

Fermented Soy-coffee Pudding Dessert Containing Probiotics: Product Formulation and Evaluation of Compositional Changes during Fermentation

Giselle Duarte^{1,2,*}, Ilana Felberg³, Veronica Calado⁴, Juliana DePaula¹,
Monalisa S. C. de Jesus³, Rosires Deliza³, Marco Antonio L. Miguel^{5,*}, Adriana Farah^{1,*}

¹Universidade Federal do Rio de Janeiro, Instituto de Nutrição Josué de Castro, Av. Carlos Chagas Filho, 373, CCS, bloco J, Cidade Universitária - Ilha do Fundão, CEP 21941-902, Rio de Janeiro, Brasil

²Instituto Federal do Rio de Janeiro, Rua Senador Furtado, 121, Maracanã, CEP 20270-021 Rio de Janeiro, Brasil

³Embrapa Agroindústria de Alimentos, Rio de Janeiro, Brasil

⁴Universidade Federal do Rio de Janeiro, Escola de Química, Centro de Tecnologia, bloco E, Cidade Universitária - Ilha do Fundão, CEP 21941-909, Rio de Janeiro, Brasil

⁵Universidade Federal do Rio de Janeiro, Instituto de Microbiologia Paulo de Góes, Av. Carlos Chagas Filho, 373, CCS, bloco I, Cidade Universitária - Ilha do Fundão, CEP 21941-902, Rio de Janeiro, Brasil

*Corresponding author: giselle.oliveira@ifrrj.edu.br, marco.miguel@micro.ufrj.br, afarah@nutricao.ufrj.br

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Abstract This study aimed at developing a probiotic fermented soymilk-based dessert containing coffee and soybean hull. Nine fermented formulations were elaborated with 10% powdered soymilk (w/v), varying percentages of sugar, arabica soluble coffee, and soy hull. They were fermented with probiotic strains of Lactobacilli and Bifidobacteria (10^6 CFU/mL). One hundred and twenty-nine adults from Rio de Janeiro/RJ and Curitiba/PR, Brazil, evaluated the acceptance of the formulated products. The final formulation was physicochemically characterized. During 6h fermentation, the probiotics count increased from 10^6 to 10^8 in both strains. The well-accepted formulation contained 15% sucrose, 1% soy hull, and 0.5 or 1.5 % soluble coffee (score: 6.6 ± 1.5 on a 9-point-scale). Alternatively, sucrose can be replaced by other types of sweeteners. Young people ($n=45$) who drank 2-4 cups of coffee per day liked the product the most (score: 7.1 ± 1.4). While fermentation did not affect the total soy isoflavones content, it decreased the content of coffee chlorogenic acids by 32.6% but produced bioavailable phenolic acids as metabolites. A decrease in the content of flatus-producing oligosaccharides was also observed. In conclusion, probiotics fermentation and the addition of arabica soluble coffee made possible the development of a well-accepted and potentially healthy beany-flavor-free, dairy-free, pudding-like dessert.

Keywords: dairy-free, probiotic, phenolic compounds, soymilk, soy hull, vegan

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1. Introduction

In recent years, consumers' awareness of the association between food, health, and sustainability has increased considerably, leading to higher consumption of foods with human and environmental health-promoting features [1]. Simultaneously, adverse food reactions are becoming an increasing public health problem in many countries. Among the available functional foods, dairy-free, lactose-free, and gluten-free alternatives and products containing probiotics show promising trends worldwide [1-5]. It has been estimated that 15% of European consumers avoid dairy products for a variety of reasons, including medical reasons such as

lactose intolerance, cow's milk allergy, cholesterol issues and phenylketonuria, in addition to lifestyle choices like a vegetarian/vegan diet, and concerns about growth hormone and antibiotic residues in cow's milk [6,7].

Epidemiological studies show that a diet including habitual consumption of soy products or coffee may reduce the risk of several chronic diseases, most probably due to bioactive phenolic compounds in these foods (chlorogenic acids present in coffee and isoflavones in soybean) [8,9]. Soy isoflavones and metabolites are not only antioxidant compounds; they may mimic the action of estrogens on target receptors and exert many health benefits against hormone-dependent diseases, including breast and prostate cancers, osteoporosis, dyslipidemia, and menopausal symptoms [10,11,12].

Soy hull is the seed coat of soybean (approximately 5-8% of the whole bean) [13,14] and one of the significant by-products generated in the soybean processing industry [14]. It has great potential to be used as a functional food ingredient due to its high dietary fiber content, including cellulose and pectin [15]. Dry soy hull may contain variable amounts of cellulose (29–51%), hemicellulose (10-25%), lignin (1-4%), pectins (4-8%), proteins (11-15%), and minor extractives [16], depending on the dehulling processing efficiency. It is also a source of polyphenols with antioxidant activity [17]. Despite its potential, soy hull is partly used as animal feed, mainly for dairy cattle, discarded as agricultural waste or burned, causing considerable disposal costs and negative environmental issues [17,18]. Little attention is given to its potential use as a food ingredient, especially for human consumption [13,18].

Because soybean isoflavones are mainly found conjugated with glucosides, they need to be hydrolyzed either in the food matrix or by the intestinal microbiota to release more bioavailable aglycones [19,20]. Considering the large interindividual variability in the gut microbiota composition [21,22,23], and that the aglycones are more readily absorbed in the small intestine, the conversion of the isoflavone-glycosides into their aglycone before consumption is attractive [24,25]. Probiotics such as *Lactobacillus spp.* and *Bifidobacterium spp.* are viable microorganisms through the digestive system and, when ingested in adequate amounts, confer health benefits and well-being to the host [26]. Among their benefits, we can mention the production of short-chain fatty acids and improvement of the intestinal microbial balance, resulting in the inhibition of bacterial pathogens, risk reduction for colon cancer, immune system stimulation, and lowering of serum cholesterol levels [27,28]. These microorganisms can release polyphenols' aglycones in the soybean and soymilk matrixes, just as the gut microbiota. Additionally, their proteases can release small bioactive peptides, which in different studies have exhibited anti-hypertensive, antioxidant, hypocholesterolemic, chemopreventive, and antidiabetic properties [29].

Probiotics are recognized mainly for their dairy product applications, particularly yogurts and fermented milk, and the market for these products is still expanding. Simultaneously, the development of lactose-free probiotic products is still needed and demanded [30]. In addition to improving digestibility and increasing bio-functional properties, fermentation of soybean and soymilk by microorganisms has also been reported to eliminate the beany off-flavor characteristic of legume seeds caused by decomposition products of soybean lipids and improve consumers' acceptance [31,32].

Adding flavorful ingredients to soymilk is another way to mask its characteristic beany off-flavor [33,34]. Coffee exhibits a pleasantly pungent flavor, which makes it suitable for enhancing the flavor of soymilk products. Additionally, coffee has been associated with many positive health outcomes and is considered functional [35].

For the reasons above, the present study aimed at developing a fermented probiotic pudding-like

soy-coffee dessert containing soybean hull and characterize it.

2. Material and Methods

2.1. Raw Materials and Other Ingredients

Olvebra Industrial SA provided powdered soymilk (Provesol® SM-N), Rio Grande do Sul, Brazil; soybean hull was provided by the Brazilian Agricultural Research Corporation, Rio de Janeiro-RJ, Brazil; probiotic lactic culture mix consisting of *Lactobacillus acidophilus* (DSM13241); *Bifidobacterium animalis* subsp. *Lactis* (DSM15954), was from Christian Hansen, São Paulo-SP, Brazil; specialty organic medium roasted soluble arabica coffee was from Native®, SP, Brazil; sugar cane-derived sucrose was commercial; stabilizing base of gelatin, carrageenan, maltodextrin, and the mix of guar and xanthan gums were from Sabor Alternativo®, Pirai do Sul/PR, Brazil.

For soybean dehulling, the beans were dried in a laboratory oven (FabbePrimar, #170, Brazil) with forced air circulation, at 100 °C, for 5 min. After heat treatment, the beans remained at room temperature for 15 min. Then, they were placed in a rice dehuller adapted to soybean, according to the methodology described by Felberg & Cabral [36].

2.2. Product Formulation

After preliminary tests which allowed establishing the adequate amount of soymilk (10%, weight/water - w/v volume), mix of guar and xanthan gums (0.1%), stabilizer (gelatin, carrageenan and maltodextrin) (0.8%), and probiotics inoculum solution (2%), nine formulations were subjected to sensory acceptance evaluation (see section 2.3), considering three variables and three central points, as follows: sugar content (10%, 12.5%, and 15%, w/v), soy bean hull (1%, 2%, and 3% w/v) and soluble coffee (0.5%, 1%, and 1.5% w/v).

The lyophilized probiotic microorganisms were hydrated in sterile distilled water (0.13 g/mL). First, soymilk (diluted powder), sugar, a mixture of gums, and a stabilizer were stirred together to prepare the formulations. These formulations were then pasteurized in a water bath at 80 °C for 10 min to reduce their microbial counts and inactivate thermolabile antinutritional factors [37]. The inoculum was added to the mixture (at 42 °C) to obtain an initial count of 10⁶ Colony Forming Unit - CFU/mL (for each bacterium). The mixture was homogenized with a stainless steel spoon and let to ferment for 6 h at 42 °C to reach pH 4.6 - 4.7. After incubation, the product was cooled for 12 h at 10 °C. Following, soluble coffee and milled soy hull cooked in a pressure cooker for (15 min) were added (Figure 1).

Microbiological analyses were performed before sensory analysis to evaluate the microbiological safety conditions (see section 2.4.1). The growth of probiotics was followed through the fermentation period (see section 2.4.2).

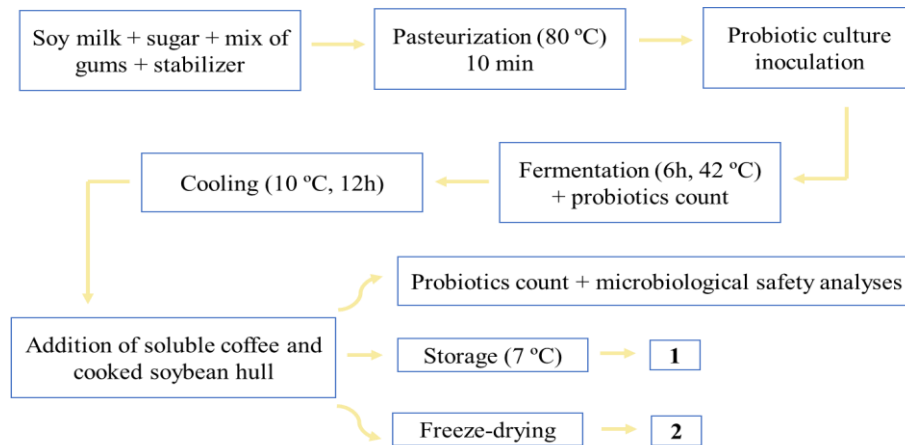


Figure 1. Experimental procedure scheme. 1: sensory analysis, titratable acidity, pH, water activity, rheological behavior, and moisture determination; sensory evaluation; 2: compositional analyses

2.3. Consumer's Acceptance Evaluation

The Research Ethics Committee of the Federal University of Paraná-UFPR, Brazil (Reg.# 243.175) approved the study. After the preliminary acceptance evaluation by a small group of people, the acceptance of nine formulated products (with two central point repetitions, totaling 11 samples) was evaluated by 129 individuals (98 female and 31 male) aged 18-66 years old who habitually consumed one or more cups of coffee per day and soy products. They were staff, students, professors, and visitors at the Federal Universities of Paraná (PR, Brazil) (n = 65) and Rio de Janeiro (RJ, Brazil) (n = 64). Before sensory analysis, assessors were asked to complete a questionnaire to provide general information (age, gender, occupation, monthly income) and habitual consumption of coffee and soy beverages or other soy products.

The acceptance test was carried out using 9-point structured hedonic scales, varying from 1 (extremely disliked) to 9 (extremely liked). Formulations were presented at 8 ± 2 °C in 30 mL plastic cups coded with three-digit numbers and monadically presented to participants, following a balanced order to prevent carryover effects [38]. Spring water was provided for mouth rinsing between samples. Assessors also evaluated the attributes “global acceptance” and how much they liked the “content of fiber”, “content of sugar”, and “content of coffee”. The acceptance test also allowed the inclusion of additional comments about the samples from participants.

2.4. Microbiological Analyses

2.4.1. Microbiological Safety Evaluation

Based on standards established by the Brazilian Health Regulatory Agency (ANVISA) [39], the following microbiological safety tests were performed in duplicate, both in the raw material and in the final product before sensory analysis: yeasts and mold count; *Escherichia coli* and thermotolerant coliforms; *Salmonella* spp., coagulase-positive *Staphylococci* and total mesophilic bacteria. Analyses followed American Public Health Association (APHA) methods [40].

2.4.2. Starter Bacteria Culture

Probiotics count was performed during fermentation as well as in the final products. Every 2 h, for 6 h, a 1mL sample was taken from the product. Serial ten-fold dilutions were prepared in peptone water - 0.1% (w/v) bacto peptone (Difco TM, New Jersey, USA), and suitable dilutions were plated on appropriate media. *L. acidophilus* and *B. animalis subsp. lactis* were inoculated on lactobacilli MRS broth (DifcoTM), supplemented with agar and maltose solution at 50% [41]. Plates were incubated at 37 °C for 72 h in anaerobic jars using the anaerobic atmosphere generator (Anaerobac®, Proback Brazil). Experiments were performed in duplicate. The identification of isolated strains was performed by Gram stain, catalase test and mass spectrometry-MALDI-TOF (Microflex LT - Brucker, Germany).

2.5. Physicochemical and Chemical Analyses

2.5.1. pH and Titratable Acidity

The pH values of samples were measured using a digital pHmeter (Macherey-Nagel®, Düren, Nordrhein-Westfalen, Germany); titratable acidity (TA) was determined by titrating samples (10 g) with sodium hydroxide (0.1 N), using 1% phenolphthalein as an indicator, according to Adolfo Lutz Institute [42]. Results were expressed as g/100g, dry basis-db. Measurements were performed in triplicate.

2.5.2. Water Activity and Rheological Behaviour

An electronic hygrometer was used for water activity (Wa) determination (Aqualab 4TE, Decagon®, São Paulo, Brazil). Reading was performed in duplicate from 0.003 to 1.000, with a precision of 0.003.

A rheometer (RVD-III – Brookfield, Middleboro, MA, USA) measured the sample's apparent viscosity as a shear rate function. The data acquisition interval was 10 seconds and the total acquisition time was 15 min. The experiments were carried out at room temperature.

2.5.3. Macro-constituents Composition

Analyses of moisture (method #972.20), ash (method #942.05), total lipids (method #983.23), and hull dietary fiber (method #985.29) were performed according to the Association of Official Analytical Chemists [43]. Crude protein was calculated as nitrogen amount times

6.25, using the micro-Kjeldahl (method#46.13) [44]. Carbohydrates and remaining constituents were calculated by difference; total sugar and dietary fiber were determined by titration, according to IAL [42]. All analyses were carried out in triplicate.

2.5.4. Sucrose and Oligosaccharides

Samples clarification and analyses of sucrose, raffinose, and stachyose were performed by HPLC-RI described in Macrae [45]. A Waters® (Milford, MA, USA) system was used, consisting of a binary pump (model #1525), RI detector (model #2414) at 30 °C and an Aminex® HPX-87H, 300 x 7.8mm (Bio-Rad Laboratories Ltd) column coupled to a cation exchange pre-column (Bio-Rad Laboratories Ltd). The mobile phase was H₂SO₄ 0.005 M, running at 0.6 mL/min, 60°C, injection volume of 20 µL. External standards were purchased from Sigma-Aldrich Chemical Co. (St. Louis, Missouri, USA), diluted in Milli-Q water, and injected in triplicate to build standard curves.

2.6. Polyphenols

2.6.1. Isoflavones

Extraction and analysis of isoflavones in the final product were performed in triplicate according to AOAC (method #2001.10) [46], using methanol/water (80:20, volume/volume - vv), followed by hydrolysis with NaOH solution, acidification, and filtration in Whatman n°1 filter paper. Analysis was carried out in an HPLC-PDA (Alliance™ 2695, Waters, Waltham, MA, USA) with a YMC-Pack Pro C18 column BDS HYPERSIL (100 x 4.6 mm, YMC, Kyoto, Japan). A gradient with acetic acid solution and methanol, running at 5.0 mL/min, was performed, with data acquisition at 260 nm. Identification and quantification of isoflavones were performed by comparing the investigated peak retention time with those of the respective standards, injected as a pool. UV spectra confirmed peak identities to avoid coelutions. Isoflavone contents were expressed as mg of aglycon/100g of sample after normalizing individual isoflavones to account for differences in molecular weight between glycoside derivatives as recommended by AOAC [46]. To calculate total isoflavones as aglycon equivalents, the total concentrations of daidzein, glycitein, and genistein were added to daidzin, glycitin, and genistin aglycon equivalent concentrations [46].

2.6.2. Chlorogenic Acids and Phenolic Acids

Extractions were performed in triplicate as in Farah et al. [47], with water/methanol (60:40, v/v), followed by precipitation of high molecular weight substances with Carrez solutions and filtration with Whatman n°1 filter paper. Identification and analyses were carried out in an LC-DAD-MS system, consisting of an LC-10AD vp quaternary pump; SPD-M10A vp DAD, and an LCMS-2010 mass spectrometer (Shimadzu, Kyoto, Japan), using a Magic C30 HPLC column (150 x 20 mm, 5 µm, 100 Å, Michrom Bioresources, Inc., Auburn, CA, USA). Spectra were acquired over the range of 190–370 nm. In addition to mass spectra, the peaks' identity was confirmed by comparison with external standards, injected as a pool, and confirmed by UV and mass spectra. For quantification, chlorogenic acid standard (Sigma-Aldrich Chemical Co) curves and molar

extinction coefficients were used as in Farah et al. [47].

2.7. Statistical Analysis

Data on global acceptance and the responses related to additional questions about the formulations were submitted to analysis of variance (ANOVA), followed by Fisher's test. Internal Preference Mapping and cluster analysis were also applied to the acceptance data.

The Acceptability Index (AI) was calculated, according to Meilgaard et al. [48], using the following equation: $AI = (X * 100) / N$, where: X = Average score given by assessors and N = Highest score given by assessors. AI equal to or greater than 70% was considered satisfactory. XLSTAT for Windows (version 2019.3, Boston, MA, USA) was used to treat the sensory analysis data. GraphPad Prism software (Version 8.4.2, Informer Technologies, Los Angeles, CA, USA) was used to treat results from chemical and microbiological analyses using ANOVA with Tukey's test. Statistica™ (version 13.1, Tulsa, OK, USA) was used to treat results from rheological analyses using ANOVA, followed by Fisher's test to obtain the model parameters, K and n. A significance level of 5% was considered for all analyses.

3. Results and Discussion

3.1. Sensory Analysis

Food safety analyses were performed on the raw material and in the final product before sensory analyses to make sure the microbial count was in accordance with the Brazilian national regulations for fermented dairy products [39]. Both the raw material and the formulations met the standards established for human consumption and for probiotic foods [39].

Considering that the same acceptance test protocol used in both educational institutions resulted in similar outcomes, with no difference in the average acceptance, both databases were treated jointly.

Results of the acceptance test revealed that the sample with 15% sucrose, 10% soymilk, 0.5% soluble coffee, and 1% soy hull, in addition to the complementary ingredients, received the highest mean score (6.6 ± 1.5), corresponding to between “liked slightly” to “liked moderately” (Figure 2). This score indicates good acceptance, considering soy products [34,49,50].

Samples with the highest soluble coffee contents (1.5%) and lowest sugar contents (10%) received the lowest scores by assessors, suggesting that the increased bitterness might have affected consumer liking. These results were confirmed when sweetness and coffee intensity were separately evaluated. The most liked samples contained 15% sugar and 0.5% soluble coffee, confirming that Brazilian consumers like sweeter products [34]. In addition, the sweetness can mask the coffee bitterness and the product's acidity. These two characteristics positively impacted global acceptance. Ferreira et al. [51] added soluble coffee to a condensed milk dessert and reported that samples with higher global acceptance scores had the lowest coffee percentage (1%), although assessors liked the coffee flavor. This percentage improved acceptance in Felberg et al. [34] when developing a soy-coffee beverage.

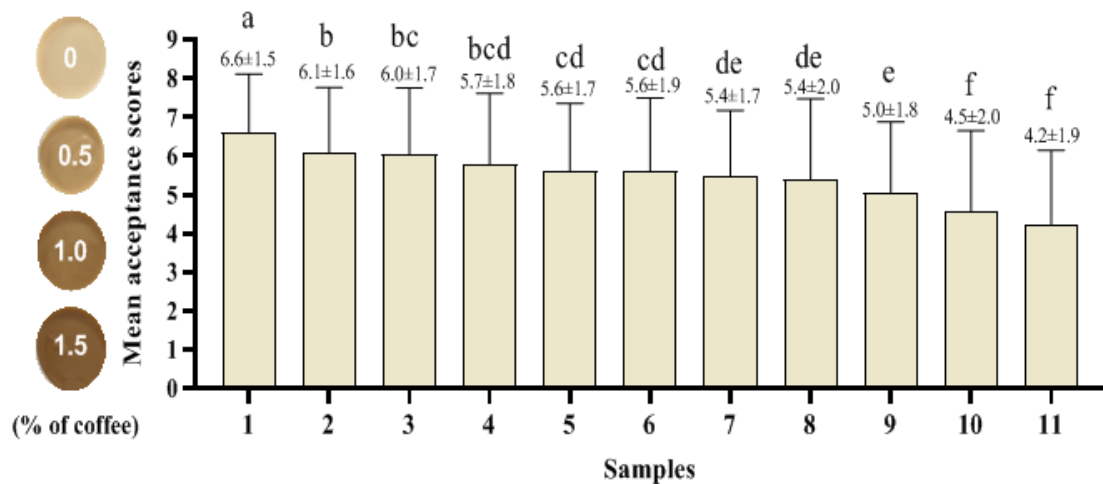


Figure 2. Average acceptance^s scores (mean for the nine fermented soymilk formulations and two central point repetitions, containing different percentages of sucrose, coffee, and soy hull. 1: 15% sugar, 1% hull and 0.5% coffee; 2: 15% sugar, 3% hull and 0.5% coffee; 3: 10% sugar, 1% hull and 0.5% coffee; 4: 12.5% sugar, 2% hull and 1% coffee; 5: 12.5% sugar, 2% hull and 1% coffee; 6: 12.5% sugar, 2% hull and 1% coffee; 7: 10% sugar, 3% hull and 0.5% coffee; 8: 15% sugar, 1% hull and 1.5% coffee; 9: 15% sugar, 3% hull and 1.5% coffee; 10: 10% sugar, 1% hull and 1.5%; 11: 10% sugar, 3% hull and 1.5%. Different letters over the bars indicate significant difference by Fisher's test ($p \leq 0.05$) § 1 = disliked extremely; 5 = nor liked neither disliked; 9 = liked extremely. Mean $\pm$ SD

The amount of fiber did not affect acceptance scores statistically, although, in the individual questions, samples with 1% fiber received higher scores (Figure 2). It is worth mentioning that assessors did not have options with lower soy hull percentages or no hull. A similar situation occurs with sugar and coffee. The statistical model did not make available the choice of 12.5% sucrose and 1% coffee, which could have been accepted as the samples with 15% sucrose and 0.5% coffee. In the preliminary acceptance test, 1% coffee received the highest mean score. Further tests are required considering the results achieved in the present study.

It is worth noting that participants did not mention beany flavor or any off-flavor common to soybean products. This fact confirms previous results by Luz et al. [52], who reported that both high-temperature extraction and soymilk fermentation reduced the beany off-flavor. According to the authors, soymilk fermentation produces similar sensory characteristics to fermented dairy products.

Meilgaard et al. [48] reported that for a product to be considered sensorially accepted, an AI equal to or higher than 70% is needed. The most well-accepted sample (15% sucrose, 0.5% coffee, and 1% hull) had an AI of 74%, indicating it achieved the required index. The study by Felberg et al. [34] reported AI of 71% for a non-fermented soy-coffee beverage. No fermented soy-coffee desserts were found in the literature for comparison.

Considering that people differ in their physiological and psychological aspects and have different backgrounds and life experiences, Internal Preference Mapping was applied to the data, as mentioned before, because this methodology considers individual preferences. Results can be seen in Figure 3. The first and second dimensions explained 42% of the variance (1st dimension 29.4%, and the 2nd 12.6%). The cluster analysis identified three segments of consumers. Figure 3a shows the position of the consumers and clusters, and Figure 3b shows the position of the samples.

Cluster 1 ($n = 45$) grouped assessors who gave the highest scores to samples with 15% sucrose, 1% soy hull, 1.5% coffee (mean score 7.3) and 12.5% sucrose, 2% soy hull and 1% coffee (mean score 7.1), with no difference between both samples. This cluster was composed of 60% assessors from Rio de Janeiro, primarily young students (18 - 25 years old) with a monthly income of 1 to 10 minimum wages. These assessors most often drank 2 to 4 cups of coffee daily.

In Cluster 2 ($n = 57$) most assessors were from Paraná, 18 - 35 years old. They preferred the sample with 15% sucrose, 1% soy hull and 0.5% coffee (mean 6.9), which did not differ from samples with 15% sucrose, 3% soy hull, and 0.5% coffee (average 6.7) and the formulation with 12.5% sucrose, 2% soy hull, and 1% coffee (average 6.7). Curiously, participants in this group declared drinking more than 4 cups of coffee a day.

Cluster 3 ($n = 25$) was the smallest segment, and 52% of assessors were from Rio de Janeiro, among which 40% were teachers (mostly 45-55 years old) and graduate students. They gave the worse acceptance scores to all samples; however, the most liked formulation was appreciated by the two other segments and had 15% sucrose, 1% soy hull, and 0.5% coffee. These assessors drank the least amount of coffee and less frequently than Clusters 1 and 2.

Considering the results of Cluster 1, who liked the product the most, the preliminary test results, the individual questions and that one of the main characteristics of the product was bringing together the bioactive compounds from soy and coffee, we chose to use 15% sugar, 1% soy hull and 1% coffee, in the final product formulation. Hence, the physical-chemical characterization of the final formulation and evaluation of the compositional changes during fermentation will be reported.

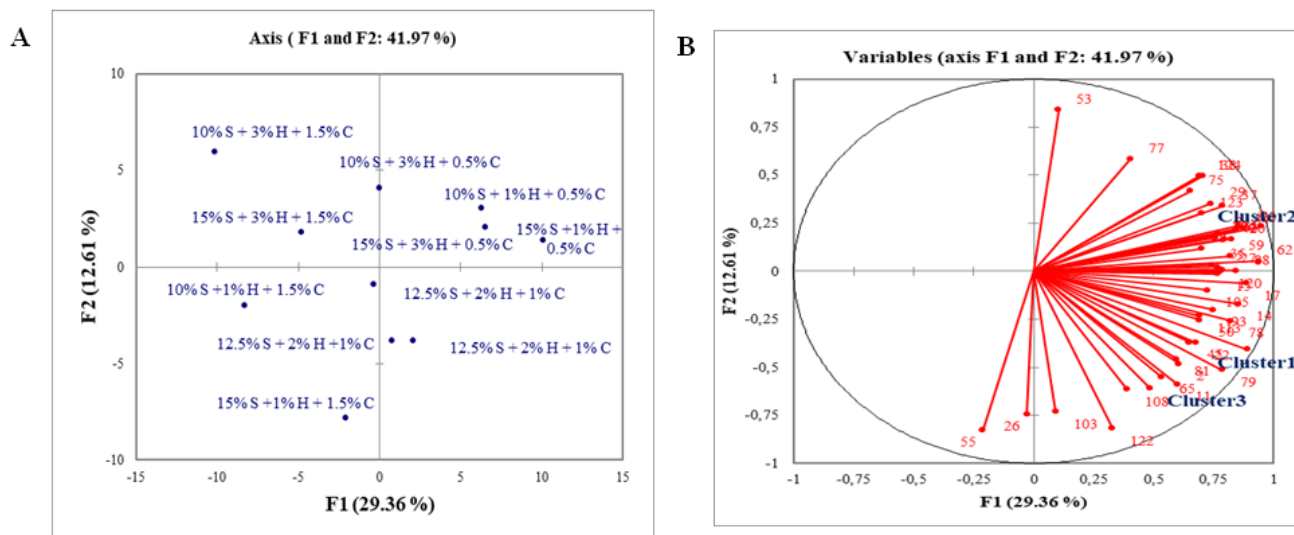


Figure 3. Internal Preference Mapping, showing: (a) the assessors' segments and (b) the samples. S: sugar; H: soy hull; C: soluble coffee

3.2. Viability of Probiotic Microorganisms in the Final Formulation

The initial inoculum count of *L. acidophilus* and *B. animalis* was 3.00 and 5.33 CFU/mL, respectively. The growth of *L. acidophilus* in soymilk occurred slower initially compared to what usually occurs with cow's milk [52] (data not shown). However, after 2-4 h of incubation, the growth of both microorganisms improved significantly ($p < 0.02$). The slow initial growth (Figure 4) possibly occurred because of the production of antimicrobial compounds by *Bifidobacterium*, such as acetic acid, as observed by Farnworth et al. [53], who investigated the

behavior of different probiotic bifidobacteria strains when searching for bacteria that could co-exist symbiotically in a soy-based yogurt type of product. An additional reason would possibly be the adaptive phase (LAG phase). The MRS media where the bacteria were activated contained glucose, while in the dessert, mostly sucrose and oligosaccharides were available as substrates. After 6 h fermentation, *L. acidophilus* and *B. animalis* count increased to 7.77 and 7.81 CFU/mL, respectively (Figure 4), in agreement with Yerlikaya [54], who used the same strains to ferment cow's milk, and with Brazilian legislation standards for cow's milk yogurt. There are no standards for the present type of product in Brazilian legislation [39].

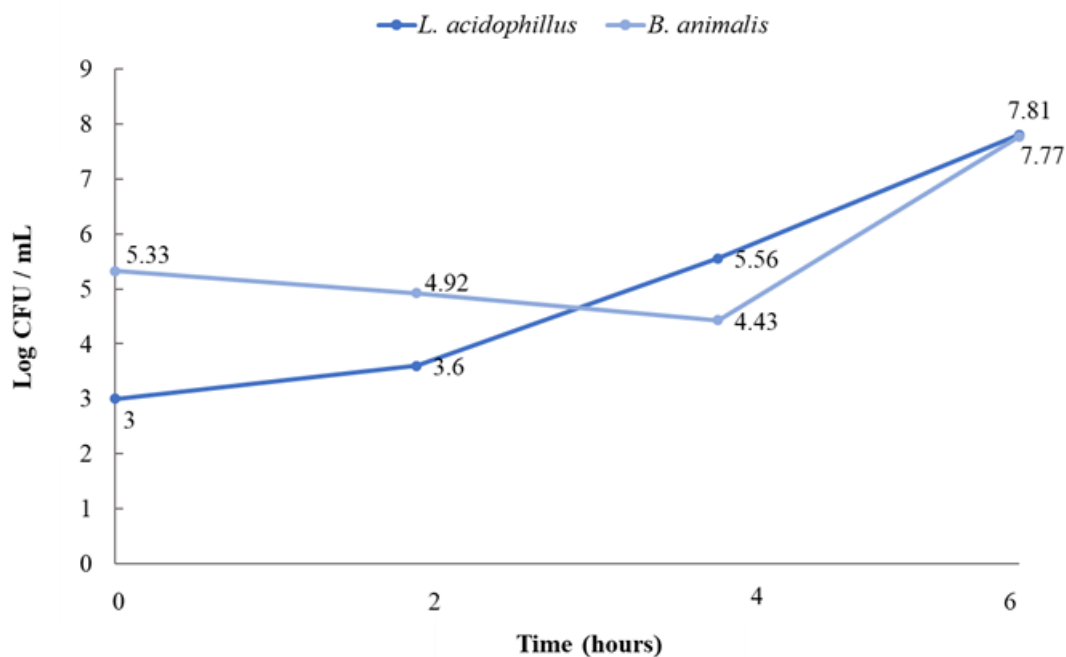


Figure 4. Growth curve of probiotic microorganisms in the fermented soy-coffee pudding dessert

3.3. Physicochemical and Chemical Analyses

3.3.1. pH and Titratable Acidity (TA)

After 6 h of fermentation, the pH and TA of the final soymilk formulation were 4.67 ± 0.03 and 0.68 ± 0.01 (g /100g lactic acid), respectively. According to Yerlikaya [54], a pH between 4.6 and 4.7 is a determinant factor for casein coagulation during cow's milk yogurt production. According to Yamamoto et al. [55], pH from 4.34–5.78 were able to coagulate soymilk in yogurt production. Hwang et al. [56] reported final pH of 4.1 to 5.1 in soy yogurts fermented by different strains. The soy protein fractions have different functional properties related to gel formation, thermal stability, and emulsification [57,58], compared to cow's milk. Soybean proteins are mainly storage globulins, especially β -conglycinin (7S) and glycinin (11S). The amino acid composition influences the gel formation in soybean proteins; containing over 31.5% non-polar amino acid residues per mol, they tend to form opaque and irreversible coagulum-type gels [59], which is very positive to produce a pudding-like dessert.

The TA value conforms with the range of values (0.6-1.5g lactic acid/100g) established by the Brazilian Ministry of Agriculture, Livestock and Food Supply [60] for cow's milk yogurts and similar products. The remaining ingredients (coffee and soy hull) did not affect pH or TA.

3.3.2. Water Activity (Wa) Determination and Rheological Properties

The Wa of the final product was 0.982 ± 0.003 . Wa affects the viability of probiotic bacteria in their environment. Available water determines the viability and functionality of living systems, and, in this case, most microorganisms cannot multiply below 0.90 Wa [61].

High concentrations of sugar added to the medium before fermentation can inhibit multiplication, increase fermentation time, and develop low acidity because of the osmotic effects of solutes in the medium and consequent low water activity [62]. Among probiotic bacteria, bifidobacteria demonstrate greater resistance to unfavorable conditions [63].

Regarding the rheological properties, the product presented a pseudoplastic behavior, following the Power Law equation $\sigma = K\dot{\gamma}^n$, where σ is the shear stress, K is the flow consistency index, $\dot{\gamma}$ is the shear rate, and n is the flow behavior index. The model parameters K and n were estimated, and their values are shown in Table 1. Three coffee concentrations were used to obtain the mathematical model, showing that adding more soluble coffee tended to decrease viscosity.

Table 1. Parameters of the power law model for the control and the three tested soluble coffee percentages (0.5%, 1.0%, and 1.5%)

Parameters	Control	0.5% Coffee	1.0% Coffee	1.5% Coffee
K	77.07 ± 1.127^a	58.95 ± 0.921^b	35.85 ± 1.017^c	32.81 ± 0.807^c
N	0.106 ± 0.003^c	0.145 ± 0.003^b	0.189 ± 0.006^a	0.196 ± 0.005^a

Different superscript letters on the same row indicate statistical difference by ANOVA followed by Fisher test at 0.05% significance level.

The viscosity curves for three coffee percentages added to the fermented soy product are presented in Figure 5. The inset shows the shear rate region characteristic of the chewing action. It is possible to note that the increase in coffee percentage decreased viscosity. However, it is worth noting that this difference increased between the groups control and 0.5% and between groups 1.0% and 1.5%, with no difference within each group.

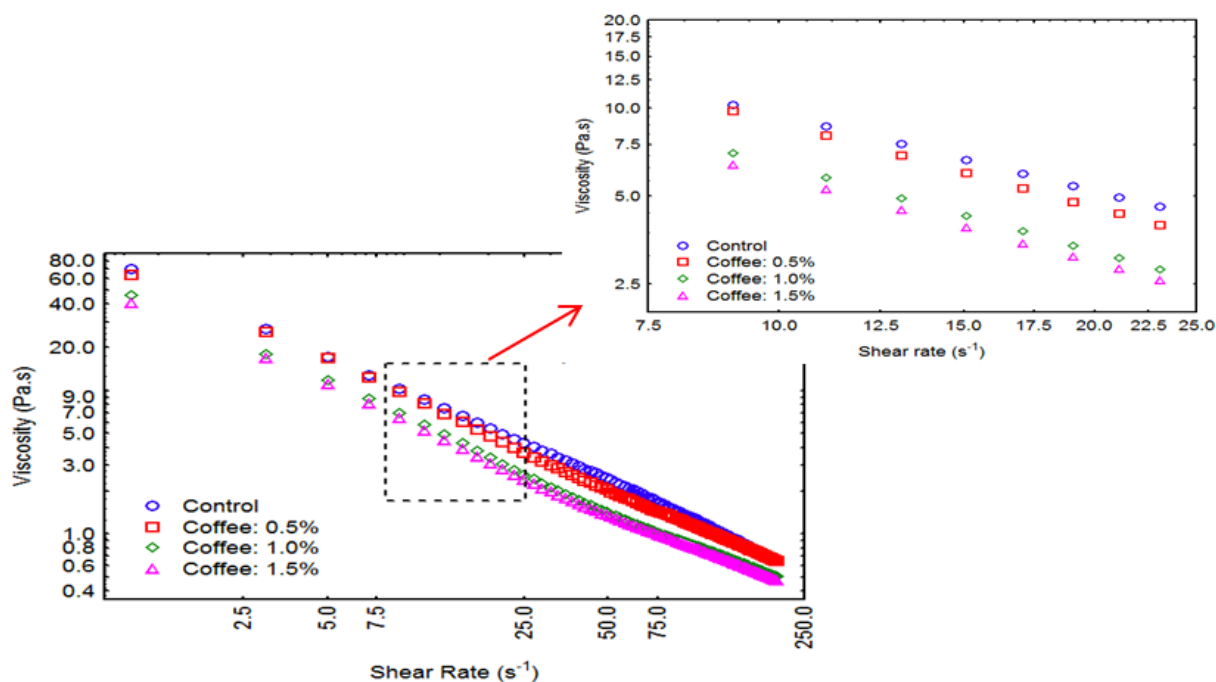


Figure 5. Viscosity curves for the control sample (no coffee added) and three tested coffee percentages (0.5%, 1.0%, and 1.5%) added to the fermented soy-coffee fermented dessert

3.3.3. Macroconstituents Composition

The macroconstituents composition of the final formulation is presented in Table 2.

Table 2. Macroconstituents composition of the final fermented soy-coffee pudding dessert

	g/100g (wb)	g/100g (db)
Water	73.6 ± 0.4	-
Protein	4.4 ± 0.4	16.7 ± 1.5
Lipids	2.6 ± 0.2	9.9 ± 0.8
Ash	0.9 ± 0.1	4.2 ± 1.2
Dietary fiber	1.4 ± 0.3	6.1 ± 0.5
Carbohydrates and other components by difference	16.7	63.1

wb – wet basis; db – dry basis.

The composition of fermented soymilk products vary considerably, and data on soymilk based dessert are rare. Genevois et al. [4] obtained 76.8% water, 6.6% protein, 4.5% lipids, 1.4% ash, 0.5% dietary fiber and 11.0% carbohydrates by difference in a slightly similar product using stevia and strawberry artificial flavor and pulp. In the present study, protein and lipids values are slightly higher than in the USDA Food Data Central compositional table for soy yogurt (3.5% and 2.0%, respectively) [64]. Fuchs et al. [65] obtained similar values to those published by USDA for a soy yogurt supplemented with oligofructose and inulin. Although soybean contains all eight essential amino acids for human nutrition, it contains relatively small amounts of methionine and cysteine [66]. Despite this, soy products' protein is comparable to high biological value proteins, such as those in milk and egg [67,68]. The fiber content is attributed to the soymilk and to the soy hull. The remaining amount is attributed to sucrose, oligosaccharides, polyphenols and other constituents not measured in this study, like saponins and other minor constituents.

3.3.4. Sucrose and Oligosaccharides

Table 3 shows the content of sucrose and α -galactosides in the formulated product before and after fermentation. The amount of sucrose in the product before fermentation is the result of the initial amount of sucrose ($2.66 \pm 0.02\%$, wb) with the addition of 15% sucrose.

Lactobacillus spp. and *Bifidobacterium spp.* can reduce different carbohydrates and accumulate final products such as lactic and acetic acids [30]. However, although these bacteria are more capable of utilizing lactose from cow's milk, they can also use sugars in soymilk like raffinose and stachyose as substrates, given that these bacteria produce α -galactosidase to break them down [69,70,71]. In this study, the amount of raffinose was reduced by 14% and stachyose by 2%, while the amount of sucrose was reduced by 8.1%, indicating that the probiotic bacteria utilized these carbohydrates as substrates for growth. The preferred type of sugar for growth has been reported to vary for different probiotic strains [72]. Martinez-Villaluenga et al. [69] reported similar α -galactosidase content for fermented soymilk (0.15% of raffinose and 0.59% stachyose, wb). Regarding the amount of sucrose, we have not found a similar fermented soy product to compare. Although this percentage seems to be high for a functional product, the

amount of sucrose added to regular pudding desserts available in the Rio de Janeiro market is about 20-22%, given that the Brazilian population inherited the preference for sweeter products from the Portuguese culture [34,73]. Therefore, our dessert is a functional alternative with lower amount of sugar. As aforementioned, decreasing the amount of sugar in preliminary studies may reveal the bitterness of coffee and the acidity produced by fermentation. Healthier options of sugar, such as coconut sugar and brown sugar (largely available in the Brazilian market), enhanced the product taste in preliminary tests and can be tried in commercial products. It could also be mixed with natural low-calorie sweeteners. We opted for using plain sucrose in this study to decrease variables that could change the probiotic's behaviour. Tests evaluating the effect of other sweeteners and probiotics growth behaviour are needed in the future.

Table 3. Content of sucrose and oligosaccharides before and after the soy-coffee pudding fermentation

	Sucrose	Raffinose	Stachyose
	(g/100g wb)		
Unfermented	17.66±0.49 ^a	0.21±0.004 ^a	0.51±0.009 ^a
Fermented	16.24±0.10 ^b	0.18±0.007 ^b	0.50±0.007 ^b
	(g/100 g db)		
Unfermented	66.90±1.86 ^a	0.80±0.02 ^a	1.93±0.03 ^a
Fermented	61.51±0.38 ^b	0.66±0.03 ^b	1.89 ±0.03 ^b

wb – wet basis; db – dry basis.

3.3.5. Content of Polyphenols

The contents of isoflavones from soymilk (sum of daidzein, glicitein, genistein, and daidzin, glicitin, genistin as aglycone equivalents), chlorogenic acids from coffee (as the sum of three caffeoylquinic acids, three feruloylquinic acids, three dicaffeoylquinic acids), and two caffeoylquinides and phenolic acids, also from coffee, were evaluated before and after the product fermentation by probiotics. Results are presented in Tables 4a-c.

The total isoflavones content (aglycones and glucosides) did not change significantly after fermentation by probiotics (Table 4a). However, a 11.7% reduction in glucosides content was observed ($p = 0.005$), while total aglycones content was raised by 31.8% ($p = 0.000$). It is known that microbial fermentation promotes the hydrolysis of glycosylated isoflavones and releases aglycones, increasing their content [74,75]. β -glycosidase produced by *L. acidophilus* and *B. lactis* was probably responsible for the breakdown of isoflavones [76]. The higher percent increase in aglycones compared to the decrease in glucosides has also been observed by Tsangalis et al. [22], who studied the changes in isoflavone and β -glucosidase activity during fermentation of soymilk using *Bifidobacterium animalis* for 24h. While total isoflavones contents did not change during fermentation, total chlorogenic acids contents were reduced by 32.6% (Table 4b). This reduction was probably caused by the utilization of their quinic acid moiety and part of the cinnamic acid moiety for growth. These results corroborate our results reported by Sales et al. [28]. In the study, chlorogenic acids were used by probiotic strains and considered potential prebiotic compounds. Intestinal microbial fermentation of chlorogenic acids is well

documented [77]. The same intestinal metabolites from chlorogenic acids were identified in the dessert (Table 4c and Figure 6). Despite the reduction in total chlorogenic acids content, the remaining amount can still contribute to polyphenols intake, and the metabolites can be readily

absorbed, given that soy proteins do not affect polyphenols absorption as in cow's milk [23,77]. Considering this reduction, the use of coffees lightly roasted to supply a higher amount of these compounds to the product is recommended.

Table 4a. Contents of isoflavones in the formulated fermented soy-coffee pudding dessert

	Product	Unfermented	Fermented	
Isoflavones (mg/100g, db)	Glucosides	Daidzin	7.07±0.31 ^b	8.36±0.66 ^a
		Glycitin	5.41±0.31 ^{ab}	5.55±0.30 ^a
		Genistin	21.32±1.08 ^a	15.95±0.90 ^b
		Total Glucosides	33.81±1.67 ^a	29.87±1.87 ^b
	Aglicones	Daidzein	3.86±0.22 ^a	4.16±0.20 ^b
		Glycitein	1.80±0.07 ^a	1.94±0.11 ^b
		Genistein	6.96±0.37 ^a	10.51±0.46 ^b
		Total Aglicones	12.61±0.67 ^a	16.62±0.77 ^b
	Total isoflavones (db)		46.4 ^a	46.5 ^a
	Total isoflavones (wb)		12.3 ^a	12.3 ^a

Results are average of triplicate extraction ± SD. Different letters in the same column indicate statistical difference by ANOVA, followed by LSD test. wb – wet basis; db - dry basis.

Table 4b. Contents of chlorogenic acids in the formulated fermented soy-coffee pudding dessert

	Product	Unfermented	Fermented	
Chlorogenic acids (mg/100g, db)	Caffeoylquinic acids (CQA)	3-CQA	7.11 ± 0.12 ^a	5.18 ± 0.22 ^b
		4-CQA	8.10 ± 1.53 ^a	6.66 ± 0.37 ^a
		5-CQA	14.82 ± 0.07 ^a	10.37 ± 0.79 ^b
		Total CQA	30.03 ± 1.48 ^a	22.21 ± 0.64 ^b
	Feruloylquinic acids (FQA)	3-FQA	0.81 ± 0.01 ^a	0.70 ± 0.04 ^b
		4-FQA	1.15 ± 0.04 ^a	1.11 ± 0.01 ^a
		5-FQA	1.86 ± 0.11 ^a	1.38 ± 0.06 ^b
		Total FQA	3.81 ± 0.06 ^a	3.18 ± 0.10 ^b
	Dicaffeoylquinic acids (diCQA)	3,4-diCQA	1.44 ± 0.12 ^a	1.09 ± 0.03 ^{bc}
		3,5-diCQA	3.77 ± 0.03 ^a	3.17 ± 0.08 ^b
		4,5-diCQA	2.08 ± 0.02 ^{ab}	2.22 ± 0.04 ^a
		Total diCQA	7.28 ± 0.37 ^a	6.48 ± 0.15 ^b
	Total CGL		0.86 ± 0.02 ^a	0.84 ± 0.01 ^a
	Total CGA (db)		42.0 ^a	32.7 ^b
	Total CGA (wb)		11.1 ^a	8.6 ^b

Total CQA: sum of 3-CQA: 3-caffeoylquinic acid + 4-CQA: 4-caffeoylquinic acid + 5-CQA: 5-caffeoylquinic acid. Total FQA: sum of 3-FQA: 3-feruloylquinic acid + 4-FQA: 4-feruloylquinic acid + 5-FQA: 5-feruloylquinic acid. Total diCQA: sum of 3,4-diCQA: 3,4-dicaffeoylquinic acid + 3,5-diCQA: 3,5-dicaffeoylquinic acid + 4,5-diCQA: 4,5-dicaffeoylquinic acid. Total CGL: sum of 3-CQL: 3-caffeoylquinolactone + 4-CQL: 4-caffeoylquinolactone. Total CGA: sum of all compounds; wb – wet basis; db – dry basis. Results are average of triplicate extraction ± SD.

Table 4c. Contents of phenolic acids in the formulated fermented soy-coffee pudding dessert

Product		Unfermented	Fermented
Phenolic acids (mg/100g, db)	Caffeic acid	0.32±0.01 ^e	0.42 ± 0.01 ^d
	Ferulic acid	0.44±0.01 ^d	0.53 ± 0.01 ^d
	<i>p</i> -coumaric acid	Tr	0.39 ± 0.02 ^d
	Gallic acid	Tr	0.24 ± 0.03 ^d
	Hippuric acid	Tr	0.70 ± 0.04 ^d
	3,4-dihydroxyphenylacetic acid	Nd	0.52 ± 0.02 ^c
	4-hydroxyphenylacetic acid	Nd	0.57 ± 0.04 ^d
	Vanillic acid	Nd	0.38 ± 0.03 ^c
	Dihydrocaffeic acid	Nd	0.48 ± 0.01 ^d
	Total phenolic acids (db)	0.76	4.23
	Total phenolic acids (wb)	0.20	1.12

Results are average of triplicate extraction ± SD. Different letters in the same column indicate statistical difference by ANOVA followed by Fisher test. Tr: traces; Nd: not detected.; wb – wet basis, db - dry basis.

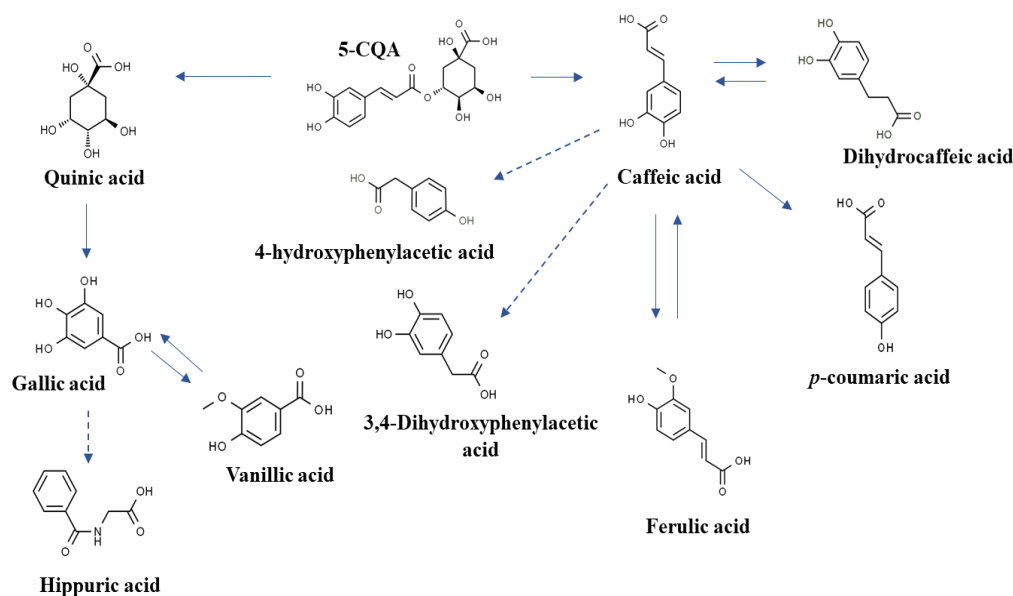


Figure 6. Microbial metabolites of chlorogenic acids (represented by 5-caffeoylquinic acid) identified in the fermented soy-coffee pudding dessert and the proposed metabolic route. Adapted from [77]

4. Concluding Remarks

An accepted, potentially healthy lactose-free, beany flavor-free, and probiotic pudding-like soy-coffee dessert containing soy hull fiber was developed. The addition of soluble arabica coffee and fermentation contributed to consumer acceptance in Rio de Janeiro/RJ and Curitiba/PR, Brazil, suggesting an improvement of the sensory characteristics of a soy-based product. Future studies must be conducted to investigate the sensory attributes and identify the drivers of liking to make available products that meet consumers' demands. The product was especially well accepted by young people who regularly drank coffee, but it can also be a good option for those who suffer from allergies to cow's milk protein. It can also serve different age groups of a growing niche in the consumer's market for non-dairy and lactose-free food products.

Sucrose can be replaced by other types of sugar or by mixtures with sugar substitutes. Despite the high-quality ingredients, its cost should be accessible, especially in Brazil, where soy and coffee are two of the most important and abundant crops.

Fermentation did not change isoflavones content, contributed to acceptance, decreased the amount of flatus-producing oligosaccharides, and increased the probiotics count. Despite the decrease in chlorogenic acids from coffee, the released phenolic acids were still available in the product for absorption. Changes during the storage of the product will be reported in the following manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

Author Contributions

G. Duarte: conceptualization, formal analysis, data curation writing - original draft, writing, - review & editing; I. Felberg: data curation, review & editing; V. Calado: formal analysis; J. DePaula: formal analysis; M.S.C de Jesus: formal analysis; R. Deliza: formal analysis, review & editing; M. A. L. Miguel: Data curation; A. Farah: conceptualization, data curation, writing - review & editing, supervision, funding acquisition. All authors have read and agreed to the published version of the manuscript.

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