pharmacological properties (i.e., antiviral, antioxidant, anti-inflammatory and anticancer activities), their occurrence in food matrices is of great relevance.

Taking into account both the nutritional value of okara and its disposal problem, different approaches have been developed to add value to it (Vong and Liu 2016). Okara itself has been increasingly used in the formulation of different food products (Radočaj and Dimić 2013; Jang et al. 2019). However, the concentration of bioactive compounds effectively released from the food matrix into the gastro-intestinal tract and available for absorption (bioaccessibility) might not be comparable to that present in the food matrix (Palafox-Carlos et al. 2011).

This underlines the importance of determining bioaccessibility of bioactive compounds in the food matrix after being consumed (Cilla et al. 2018). Hence, to get an insight into the potential benefits of okara phenolic compounds on human health, it becomes necessary to determine not only how processing affects their stability but also how the digestion process does, as temperature, pH and digestive enzymes expose polyphenolic compounds to physico-chemical changes that may result in changes of their bioaccessibility (Gullon et al. 2015; Vital et al. 2018). It is important to assess the stability and absorption of these compounds when exposed to digestive conditions, to better understand their potential biological properties (Wang et al. 2016; Santos et al. 2018).

Fermentation of okara appears as an interesting strategy to preclude spoilage, thus providing a more stable matrix that can also be incorporated as functional ingredient (Quintana et al. 2017; Espinosa-Martos and Rupérez 2009; Vong and Liu 2016). Fermentation with yeasts, fungi or bacteria is an efficient strategy to enhance the antioxidant properties of different food products, namely soymilk, soybeans, black beans or chickpeas (Lee et al. 2008; Marazza et al. 2012; Xiao et al. 2014), also playing an important role in the release, transformation and absorption of bioactive compounds during gastro-intestinal digestion (Parada and Aguilera 2007). In particular, fermenting okara with *Saccharomyces cerevisiae* increases the phenolic contents and the antioxidant capacity, probably due to the hydrolysis of glycosides (mainly of isoflavones) to the corresponding aglycones (Santos et al. 2018; Marazza et al. 2012). Although okara has also been fermented with lactic acid bacteria before, experiments were mainly focused on the effect of these microorganisms on the consumption and hydrolysis of fiber (Espinosa-Martos and Rupérez 2009; Pérez-López et al. 2016), and the outcome of fermentation on the antioxidant properties and bioaccessibility of polyphenols was scarcely addressed.

For this reason, the goal of this work was to evaluate the influence of fermentation on the bioaccessibility of okara antioxidants. To achieve this aim, the phenolic compounds (total phenolic and total flavonoid compounds, TPC and TFC, respectively) as well as the antioxidant properties ($ABTS^{\cdot+}$, $DPPH^{\cdot}$, $O_2^{\cdot-}$) of okara and okara fermented with *Lactobacillus plantarum* CIDCA 83114 were determined both before and after exposure to simulated gastro-intestinal conditions. *L. plantarum* CIDCA 83114 is a specially interesting strain because of its technological and inhibitory properties. In fact, it has demonstrated inhibitory properties against *E. coli* O157:H7, *Shigella* and *Salmonella* (Golowczyc et al. 2011; Kakisu et al. 2013). From a technological viewpoint, its behavior towards dehydration processes has been assessed, and it has also been incorporated into functional foods exposed to high temperatures (Tavera-Quiroz et al. 2015). More specifically, it was demonstrated that it is able to grow in okara (Quintana et al. 2017) and be encapsulated using okara oil as emulsifier (Quintana et al. 2018). Besides the potential effect on antioxidant properties, fermentation of okara with lactic acid bacteria may contribute to improve its sensorial properties, avoiding spoilage and improving its nutritional value.

Materials and methods

Materials

Okara was obtained from Soyana S. H. (San Martin, Argentina). Sulfuric acid, methanol, chloroform, acetone, butanol, isooctane, ethanol and isopropanol (Merck, Darmstadt, Germany), sodium thiocyanate, ferrous chloride and cumene hydroperoxide (Sigma-Aldrich, St. Louis, MO, USA), MRS broth (Biokar Diagnostics, France) and agar (Parafarm, Argentina) were used.

Folin-Ciocalteu's phenol reagent, calcium chloride (99–105%), potassium chloride (99.5–100.5%), and potassium acetate (N99.5%) were purchased from Panreac (Barcelona, Spain). 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$), 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Fluka (Lisbon, Portugal). N-(1-naphthyl)ethylene-diamine dihydrochloride (≥ 98%), phenazine methosulfate (PMS, ≥ 90%), sulfanilamide (≥ 99%), β-nicotinamide adenine dinucleotide reduced form (≥ 94%), potassium persulfate (99%), hydrochloric acid (37%), potassium dihydrogen phosphate (99.5%), disodium hydrogen phosphate (99%), ammonium chloride (99.8%), sodium carbonate, mucin (type II; from porcine stomach), α-amylase (porcine pancreas, type VI-B), pepsin (porcine gastric mucosa), pancreatin (porcine pancreas), lipase (type II; from porcine pancreas) and porcine bile extract (contains

glycine and taurine conjugates of hydrodeoxycholic acid and other bile salts) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Nitroblue tetrazolium chloride (NBT, 90%) was acquired from Acros Organics (Geel, Belgium) and o-phosphoric acid (85%) from BDH AnalaR (Poole, Finland), Magnesium chloride hexahydrated (99%) was purchased from Riedel–de Haen (Seelze, Germany). Urea (99%) was acquired from Merck (Darmstadt, Germany). Ethylenediaminetetraacetic acid (N99%) was acquired from Merck (Darmstadt, Germany).

Methods

Preparation of samples

Immediately after being received, okara was centrifuged five times to remove the excess of water. The sediment was frozen at − 80 °C for 48 h and freeze-dried on a Heto FD4 equipment (Heto Lab Equipment, Denmark) for 48 h (temperature of condenser: − 50 °C; chamber pressure: 0.04 mbar). Freeze-dried okara was suspended in distilled water (5% w/v) and autoclaved for 15 min at 121 °C. *L. plantarum* CIDCA 83114 was isolated from kefir grains (Garrote et al. 2001) and maintained frozen at − 80 °C in 120 g/L non-fat milk solids (Difco, USA). Microorganisms were cultured twice in MRS broth (de Man et al. 1960) at 37 °C in aerobic conditions. Cultures in the stationary phase ($\sim 1\times10^{13}$ CFU/mL) were used to inoculate 100 mL of okara suspensions (concentration of inoculum, 2% v/v). Cultures were incubated at 37 °C for 24 h, with shaking (stationary phase) (Quintana et al. 2017). Cultures were harvested by centrifugation at 8000 rpm for 10 min at 4 °C. Supernatants were removed and the sediments were freeze-dried following the same procedure as for okara. Therefore, two different types of samples were obtained, namely freeze-dried okara and freeze-dried fermented okara.

In vitro digestion

An in vitro gastro-intestinal digestion procedure was used to simulate the human digestion process, using the methodology described by Pinto et al. 2017, with slight modifications. The detailed composition of digestive juices (saliva, gastric, intestinal, and bile) is provided in Table 1, and mimics that of each of the simulated gastro-intestinal compartment, with site specific salts, enzymes, and pH. The process was sequential and was divided into three stages, namely oral, gastric and intestinal digestion. Several experiments were conducted, with different incubation times, to simulate gastric (oral + gastric steps) or total (oral + gastric + intestinal steps) digestions. Two independent replicated digestions were performed for each step.

In 100 mL flasks, approximately 2.5 g of freeze-dried material (see "Preparation of samples" section) was mixed with 15 mL of simulated saliva solution and incubated in a shaker (37 °C) with agitation (190 rpm) (MaxQ 4000, Thermo Scientific, USA) for 5 min, protected from light. Then, 30 mL of gastric solution were added to the flask. Samples were incubated for 2 h at 37 °C under continuous shaking (gastric digestion stops here). Afterwards, 30 mL of duodenal and 15 mL of bile solutions were added, and samples were incubated for another 2 h at 37 °C (total digestion).

In parallel assays, *L. plantarum* CIDCA 83114 in the stationary phase ($\sim 1\times10^{13}$ CFU/mL) was incorporated into the intestinal medium for 2 h at 37 °C under continuous shaking, to investigate the effect of a potential intestinal probiotic strain on the phytochemicals.

At the end of each digestion experiment, samples were centrifuged (4000 rpm, 10 min), the supernatant was collected, freeze-dried (as described above) and stored until analysis.

Extraction of phytochemicals compounds

The extraction of phytochemicals from okara and fermented okara and from the digestion freeze-dried supernatants was carried out by homogenizing 5 g of each sample in 200 mL of 80:20 ethanol:water (extraction solvent). Samples were sonicated (Bandelix Sonorex, Germany) for 1 h at 25 °C (frequency 35 kHz, power 200 W) to facilitate the extraction of phytochemicals. This step was performed twice. Ethanol from homogenates was removed at 40 °C with a rotary evaporator (Büchi Rotavapor, Flawil, Switzerland). Then, the liquid extracts were freeze-dried to remove the remaining water and stabilize phytochemicals before analysis.

Total phenolic and flavonoid contents

Total phenolic content Total phenolic content (TPC) was determined according to Pinto et al. (2017), with slight modifications. Briefly, dry extracts were dissolved in ethanol, to achieve a final concentration of 100 mg/mL. Fifty µL of each extract solution were mixed with 1.25 mL Folin-Ciocalteu reagent (1:10 dilution with distilled water) and 1 mL of 7.5% w/v sodium carbonate solution. The mixture was incubated at 25 °C for 30 min and the absorbance at 765 nm was read (Perkin Elmer UV–Vis Lambda 2, Germany). TPC data was expressed as milligrams of gallic acid equivalents (GAE) per gram of dry extract (mg GAE/g dry extract), based on the gallic acid calibration curve (50–600 mg/L).

Table 1 Composition of simulated gastrointestinal juices. Adapted from Pinto et al. (2017)

Stock solutions	Saliva	Gastric	Duodenal	Bile
Distilled water	100 mL	100 mL	100 mL	100 mL
NaCl	11.70 mg	0.55 g	1.40 g	1.05 g
KCl	14.90 mg	0.16 g	0.11 g	0.08 g
$NaHCO_3$	0.21 g	–	0.68 g	1.16 g
$CaCl_2.H_2O$	–	0.08 g	–	–
NaH_2PO_4	–	0.053 g	–	–
KH_2PO_4	–	–	16.06 mg	–
NH_4Cl	–	0.061 g	–	–
$MgCl_2$	–	–	10.08 mg	–
Urea	0.04 g	0.02 g	0.02 g	0.05 g
Concentrated HCl	–	1.30 mL	0.03 mL	0.03 mL
Adjuncts	0.10 g mucin	0.50 g pepsin	1.80 g pancreatin	2.40 g bile salts
	0.21 g α-amylase	0.60 g mucin	0.30 g lipase	–
pH	6.8 ± 0.2	1.30 ± 0.02	8.1 ± 0.2	8.2 ± 0.2

Total flavonoid content Total flavonoid content (TFC) was determined using the aluminum chloride method, according to Pinto et al. 2017. In a 5-mL flask, 0.5 mL of extract solution (100 mg/mL in ethanol), 1.5 mL of methanol, 2.8 mL of distilled water, 0.1 mL of potassium acetate solution (1 M) and 0.1 mL of aluminum chloride (10% in methanol) solution were added and mixed. After incubation for 30 min at 25 °C (protected from light), the absorbance was read at 415 nm (Perkin Elmer UV–Vis Lambda 2, Germany). Results were expressed as milligrams of rutin equivalent (RUE) per gram of dry extract (mg RUE/g dry extract), based on the rutin calibration curve (10–200 mg/L).

In vitro antioxidant assays

ABTS radical cation ($ABTS^{\cdot+}$) scavenging assay The antioxidant activity was evaluated by the method of decolorization of $ABTS^{\cdot+}$ solution. First, 50 mL of ABTS aqueous solution (2 mM) was reacted with 200 µL of potassium persulfate solution (70 mM) (Re et al. 1999). This mixture was kept in the dark for at least 16 h at 25 °C and was stable in this form for 2 days.

The $ABTS^{\cdot+}$ solution was diluted with phosphate buffered saline solution (K_2HPO_4 0.144 g/L; NaCl 9.00 g/L; Na_2HPO_4 0.795 g/L, pH 7.4) (10 mM) (PBS) to an initial absorbance of 0.700 ± 0.021 at 734 nm, before use. This solution was freshly prepared for each analysis. For the evaluation of the radical scavenging activity, 40 µL of extract solution (100 mg/mL in ethanol) was added to 1.96 mL of $ABTS^{\cdot+}$ solution. PBS was used as a blank. The decrease of absorbance at 734 nm (Perkin Elmer UV–Vis Lambda 2, Germany) was measured during 6 min (Pinto et al. 2017). Results were expressed based on the Trolox calibration curve (0.5–0.7 mM) as micromol of Trolox equivalents per gram of dry extract (µmol TE/g dry extract).

$DPPH^{\cdot}$ radical scavenging activity Decolorization of the $DPPH^{\cdot}$ radical solution was determined according to Pinto et al. 2017. For each determination, 100 µL of extract solution (100 mg/mL in ethanol) was added to 3.5 mL of $DPPH^{\cdot}$ solution (0.06 mM in ethanol). Absorbance was measured at 516 nm (Perkin Elmer UV–Vis Lambda 2, Germany), after 30 min of reaction in the dark (25 °C). Ethanol was used as blank control. The radical scavenging activities were expressed as µmol TE/g dry extract, based on the Trolox calibration curve (0.2–1.2 mM).

Superoxide anion ($O_2^{\cdot-}$) radical scavenging activity Superoxide anion radicals ($O_2^{\cdot-}$) were generated by the NADH/phenazine methosulfate system, according to Pinto et al. 2017. In a 96 well-plate, 25 µL of extract solutions (100 mg/mL) were mixed with 200 µL of a solution composed of 0.1 mM EDTA, 62 µM nitroblue tetrazolium chloride and 98 µM NADH. The reaction was initiated by the addition of 25 µL of phenazine methosulfate (33 µM, containing 0.1 mM EDTA) to each well. All solutions were prepared in 0.1 M PBS. After 5 min, the absorbance was measured at 550 nm (Victor^3 1420 multilabel plate counter, Perkin-Elmer). For the individual blanks, buffer was used instead of phenazine methosulfate solution. Data were expressed as µmol TE/g dry extract, based on the Trolox calibration curve (4–40 mM).

Reproducibility of results

Statistical analysis was performed using SPSS Statistics software v.20 (IBM SPSS Statistics for Windows, IBM Corp., USA). Data of all analysis, in triplicate, are

expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) was performed to determine whether there are any statistically significant differences among parameters experimentally determined, followed by Tukey's HSD post hoc test. A 5% significance level was considered for all tests. Pearson correlation coefficients (r) were determined to corroborate relationships between TPC and TFC with the antioxidant activity. Correlation was significant for $p < 0.05$.

Results and discussion

Total phenolic compounds (TPC)

Figure 1 shows the content of TPC of okara and fermented okara before (just extraction) and after exposure to gastric and intestinal simulated conditions in the absence or presence of *L. plantarum* CIDCA 83114. Okara showed higher TPC values than fermented okara ($p < 0.05$). In a previous work, Sanjukta et al. 2015 reported that fermentation of soybeans with *Bacillus subtilis* results in a dramatic increase of phenolic contents (up to 78%), but okara is a very different matrix, a sub-product and many (variable) processes happen during its production. For instance, soybeans must be soaked and that would solubilize a great amount of the glycosides, so the polyphenolic contents is lower to start with. The fermentation includes a centrifugation step in which the supernatant is discarded and the sediment retained. Polyphenols were determined on the sediment only, so a lower recovery is achieved. The observed difference to published data can also be related to the use of different genera of microorganisms (*Bacillus subtilis* vs. *L. plantarum*), as *Bacillus subtilis* strains produce a large amount of enzymes, leading to an increase of free phenolic compounds and an improvement of their bioavailability. Moreover, *B. subtilis* strains are excellent cellulase producers (Sreen and Sebastian 2018; Singh et al. 2004), which could facilitate the release of phenolic compounds from okara fiber. On the contrary, there is no concluding information for *L. plantarum* in this regard, as most publications dealing with cellulolytic activity report cellulase production under certain circumstances (i.e., recombinant strains, bacterial consortia or specific substrates (Rossi et al. 2001; Morais et al. 2013; Frediansyah and Kurniadi 2017). Furthermore, a decrease in TPC has been reported for fruits and vegetables fermented with *L. plantarum* strains, although the matrices were different from okara (Hashemi and Mahmoodi 2017; Othman et al. 2009; Mousavia et al. 2013).

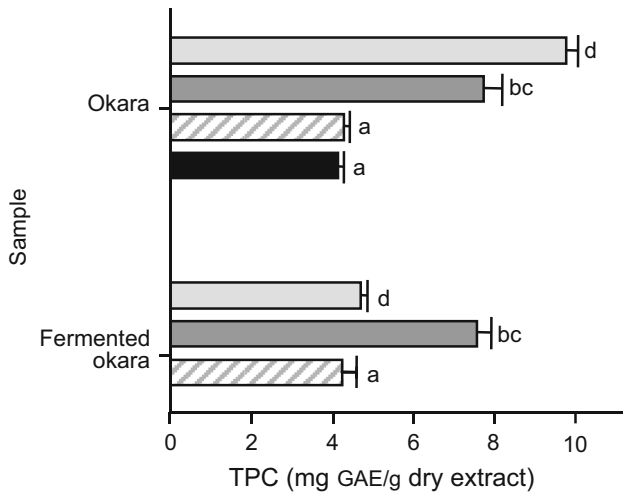

Fig. 1 Total phenolic content of okara and fermented okara before and after exposure to sequential simulated gastro-intestinal conditions. (1) fresh samples (before digestion, ▭); (2) after exposure to gastric conditions (▭); (3) after exposure to intestinal conditions (▨); (4) after exposure to intestinal conditions in the presence of *L. plantarum* CIDCA 83114 (■). Bars with the same pattern denote similar digestive conditions. Different letters indicate significant differences ($p < 0.05$)

TPC of the gastric digested okara is lower than the initial sample, meaning that the recovery of polyphenols was incomplete. It must be pointed out that the ethanol:water extraction of okara was performed on the whole matrix, where polyphenol are supposed to be bound to proteins, polysaccharides and/or lignins. Digestion leads to the formation of a liquid phase (simulating the chyme) and a solid phase containing all the debris (insoluble materials, enzymes…). Polyphenols will be partitioned between the two. Gullon et al. (2015) determined the percentage of recovery on pomegranate peel flour, evaluating TPC on both the solid and the liquid phase and adding the two, obtaining values close 100% recovery after gastric digestion. However, the polyphenols in the solid part were not bioaccessible, i.e., released from the food matrix and solubilized, so in the present work they were not determined. The observed decrease in TPC should be a balance between a lower amount caused by poor recovery and the expected higher amount of unbound polyphenols. This decrease will be also reflected on TFC and on the antioxidant properties.

The TPC recovery of okara samples after gastric digestion was 79% of the pre-digested samples. Similar results were reported for phenolic compounds released from soybean meal (extruded soybeans from oil refinery industries) after gastric digestion (Freitas et al. 2019). However, the gastric digestion of soymilk increases (70–152%) the phenolic contents after gastric phase (Ma et al. 2014; Rodríguez-Roque et al. 2013). It seems that the extraction of phenolic compounds following simulated gastro-intestinal digestion is mainly achieved during the gastric phase. The exposure to low pH and enzymatic

activity (pepsin) favors the hydrolysis of phenolic compounds bound to other matrix components (proteins, carbohydrates, celluloses, lignin), therefore improving their extraction (Ma et al. 2014; Rodríguez-Roque et al. 2013; Freitas et al. 2019). Based on these results, the release of phenolic compounds under gastric digestion seems to be dependent on the pH and on the food matrix. In this work, the release of phenolic compounds from okara represented a lower percentage than that of soymilk (Ma et al. 2014; Rodríguez-Roque et al. 2013), which is probably related to the soybeans matrix characteristics. In what concerns fermented okara, it is interesting to note that although before digestion it had a significantly lower TPC compared with okara, when exposed to gastric conditions such content was comparable to that of okara samples exposed to the same conditions, apparently closer to the literature data for soymilk. The simultaneous presence of *L. plantarum* and gastric fluids may facilitate the solubility of polyphenols into the aqueous ethanol extracting solvent.

After exposure to intestinal conditions, TPC significantly decreased for all the samples (32–45%). Again, no differences were observed in the phenolic content between samples ($p > 0.05$; slash gray bars in Fig. 1). Similar results of TPC were observed in other works (Ma et al. 2014; Freitas et al. 2019) for soybean meal and soymilks after intestinal digestion in comparison to the gastric phase. Rodríguez-Roque, et al. (2013) reported a bioaccessibility of 16% for total flavonoids present in soymilk and found that total phenolic acids were not bioaccessible after gastrointestinal digestion. Incorporating *L. plantarum* CIDCA 83114 strain into the intestinal medium had no influence in the TPC of okara (black bars in Fig. 1). Phenolic compounds are highly unstable in the alkaline conditions of the small intestine. In such conditions, vegetal structures are degraded, favoring the release of phenolic compounds present in okara, namely *p*-hydroxybenzoic acid, salicylic acid, *p*-coumaric acid and ferulic acid (Kim et al. 2006). The intestinal environment promotes several changes in their chemical structure/weight (hydrolysis, oxidation, epimerization and even degradation), which affects their bioavailability (Bermúdez-Soto et al. 2007; Bouayed et al. 2011).

Total flavonoid content (TFC)

Figure 2 shows the effect of in vitro gastro-intestinal digestion on the TFC. Before digestion, okara showed the highest TFC among samples (Fig. 2). The considerations presented on the discussion of TPC also apply here, after all flavonoids are polyphenols compounds, and reportedly the most abundant type.

After exposing okara samples to simulated gastric conditions, again a decrease of TFC was observed (dark gray bars, Fig. 2). It is interesting to note that the trend observed in fermented okara (lower contents than okara before digestion, increasing with exposure to gastric conditions attaining values that were comparable with those of non-fermented okara exposed to the same conditions) observed for TPC was also observed with TFC (Figs. 2), reinforcing the discussion previously presented. Contradictory literature data were found about this issue. It was reported that the flavonoids content of soybean meal (analyzed by HPLC) decreases 21.6% during the gastric phase (Freitas et al. 2019). Other authors informed an increase of TFC of soymilks after gastric digestion (4–33%) (Ma et al. 2014; Rodríguez-Roque et al. 2013). These contradictions have been discussed in terms of higher or lesser extension on the hydrolysis of glycosylated isoflavones by gastric juices but it seems that differences on experimental methodology and processing over food matrices may be more relevant. Those water soluble compounds should be extensively lost in the processes leading to okara obtention as a residual product. Also terminology may be part of the reported variations since some authors are indeed referring to bioaccessibility while others are mentioning recovery, not always distinguishing between the two concepts.

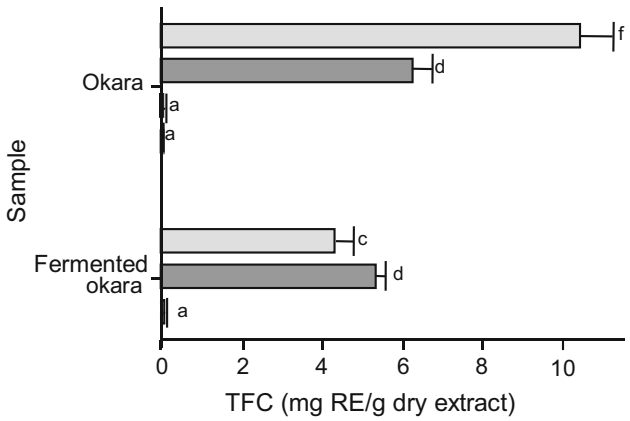

Fig. 2 Total flavonoid content of okara and fermented okara before and after exposure to sequential simulated gastro-intestinal conditions. (1) fresh samples (before digestion, ▭); (2) after exposure to gastric conditions (▭); (3) after exposure to intestinal conditions (▨); (4) after exposure to intestinal conditions in the presence of *L. plantarum* CIDCA 83114 (■). Bars with the same pattern denote similar digestive conditions. Different letters indicate significant differences ($p < 0.05$)

As in TPC, the exposure to intestinal conditions led to a dramatic decrease (> 97%) of TFC in both samples (slashed bars, Fig. 2). The presence of intestinal juices and bile might have affected the stability of soybeans flavonoids in the intestinal medium. The content of flavonoids in soybeans meal was reduced up to 57.93%, compared to gastric digestion (Freitas et al. 2019). Additionally, the presence of *L. plantarum* CIDCA 83114 in the intestinal medium did not lead to significant differences compared to samples

exposed to intestinal medium without bacteria (black bars, Fig. 2).

After the gastric and intestinal phases, the amounts of phenolic compounds, as determined by the TPC and TFC assays, were significantly lower than those determined before digestion. Flavonoids seem to be more affected (− 99%) by the harsh intestinal conditions than non-flavonoids compounds (the reduction was within 40–56%). A previous work reported that chemical extraction (using organic solvents) could lead to overestimation of the availability of phenolic compounds, when compared to gastro-intestinal extraction (Bouayed et al. 2011). The gastro-intestinal tract may be considered as an extractor where both the mechanical action and the chemical action during the digestive phase contribute to the solubilization of phenolic compounds. However, the amounts of extractable compounds were still significantly lower when compared to those obtained by chemical extraction (using 80% aqueous ethanol), which indicates incomplete release, difficult recovery or degradation of phenolic compounds (Bouayed et al. 2011). Phenolic compounds from soybeans and their by-products are usually extracted using different organic solvents (i.e., ethanol, methanol, acetone) or in combination with water in different proportions (Jankowiak et al. 2014a, b; Kumar et al. 2010; Freitas et al. 2019). Generally, phenolic contents obtained from water extractions are lower than those obtained with organic solvents. From an optimization study, 80% aqueous ethanol extracted up to 2.52 times more phenolic compounds in comparison with water (data not shown). Similar results were obtained by other authors (Jankowiak et al. 2014a, b). Ethanol promotes the release of phenolic compounds and reduces co-extraction of other components, leading to higher yields and purity (Jankowiak et al. 2014a).

Antioxidant activity

The phenolic content of plant material is correlated with their antioxidant activity (Ambawat and Khetarpaul 2018; Kumar et al. 2010; Ma et al. 2014). Figure 3a–c shows the evolution of antioxidant activity before and after in vitro gastro-intestinal digestion, as determined by the $ABTS^{\cdot+}$, $DPPH^{\cdot}$ and $O_2^{\cdot-}$ assays, respectively. Non-fermented okara showed greater antioxidant activity than the counterpart fermented (regardless of the assay) indeed paralleling the lower contents of polyphenols. Soybeans and their by-products have been shown to possess antioxidant activity (Ambawat and Khetarpaul 2018; Kumar et al. 2010; Ma et al. 2014; Freitas et al. 2019; Sanjukta et al. 2015; Vital et al. 2018) and fermentation of soybeans with *B. subtilis* was reported to increase the radical scavenging activity ($DPPH^{\cdot}$ and $O_2^{\cdot-}$) (3.1–24 folds) (Sanjukta et al. 2015).

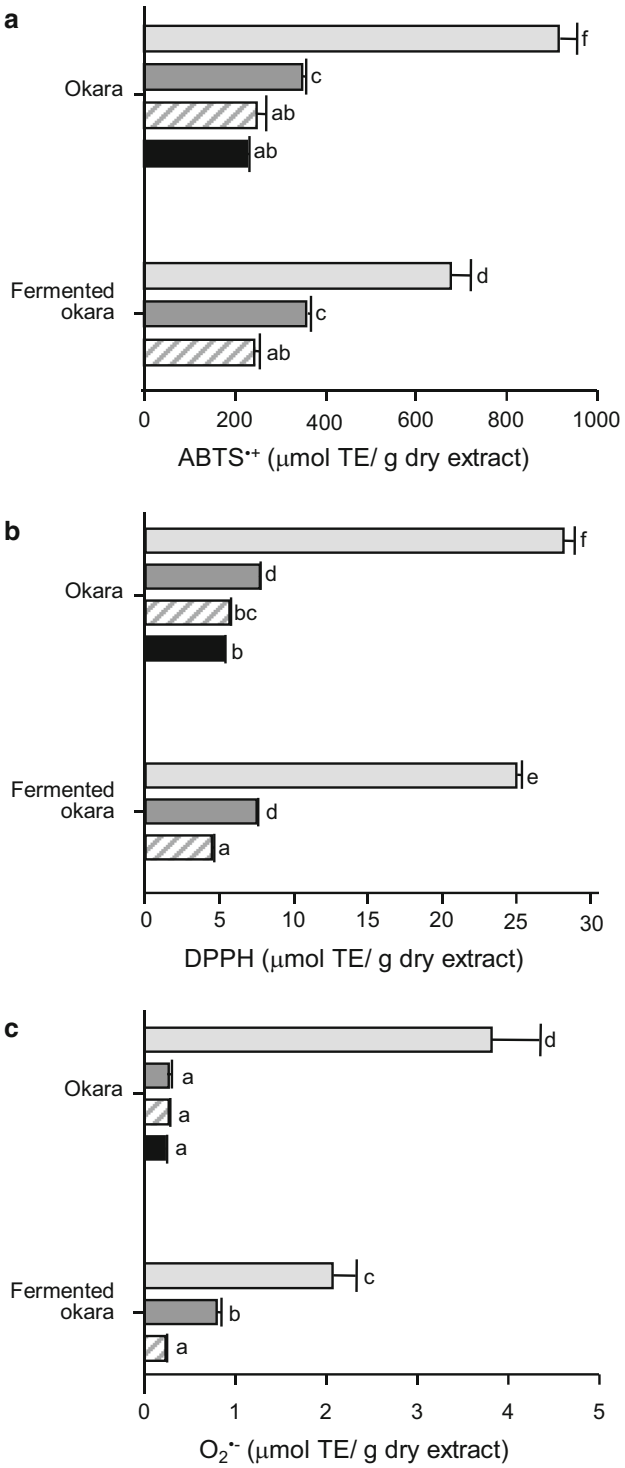

Fig. 3 Antioxidant activity assays of okara and fermented okara before and after exposure sequential to simulated gastro-intestinal conditions. (1) fresh samples (before digestion, ▭); (2) after exposure to gastric conditions (▬); (3) after exposure to intestinal conditions (▨); (4) after exposure to intestinal conditions in the presence of *L. plantarum* CIDCA 83114 (■). Bars with the same pattern denote similar digestive conditions. Different letters indicate significant differences ($p < 0.05$). **a** $ABTS^{\cdot+}$ scavenging assay on; **b** $DPPH^{\cdot}$ scavenging activity; **c** $O_2^{\cdot-}$ scavenging activity

The antioxidant activities were also significantly influenced by in vitro gastro-intestinal digestion. When exposed to gastric conditions, a drastic decrease of the antioxidant activity was observed. In fact, the antioxidant activity ($ABTS^{\cdot+}$ assay) of okara after exposure to gastric conditions was 38% of that before digestion. In turn, the remaining antioxidant activity as determined by the $DPPH^{\cdot}$ assay was 27% (Fig. 3b). Regarding the $O_2^{\cdot-}$ scavenging activity, it was the most affected one by gastric conditions, as in none of the cases it was greater than 8% of the values before digestion (Fig. 3c). After in vitro gastric digestion, the antioxidant activity of fermented okara was not significantly different from that of non-fermented okara, thus representing just a 53, 30 and 38% of the initial antioxidant activity as determined with the three assays (Fig. 3a–c).

Finally, the intestinal medium led to a further decrease of antioxidant activity. The presence of *L. plantarum* CIDCA 83114 in the intestinal medium did not lead to significant differences compared to samples exposed to intestinal medium without bacteria (Fig. 3a–c). These results are in agreement with other studies (Ma et al. 2014; Rodríguez-Roque et al. 2013; Freitas et al. 2019). For example, the antioxidant activity of soymilks (measured via $DPPH^{\cdot}$ assay) is reduced after simulated gastro-intestinal digestion (74–99%) (Ma et al. 2014; Rodríguez-Roque et al. 2013).

Overall, the decrease of antioxidant capacity observed in the present study can result from the considerable decrease of TPC and TFC. It seems that the stability and antioxidant activity of phenolic compounds depend on the physicochemical conditions of the digestive tract (pH, temperature and enzyme activities) as well as on the nature of the food matrix (Bouayed et al. 2011). It was reported that the acidic environment in the gastric phase enables the hydrolysis and the release of phenolic compounds linked to lignins, proteins and carbohydrates (Ma et al. 2014). The acid treatment mainly breaks down glycosidic bonds and solubilizes sugars, thus releasing phenolic compounds and increasing antioxidant activity (Freitas et al. 2019). However, this is not an equilibrium process and the nature of okara as a byproduct of food industry leads to its depletion of water soluble compounds during processing. The fibers present in high amounts interact strongly with these remaining compounds, leading to a decrease of their bioavailability and antioxidant activity. The loss of antioxidant activity under alkaline intestinal conditions might be attributed to the degradation of some phenolic compounds, that could also be transformed into different structural forms with less reactivity (Bermúdez-Soto et al. 2007).

Despite their simplicity, $ABTS^{\cdot+}$ and $DPPH^{\cdot}$ assays have been criticized for their lack of biological relevance, since they make use of artificial radicals (López-Alarcón and Denicola 2013). $O_2^{\cdot-}$ is a precursor of active free radicals that have potential to induce tissue damage by reacting with biological macromolecules (i.e., lipids, proteins and DNA) (Sanjukta et al. 2015). Hence, this assay seems to provide more relevant information since this radical is found in biological systems.

Table 2 Correlation coefficients (r) observed among TPC, TFC and in vitro antioxidant activity assays

Parameters	$ABTS^{\cdot+}$	$DPPH^{\cdot}$	$O_2^{\cdot-}$
TPC	0.627	0.644	0.622
TFC	0.883	0.816	0.842

In the present study, poor correlations between TPC and antioxidant activity assays were observed (Table 2). On the contrary, good correlations were found for TFC and the assayed free radicals scavenging activities. Similarly, the TFC of soymilk was also significantly correlated with the $DPPH^{\cdot}$ scavenging activity (Ma et al. 2014). The low correlations obtained with TPC could be related to the fact that the Folin-Ciocalteu reagent is not specific for phenolic compounds. Other substances (reducing sugars, vitamins, carbohydrates, aromatic amines, organic acids and proteins) can also reduce the reagent, thus leading to overestimated values (Rodríguez-Roque et al. 2013). Therefore, the observed correlations suggested that phenolic compounds, mainly flavonoid type, played an important role as antioxidants. In fact, according to literature, genistein, daidzein and glycitein are the main components of soybeans and okara (Jankowiak et al. 2014a, b; Freitas et al. 2019).

Conclusion

The food industry has an increasing number of disposal products, and this is particularly relevant in the case of okara, as 1 kg of processed soybeans generate about 1.1 kg of okara. Fermentation of okara with probiotic microorganisms represents a valuable strategy to add it value and avoid spoilage. The present work has shown that both okara and fermented okara are important sources of antioxidant compounds. The in vitro digestion procedure provided a simple and rapid method to assess the stability of phytochemicals. These results revealed that the content of bioaccessible health-related compounds was not very different from that of soybeans. Considering the large production of this by-product, fermenting it with *L. plantarum* CIDCA 83114 appears as an important strategy to add it value, by incorporating a probiotic strain and retaining the antioxidants content. As a whole, fermentation provides a cost-effective strategy, providing a functional ingredient with antioxidant properties, to be incorporated in the formulation of novel food products.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

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