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Fermented Soymilk Supplemented With Soybean Germ: Optimisation of *Lactobacillus reuteri* LR-92 Growth and Bioactive Compounds

Extrato de Soja Fermentado Suplementado com Gérmen de Soja: Otimização do Crescimento de *Lactobacillus reuteri* LR-92 e Compostos Bioativos

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Abstract

This work aimed to use soy germ in soymilk supplementation and to verify the impact on the development of a fermented product. During the fermentation process, the *L. reuteri* growth, the bioactive compounds and antioxidant activity were determined. The Central Composite Rotational Design (CCRD) was applied to evaluate the independent variables: fermentation time and temperature in the fermentation process of the soybean germ-soymilk. In addition, the texture profile of the product and the influence of fermentation on the isoflavones profile and phenolic compounds content were evaluated during 30 days under refrigeration. The fermented soy germ-soymilk (FSG) reached the maximum response for growth in 24 h at 36 ºC. The counts of *L. reuteri* reached values of 8.44 ± 0.05 log CFU.g^{-1} in the FSG. FSG had a higher content of total isoflavone (15.74 mg.g^{-1}) and phenolic compounds (303.37 mg EAG.100 g^{-1}) compared to fermented soymilk (FS). The antioxidant activity of FSG and FS based on the scavenging of the radicals DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ was higher than in soymilk. FSG showed microbiological and functional stability during storage at 4ºC for 30 days. The findings suggest that the supplementation of soymilk with germ increases beneficial compounds and antioxidant properties.
Keywords: Antioxidant. Aglycones. Functional Food. Isoflavones. Probiotic.

Resumo

Este estudo teve como objetivo incorporar gérmen de soja na suplementação de extrato de soja e verificar o impacto no desenvolvimento de um produto fermentado. Durante o processo de fermentação foi determinado o crescimento de L. reuteri, os compostos bioativos e a atividade antioxidante. Um Planejamento Central Composto Rotacional (DCCR) foi utilizado para avaliar as variáveis independentes: tempo de fermentação e temperatura no processo de fermentação do extrato de soja contendo gérmen de soja (FSG). Além disso, foi avaliado o perfil de textura do produto e a influência da fermentação no perfil das isoflavonas e no conteúdo dos compostos fenólicos durante 30 dias sob refrigeração. O extrato de soja fermentado com gérmen (ESGS) obteve a resposta máxima de crescimento em 24 h a 36 ºC. com 8,44 ± 0,05 log UFC.g^{-1}. O ESGS apresentou maior conteúdo de isoflavonas totais (15,74 mg.g^{-1}) e compostos fenólicos (303,37 mg de EAG.100 g^{-1}) em comparação com o extrato de soja fermentada (FS). A atividade antioxidante de FSG e FS baseada na eliminação dos radicais DPPH$^{\bullet}$ e ABTS$^{\bullet+}$ foi maior no FSG e mostrou estabilidade microbiológica e funcional durante o armazenamento a 4 ºC durante 30 dias. Os resultados sugerem que o FSG resultou em maiores concentrações de compostos bioativos e atividade antioxidante.
Palavras chaves: *Antioxidante. Agliconas. Alimento Funcional. Isoflavonas. Probiótico.*

1 Introduction

Traditionally, Asian populations have consumed many soybean products over the centuries. Epidemiological studies have shown that fermented soy foods exhibit potent anti-carcinogenic effects related to the antioxidant properties of fermented soybeans (Applegate *et al.,* 2018; He; Chen, 2013; Moraes Filho *et al.,* 2016;).

The production of functional drinks is increasingly being used in industry. This product is very well accepted and consumed by individuals looking for a healthier diet, rich in nutrients and proteins. (Saviani; Sifuente, 2020). The soybean is considered a functional food because of its high protein and fibre content, as well as the amount of bioactive compounds, such as isoflavones. There are twelve different forms of isoflavones found in soybeans that can be divided into four groups according to their chemical structures: aglycones (daidzein, genistein and glycitein), β-glucosides (daidzin, genistin and glycitin), acetylglucosides (6”-O-acetyldaidzin, 6”-O-acetylgenistin and 6”-O-acetylglycitin) and malonylglucosides (6”-O-malonyldaidzin, 6”-O-malonylgenistin, 6”-O-malonylglycitin) (Bolzan; Liberali; Coutinho, 2011; De Oliveira Silva *et al.,* 2018; Rizzo; Baroni, 2018; Yoshiara *et al.* 2018).

The aglycone form does not have the glucose molecule linked to the flavonoid moiety and represents the minor fractions of isoflavones present in the grain. However, the aglycone fraction represents the constituents of greatest interest to the consumption of soy and derivates, because aglycones are absorbed in higher and faster amounts than their glycosylated forms. Many steps can be used to change the isoflavones profile, such as germination or fermentation

process (Izumi *et al.,* 2000; Bolzan; Liberali; Coutinho, 2011; Yoshiara *et al.* 2018). The isoflavone aglycones are considered more bioactive than other isoflavones because of their chemical structure and low molecular weight. These aglycones show antioxidant activity and may combat oxidation-related diseases, including atherosclerosis, hypertension and breast cancers (Chen, 2012; Wong *et al.*, 2012).

Soy germ contains high levels of isoflavones and other bioactive phytochemicals, it is considered one of the most nutritious parts of soybean and have the highest glycitein content**.** The isoflavones have molecular similarities to estradiol and can play an important role in the prevention of cardiovascular diseases, osteoporosis, menopausal symptoms and hormone-dependent cancers (Carbonel *et al.* 2017; Zhan *et al.* 2018). Moreover, soy germ has been suggested to have a preventive effect against breast cancer, associated to the presence of isoflavones (Watanabe; Uehara, 2019).

Potentially probiotic microorganisms have been used for soybean fermentation to achieve beneficial effects on human health when consumed at concentrations of 10^{8-9} CFU per daily portion of the product (Brasil, 2008; Janpaeng *et al.*, 2018; Fino *et al.* 2020). *Lactobacillus reuteri* is a Gram-positive bacteria naturally present in the gut microbiota. This bacteria was described in 1980 and is currently used as probiotic because of its beneficial properties. Studies have shown the effect of ingestion of this probiotic to reduce abdominal pain and acute diarrhea in children (Urbańska; Gieruszczak-Białek; Szajewska, 2016; Mantegazza *et al.* 2018). Furthermore, when *Lactobacillus* sp. was used to ferment soymilk, the isoflavone aglycone content increased (Delgado *et al.* 2019; Lim *et al.*, 2020). Mendoza-Avendaño *et al.* (2019) found that the fermentation of soymilk by genus *Lactobacillus* could be an alternative to increase the concentration of bioactive compounds producing *β*-glucosidase, converting β-glucosides into their corresponding aglycones, with an increase of 420 to 490%.

In this context, the aim of this work was to develop a novel fermented soymilk product supplemented with soy germ. To achieve this, the growth of *L. reuteri* LR-92 in soy germ-soymilk was optimized using a Central Composite Rotational Design (CCRD) to study the effects of fermentation time and temperature. Additionally, the content of isoflavones, phenolic compounds, and antioxidant activity in fermented soy germ-soymilk (FSG) and fermented soymilk (FS) stored at 4°C for 30 days were evaluated.

2 Material and Methods

2.1 Soy grain, soy germ and culture

Soybeans BRS 257, cultivated in Mauá da Serra (Paraná, Brazil), were provided by Sementes Paraná Ltda., while commercial soy germ (in flour form) was supplied by Herborisa Indústria e Comércio Ltda. (Londrina, Brazil). The lyophilized culture of *L. reuteri* LR-92 Clericci-Sacco® (Cadorago, Italy) was utilized in this study.

2.2 *L. reuteri* LR-92 inoculum preparation

The lyophilised culture of *L. reuteri* LR-92 was mixed with sterile pure soymilk (1 $g.L^{-1}$). The inoculum was stored with 10% (v/v) of sterile glycerol at -20 ºC.

2.3 Soymilk production and inoculation with *L. reuteri* LR-92

Soymilk was prepared according to the previously described methods of Rosenthal *et al.* (2003) and Ciabotti *et al.* (2006), with minor modifications. Whole soybean lipoxygenase-free BRS 257 was heated with distilled water in a proportion of 1:3 (w/v) at 95 ºC for 5 min, followed by draining and thermal shock in distilled water at 25 ºC. The soybeans were grinded with distilled water (1:8 w/v) at 90 °C for 3 min. The slurry was filtered using a specific cheesemaking cloth. The residue was discarded, and the soymilk was mixed with 3% (w/v) soy germ. The soy germ-soymilk was dispensed in glass bottles of 200 mL and was then heat-treated at 95 °C for 10 min and cooled at 35 °C. The soy germ-soymilk was inoculated with 4% (v/v) of *L. reuteri* LR-92 (previously thawed), resulting in an initial viable cell count of approximately 10^5 $CFU.g^{-1}$. For comparison, samples of non-germ-added soymilk and germ-added soybean milk and 12% sucrose (added before pasteurization) were produced.

2.4 Experimental design and statistical analysis of experimental growth conditions

The statistical analysis of viable cell counts in the Fermented Soy Germ-Soymilk (FSG) was performed using Statistica version 8.0. A Central Composite Rotational Design (CCRD) was used to study the interaction of the process variables and to predict the optimum process conditions for bacterial growth by Response Surface Methodology (RSM). The range and coded levels of the process variables are listed in Table 1, selected based on preliminary experiments. The two variables were incubation temperature (X_1) and incubation time (X_2). Each variable consisted of 5 different levels: low (-1), medium (0), high (+1), and axial points (-α, +α). The 14 experiments were divided into 2 blocks, with 3 repetitions made at the medium point (0) in each block to evaluate the pure error. The viable counts of *L. reuteri* LR-92 were assumed to be a response to the experimental design.

Table 1 - Values used in CCRD for the fermentation of soymilk with 3% (w/v) of soybean germ

	Levels				
	-α	**-1**	**0**	**+1**	**+α**
Temperature (ºC)	30.36	32	36	40	41.64
Time (h)	12.72	16	24	32	35.28

Source: research data.

A second-order model was adopted to fit the experimental responses to the following six parameters: $y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_{12} + \beta_{22} X_{22} + \beta_{12} X_1 X_2$ (eq. 1). In this equation, X_1 and X_2 stand for the coded variables. β_0, β_1, β_2 and β_{12} stand for the regression model parameters, as estimated by the least squares method. Analysis of variance (ANOVA) was employed to evaluate the empirical mathematical model at a 5% significance level. The statistical significance was determined by an F-value, and the F-value calculated should be greater than the F-value tabulated.

2.5 Viable cell count in fermented soymilks

The pour-plate method was used for the viable counts of *L. reuteri* LR-92 in Fermented Soy Germ-Soymilk (FSG) and Fermented Soymilk (FS), using De Man, Rogosa and Sharpe agar (Difco, Detroit, Michigan, USA). The plates in triplicate were incubated anaerobically at 37 °C for 48 h. Plates containing 25 to 250 visible colonies were counted, and their numbers were recorded as log CFU.g^{-1} of the sample.

2.6 Analytical Methods

2.6.1 pH and titratable acidity determination

The pH of the samples was measured with a pH meter (Hanna HI 223). The titratable acidity was determined by titration with NaOH 0.1 M and expressed as g of lactic acid.100 g^{-1} of fresh weight.

2.6.2 Extraction and determination of the isoflavones

The samples were previously lyophilised (Christ Alpha 2-4 LD plus, Osterodeam Harz, Germany) and defatted with hexane (1:10 w/v) at 25 ºC for 60 min by continuous rotatory agitation at 200 rpm and then vacuum filtered. The isoflavone extraction was performed using a mixed solvent extractor (ultra-pure water, ethanol and acetone) in equals proportion as described by Yoshiara *et al.* (2012) and Handa *et al.* (2014). The extraction was performed in triplicate with 0.3 g of defatted samples and 6 mL of solvent extractor and then stirred for 15 min each over 1 h at 25 °C.

The mixture was placed in an ultrasonic bath at 25 ºC for 15 min and centrifuged (2500 x*g* at 4 ºC for 15 min) (Centrifuge 5804R - Eppendorf, Hamburg, Germany), and the supernatant was filtered (Millex filter-H 0.22 μm). The assays were performed according to Handa *et al.* (2014). The analyses were conducted using Ultra-Performance Liquid Chromatography UPLC (Acquity UPLC® System, Waters, USA) with an autosampler and diode array detector (Waters) at the wavelength of 260 nm. A reverse-phase column (Acquity model - UPLC BEH C18, Waters, USA) with the dimensions of 2.1 mm x 50 mm and particle size of 1.7 µm was used to separate the isoflavone isomers. The elution was performed with a non-linear gradient using mobile phase A with acidified ultra-pure water at pH 3.0; for mobile phase B, acetonitrile was applied at a flow of 0.70 mL.min^{-1} at 35 °C. Standard solutions for each calibration curve were used [daidzin, glycitin, genistin, daidzein, glycitein, genistein (Sigma-Aldrich), malonyl-daidzin, malonyl-glycitin, malonyl-genistin, acetyl-daidzin, acetyl-glycitin, acetyl-genistin (Wako Pure Chemical Industries, Japan). The isoflavone concentrations were calculated and expressed in mg of isoflavones per g of defatted sample on a dry basis.

2.6.3 Extraction of total phenolic and antioxidant compounds

The samples previously lyophilised was extracted with an 80% ethanol solution (1:10 w/v) by a continuous rotatory agitation at 200 rpm for 20 min, according to the description of Hung *et al.* (2009). After centrifugation (2200x*g* at 4 ºC for 10 min), the supernatant was collected and concentrated in a rotative roto-evaporator at 50 ºC (Marconi MA 120, São Paulo, Brazil) until 10 mL of final volume was attained. The extracts were stored at -20ºC until use.

2.6.4 Total phenolic compound assay

Total phenolic compound content was estimated as gallic acid equivalents, as described by Swain and Hills (1959). Briefly, 0.5 mL of each extract was mixed with 2.5 mL of 10% Folin-Ciocalteau reagent and 2 mL of 7.5% sodium carbonate. After a reaction at 50 ºC for 5 min, the absorbance was determined at 760 nm by UV visible spectrophotometer (UV-Libra S22, Biochrom®, UK). The reactions were conducted in triplicate, and the results are expressed as milligrams of gallic acid equivalent (GAE) per 100 g of sample on dry basis.

2.6.5 Antioxidant activity assays

2.6.5.1 DPPH$^{\bullet}$ radical scavenging activity

The scavenging capacity against DPPH$^{\bullet}$ (2.2-diphenyl-1-picrylhydrazyl) was measured according to the methods described by Brand-Williams *et al.* (1995). Briefly, 50 µL of diluted extract was mixed with 1 mL acetate buffer 100 µM, 1 mL absolute ethanol and 0.5 mL DPPH solution 250 µM. The mixture was shaken vigorously and kept in the dark for 30 min, and the absorbance was determined at 517 nm. The results were expressed in Trolox equivalents per gram of sample on a dry basis.

2.6.5.2 ABTS$^{\bullet+}$ radical scavenging activity

The scavenging capacity against ABTS$^{\bullet+}$ [2.2'-azinobis (3-ethylbenzothiazoline 6-sulfonic acid)] was measured according to Sanchez-Gonzales *et al.* (2005). Briefly, a working solution ABTS$^{\bullet+}$ was prepared by oxidising ABTS to ABTS$^{\bullet+}$ using potassium persulfate. The mixture was diluted to reach an absorbance of 700-734 nm, and the working solution ABTS$^{\bullet+}$ was obtained. For this reaction, 3 mL of the working solution ABTS$^{\bullet+}$ was mixed with 30 µL of the diluted extract, and the absorbance was determined after 5 min of

reaction at 734 nm. The scavenging capacity of $ABTS^{\bullet+}$ was expressed in Trolox equivalents per gram of sample on a dry basis.

2.6.6 Texture profile analysis of fermented soy germ-soymilk

A texture profile analysis (TPA) test was performed by a TA XT2 texture analyser (Stable Micro Systems Ltd, Godalming, Surrey, UK). A probe (A/Be/35 mm) was used to measure, in triplicate, the TPA of samples at room temperature. During the pre-test, for the compression and relaxation of a sample, the probe speed was 1.0 $mm.s^{-1}$; the applied force was 0.09 N; and the samples were compressed to 30 mm of depth. The data were analysed using the Exponent Stable Micro Systems V 6 software.

2.7 Statistical Analysis – physical and chemical determinations

The data are reported as the means ± standard deviation (SD) for quintuplicate determinations. The analysis of variation (ANOVA) and Tukey's test were employed to identify the differences between means. Statistical significance was considered present when $p \leq 0.05$. All statistical analyses were performed using Statistic version 8.0.

3. Results and Discussion

3.1 Effect of temperature and fermentation time on the growth of *Lactobacillus reuteri* LR-92

The complete matrix of CCRD and the interaction effects of independent variables for the response function (*L. reuteri* LR-92 viable counts) are shown in Table 2.

Table 2 - CCRD with independent variables and response function

Assay	Temperature (ºC)	Time (h)	*L. reuteri* viable counts ($CFU.g^{-1}$)
1	32	16	8.24
2	40	16	8.75
3	32	32	8.79
4	40	32	8.35
5	36	24	9.23
6	36	24	9.07
7	36	24	9.49
8	30	24	9.23
9	41.5	24	8.28
10	36	12.7	8.56
11	36	35.3	9.37
12	36	24	9.52
13	36	24	9.46
14	36	24	9.24

Source: research data.

Data indicated that the differing levels of the independent variables contributed to the variation of the response function. The proposed quadratic model was established using the mathematical equation (Eq. 1). The model for the *L. reuteri* LR92 viable counts are shown in Eq. 2.

$$y = 9.3350 - 0.1592x_1 - 0,3719x_1^2 + 0.1619x_2 - 0,2669x_2^2 - 0,2375x_1x_2 \text{ (Eq. 2)}$$

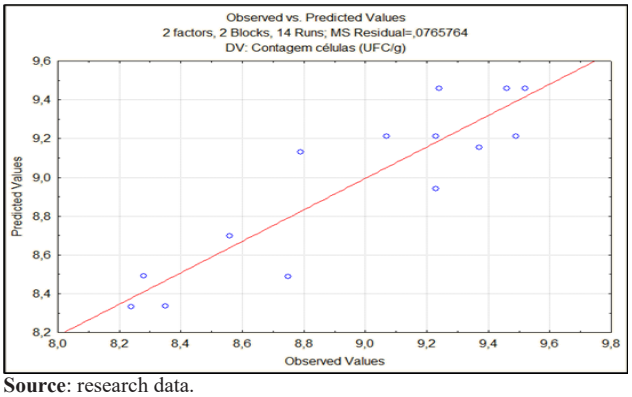
The equation was validated by the F test (Table 3). The F value found for the regression (15.49) was higher than the tabulated F value (3.71) at the 5% significance level. The linear effect of the variables, although not significant, remained in the model, due to their contribution to the data adjustment. The quadratic effect of two variables were significant. The optimum response for the growth of each microorganism was achieved in the experimental data, with no significant lack of fit. The parity plot (Figure 1) compares the predicted values versus the experimental cell growth observed for *L. reuteri* LR-92.

Table 3 - ANOVA for *Lactobacillus reuteri* LR-92 viable cell count

Source of Variation	SQ	DF	QM	F_{cal}	F_{tab}	R^2
Regression	1.5471	3	0.5157	15.49*	3.71	0.8108
Residual	1.2863	10	0.1286			
Lack-of-fit	1.1530	6	0.1922	5.77	6.16	
Pure error	0.1333	4	0.0333			
Total	2.8334	13				

Source: research data.

Figure 1 - Predicted and observed values for *L. reuteri* viable count in soy germ-soymilk fermented



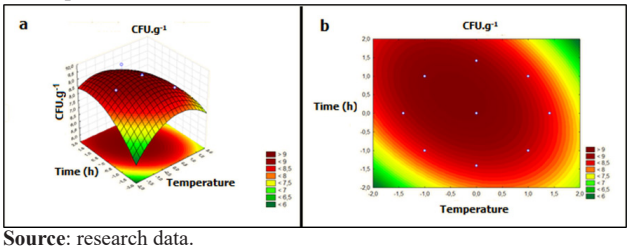
Source: research data.

The predicted values and experimental values were found to be relatively close, indicating a good equation fit. The coefficient of determination (R^2), which is an indicator of how well the model fits the data, was greater than 80%, suggesting that the proposed model was adequate for the response variable (Table 3). The second-order equation was evaluated by the ANOVA F-test and the F-value (15.49) was higher than the tabulated F-value (3.71) at 5% significance. There was a significant effect on the growth of *L. reuteri* LR-92 caused by the incubation parameters, and the empirical model fits the experimental data adequately. Therefore, this model was significant for the process in question.

The empirical model is plotted as a dimensional surface representing the response function (*L. reuteri* LR-92 viable counts) as a function of two factors within the tested

parameters (Figure 2). The contour plot (Figure 2b) illustrates the interaction of time and temperature incubation on bacterial growth. The increment of cell numbers of *L. reuteri* LR-92 with the increase of time and temperature incubation, up to a critical point, can be observed in Figure 2a. However, above the optimal conditions, the cell number decreased, which could be due to a limitation in the bacterial growth due to the nutritional depletion in the soymilk. The region of interest for the incubation time (24-32 h) and incubation temperature (32-36ºC) fell within the RSM. In these conditions the *L. reuteri* LR-92 growth reached counts above 9 log CFU.g^{-1}.

Figure 2 - Response surface (a) and contour plot (b) as a function of temperature and time for viable cell count of *L. reuteri*



Source: research data.

Optimization techniques such as response surface methodology are widely used to optimize production processes and predict response variables (Güldane, 2023). For comparison, Hekmat, Soltan and Reid (2009) studying the growth of *Lactobacillus reuteri* in yogurt reported counts below 10^8 CFU.mL^{-1} after fermentation, lower than those obtained under optimized conditions in this work. Mauro and Garcia (2019) when studying a coconut milk beverage fermented by *Lactobacillus reuteri*, reported maximum counts of approximately 8.7 CFU.mL^{-1}. In the authors work, it was possible to highlight the influence of incubation temperature and constitution of the medium in the fermentation, similar characteristics to those observed in our work. Champagne *et al.* (2016) reported that by inoculating 10^7 CFU.mL^{-1} of *Lactobacillus reuteri* in milk, after 4 to 6 h of fermentation the bacteria reached counts close to 8.0 log CFU.g^{-1}. Despite different mediums of fermentation, we emphasize that the fermentation in this study started from an initial count of 10^5 CFU.g^{-1} and after optimized conditions, settled above 9 log CFU.g^{-1}, demonstrating that high counts can be achieved when fermentation conditions are optimized.

3.2 Texture profile analysis of Fermented Soy Germ-Soymilk (FSG)

The texture profile analysis simulated the conditions in the mouth by compressing the product twice. The results presented in Table 4 show that, except for cohesiveness, the FSG significantly altered the textural characteristics of fermented soymilk (FS) ($p \leq 0.05$). The FSG was fermented until it reached the consistency of a thicker soymilk.

As a critical parameter to evaluate the texture characteristics of the food, firmness is used to estimate the maximum strength at first compression. FSG presented higher firmness among the other samples (Table 4), due to the presence of soybean germ. FSU, although containing germ, showed lower firmness values than FSG, resulting from the addition of sucrose.

Table 4 - Texture profile analysis of FS, FSG and FSU

Samples	**Firmness (g)**	**Consistency (g.s)**	**Cohesiveness**
FS	$22.84^{c} \pm 1.00$	$2.271^{c} \pm 0.05$	$-0.146^{a} \pm 0.01$
FSG	$142.25^{a} \pm 2.00$	$12.844^{a} \pm 0.61$	$-0.379^{a} \pm 0.08$
FSU	$90.70^{b} \pm 3.00$	$7.991^{b} \pm 0.57$	$-0.175^{a} \pm 0.06$

Source: research data.

Means values±SD in the same column followed by the same letter are not significantly different. FS: fermented soymilk; FSG: Fermented soy germ-soymilk; FSU: Fermented soy germ-soymilk added with 12% of sucrose.

Consistency is the speed at which a deformed material returns to the undeformed condition after removal of the deforming force (Szczesniak; Brandt; Friedman, 1963). The values obtained for this parameter were FSG > FSU > FS, following the firmness values (Table 4).

Cohesiveness is determined as the extent to which a material can be deformed before rupture (Szczesniak; Brandt; Friedman, 1963). In this parameter, the three samples were statistically equal ($p \geq 0.05$), indicating that the addition of germ or sugar did not alter the strength of internal bonds in the sample structure.

Yang and Li (2010) performed texture analysis on samples of germinated and fermented soymilks and found values close to consistency (7.59-12.15) and cohesiveness (0.44-0.45). For firmness, the values were lower than those obtained in this study (16,89-26,71), indicating that the addition of soybean germ provides a product with greater firmness than fermented soybean extracts, probably the increase in macronutrient content such as proteins, lipids that contribute to these parameters. According to Damodaran, Parking and Fennema (2010), soluble polymers such as proteins aid in viscosity, with an exponential increase according to concentration. Kovalenko and Briggs (2002) worked with soy protein gel and reported that viscosity was dependent on protein concentration. Also, according to Damodaran, Parking and Fennema (2010), proteins have a good water retention capacity. Wang *et al.* (2018), reported that the addition of polymerized whey protein (PWP) in fermented goat milk increased the texture parameters of the product.

3.3 Viable cell count during fermentation and storage

To modulate the balance of the intestinal microbiota and to enhance the biotransformation of isoflavones into aglycones effectively, probiotics must be present in sufficient numbers at the time the product is consumed. The viable cell counts, pH and titratable acidity in the FSG and FS during storage at 4°C for 30 days are shown in Table 5. According to the CCRD, the highest viable count was found at 32-36°C for 24-32 h. After

inoculation using 4% (v/v) of *L. reuteri* LR-92, the soy germ-soymilk showed initial cell counts of 5.81 ± 0.09 log CFU.g^{-1}. After incubation at 36ºC for 24 h, the FSG reached values of viable counts of 8.44 log CFU.g^{-1}, pH 3.43 and titratable acidity of 0.67 mg lactic acid.100 g^{-1}.

Table 5 - *L. reuteri* viability in fermented soymilks with or without soy germ addition (3% w/v) in 1, 15 e 30 days of storage at 4 ºC

Sample	*L. reuteri* Viability (CFU.g^{-1})	pH	Titratable Acidity (g of Lactic Acid.100 g-1)
FS 1	$7.71^{a} \pm 0.36$	$3.73^{a} \pm 0.01$	$0.53^{e.f} \pm 0.03$
FS 15	$7.69^{a} \pm 0.25$	$3.25^{b.c} \pm 0.01$	$0.64^{d.e} \pm 0.01$
FS 30	$7.88^{a.b} \pm 0.21$	$3.27^{b.c} \pm 0.02$	$0.69^{c.d} \pm 0.01$
FSG 1	$8.44^{c} \pm 0.05$	$3.43^{b} \pm 0.01$	$0.67^{d} \pm 0.05$
FSG 15	$8.45^{c} \pm 0.06$	$3.25^{b.c} \pm 0.01$	$0.77^{b.c.d} \pm 0.01$
FSG 30	$8.30^{b.c} \pm 0.04$	$3.29^{b.c} \pm 0.03$	$0.89^{a.b} \pm 0.03$

Source: research data.

Means values±SD in the same column followed by the same letter are not significantly different. FS: fermented soymilk; FSG: Fermented soy germ-soymilk.

The FS showed viable counts of 7.71 log CFU.g^{-1}, pH 3.73 and titratable acidity of 0.53 mg lactic acid.100 g^{-1}. After storage, *L. reuteri* LR-92 showed stability in both the FS and FSG, indicating that the microorganism was well-adapted to the soymilk and that the 3% (w/v) of soy germ inclusion in the soymilk did not affect the stability or viability of *L. reuteri* LR-92, but it promotes the growth of the probiotic in the system.

There was a decline in the pH and an increase in the titratable acidity in FS and FSG at the end of the storage period. The viable counts of FSG were in accordance with Brazilian regulations (Brasil, 2008). Thus, *L. reuteri* LR-92 showed satisfactory potential for incorporation into soy products.

Wang *et al.* (2009) observed, in *L. casei* fermented soymilk an initial titratable acidity between 0.44-0.56%, which increased to 0.86-0.98% after 28 days of storage at 4 ºC. The accumulation of organic acids during storage and the decrease in pH are characteristic of fermented products and may contribute to the reduction of cell viability. Niyibituronasa *et al.* (2019) reached counts around 9 log CFU.mL^{-1} for *L. reuteri* in soymilk after fermentation for 24 h. According to the authors, the pH values reached 4.78 after the fermentation process, with titratable acidity ranging from 0.2 to 0.42. The pH value was higher than the pH observed in this work, resulting in less intense final acidity.

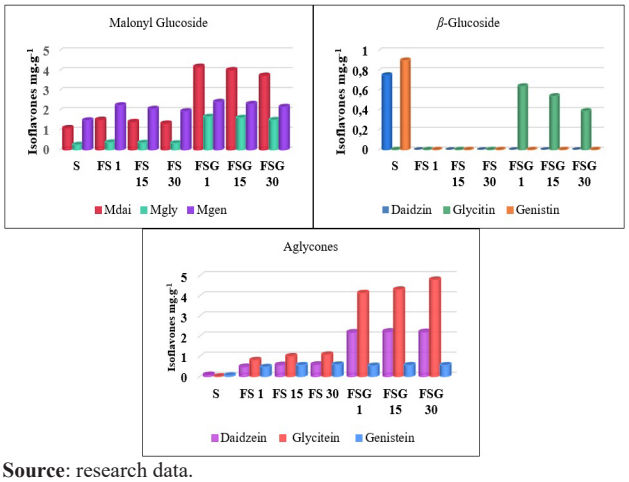
3.4 Stability of isoflavones in fermented soy germ-soymilk during storage

The changes in the isoflavone contents (on a defatted, dry basis) of soymilk (S), FS and FSG during storage at 4 ºC for 30 days were studied. As shown in Figure 3, the non-fermented soymilk (S) was marked by 4.60 mg.g^{-1} of total isoflavones and the presence of *β*-glucosides (daidzin and genistin). After fermentation at 36 ºC for 24 h, the FS showed a higher content of total isoflavones and isoflavone aglycones than S ($p \leq 0.05$). According to Wei *et al.* (2007) and Marazza and De Giori (2009) there was a significant increase in the isoflavone aglycones after soymilk fermentation at 37 °C for 24 h using lactic acid bacteria.

There were no detectable *β*-glucosides in the FS, indicating that the fermentation process was effective in converting β-glucosides into aglycones. After fermentation, glycitin was observed in FS. During storage, there was a significant increase in the isoflavone aglycones ($p \leq 0.05$), but the amount of total isoflavones remained stable.

The genus *Lactobacillus* is widely used and reported as an isoflavone bioconverter. The results observed here corroborate this statement. The improvement in aglycones levels is result of β-galactosidase activity and the aglycones forms are more susceptible to promote health benefits (Liu *et al.*, 2018). According to Murphy *et al.* (2002) fermented soy foods have a high content of aglycones due to the endogenous enzymatic activity of soybeans and the enzymatic activities of fermentative microorganisms, both of which promote *β*-glucosides hydrolysis and conversion into aglycones. The FSG indicated a total isoflavone content (15.74 mg.g^{-1}) 3 times greater than that of FS ($p \leq 0.05$) and the presence of glycitin due to the inclusion of soy germ in the soymilk, leading to high content of malonyl-glycitin and glycitin (data not shown).

Figure 3 - Mean values for isoflavone fraction (mg.g^{-1}) in soymilk (S), fermented soymilk (FS), Fermented soy germ-soymilk (FSG) during 1, 15 and 30 days of storage under refrigeration at 4 ºC



Source: research data.

After storage, the FSG showed an aglycone content (7.57 mg.g^{-1}) 3 times greater than that of FS (2.26 mg.g^{-1}) and glycitein content 4 times greater than that of FS ($p \leq 0.05$). According to Song *et al.* (1998), Song, Hendrich and Murphy (1999) and Wiseman *et al.* (2002), glycitein has shown a greater estrogenic potential than others isoflavone aglycones (glycitein > genistein > daidzein), contributing to the treatment of hormone-dependent diseases and to hormone replacement

therapy in post-menopausal women. Based on these results, the FSG has a total isoflavone content of 155 mg.100 g^{-1} (wet weight), whereas the FS has a content of 42 mg.100 g^{-1} (wet weight). According to Taku *et al.* (2007), a daily consumption of 100 mg of isoflavones may reduce the total cholesterol and LDL fractions after 3 months of supplementation. Supplementation of patients with type 2 diabetes with soybean germ paste containing isoflavones aglycone has shown effects on endothelial functions, reducing oxidative effects that contribute to risk markers of cardiovascular problems (Clerici *et al.,* 2011).

3.5 Stability of total phenolic and antioxidant activity in fermented soy germ-soybean during storage

For soybean germ, the phenolic content was higher (442.73 mg EAG.100 g^{-1}) than soybean grain, which was expected due to the concentration of isoflavones in the soy germ (Table 6). Barbosa *et al.* (2006) found, in the soybean germ analyzed a phenolic content twice higher than that of soybean grain, while Bolanho and Beléia (2011) determined, respectively in the germ and soybean BRS 267, phenolic contents of 213,9 and 187.8 mg GAE.100 g^{-1}.

Table 6 - Values for phenolic compounds and antioxidant capacity by DPPH· and ABTS+· of soybean grain BRS 257, soy germ and fermented soymilks with 1, 15 and 30 days of storage at 4 ºC

Samples	Phenolic (mg GAE.100g^{-1})	DPPH· (µmol Trolox.g^{-1})	ABTS+ (µmol Trolox.g^{-1})
Soy Grain BRS 257	$104.01^{j} \pm 0.07$	$3.06^{b} \pm 0.03$	$1.04^{f} \pm 0.00$
Soy Germ	$442.73^{a} \pm 0.78$	$3.67^{a} \pm 0.02$	$5.00^{a} \pm 0.09$
S	$146.56^{g} \pm 0.08$	$2.06^{e} \pm 0.06$	$2.52^{d} \pm 0.01$
FS 01	$161.86^{f} \pm 0.17$	$2.51^{e,d} \pm 0.07$	$3.09^{c} \pm 0.00$
FS 15	$171.11^{e} \pm 0.62$	$2.95^{b} \pm 0.04$	$3.12^{c} \pm 0.02$
FS 30	$193.74^{d} \pm 0.67$	$3.11^{b} \pm 0.15$	$3.10^{c} \pm 0.00$
FSG 01	$303.37^{b} \pm 0.40$	$2.27^{d,e} \pm 0.02$	$3.32^{b,c} \pm 0.00$
FSG 15	$271.38^{c} \pm 0.33$	$2.79^{b,c} \pm 0.03$	$3.55^{b} \pm 0.01$
FSG 30	$273.40^{c} \pm 0.77$	$2.80^{b,c} \pm 0.11$	**$3.52^{b} \pm 0.06$**

Source: research data.

Means values±SD in the same column followed by the same letter are not significantly different. GAE: Gallic Acid Equivalent; Trolox: Trolox Equivalent; S: Soymilk; FS: fermented soymilk; FSG: Fermented soy germ-soymilk.

The antioxidant capacity, determined by sequestering DPPH• radicals was 3.06 µmol Trolox.g^{-1} in soybean and 3.67 µmol Trolox.g^{-1} in soybean germ (Table 6). Bolanho and Beléia (2011) found values of 4.0 and 3.4 µmol Trolox.g^{-1} for the BRS 267 soybean and in the germ. Barbosa *et al.* (2006) found respective values of 3.7 µmol Trolox.g^{-1} and 5.1 µmol Trolox.g^{-1}. Regarding the antioxidant capacity determined by $ABTS^{\bullet+}$ radical uptake, the values were 1.04 µmol Trolox.g^{-1} for grain and 5.0 µmol Trolox.g^{-1} for soybean germ. By analysing the antioxidant capacity of 13 soybean samples, Zhang *et al.* (2012) found values between 2-4 µmol Trolox.g^{-1}.

The FSG had the highest phenolic content (303.37 mg GAE.100 g^{-1}), followed by FS (161.86 mg GAE.100 g^{-1}) and soymilk (146.56 mg GAE.100 g^{-1}). According to Xu and Chang (2009) and Tyug *et al.* (2010), soymilk has shown a phenolic content of 96-320 mg GAE.100 g^{-1}, depending on the type of soybean used and the soymilk processing conditions. Compared to soymilk, the phenolic content of fermented soymilk was 10.4% higher.

Lai *et al.* (2013) found that the phenolic content in unfermented soybean extract was 113.3 mg GAE g^{-1}, while in fermented soybean extract it was 126.1 mg GAE g^{-1}. This indicates an 11.3% increase in phenolics after fermentation by lactic acid bacteria, a result similar to what was observed in the present study. After storage, the phenolic compounds increased significantly in FS ($p \leq 0.05$) and decreased in FSG. In relation to the antioxidant activity via the scavenging of the radicals DPPH• and $ABTS^{\bullet+}$ the FS and FSG showed a higher activity than that of soymilk.

A significant increase in the antioxidant activity of soymilk fermented by *L. rhamnosus* compared to that of unfermented soymilk was observed by Marazza *et al.* (2009). The increase in the isoflavone aglycones after fermentation can contribute to the increased antioxidant effects in soymilk.

According to Marazza *et al.* (2009), daidzein and genistein may act as hydrogen donors, effectively reacting against the DPPH• radical. The FSG showed an antioxidant activity statistically similar to that of FS ($p \geq 0.05$). Although the soy germ addition does increase the content of isoflavone aglycones in FSG, according to Roginsky and Lissi (2005), some antioxidant compounds may react slowly or be inert in the presence of DPPH• radicals. The antioxidant capacity assays according to Zhang *et al.* (2012), involve different chemical mechanisms, which have no significant correlation with each other. In any case, isoflavones aglycones are more active anti-radical compounds than their precursors *β*-glucosides and increase the antioxidant activity of fermented foods.

4 Conclusion

The incubation conditions of 36 °C for 24 hours result in higher counts of *L. reuteri*, with viable cells remaining stable at 4 °C for up to 30 days. The FSG exhibit a higher content of total isoflavones, and phenolic compounds compared to the FS. Soy germ contributes to increased hardness and gumminess in the fermented soymilk. Fermentation by *L. reuteri* LR-92 enhances the antioxidant activity in the fermented soymilk compared to regular soymilk, due to the increased isoflavone aglycone content.

References

APPLEGATE, C.C. *et al.* Soy consumption and the risk of prostate cancer: An updated systematic review and meta-analysis, Nutrients, v.10, n.1, p.457-464, 2018.

BARBOSA A.C.L. *et al.* Teores de isoflavonas e capacidade antioxidante da soja e produtos derivados. Ciênc. Tecnol. Alim., v.26, n.4, p.921-926, 2006.

BOLANHO, B.C.; BELÉIA, A.D.P. Bioactive compounds and antioxidant potential of soy products. Alim. Nutr. Araraquara, v.22, p.539-546, 2011.

BOLZAN, M.S.; LIBERALI, R.; COUTINHO, V.F. EFEITOS DAS ISOFLAVONAS NO PERFIL LIPÍDICO DE MULHERES NA MENOPAUSA: UMA REVISÃO SISTEMÁTICA. ENSAIOS E CIÊNCIAS, V.15, N.6, 2011. DOI: HTTPS://DOI.ORG/10.17921/1415-6938.2011V15N6P%25P

BRAND-WILLIAMS, W.; CUVELIER, M.E.; BERSET, C. Use of free radical method to evaluate antioxidant activity. LWT - Food Sci. Technol., v.28, p.25-30, 1995.

BRASIL. Ministério da Saúde. Agência Nacional de Vigilância Sanitária. (2008). Alimentos com alegações de propriedades funcionais e ou de saúde, novos alimentos/ingredientes, substâncias bioativas e probióticos. Retrieved on October 15, 2019

CARBONEL, A.A.F. *et al.* The effects of soybean isoflavones and 17 β-estradiol in uterus and mammary glands of diabetic rat models. Gynecol. Endocrinol., v.34, n.4, p.314-319, 2017.

CHAMPAGNE, C.P. *et al.* Growth of *Lactobacillus reuteri* NCIMB 30242 during yogurt fermentation and bile salt hydrolysis activity in the product. Dairy Sci. Technol., v.96, n.2, p.173-184, 2016.

CHEN, K.I. *et al.* Soyfoods and soybean products: from traditional use to modern applications. Appl. Microbiol. Biotechnol., v.96, p.9-22, 2021.

CIABOTTI, S. *et al*. Avaliações químicas e bioquímicas dos grãos, extratos e tofus de soja comum e de soja livre de lipoxigenase. Ciênc. Agrotec., v.30, p.920-929, 2006.

CLERICI, C. *et al.* Novel soy germ pasta improves endothelial function, blood pressure, and oxidative stress in patients with type 2 diabetes. Diabetes Care, v.34, n.9, p.1946-1948, 2011.

DAMODARAN, S.; PARKIN, K.L.; FENNEMA. O.R. Química de alimentos. Porto Alegre: CRC Press, 2010.

DE OLIVEIRA SILVA, F. *et al.* Soybean meal and fermented soybean meal as functional ingredients for the production of low-carb, high-protein, high-fiber and high isoflavones biscuits. LWT - Food Sci. Technol., v.90, p.224-231, 2018.

DELGADO S. *et al.* Fermentation of commercial soy beverages with lactobacilli and bifidobacteria strains featuring high β-glucosidase activity. Innov. Food Sci. Emerg. Technol., v.51, p.148-155, 2019.

FINO, L.C. *et al.* Digestão simulada de bebida esportiva contendo diferentes probióticos. Ens. Ciênc., v.24, n.1, p.47-53, 2020. doi: https://doi.org/10.17921/1415-6938.2020v24n1p47-53

GÜLDANE, M. Optimizing foam mat drying process for cornelian cherry pulp using response surface methodology and artificial neural networks. Rev. Mex. Ing. Quim., v.22, n.3, Alim., 2023.

HANDA, C.L. *et al.* Optimisation of soy flour fermentation parameters to produce β-glucosidase for bioconversion into aglycones. Food Chem., v.152, p.56-65, 2014.

HE, F. J.; CHEN, J. Q. Consumption of soybean, soy foods, soy isoflavones and breast cancer incidence: differences between Chinese women and women in Western countries and possible mechanisms. Food Sci. Hum. Well., v.2, p.146-161, 2013.

HEKMAT, S.; SOLTAN, H.; REID, G. Growth and survival of *Lactobacillus reuteri* RC-14 and *Lactobacillus rhamnosus* GR-1 in yogurt for use as a functional food. Innov. Food Sci. Emerg. Tech., v.10, n.2, p.293-296, 2009.

HUNG, P. V. *et al*. Total phenolic compounds and antioxidant capacity of wheat graded flours by polishing method. Food Res. Int. v.42, p.185-190, 2009.

IZUMI, T. *et al.* Soy isoflavone aglycones are absorbed faster and in higher amounts than their glucosides in humans. J. Nutr., v.130, n.7, p.1695-1699, 2000.

JANPAENG, J. *et al.* Effect of probiotic fermentation on bioactivity in fermented soy milk. The International Conference on Food and Applied Bioscience proceeding book. Thailand: Agro-Industry, Chiang Mai University, 2018.

KOVALENKO, I. V.; BRIGGS, J. L. Textural characterization of soy-based yogurt by the vane method. J. Texture Stud., v.33, n.2, p.105-118, 2002.

LAI, L-R. *et al*. Effect of lactic fermentation on the total phenolic, saponin and phytic acid contents as well as anti-colon cancer cell proliferation activity of soymilk. J. Biosci. Bioeng., v.115, p.552-556, 2013.

LIM, Y. *et al.* Deglycosylation patterns of isoflavones in soybean extracts inoculated with two enzymatically different strains of Lactobacillus species. Enzyme Microb. Technol., v.132, 2020.

LIU, W.S.; YANG, C.Y.; FANG, T.J. Strategic ultrasound-induced stress response of lactic acid bacteria on enhancement of β-glucosidase activity for bioconversion of isoflavones in soymilk. J. Microbiol. Methods, v.148, p.145-150, 2018.

MANTEGAZZA, C. *et al.* Probiotics and antibiotic-associated diarrhea in children: A review and new evidence on *Lactobacillus rhamnosus* GG during and after antibiotic treatment. Pharmacol. Res., v.128, p.63-72, 2018.

MARAZZA, J.A.; GARRO, M.S.; DE GIORI, G.S. Aglycone production by *Lactobacillus rhamnosus* CRL981 during soymilk fermentation. Food Microbiol., v.26, p.333-339, 2009.

MAURO, C.S.I.; GARCIA, S. Coconut milk beverage fermented by *Lactobacillus reuteri*: optimization process and stability during refrigerated storage. J. Food Sci. Technol., v.56, n.2, p.854-864, 2019.

MENDOZA-AVENDAÑO, C. *et al.* Evaluation of bioactive and anti-nutritional compounds during soymilk fermentation with *Lactobacillus plantarum* BAL-03-ITTG and *Lactobacillus fermentum* BAL-21-ITTG. Rev. Mex. Ing. Quim., v.18, n.3, p.967-978, 2019.

MORAES FILHO, M.L.; BUSANELLO, M.; GARCIA, S. Optimization of the fermentation parameters for the growth of Lactobacillus in soymilk with okara flour. LWT - Food Sci. Technol., v.74, p.456-464, 2016.

MURPHY, P.A.; BARUA, K.; HAUCK, C.C. Solvent extraction selection in the determination of isoflavones in soy foods. J. Chromatogr. B, v.777, p.129-138, 2002.

NIYIBITURONASA, M. *et al.* The growth of different probiotic microorganisms in soymilk from different soybean varieties and their effects on antioxidant activity and oligosaccharide content. J. Food Res., v.8, p.41-51, 2019. doi: 10.55391/jfr.v8n1p41

RIZZO, G.; BARONI, L. Soy, soy foods and their role in vegetarian diets. Nutrients, v.10, n.1, 2018.

ROGINSKY, V. & LISSI E. A. Review of methods to determine chain-breaking antioxidant activity in food. Food Chem., v.92, n.2, p.235-254, 2005.

ROSENTHAL, A. *et al.* Effect of enzymatic treatment and filtration on sensory characteristics and physical stability of soymilk. Food Control, v.14, p.187-192, 2003.

SANCHES-GONZALES, I.; JIMÉNEZ-ESCRIG, A.;

SAURA-CALIXTO, F. In vitro antioxidant activity of coffee brewed using different procedures (Italian, Expresso and Filter). Food Chem., v.90, p.133-139, 2005.

SAVIANI TRENTIN, R.; SIFUENTES DOS SANTOS, J. Benefícios do emprego de substâncias funcionais no desenvolvimento de produtos. Ensaios e Ciência, v.24, n.3, p.283–288, 2020. doi: https://doi.org/10.17921/1415-6938.2020v24n3p283-288

SONG, T. *et al.* Soy isoflavone analysis: quality control and a new internal standard. Am. J. Clin. Nutr., v.68, p.1474S-1479S, 1998.

SONG, T.; HENDRICH, S.; MURPHY, P. Estrogenic Activity of glycitein, a soy isoflavone. J. Agric. Food Chem., v.47, p.1607-1610, 1999.

SZCZESNIAK, A.S.; BRANDT, M.; FRIEDMAN, H.H. Development of standart rating scales for mechanical parameters of texture and correlation between the objective and the sensory methods of texture evaluation. J. Food Sci., v.28, p.397-403, 1963.

SWAIN, T.; HILLIS, W. E. The phenolic constituents of *Prunus domestica:* the quantitative analysis of phenolic constituents. J. Sci. Food Agric., v.10, p.63-68, 1959.

TAKU, K. *et al.* Soy isoflavones lower serum total and LDL cholesterol in humans: a meta-analysis of 11 randomized controlled trials. Am. J. Clin. Nutr., v.85, p.1148-1156, 2007.

TYUG, T.S.; PRASAD, K.N.; ISMAIL, A. Antioxidant capacity, phenolics and isoflavones in soybean by-products. Food Chem., v.123, p.583-589, 2010.

URBAŃSKA, M.; GIERUSZCZAK-BIAŁEK, D.; SZAJEWSKA, H. Systematic review with meta-analysis: *Lactobacillus reuteri* DSM 17938 for diarrhoeal diseases in children. Aliment. Pharmacol. Ther., v.43, n.10, p.1025-1034, 2016. doi: https://doi.org/10.1111/apt.13590

XU, B.; CHANG S.K.C. Phytochemical profiles and health-promoting effects of cool-season food legumes as influenced by thermal processing. J. Agric. Food Chem., v.57, p.10718-10731, 2009.

WANG, C. *et al.* Effects of polymerized whey protein on goaty flavor and texture properties of fermented goat milk in comparison with β-cyclodextrin. J. Dairy Res, v.85, n.4, p.465-471, 2018.

WANG, J. *et al.* Fermentation characteristics and transit tolerance of probiotic *Lactobacillus casei* Zhang in soymilk and bovine milk during storage. J. Dairy Sci., v.92, n.6, p.2468-2476, 2009.

WATANABE, S.; UEHARA, M. Health Effects and Safety of Soy and Isoflavones, In: WATSON, R.; SINGH, R.; TAKAHASHI, T. The role of functional food security in global health. [s.l.]: Academic Press, 2019. p.379-394.

WEI, Q. K.; CHEN, T. R.; CHEN J. T. Using of Lactobacillus and Bifidobacterium to product the isoflavone aglycones in fermented soymilk. Int. J. Food Microbiol., v.177, p.120-124, 2007.

WISEMAN, H. *et al.* Isoflavone aglycone and glucoconjugate content of high- and low-soy U.K. foods used in nutritional studies. J. Agric. Food Chem., v.50, p.1404–1410, 2002.

WONG, J. M. W. *et al*. Equol status and blood lipid profile in hyperlipidemia after consumption of diets containing soy foods. Am. J. Clin. Nutr., v.95, p.564-571, 2012.

YANG, M.; LI, L. Characteristics of probiotic soy yogurt. Food Technol. and Biotech., v.48, p.490-496, 2010.

YOSHIARA, L.Y. *et al.* Optimization of soy isoflavone extraction with different solvents using the simplex-centroid mixture design. Int. J. Food Sci. Nutr., v.63, p.978-986, 2012.

YOSHIARA, L. Y. *et al.* Germination changes the isoflavone profile and increases the antioxidant potential of soybean. J. Food Bioact., v.3, p.144-150, 2018.

ZHAN, H. C. *et al.* Chemical Constituents of Soy Germ. Chem. Nat. Cpd., v.54, n.3, p.425-428, 2018.

ZHANG, X. *et al.* Chemical composition of 13 commercial soybean samples and their antioxidant and anti-inflammatory properties, J. Agric. Food Chem., v.60, p.10027-10034, 2012.