DOI: 10.1111/jfbc.13149

Received: 6 July 2019 | Revised: 18 December 2019 | Accepted: 27 December 2019

DOI: 10.1111/jfbc.13149

FULL ARTICLE

Journal of Food Biochemistry WILEY

Biotransformation processes in soymilk isoflavones to enhance anti-inflammatory potential in intestinal cellular model

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Funding information
Conselho Nacional de Desenvolvimento Científico e Tecnológico, Grant/Award Number: 130629/2018-9; Fundação de Amparo à Pesquisa do Estado de São Paulo, Grant/Award Number: 25107-6/2018

Abstract

The present study investigated, in in vitro cellular model, the modulation of intestinal inflammation by biotransformed soymilk with tannase and probiotic strains. The ability to reduce the generation of intracellular reactive oxygen species (ROS) and the antioxidant power of soy extracts were also evaluated. The results showed changes in isoflavones profile after biotransformation processes, with a significative enhancement in aglycones content. Reduction in intracellular ROS production and improvement in antioxidant capacity were observed. Anti-inflammatory responses in Caco-2 cells were also expressive. A significative decrease in interleukin 8 (IL-8) level was detected for all biotransformed samples, especially for extracts with tannase. The biotransformed soy extracts by tannase have a great potential to improve health conditions, defending the intestinal cells of oxidative damage, and acting as a possible adjuvant in inflammatory process.

Practical applications

Soy isoflavones have been explored owing to health benefits. Only glycosylated forms are found in high concentrations in soybeans. So, microbial and enzymatic biotransformation processes aiming to increase aglycones and metabolites appear as an attractive option to enlarge the bioactivity of soy products. The present study showed a positive impact of biotransformed soymilk on antioxidant defenses systems and modulation of intestinal inflammation and could act as a nutraceutical agent.

KEYWORDS

antioxidant activity, biotransformation, inflammation, isoflavones, probiotics, tannase

1 | INTRODUCTION

Soybeans and derivatives are considered a great source of phenolic compounds. Isoflavones are found in elevated amounts in these seeds and have been associated with beneficial health effects (Gaya, Peirotén, Medina, & Landete, 2016; Shao et al., 2011). However, the bioactive chemical forms of isoflavones, such as aglycones and metabolites, are available in low content or absent in soybeans (Izumi et al., 2000). So, biotransformation processes aiming to enlargement the bioactivity of soy products have attracted attention.

In this context, our research group has investigated enzymatic and fermentative bioprocesses for the purpose to improve the soy extracts bioactivity. So, owing to positive results previously observed (de Ávila et al., 2018; de Ávila, de Queirós, Ueta, Macedo, & Macedo, 2019; de

Queirós, Macedo, & Macedo, 2016), the evaluation of biological potential of biotransformed soy extracts deserve further investigations.

Bioactive isoflavones and metabolites, such as equol, have been related to health benefits, involving antioxidant and anti-inflammatory properties. In this way, studies have reported a positive role of bioactive compounds to modulate the intestinal inflammation (Kinger, Kumar, & Kumar, 2018; Ozdal et al., 2016; Selma, Espín, & Tomás-Barberán, 2009).

Inflammatory bowel disease (IBD) is considered a chronic disease, represented by a group of colon and small intestine inflammatory conditions characterized by Crohn's disease and ulcerative colitis. IBD is associated with the major cause of morbidity in Western countries and with an increased incidence in developing world (Ramos & Papadakis, 2019). The development of IBD may be result of various interactions between the gastrointestinal microbiota and intestinal immune system in genetically vulnerable hosts, and it can be also involved with one or several environmental factors (Wright, Ding, & Niewiadomski, 2018). According to Garg, Lule, Malik, and Tomar (2016), oxidative stress is related to development of chronic diseases owing to an imbalance of reactive oxygen species (ROS) formation. ROS present a relevant role in protection of the body against pathogens; however, excessive ROS production by neutrophils and macrophages in the intestinal mucosa can lead to IBD (Asakura & Kitahora, 2018). Thus, alternatives based on dietary components, like phenolic compounds, have been considered with the purpose to improving inflammatory clinical conditions.

Based on this information, this study investigated the role of biotransformed soymilk by enzymatic and fermentative processes in order to modulate the intestinal inflammation using an in vitro cellular model.

2 | MATERIALS AND METHODS

2.1 | Materials

Soybeans (Natu's, 0706 lot) were obtained at a local supermarket (Campinas, Brazil). Gallic acid, Folin–Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), Trolox, daidzin, daidzein, genistin, genistein, glycitin, glycitein, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), interleukin 1-beta (IL-1β), 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate probe (CM-H_2DCFDA) and Hank's balanced salt solution (HBSS) were purchased from Sigma-Aldrich (Steinheim, Germany). All other chemicals were purchased in the commercial grade available.

2.2 | Soymilk preparation

Soybeans were soaked in distilled water (1:3 w/v) for 6 hr. Then, the seeds were boiled (1:2 w/v) during 5 min and blended (1:4 w/v) for 3 min. After that, the mixture was centrifuged at 9,630× *g* for 15 min at room temperature (Megafuge 16R, Thermo Electron Led GmbH, Germany). Soymilk, corresponding to supernatant, was pasteurized at 85°C for 30 s, freeze-dried, and maintained at −20°C until the analyses (Mandarino & Carrão-Panizzi, 1999).

2.3 | Tannase

Tannase from *Paecilomyces variotii* was obtained according to Battestin and Macedo (2007). The enzymatic activity was measured as described by Sharma, Bhat, and Dawra (2000). One unit (U) of activity was corresponding to the amount of tannase that released 1 μmol of gallic acid/minute of reaction. The protein content (Bradford, 1976) was measured for specific enzymatic activity calculation.

2.4 | Biotransformation processes

For all processes, soymilk was used at concentration of 200 mg of lyophilized powder extract/ml of sterilized water, according to de Ávila et al. (2018). Biotransformation was performed in triplicate and, at the end of each process, soymilk was freeze-dried and maintained at −20°C.

2.4.1 | Process I—Fermentative process

For microbial biotransformation, two probiotic strains were used together: *Bifidobacterium lactis* (BLC1) and *Lactobacillus casei* (MB151), kindly donated by Sacco Brazil® and by Chemical, Biological, and Agricultural Pluridisciplinary Research Center (CPQBA – UNICAMP), respectively. For reactivation, the lyophilized cultures were individually cultivated in De Man, Rogosa, and Sharpe (MRS) broth (1% v/v) at 37°C for 48 hr.

Activated cultures were used at a concentration of 1% v/v (0.5% of BLC1 and 0.5% of MB151) in sterilized soymilk (20 ml aliquot). Inoculated soymilk was conditioned in an anaerobic jar (Anaerobac, Probac) at 37°C and 50 rotations per minute (rpm), for 24 hr (de Queirós et al., 2016).

2.4.2 | Process II—Fermentative process followed by enzymatic biotransformation

Fermented soymilk (20 ml aliquot) obtained in Process I was incubated with 3.8 U/ml of tannase at 50°C and 100 rpm for 45 min (de Queirós et al., 2016). The reaction was interrupted by placing the sample for 15 min in an ice bath.

2.4.3 | Process III—Enzymatic biotransformation

Soymilk (20 ml aliquot) was incubated with 3.8 U/ml of tannase at 50°C in a thermostated bath (model B12D, Micronal, Brazil) at

100 rpm for 45 min (de Ávila et al., 2018; de Queirós et al., 2016). The biotransformation process was stalled by putting the sample for 15 min in an ice bath.

2.4.4 | Process IV—Enzymatic biotransformation followed by fermentative process

The probiotic strains (final concentration of 1% v/v – 0.5% of BLC1 and 0.5% of MB151) were inoculated in enzymatically biotransformed soymilk (20 ml aliquot) obtained in Process III. Inoculated soymilk was maintained at 37°C and 50 rpm in anaerobic conditions for 24 hr.

2.5 | Identification and quantification of isoflavones

For isoflavones extraction, 10 mg of each freeze-dried sample, biotransformed and control, were diluted in 1 ml 70% of methanolic solution and blended at 5,000 rpm for 15 min at 25°C. After centrifugation at 5,790× *g* for 10 min, the supernatants were filtered in 0.45 μm membrane (Aguiar, Suzuki, Paredes-Guzmán, Alencar, & Park, 2003).

High Performance Liquid Chromatography (HPLC) analyses were carried out on a liquid chromatograph system (Dionex UltiMate 3000, Germany) with a RP18 XTerra® column (Waters, 5 μm, 4.6 × 150 mm) maintained at 30°C and using an ultraviolet/visible (UV/VIS) diode array detector (DAD-3000). The mobile phase solutions were formulated as follows: water/formic acid at 99.9:0.1, v/v (A) and 100% of methanol (B). Elution with a flow rate of 0.5 ml/min was performed as follows: 0–15 min of 20% B; 15–75 min of 20%–80% B; 75–80 min of 80%–100% B; 80–90 min of 100%–20% B; and 90–95 min of 20% B. The injected volume was 20 μl and the analyses were performed in triplicate. The absorption spectra were obtained at 190 and 480 nm. The chromatograms were processed at 254 nm. The identification of isoflavones was carried out by the retention time and UV–VIS spectra. Individual compounds were quantified using calibration curves of standards (daidzin, genistin, glycitin, daidzein, genistein, and glycitein). Values were expressed as μg of each isoflavone/ml of sample (Maubach et al., 2003).

2.6 | Total phenols and antioxidant capacity

Total phenols of samples were assessed in triplicate by Folin–Ciocalteu assay (Swain & Hillis, 1959). A gallic acid curve was designed in a range from 16–300 μg/ml and results were expressed as μmol of gallic acid equivalent/mg of sample. Antioxidant potential was investigated by Oxygen Radical Absorbance Capacity (ORAC) assay, as described by Dávalos, Gómez-Cordovés, and Bartolomé (2004) and adapted by Macedo, Battestin, Ribeiro, and Macedo (2011); by DPPH assay, following the methodology established by Peschel et al. (2006) and adapted by Macedo et al. (2011); and by Ferric Reducing Antioxidant Power (FRAP) assay, as described by Benzie and Strain (1996). All measurements were performed in triplicate and results were expressed as μmol Trolox equivalent/mg of sample.

2.7 | Cell assays

2.7.1 | Cell culture

Human colon adenocarcinoma-derived Caco-2 cells were obtained from Rio de Janeiro Cell Bank. The cells were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% of fetal bovine serum and 1% of antibiotics (penicillin and streptomycin) at 37°C in a 5% of CO_2 humidified atmosphere.

2.7.2 | Cell viability assay

Caco-2 cells (inoculation density of 4×10^5 cells/ml) were seeded in 96-well plates and exposed to different concentrations of samples (100–50,000 μg/ml), in triplicate, during 24 hr. After that, the medium was replaced by MTT solution (5 mg/ml). After incubation for 3 hr at 37°C, 50 μl of dimethyl sulfoxide were added in each well and mixed on a shaker table (TS-2000A VDRL Shaker, Biomixer, Brazil) for 5 min. The optical density was measured at 570 nm on a FLUOstar OPTIMA plate reader (BMG Labtech, Germany) and values were expressed as percentage compared to cell control (Mosmann, 1983).

2.7.3 | Reactive oxygen species

Intracellular ROS generation was investigated using a fluorescent probe (Wang & Joseph, 1999). Caco-2 cells were inoculated at 4×10^4 cells/well in 96-well black plates with clear flat bottom. After confluence around 80%, the medium was removed and substituted by HBSS plus 10 μmol/L CM-H_2DCFDA, followed by incubation for 20 min at 37°C. After washed with phosphate-buffered saline (PBS) solution, the cells received the biotransformed and control samples at concentrations of 100, 500, and 1,000 μg/ml, diluted in HBSS. ROS inducer (50 μmol/L AAPH) was added to sample wells and positive control. Fluorescence was measured at intervals of 60 min at wavelengths of 490 nm (excitation) and 520 nm (emission) at 37°C during 5 hr in a FLUOstar OPTIMA plate reader. The assay was performed in triplicate and values were expressed as ROS production percentage in comparison to positive control.

2.7.4 | Caco-2 inflammatory assay

Caco-2 cells (inoculation density of 3×10^5 cells/well) were cultured in 24-well plates during 24 hr. After 24 hr IL-1β stimulation (5 ng/ml), the cells received the biotransformed and control

samples at concentration of 1,000 μg/ml for more 24 hr. After treatment, supernatants were collected and assessed for interleukin 8 (IL-8) levels with kit Human IL-8 Enzyme Linked Immune Sorbent Assay (ELISA) Set (BD OptEIA™) according to the manufacturer's instructions. The absorbance was measured at wavelengths of 450 nm (excitation) and 570 nm (emission) in a FLUOstar OPTIMA plate reader. The assay was performed in triplicate and results were calculated using a standard curve of IL-8 and expressed in ρg/ml.

2.8 | Statistical analysis

Values were expressed as means ± standard deviation (*SD*). Analysis of variance was executed followed by Dunnett's or Tukey's tests. Differences were considered statistically significative when p value <.05. The GraphPad Prism software version 5.0 was used for all analyses.

3 | RESULTS AND DISCUSSION

3.1 | Soymilk characterization

Changes in isoflavones profile in soy extracts after enzymatic and fermentative processes were demonstrated by HPLC-DAD analyses (Figure 1). In general, all biotransformation processes were effective to enlarge the bioactivity of samples, increasing the aglycone forms.

Studies have suggested that modifications in chemical structure of isoflavones can be promoted by distinct processing (Chen et al., 2013; Park, Alencar, Nery, Aguiar, & Pacheco, 2001; Yang, Wang, Yan, Jiang, & Li, 2009). In this context, biotransformation processes with enzymes and/or probiotic strains appear as an alternative strategy to convert glycosylated forms of isoflavones into their respective bioactive aglycones and metabolites (de Ávila et al., 2018; de Queirós et al., 2016).

Our results showed that the biotransformation processes were able to bioconvert glycosides into aglycones, reducing the glycosidic forms to undetectable levels (Table 1). Genistin, a glycosylated form, was detected only in process I but in minor concentration compared to soymilk control (standard process). However, process I was also responsible for hydrolysis of glycitin in their respective aglycone (glycitein), increasing the bioactivity of soy extract. Although in low concentration, glycitein was also quantified in process II.

As observed in Table 1, enlargement in aglycones content was detected for "soymilk + fermentative process" (SF) sample, corresponding to process I. The concentration of daidzein, genistein, and glycitein increased around 10%, 225%, and 36%, respectively, compared to control. These results suggest that fermentative process with probiotic strains was able to convert glycosides and other substrates present in soy extract into aglycones, probably due to the β-glycosidase activity of microorganisms during fermentation (Dueñas et al., 2012).

Gaya et al. (2016) investigated the biotransformation of soy extracts and verified that isoflavones metabolism is limited to hydrolysis of glycosidic forms by most of probiotic strains. The authors reported that bifidobacteria and lactic acid bacteria, such as *Lactobacillus*, have more affinity for conversion of genistin into genistein. Our results are in agreement with these data since the biotransformation of soy extract with probiotic strains (process I) was responsible for a significative increase in genistein content compared to control.

Biotransformation processes involving tannase (process II, III, and IV) presented the highest increase in aglycones content compared to soymilk control. Daidzein and genistein content enhanced by 1.61–1.85 and 6.08–7.48 times, respectively. For "soymilk + fermentative process + tannase" (SFT) and "soymilk + tannase + fermentative process" (STF) samples, the initial order of the process did not interfere on bioactive isoflavones content. Our hypothesis for this higher increase in aglycones is the broader action of tannase enzyme. In addition to its β-glycosidase activity, previously reported by de Ávila et al. (2018), tannase could act in other desesterification and depolymerization reactions, generating aglycones and metabolites from other substrates present in soy extracts.

Similar to our findings, an increase in aglycone levels after enzymatic and fermentative biotransformation of soymilk was observed by de Queirós et al. (2016). The authors studied the alterations in isoflavones profile, antioxidant capacity, and equol generation by three processes: (I) fermented soymilk with probiotic lactic bacteria, (II) fermented soymilk with tannase, and (III) soymilk with tannase. All processes were effective to convert glycosides into aglycone forms and to generate equol. The best performance was attributed to enzymatic process with tannase (process III), resulting in the highest content of aglycones and equol. Likewise, de Ávila et al. (2018) investigated the soymilk biotransformation by tannase and β-glycosidase enzymes and concluded that tannase in free form presented the highest raise in aglycone levels and it was able to generate equol.

Biotransformation of glycoside isoflavones into aglycone forms is relevant since aglycones are compounds with more bioactivity, directly associated with higher bioavailability. Besides that, aglycones can generate metabolites, such as *O*-desmethylangolensin and equol (Gaya et al., 2016; Selma et al., 2009; Setchell et al., 2002). In the present work, equol metabolite was not identified. In the same way, Bravo, Peirotén, Álvarez, and Landete (2017) obtained similar results when investigated the presence of metabolite in fermented soy extracts by *Lactobacillus* and bifidobacteria.

3.2 | Total phenols and antioxidant capacity

Soymilk after enzymatic and fermentative bioprocesses were assessed for total phenols and antioxidant capacity. The results indicated that all biotransformation processes were able to increase the total phenols. The highest values were attributed to SFT and STF, correlating with HPLC analyses. For processes II and IV, it was

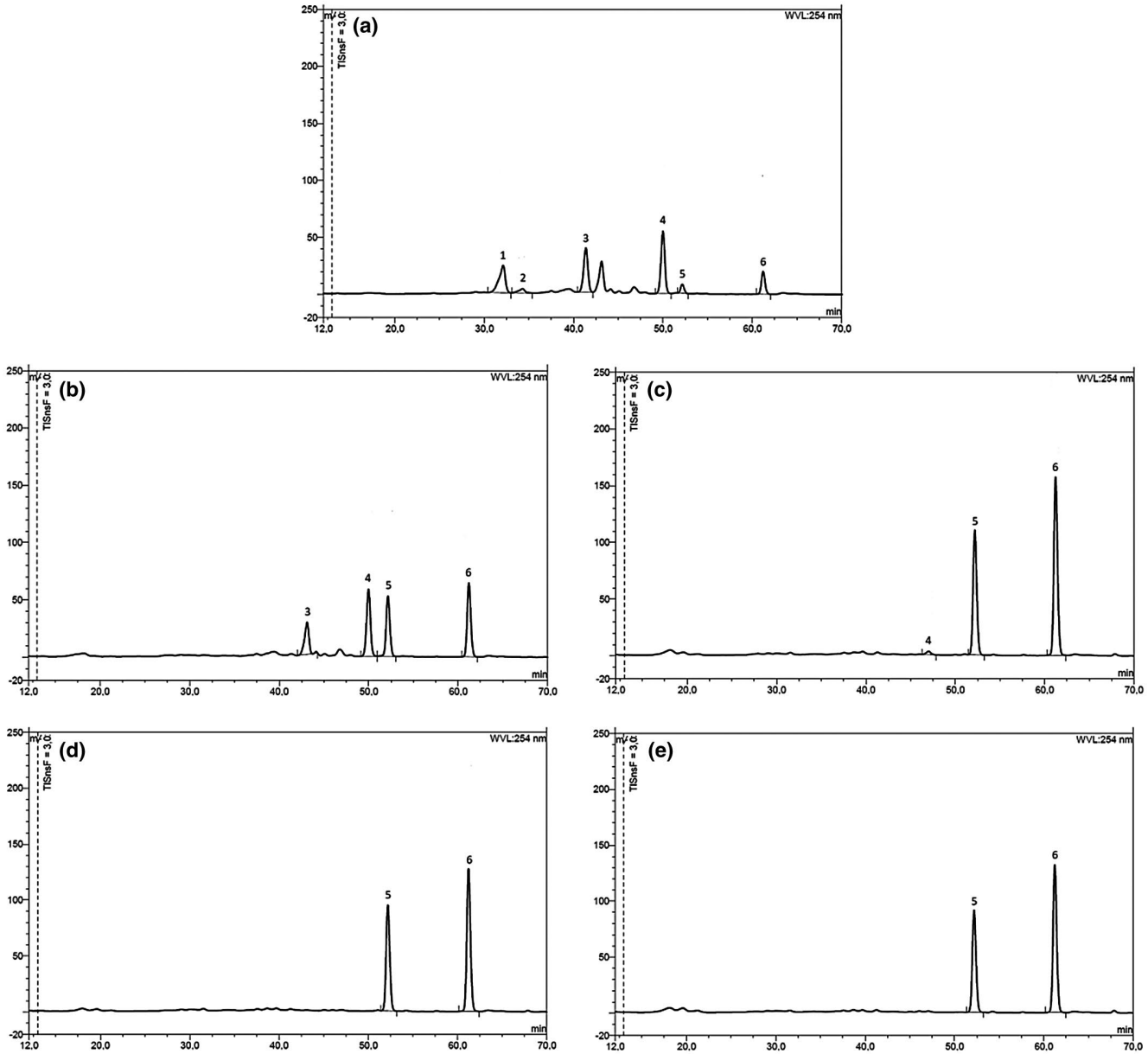

FIGURE 1 Chromatograms obtained by HPLC-DAD. (a) Soymilk control (SC); (b) soymilk + fermentative process (SF); (c) soymilk + fermentative process + tannase (SFT); (d) soymilk + tannase (ST); (e) soymilk + tannase + fermentative process (STF); 1—daidzin; 2—glycitin; 3—genistin; 4—glycitein; 5—daidzein; 6—genistein

observed a significative increase by 30% and 39%, respectively, compared to soymilk control (SC), and significative difference between the samples was not noted (Table 2).

The results suggest a mutual interaction between probiotic strains and enzyme leading to an enhancement in total phenolic content. Similar findings were reported by de Queirós et al. (2016) after fermentative process of soymilk with probiotic bacteria. The authors verified an increase in total phenols by two and three times to fermented soymilk and fermented soymilk with tannase, respectively, compared to untreated sample. In the same way, de Ávila et al. (2018) showed that biotransformed soymilk with tannase in free form was responsible for a rise of 7% in total phenolics compared to control. The researchers suggested that bioprocesses with tannase enlarge the release of phenolic compounds from soy extracts matrix, causing an increase in total phenols content.

The antioxidant power of biotransformed and control soymilk was assessed by DPPH, ORAC, and FRAP methods. As shown in Table 2, it was not observed a significant increase in antioxidant capacity by DPPH assay after biotransformation processes. The highest enlargement was noted for STF, corresponding to 2.5% compared to SC; however, there was no significative difference between SC and STF samples. For the other samples [SF, SFT, and "soymilk + tannase" (ST)], a decrease in antioxidant power can be observed when compared to soymilk control.

According to ORAC assay, the highest values of antioxidant capacity were noted to biotransformed samples with tannase (SFT, ST,

TABLE 1 Isoflavones quantification (μg/ml) of biotransformed and control samples by HPLC-DAD

		Isoflavones content					
		Glycosides			Aglycones		
Processes	Samples	Daidzin	Genistin	Glycitin	Daidzein	Genistein	Glycitein
Standard	SC	0.883 ± 0.024	0.709 ± 0.022^{a}	0.136 ± 0.001	0.655 ± 0.012^{b}	0.170 ± 0.004^{d}	0.727 ± 0.021^{b}
I	SF	n/d	0.550 ± 0.001^{b}	n/d	0.718 ± 0.023^{b}	0.553 ± 0.024^{c}	0.990 ± 0.006^{a}
II	SFT	n/d	n/d	n/d	1.209 ± 0.088^{a}	1.254 ± 0.089^{a}	0.022 ± 0.004^{c}
III	ST	n/d	n/d	n/d	1.056 ± 0.034^{a}	1.033 ± 0.034^{b}	n/d
IV	STF	n/d	n/d	n/d	1.209 ± 0.134^{a}	1.272 ± 0.147^{a}	n/d

Note: Results are expressed as means ± *SD* (n = 3). For each column (evaluated compound), values with different lowercase letters are statistically different at $p < .05$ by Tukey's test.

Abbreviations: n/d, not detected; SC, soymilk control; SF, soymilk + fermentative process; SFT, soymilk + fermentative process + tannase; ST, soymilk + tannase; STF, soymilk + tannase + fermentative process.

TABLE 2 Total phenolic content (μmol gallic acid equivalent/mg sample) and antioxidant capacity (μmol Trolox equivalent/mg sample) of soy extracts

Processes	Samples	Total phenolics	DPPH	ORAC	FRAP
Standard	SC	9.97 ± 0.31^{c}	14.91 ± 0.43^{a}	95.55 ± 18.66^{bc}	27.76 ± 0.76^{e}
I	SF	10.78 ± 0.42^{b}	9.76 ± 1.20^{ab}	83.04 ± 26.52^{c}	32.37 ± 1.34^{d}
II	SFT	12.92 ± 0.28^{a}	8.16 ± 1.66^{b}	178.57 ± 54.68^{ab}	37.13 ± 1.38^{c}
III	ST	10.31 ± 0.13^{bc}	12.91 ± 4.36^{ab}	125.14 ± 12.42^{abc}	42.06 ± 1.67^{b}
IV	STF	12.87 ± 0.28^{a}	15.28 ± 0.58^{a}	189.60 ± 26.38^{a}	50.37 ± 2.71^{a}

Note: Results are presented as means ± *SD* (n = 3). For each column (assay performed), values with different lowercase letters are statistically different at $p < .05$ by Tukey's test.

Abbreviations: SC, soymilk control; SF, soymilk + fermentative process; SFT, soymilk + fermentative process + tannase; ST, soymilk + tannase; STF, soymilk + tannase + fermentative process.

and STF) with a correspondent increase of 87%, 31%, and 98%, respectively, compared to SC. For SF, a reduction of 13% in antioxidant power was detected in comparison to SC.

In opposition, a distinct profile was detected by FRAP assay. All biotransformation processes significantly increased the antioxidant capacity and statistically differed from each other and from soymilk control. Increase around 17%, 34%, 52%, and 81% were observed to SF, SFT, ST, and STF, respectively, compared to SC. In this assay, the biotransformation processes involving tannase, alone or associated with probiotic strains, were responsible for a significative rise in antioxidant power of soy extracts. The results obtained by FRAP assay also suggest that the initial step of biotransformation process with tannase and microorganisms can interfere in the antioxidant power of extracts.

An increase in antioxidant capacity of phenolic compounds mediated by enzymes was also described by Martins, Roberto, Blumberg, Chen, and Macedo (2016), that investigated the role of tannase, pectinase, and cellulase in grape pomace biotransformation. The authors verified that the treatment with tannase presented the highest enhancement in antioxidant power by FRAP, ORAC, and DPPH assays compared to other enzymes. In another work developed by Ferreira, Macedo, Ribeiro, and Macedo (2013), the antioxidant activity of biotransformed orange juice with tannase from *Paecilomyces variotti* was studied. After biotransformation, the researchers observed a rise in antioxidant capacity around 50% and 70% by ORAC and DPPH assays, respectively, compared to control. Similarly, all treatments with tannase in our study showed a satisfactory enlargement in antioxidant power by FRAP and ORAC assays.

Our data are also consistent with results reported by de Ávila et al. (2019). The authors investigated the soymilk biotransformation and verified a significative rise in antioxidant power by free tannase, equivalent to 1.25 and 2.3 times by FRAP and ORAC assays compared to untreated extract. Likewise, de Queirós et al. (2016) observed an enhancement in antioxidant activity by DPPH and ORAC assays for soy extracts after biotransformation (enzymatic and fermentative processes), especially for biotransformed soymilk with tannase, that increased around four and eight times, respectively, compared to control.

3.3 | Intracellular ROS production and anti-inflammatory potential

The MTT assay showed that samples were safe to Caco-2 cells in the tested concentrations after 24 hr of exposure (Figure 2). So,

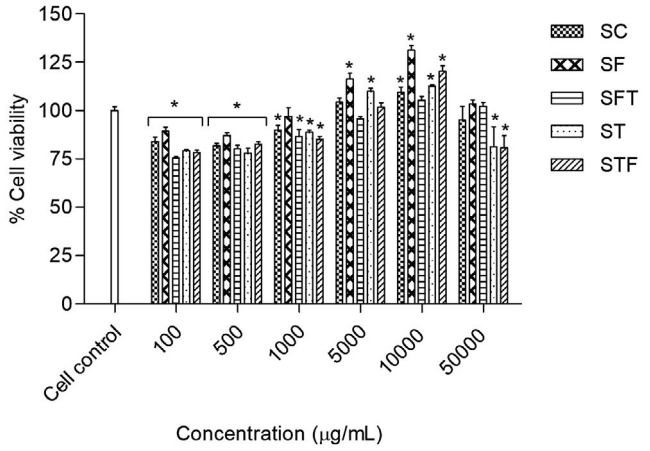

FIGURE 2 Cell viability (% of cell control) of Caco-2 treated with biotransformed soymilk according to MTT assay. Values are expressed as means ± *SD* (n = 3). Asterisks (*) indicate results with significant statistical difference compared to cell control by Dunnett's test (p < .05). SC, soymilk control; SF, soymilk + fermentative process; SFT, soymilk + fermentative process + tannase; ST, soymilk + tannase; STF, soymilk + tannase + fermentative process

the following cellular assay was carried out using the 100, 500, and 1,000 μg/ml concentrations for all the samples.

To evaluate the antioxidant effects in Caco-2 cell line, intracellular ROS production was investigated using a fluorescent probe. After incubation for 5 hr with CM-H_2DCFDA, ROS levels increased by 139% in positive control (with AAPH) compared to negative control (without AAPH), confirming the occurrence of oxidative stress by ROS inducer (Table 3). All treatment of samples, at three tested concentrations, caused an expressive decrease in ROS content compared to positive control (p < .05). Significative difference among the samples was not observed at concentration of 500 μg/ml. At 100 and 1,000 μg/ml, the highest reductions were observed by STF (24%) and SF (37%), respectively, compared to cells stimulated with AAPH alone.

The efficiency of tannase on generating phenolic extracts with higher potential of ROS reduction was tested by Martins et al. (2017) when evaluated biotransformed grape extracts in Caco-2 cell line. Cells were induced by AAPH for 5 hr and exhibited an increase of 142% in ROS expression compared to cell control (without ROS induction). After treatment of samples, the authors verified that both grape extracts, in the presence or absence of tannase, showed an expressive decrease in ROS levels. In the present study, we observed the same behavior with our samples, since soy extracts (biotransformed or control) were effective to decrease ROS content independent of the tested concentration.

In agreement with our findings, Li, Long, Pan, Zhao, and Song (2018) also investigated the antioxidant capacity and cytoprotective power of fermented soy extract by *Lactobacillus plantarum* YS-1 and *Lactobacillus bulgaricus* in Caco-2 cells stimulated by hydrogen peroxide (H_2O_2). After adding H_2O_2, cells shown an increase in ROS production by 247.5%. The authors verified that both fermented

TABLE 3 Intracellular reactive oxygen species (ROS) expression (%) in Caco-2 cells induced by AAPH and treated with soy extracts after incubation for 5 hr

	Negative control[a]	Positive control[b]	Sample concentrations (μg/ml)	SC	SF	SFT	ST	STF
ROS	141.40 ± 0.58	338.34 ± 1.23	100	280.26 ± 8.82^{*ab}	264.14 ± 5.05^{*b}	265.87 ± 2.73^{*ab}	285.76 ± 12.72^{*a}	258.82 ± 6.77^{*b}
			500	241.54 ± 4.70^{*a}	262.06 ± 1.56^{*a}	262.06 ± 14.28^{*a}	248.00 ± 8.79^{*a}	242.76 ± 5.12^{*a}
			1,000	235.01 ± 21.27^{*b}	214.82 ± 4.04^{*b}	286.88 ± 4.64^{*a}	300.46 ± 4.52^{*a}	228.54 ± 13.61^{*b}

Note: Results are presented as means ± *SD* (n = 3). Different lowercase letters indicate statistical difference among the samples in the same concentration by Tukey's test (p < .05).

Abbreviations: SC, soymilk control; SF, soymilk + fermentative process; SFT, soymilk + fermentative process + tannase; ST, soymilk + tannase; STF, soymilk + tannase + fermentative process.

[a]Without AAPH.

[b]With AAPH.

*Results with significant statistical difference compared to positive control by Dunnett's test (p < .05).

samples at 10 and 100 μg/ml were able to effectively reduce the ROS expression. In the same way, Suo et al. (2016) evaluated the antioxidant capacity of free radicals and the effects of fermented soy extract by two strains of *Lactobacillus* in proximal renal tubule cells induced by H_2O_2. The treatment with H_2O_2 was responsible for a significant rise in ROS levels, around 198%. At 100 μg/ml, the fermented samples were able to reduce ROS expression around 141%–153% compared to cells stimulated with only H_2O_2.

According to Teixeira (2013), high concentrations of ROS and reactive nitrogen species (RNS) were detected in intestinal epithelial cells and peripheral lymphocytes in patients with IBD. Additional studies also suggest that during the inflammatory process, cells release large amounts of ROS and RNS, as well as other toxic substances (Baumgart & Carding, 2007; Maloy & Powrie, 2011).

Therefore, the present study evaluated the biotransformed soy extracts by enzymatic and fermentative pathways to evidence their ability to modulate the intestinal inflammation. As observed in Figure 3, all biotransformation processes caused an expressive decline in IL-8 secretion. SF, SFT, ST, and STF led to a reduction in cytokine levels around 52%, 81%, 91%, and 83%, respectively, compared to positive control (cells stimulated with IL-1β). There was no significative difference in IL-8 expression between SC and positive control.

Our results demonstrated that biotransformed soymilk with tannase (SFT, ST, and STF) promoted an anti-inflammatory response more effective in intestinal cells, reducing the cytokine IL-8 in higher levels. Similar data were reported by Martins et al. (2017) when using biotransformed grape extracts with tannase to evaluate IL-8 expression by IL-1β-induced Caco-2 cell line. At the highest tested concentration, grape pomace treated with tannase inhibited the cytokine expression in 83% after pre-treatment; in the same conditions, a decrease of 70% was observed in pre/co-treatment. Likewise, in research conducted by Paradkar, Blum, Berhow, Baumann, and Kuo (2004), a diet containing isoflavones was responsible to control the inflammatory process in liver and intestine of mice after injection of lipopolysaccharide. The authors also described that the anti-inflammatory power of genistein in in vitro assays with Caco-2 cells was consistent with in vivo observation.

According to our assays, biotransformed samples by enzymatic and fermentative processes caused a positive effect in inhibition of ROS generation and IL-8 expression. Although the results are preliminary, the type of experimental modeling used in this study is useful and allows an accurate screening of bioactive potentials. Thus, the satisfactory data obtained indicate that these extracts are potential candidates for clinical trials and deserve further investigations.

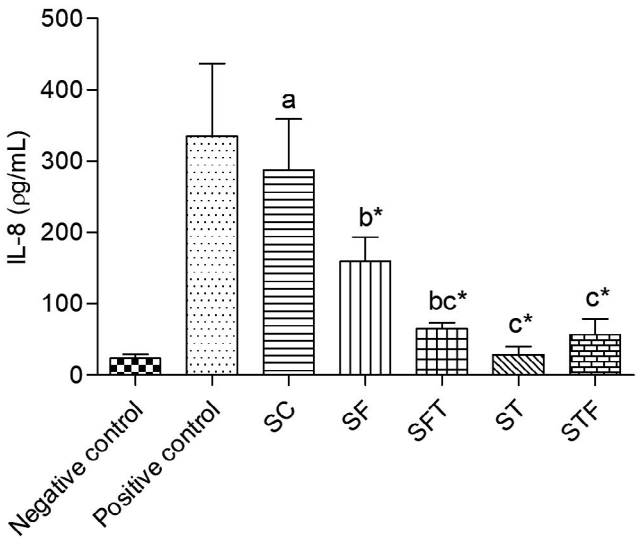

FIGURE 3 IL-8 (ρg/ml) expression in IL-1β-induced Caco-2 supernatant after 24 hr of treatment with samples at 1,000 μg/ml. Asterisks (*) indicate results with significant statistical difference compared to positive control by Dunnett's test ($p < .05$). Different lowercase letters indicate statistical difference among the samples by Tukey's test ($p < .05$). Negative control, cells without IL-1β stimulus or extract; positive control, cells stimulated with IL-1β and without samples; SC, soymilk control; SF, soymilk + fermentative process; SFT, soymilk + fermentative process + tannase; ST, soymilk + tannase; STF, soymilk + tannase + fermentative process

4 | CONCLUSION

Enzymatic and fermentative bioprocesses improved soymilk bioactivity. The highest antioxidant capacity was observed to STF by FRAP, ORAC and DPPH methods and all the samples caused a significant decrease in intracellular ROS content. Anti-inflammatory responses were also expressive. It was observed a significant reduction in IL-8 secretion for all biotransformed samples, especially for extracts treated with tannase (SFT, ST, and STF). Based on our results, biotransformed soymilk by tannase has a great potential to improve health conditions, defending the intestinal cells of oxidative damage and acting as a possible natural anti-inflammatory agent.

ACKNOWLEDGMENTS

This work was supported by a master scholarship received from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) [grant number 130629/2018-9] and by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) [grant number 25107-6/2018].

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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How to cite this article: Hiramatsu EY, de Ávila ARA, Gênova VM, et al. Biotransformation processes in soymilk isoflavones to enhance anti-inflammatory potential in intestinal cellular model. *J Food Biochem*. 2020;00:e13149. https://doi.org/10.1111/jfbc.13149