Okara residue as source of antioxidants against lipid oxidation in milk enriched with omega-3 and bioavailability of bioactive compounds after in vitro gastrointestinal digestion

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Abstract The residue from soy processing (okara) was evaluated for phenolic compounds, flavonoids, radical scavenging activity and reducing power ability. The ability of okara to decelerate the oxidation of omega-3 fatty acids caused by light exposure in a milk matrix, as well as its bioavailability after in vitro digestion, were investigated. Okara contained phenolic compounds (106.7 mg gallic acid equivalents (GAE)/100 g) and flavonoids (32.7 mg quercetin equivalents/100 g) and showed antioxidant activity. The addition of okara to omega-3 fatty acids-enriched milk inhibited the production of conjugated dienes and malonaldehyde during 8 days of storage compared to the control (without okara), particularly at the higher (3 g/L) than lower inclusion concentrations studied (1 and 2 g/L). The bioavailability assay demonstrated that polyphenols (41 mg GAE/100 g) were still present after in vitro digestion and had antioxidant activity. Okara, which is considered a residue and is discarded, can potentially be used as an antioxidant ingredient for the enrichment of foods.

Keywords Natural antioxidant · Bioaccessibility · Photooxidation · Conjugated dienes · Thiobarbituric acid reactive substances

Introduction

The industrial practice of discarding food by-products leads to environmental problems and economic losses. Thus, the search for alternative uses and value aggregation has gained attention in recent years (Scialabba 2014). Soybean residue (okara) is the insoluble residue obtained after the manufacture of soy-based food products, such as bean curd (tofu) and soymilk, from which the water-soluble components are previously extracted (Vong et al. 2016).

Okara is commonly utilized for animal feed or discarded, due to its undesirable flavor, texture and high perishability. However, okara still has significant nutritious components: 40–60% carbohydrates, 20–30% protein and 10–20% lipid (all on a dry basis) (Li et al. 2012). Consequently, it is of great interest to add value to this by-product and find new alternatives to its use. For instance, the incorporation of wet okara into human foods is possible but still limited, whereas, dried okara can be used as an ingredient in various food products (Vong and Liu 2016).

Consumers are increasingly demanding beneficial health foods. Even though okara has been discarded in industrial processes, studies have indicated that this by-product is a source of bioactive compounds and presents prebiotic and antioxidant activity. It could, therefore, be valuable to improve and protect the gut environment and, as a dietary weight-loss supplement, it could benefit the plasma lipid levels (Préstamo et al. 2007; Villanueva-Suárez et al. 2013; Villanueva et al. 2011).

Antioxidants, such as phenolic compounds, can reduce the risk of non-communicable diseases (Granato et al. 2016). The omega-3 fatty acids can also be used in the prevention and treatment of diseases (Simopoulos 1991), being of great interest for human nutrition. However, the addition of omega-3s into processed products is limited by

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their high susceptibility to oxidative degradation during processing and storage. Lipid oxidation is one of the main non-microbial factors responsible for the quality deterioration in foods and consumer rejection (Palermo et al. 2012; Pouzo et al. 2016). The oxidized fatty acids and their degeneration products, when absorbed, are harmful to animals and humans and can cause adverse biological effects (Fennema et al. 2010).

The balance between pro-oxidant and antioxidant mechanisms largely influence the stability of lipid oxidation kinetics (Shahidi and Zhong 2010). In this context, the incorporation of okara into an omega-3-enriched product may act both in inhibiting the lipid oxidation of omega-3s (natural antioxidant) and, providing it is still available after the digestive process, function to protect the body against free radicals. The addition of omega-3s and antioxidants into dairy products, such as cheese, butter and yogurts has been studied (Giroux et al. 2008; Goodridge et al. 2001). The enrichment of dairy products with okara, as a source of antioxidants, may be an alternative to avoid lipid oxidation, reduce costs and develop functional products.

Therefore, the current study aimed to evaluate the antioxidant properties of soy residue (okara) in milk enriched with omega-3 fatty acids, as well as evaluate the bioaccessibility of okara's bioactive compounds after in vitro digestion.

Materials and methods

Material

Okara was kindly provided by a soy foods industry (Maringá, Paraná, Brazil). Samples were freeze-dried, ground to a powder and then stored protected from light at room temperature. Skim milk powder (Elegê, Brazil) and omega-3 fatty acids (linseed oil) were purchased from a local market. Folin–Ciocalteu reagent, gallic acid, quercetin, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), phosphate buffer, sodium carbonate and potassium persulfate were purchased from Sigma–Aldrich. Aluminum chloride, potassium ferricyanide and ferric chloride (FeCl₃) were bought from Vetec. All reagents used were of analytical grade.

Okara characterization

Extraction of okara for analysis of bioactive compounds and antioxidant activity

The okara powder was mixed with acetone (50%) for 30 min (Chung et al. 2010). The mixture was then

centrifuged (900×g; 10 min), and the supernatant was used directly for the measurement of total phenolic compounds (TPCs), flavonoid content (FC) and antioxidant activity.

Total phenolic compounds (TPC)

TPC were determined according to Singleton and Rossi (1965) with some modifications. An aliquot of the supernatant (125 µL) was mixed with 125 µL Folin–Ciocalteu reagent (1:1 ratio with deionized water) and 2250 µL sodium carbonate (28 g/L). The samples were incubated in the dark at 25 °C for 30 min, after which the spectrophotometric absorbance at 725 nm was recorded (Evolution™ 300, Thermo Scientific). A gallic acid (0–300 mg/L) standard curve was constructed, and the results were expressed as mg gallic acid equivalents (GAE)/100 g okara.

Flavonoid content (FC)

The FC was established by using the aluminum chloride method (Buriol et al. 2009) with modifications. Briefly, 300 µL of the extract was mixed with 150 µL aluminum chloride (5% w/v in methanol), and the volume made to 3000 µL with methanol. The mixture was left at room temperature in the dark for 30 min. The spectrophotometric absorbance was measured at 425 nm. A quercetin standard curve was prepared, and the results were expressed as mg quercetin equivalents (QE)/100 g okara.

Antioxidant activity

Ferric reducing antioxidant power (FRAP) assay

FRAP assay was conducted as described by Zhu et al. (2002), with slight modifications. An aliquot (0.25 mL) of the extract was mixed with 1.25 mL phosphate-buffered saline (0.01 mol/L, pH 7.4) and 1.25 mL of 1% potassium ferricyanide. The samples were incubated at 50 °C for 20 min and then cooled down at room temperature (20 ± 1 °C). Next, 1.25 mL of 10% trichloroacetic acid was added, followed by centrifugation at 900×g for 10 min. The supernatant was mixed with 500 µL of 0.1% FeCl₃, and the absorbance was measured at 700 nm. A gallic acid standard curve was established, and the results were expressed as mg GAE/100 g okara.

DPPH free-radical-scavenging assay

DPPH scavenging activity was assayed using the protocol detailed by Li et al. (2009) with slight modifications. The extract (200 µL) was mixed with 3800 µL of the

methanolic solutions containing 60 $\mu\text{mol/L}$ DPPH radicals. Each mixture was kept in the dark for 30 min, after which, the absorbance was measured at 515 nm against pure methanol (blank). The antioxidant activity was calculated by the following equation:

$$\text{DPPH radical scavenging activity (\%)} \\ = (1 - (A_t/A_0)) \times 100$$

where A_0 and A_t are the absorbances of the sample at time zero and 30 min, respectively.

ABTS free-radical-scavenging

The antioxidant activity of okara was also evaluated by its ability to scavenge ABTS radicals. Based on Re et al. (1999), $\text{ABTS}^{\bullet+}$ cations were generated from the interaction of 7 mmol/L ABTS (5 mL) with 140 mmol/L potassium persulfate (88 μL). The cations were incubated in the dark at room temperature for 16 h. The ABTS-activated radicals were diluted with ethanol to an optical density of 0.70 ± 0.02 . An aliquot of the sample (40 μL ; 1:1000 v/v methanol) was mixed with 1960 μL ABTS solution and the radical scavenging ability (%) was estimated, by recording the absorbance at 734 nm after 6 min. The percentage inhibition was calculated as follows:

$$\text{ABTS radical scavenging activity (\%)} \\ = (1 - (A_t/A_0)) \times 100$$

where A_0 and A_t are the absorbances of the sample at time zero and 6 min, respectively.

Mineral elements

Mineral elements were measured by the nitric-perchloric digestion method. Samples were placed in digestion tubes (500 mg), and 6 mL of a mixture of HNO_3 and HClO_4 (2:1 v/v) was added. The temperature was increased to 160 °C, then cooled to 80 °C, followed by another increase to 210 °C, until the samples were colorless. Once cooled, the samples were transferred to a 50-mL volumetric flask, and the volume was adjusted with deionized water. Calcium, magnesium, iron, copper, zinc, potassium, phosphorus and sodium contents were measured by using a GCB 932 atomic absorption spectrophotometer (GCB 932 AA).

Photooxidative effects and antioxidant responses after light exposure

Milk preparation

Skim milk powder (340 g/kg protein) was dissolved in 100 mL deionized water and supplemented with Fe (as

FeSO_4) at 0.1 mg/100 g and an anti-foaming agent, as explained by Giroux et al. (2008). Sodium azide (0.2 g/kg) was added as a microbial growth inhibitor. Okara was added at three concentrations (1, 2 and 3 g/L), and each mixture was stirred for 60 min. After overnight storage (18 h) at 4 °C, the milk samples were enriched with omega-3 fatty acids (linseed oil), to give a final concentration of 20 g/kg. The milk samples were preheated to 40 °C and then homogenized at $900 \times g$ for 5 min, using an Ultra-Turrax. Then, 7 mL of each sample was transferred to glass tubes. A control sample (without okara) was also prepared.

Conjugated diene values (CD)

The measurement of CD hydroperoxides was performed (Kiokias et al. 2006), with minor modifications. Milk samples (50 μL) were diluted in 2.5 mL of a 2,2,4-trimethylpentane:2-propanol (2:1 v/v) mixture. After blending in a capped plastic tube for 10 s, the contents were filtered through a 0.45 μm polytetrafluoroethylene filter. The absorbance of the filtrate was measured at 232 nm against a trimethylpentane: 2-propanol blank. The CD concentration was calculated using the molar absorptivity of linoleic acid ($\epsilon = 26000$) and expressed as mmol per kg fat.

Measurement of thiobarbituric acid reactive substances

The malondialdehyde (MDA) content in milk was quantified using the TBARs assay (Souza et al. 2011). The sample (500 μL) was mixed with TBARs reagent (2 mL of 1% thiobarbituric acid, 562.5 $\mu\text{mol/L}$ HCl, 15% trichloroacetic acid). The mixture was boiled (100 °C) for 15 min, cooled down and centrifuged ($900 \times g$ for 10 min). The spectrophotometric absorbance of the supernatant was measured at 538 nm and compared to an MDA standard curve, ranging from 0 to 60 $\mu\text{mol/L}$.

Milk color

The L^* (lightness), a^* (redness) and b^* (yellowness) values were measured using a CR-400 chromameter (Konica Minolta) at 1 and 8 days of storage.

In vitro digestion

The in vitro digestion method of Versantvoort et al. (2005) was used to simulate the digestion process of milk. The milk (without omega-3 fatty acids) was prepared using the light oxidation process described above, at 3 g okara/L milk. In order to replicate fed conditions, 4.5 g milk was used per digestion. All digestive juices were heated to 37 ± 2 °C.

The digestion was initiated by adding 6 mL saliva, followed by incubation for 5 min. Afterward, 12 mL gastric juice was added, and the mixture was rotated for 2 h. Finally, 12 mL duodenal juice, 6 mL bile and 2 mL bicarbonate solution (1 mol/L) were added simultaneously, followed by mixing in an automatic rotator for 2 h. After the intestinal phase, a dialysis tube (D-TubeTM; 3.5 kDa) was used to concentrate the available polyphenols. The polyphenol contents were analyzed using the Folin–Ciocalteu agent, with modifications. Polyvinylpyrrolidone (0.02 g) was mixed with the milk supernatant, homogenized for 3 h and then centrifuged at $900\times g$ for 30 min. The supernatant from this extraction was used for the analyses. The antioxidant activity was determined by the ABTS and DPPH radical scavenging activities (%), as described above.

Statistical analysis

All the three experiments were done in triplicate. Data analysis was conducted using Statistical Analysis System (SAS) version 9.1 (SAS Institute Cary, NC, USA). Following analysis of variance (ANOVA), the significance of differences between samples was assessed by post hoc comparisons of the means using Tukey's test. Differences were considered significant at $p \leq 0.05$. Results were expressed as mean and standard error (SE) or standard deviation (SD). Regression analyzes of the TBARS and CD were also performed.

Results and discussion

Okara characteristics

TPC and antioxidant properties of okra presented in Table 1. TPC concentration was 130.5 mg GAE/100 g (dry weight basis), with 2% flavonoids. Earlier study, Tyug et al. (2010) analyzed the antioxidant activity, phenolic and isoflavones in soybean by-products (soy husk) and reported a TPC by the Folin–Ciocalteu method of 62.4 mg GAE/100 g wet weight. Considering the moisture content also mentioned in their study, this result is similar to that found in the present work.

Studies have shown that various soybean genotypes present total phenolic contents in a range of 96–535 mg GAE/100 g soybean (Kumar et al. 2010). Therefore, the okara under study did not lose much phenolic content after soybean processing.

The reducing power ability of okara was 24.5 mg GAE/100 g dry weight. The percentages of free radical inhibition were 34.7% DPPH at 30 min and 37.8% ABTS at 6 min.

Based on its mineral content (Table 2), okara offers good amounts of macro- and microelements. In this study,

Table 1 Total phenolic compounds (TPC), flavonoids content (FC) ferric ion reducing power (FRAP), DPPH and ABTS radical scavenging capacity of okara

TPC (mg GAE/100 g)	130.50 ± 10.20
FC (mg QE/100 g)	2.50 ± 0.10
FRAP (mg GAE/100 g)	24.50 ± 1.50
DPPH (%)	34.70 ± 2.04
ABTS (%)	37.84 ± 3.14

Results are expressed as mean ± standard deviation

GAE gallic acid equivalent, QE quercetin equivalent

potassium was found at 1232 mg/100 g, iron at 8.27 mg/100 g, zinc at 3.02 mg/g, copper at 0.97 mg/100 g, phosphorus at 353.50 mg/dm³ and others in smaller quantities. These values concurred with those of Vong and Liu (2016), who reviewed the literature values of micronutrients in okara.

Antioxidant responses after light exposure

Milk lipid oxidation

During the first phase of lipid oxidation, CD hydroperoxides are produced by the reaction between peroxy radicals and triplet oxygen (³O₂) (Frankel 1980). The TPCs are great metal chelators and scavengers that may interrupt the lipid oxidation reaction, reducing or avoiding the formation of CDs and MDA (Farhoosh et al. 2012). The milk control and samples enriched with okara residue (1, 2 and 3 g/L) and omega-3 fatty acids (linseed oil) were allowed to oxidize under light exposure. The results showed that okara residue (3 g/L) effectively reduced the formation of CDs after storage for 2 days (Fig. 1). From days 4 and 6, all formulations with okara showed lower CD values compared to the control ($p < 0.05$). At the end of the analysis, the samples enriched with okara at 3 g/L, showed the best CD inhibition value.

Table 2 Mineral composition of okara

Mineral	Concentration
Phosphorus (mg/dm ³)	353.40 ± 10.53
Potassium (mg/100 g)	1232 ± 69.28
Sodium (mg/100 g)	0.50 ± 0.02
Calcium (mg/100 g)	2220 ± 68.45
Magnesium (mg/100 g)	1590 ± 53.85
Iron (mg/100 g)	8.27 ± 0.66
Copper (mg/100 g)	0.97 ± 0.01
Zinc (mg/100 g)	3.02 ± 0.26

Results are expressed as mean ± standard deviation

Fig. 1 Conjugated diene (mmol/kg fat) in milk enriched with okara at 0, 1, 2 and 3 g/L and omega-3 fatty acids

TBARs are secondary lipid oxidation products, and the measurement of their concentration is widely used as a technique for lipid oxidation evaluation (Beltran et al. 2003). In this study, the TBARs levels (as μM MDA) revealed that from day 4 of storage, the samples with added okara residue had undergone less oxidation compared to the control ($p < 0.05$) (Fig. 2). At 4, 6 and 8 days of storage, the okara residue (2 and 3 g/L) protected the milk from oxidation, when compared with the control ($p < 0.05$). The formulations with okra at 1 and 2 g/L did not differ at days 6 and 8, while the milk with 3 g/L presented the least lipid oxidation regarding MDA production ($p < 0.05$).

The regression analysis results indicated that the model is explanatory, as it presented a coefficient of determination (R^2) close to 1 ($R^2 > 0.947$ for CD and $R^2 > 0.960$ for TBARs). Also, an excellent correlation between CD and TBARs with storage time ($R^2 > 0.973$ for CD and $R^2 > 0.983$ for TBARs) was observed. The linear regression relationship between these parameters was statistically significant ($p < 0.05$) for all treatments analyzed.

Milk color during storage

Oxidation during storage or processing of milk and dairy products may lead to the development of undesirable characteristics, such as off-flavors, discoloration and other adverse changes to the final product (Dalsgaard et al. 2008).

At 8 days of storage under light exposure, the a^* value (redness) of milk had increased while the b^* (yellowness) parameter had decreased (Fig. 3). The elevation in a^* value is probably a result of browning, due to tyrosine and tryptophan degradation during the light exposure (Toba et al. 1980). The decline in the b^* value is possibly caused

Fig. 2 Thiobarbituric acid reactive substances (TBARs) (μM malonaldehyde) in milk enriched with okara at 0, 1, 2 and 3 g/L and omega-3 fatty acids

by the deterioration of the yellowish compounds, e.g., riboflavin, vitamin A and β -carotene (Lee et al. 1998; Toba et al. 1980). Mestdagh et al. (2005) studied the influence of different packaging materials on the light oxidation of milk and found the same behavior regarding the a^* and b^* values.

Although all samples exhibited the same trend, milk samples containing okara were more effective in inhibiting the changes ($p < 0.05$), particularly at 3 g okara/L milk. The L^* (lightness) values were not significantly different at day 0 ($p > 0.05$). However, the L^* value was influenced by illumination during storage, showing an increase ($p < 0.05$) after storage for 8 days. This increased lightness may be related to the color degradation of the linseed oil (omega-3 fatty acids) added to milk. Normally, L^* increases with the reduction in oil pigment content (Crisado et al. 2008).

Total phenolic compounds and antioxidant bioavailability

Food matrices enriched with polyphenols can enhance or prevent the stability and release of these compounds, thereby affecting their efficiency during digestion. When assessing the beneficial effects of polyphenols, the metabolism, absorption and bioavailability are indicative concepts to distinguish if a compound remains available to be used by the organism when it reaches the systemic circulation (Pineda-Vadillo et al. 2016). The extent of polyphenols solubilized in the digestive fluids released by the food matrix (bioaccessibility) is an important step, considering only bioaccessible polyphenols may be absorbed and remain bioavailable. Even though it is difficult to simulate digestion *in vivo* accurately, such models

Fig. 3 Effect of Okara addition on milk color: L* (Lightness), a* (redness) and b* (yellowness)

are considered satisfactory for these types of studies (Pineda-Vadillo et al. 2016).

Milk containing 3 g okara residue/L presented the best inhibition of lipid oxidation. Therefore, it was submitted to simulated gastrointestinal digestion. The TPC concentration and antioxidant activity in the digested and dialyzed sample were determined. The results (Table 3) showed a TPC value of 0.416 and 0.139 mg GAE/mL for milk, with and without okara, respectively. According to the data, it is possible to observe that even after digestion, the polyphenol contents in milk with okara were significantly higher than in the absence of okara ($p < 0.05$).

Table 3 Antioxidants properties of milk enriched with 3 g/L okara residue and without okara after in vitro digestion

	Milk with okara	Milk without okara
TPC (mg GAE/mL)	0.41 ± 0.04 ^a	0.13 ± 0.00 ^b
DPPH (%)	29.56 ± 3.00	n.d.*
ABTS (%)	29.42 ± 0.95 ^c	16.34 ± 1.03 ^b

Results are expressed as mean ± standard deviation

TPC total phenolic compounds, GAE gallic acid equivalent, DPPH free radical scavenging activity, ABTS free radical scavenging activity, *n.d. no detected

Different letters indicate that means are significantly different ($p < 0.05$)

The ABTS•+ scavenging activity of milk without okara was significantly lower than the milk enriched with this ingredient (Table 3). Similarly, there was a significant difference in the DPPH activity between the samples. In contrast to the DPPH values of 29.56% for milk enriched with okara, no DPPH radical scavenging activity was observed for digested milk without okara. The ABTS•+ scavenging properties also showed significant differences between the enriched and non-enriched milk (43.16 and 16.34%, respectively). In milk, the antioxidant activity is mainly due to caseins, which are the major radical scavengers. During digestion, an increase in milk antioxidant activity depends on protein hydrolysis, which releases compounds with antioxidant activity, like peptides and free amino acids (Clausen et al. 2009).

The results demonstrated that at the end of the digestion, the milk containing okara showed the highest antioxidant activity and the polyphenols remained bioavailable. Thus, due to the improved antioxidant activities and bioavailability, milk with okara residue may be efficient in the protection of organisms from free radical injury.

Conclusion

The okara residue displayed antioxidant capacity and decreased lipid oxidation in milk enriched with omega-3 fatty acids during light exposure. After the digestion process, milk with added okara exhibited the highest content of phenolic compounds with antioxidant activity. These bioactive compounds present in the milk may act against the free radicals present in humans. Therefore, despite being a residue, okara can potentially be considered as an antioxidant ingredient and offers a cheap and efficient source of antioxidants for food products.

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