

# Fungal-Mediated Biotransformation of Soybean Supplemented with Different Cereal Grains into a Functional Compound with Antioxidant, Anti-Inflammatory and Antitumoral Activities

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**Abstract:** The aim of this study was to select fungal strains and alternative substrates to increase the production of bioactive compounds by solid-state bioprocessing using soybeans. Initially, from a total of 17 fungi strains, *R. oligosporus* NRRL 3267, *R. oligosporus* NRRL 2710, and *R. arrhizus* NRRL 2582 were pre-selected for presenting the greatest antioxidant activities during soybean fermentation. The three *Rhizopus* strains were cultured in soybeans supplemented with different cereal grains (brown rice, wheat, corn, and oat) aiming to achieve a higher antioxidant activity. Soybean supplementation with brown rice increased the concentration of phenolic compounds (0.697 to 6.447 mg GAE g<sup>-1</sup>) significantly compared with the only use of soybean (1.792 to 3.10 mg GAE g<sup>-1</sup>), using *R. oligosporus* NRRL 2710. The improved fungal-mediated biotransformation process (*R. oligosporus* NRRL 2710 with soybeans and brown rice) generated an isoflavone aglycone-rich product, containing different antioxidant compounds, such as trans-cinnamic acid, gallic acid, myricetin, quercetin, and kaempferol. The fermented substrate also showed great potential to inhibit hyaluronidase enzyme (anti-inflammatory activity) and against CaCo-2 tumor cells growth (antitumor activity). The resulting compound could serve as a value-added food and/or feed ingredient and a rich source of bioactive compounds.

**Keywords:** fermentation; antioxidants; anti-inflammatory; polyphenolic compounds; trans-cinnamic acid; *Rhizopus* sp.

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## 1. Introduction

Antioxidants are among the main health components that consumers expect in their food products, due to beneficial effects in the prevention of many diseases caused by oxidative cell processes and species oxygen-reactive, such as aging, cataracts, coronary heart diseases,

arteriosclerosis, arthritis, diabetes and cancer [1-3]. Phenolic compounds are an important class of phytochemicals antioxidants. These compounds reach at least 8000 phenolics structures known to date [4], acting as reducing agents (free radical terminators) and metal chelates and singlet oxygen quenchers [5]. Extensively studied sources of natural antioxidants are fruits, vegetables, seeds, cereals, berries, wine, tea, onion bulbs, olive oil, coffee, and aromatic plants [6]. However, natural antioxidants can also be released via biotechnological processes by submerged or solid-state microbial fermentation. Solid-state fermentation (SSF) has some facilities related to the recovery of bioproducts and the use of natural substrates as nutritional support. Since 1986 in Brazil, a series of research projects for the value-addition of tropical agricultural products and sub-products by SSF has been developed due to the generation of large amounts of agricultural residues [7]. Then, in the last years, SSF has been employed to increase the content of phenolic compounds in certain food products, thus enhancing their antioxidant activity [8–10].

Soybean *Glycine max L. Merrill* is naturally known for presenting health benefits and high protein content. It has been successfully cultivated around the world and is largely produced by the world's top producers United States, Brazil, Argentina, China, and India. It is widely known that soybeans fermented by filamentous fungi possess enhanced nutritional value, such as antioxidants, vitamins, phytochemicals, and essential fatty acids [11–15]. Other agroindustrial grains are also good sources of antioxidants constituents but very little explored in fermentation processes [16, 17].

Soybeans are the richest sources of isoflavones (phytoestrogen compounds with estrogenic activity) in the human diet [18–20]. Isoflavones are divided into two basic phytochemical categories: glycosides and aglycones [21]. The aglycone forms are characterized by being more quickly absorbed by the organism and have the higher estrogenic and antioxidant potential [22, 23]. The percentage of aglycones in non-fermented soybeans ranges between 10 and 40%. In contrast, the percentage of aglycones in fermented soy products ranges between 40% and 100% [24, 25]. Thus, the consumption of fermented soy products in Eastern countries is associated with the reduction of chronic diseases, because the consumption of natural antioxidants is effective in reducing the deleterious effects of reactive oxygen species (ROS) and restoring the body's antioxidant load [26–29].

The aim of the present study was to select fungi strains and alternative substrates to increase the production of bioactive compounds by solid-state bioprocessing using soybean supplemented with different cereal grains, as well as to evaluate the anti-inflammatory and antitumor effects against CaCo-2 and MCF-10 cells of the obtained substrate.

## 2. Materials and Methods

### 2.1. Microorganisms.

Seventeen fungi strains of the genus *Rhizopus* spp. (Figure 1) were used in this study, belonging for ARS Culture Collection of the Northern Regional Research Laboratory (NRRL, USA). The strains were maintained in Potato Dextrose Agar (PDA) slants at 4°C.

### 2.2. Substrate treatment.

All the substrates used in this study (soybean, brown rice, wheat, corn, and oat) were pre-soaked in deionized water containing 5% v/v of acetic acid. After 12 h, the substrates were

thermally treated under fluent steam at 100°C for 15 min. The excess water was removed, and the substrates cooled inoculation temperature of 30°C.

### 2.3. Selection of fungi strains.

The fungal strains were pre-selected for antioxidant activity under SSF using soybean (SB) as a substrate. The substrate thermally treated was inoculated with  $10^7$  spores per gram of dry matter. The experiments were carried out in perforated trays at 30°C for 72 h. At the end of the 72 h of fermentation, antioxidant activity was evaluated as described below. Non-fermented soybean was used as a control.

### 2.4. Antioxidant activity in SB supplemented with different cereal grains.

#### 2.4.1. Substrate selection.

SB supplementation tests were performed with fungal strains presenting higher antioxidant activity, viz., *R. oligosporus* NRRL 3267, *R. oligosporus* NRRL 2710, and *R. arrhizus* NRRL 2582. The cereal grains were brown rice (BR), wheat (W), corn (C), and oat (O). Before supplementation, selected grains were prepared by a thermal treatment, as previously described. Five experiments were conducted supplementing the SB by the grains in the proportion of 1:1 and inoculated with  $10^7$  *Rhizopus* spp. spores  $g^{-1}$  of a dry substrate. The experiments were carried out in perforated trays at 30°C for 72h. The antioxidant activity of supplemented treatments was assessed, and non-fermented soybean was used as a control.

#### 2.4.2. *Rhizopus* selection.

The kinetic behavior of the best treatments (SB + BR fermented by *R. oligosporus* NRRL 2710 or *R. arrhizus* NRRL 3267) was evaluated through 120 h of the fermentation process. Samples were taken at an interval of 24 h for antioxidant activity and phenolic content determination, as described below. From these results, *R. oligosporus* NRRL 2710 was selected for further characterization of isoflavone content and  $\beta$ -glucosidase, anti-inflammatory and antitumor activities.

### 2.5. Analytical procedures.

#### 2.5.1. Preparation of ethanol extracts.

Alcoholic extraction of fermented substrates and control (non-fermented soybean) was performed according to the method described by [30]. A 1-g freeze-dried sample was crushed in a kitchen blender (Turbo Blender) for 5 min. Dry powder was extracted with 70% (v/v) for one hour in a shaking water bath at 120 rev/min. The extracts were collected and centrifuged at 2268xg for 15 min. Supernatants were stored at -18°C for further analysis.

#### 2.5.2. Measurement of DPPH radical-scavenging activity.

The antioxidant activity of the alcoholic extracts was assessed based on the radical scavenging effect of the stable DPPH-free radical activity (1,1-diphenyl-2-picrylhydrazyl) (Sigma-Aldrich, USA), as described by [31]. A methanol solution of DPPH (0.004% w/v) was prepared. BHA (butylated hydroxyanisole) (Sigma-Aldrich, USA) was used as a standard, prepared in concentrations from 0.02 to 1.00 mg of BHA  $mL^{-1}$ . One milliliter of the DPPH

methanolic solution was added to 250  $\mu\text{L}$  sample extracts at different concentrations (50; 100; 150; 200; 250  $\mu\text{L } \mu\text{L}^{-1}$ ). After dark incubation at ambient temperature for 30 min, the absorbance was measured at 517 nm (Spectrum band, UV-VIS). Ethanol solution without the sample was considered as a control.  $\text{IC}_{50}$  value (index representing the concentration of the antioxidant, which can reduce 50% of free radicals) was obtained using a standard curve. The inhibition rate (%) was calculated to the following equation:

$$\text{inhibition (\%)} = \left[ \frac{A^{\text{control}} - A^{\text{extract}}}{A^{\text{control}}} \right] \times 100$$

Where:

$A^{\text{control}}$  = DPPH absorbance (diluted with ethanol solution)

$A^{\text{extract}}$  = DPPH + sample extract absorbance (diluted with ethanol solution)

The percent antioxidant activity was plotted against log absorbance values to generate a straight line to calculate the half-inhibitory concentration ( $\text{IC}_{50}$ ) in  $\text{mg mL}^{-1}$ . The anti-free radical activity (expressed as  $\text{IC}_{50}$ ) was defined as the quantity of antioxidant substances needed to reduce 50% of the initial concentration of DPPH. The higher the power anti-free radical, the most effective antioxidant, and, consequently, lower the  $\text{IC}_{50}$  value by [31].

#### 2.6. Determination of phenolic content.

Total phenol content [32] was determined by the Folin-Ciocalteu method [33]. 0.1 mL of the alcoholic extracts was mixed with 0,4 mL of Folin-Ciocalteu reagent (10% v/v) and 0,4 mL of sodium carbonate (7,5% w/v). Samples, protected from light, were shaken by 1 h. Ethanol was used as a control. The samples were analyzed in triplicate. A standard curve prepared using gallic acid monohydrate was used to calculate the total phenol content, which was expressed as gallic acid equivalent in  $\text{mg L}^{-1}$  of the extract [34–36].

#### 2.7. Determination of $\beta$ -glucosidase activity.

$\beta$ -glucosidase activity of ethanol extracts from *R. oligosporus* NRRL 2710 fermentation of SB + BR (0, 24, 48 and 72 h) was assayed by monitoring the release of p-nitrophenol (pNP) using p-Nitrophenyl- $\beta$ -D-glucopyranoside (pNPG) as the substrate, according to the method described by Zhang et al., 2012. A total of 20  $\mu\text{l}$  of appropriately diluted enzyme solution and 280  $\mu\text{l}$  of 1.785 mM pNPG in 100 mM acetate buffer pH 4.8 were incubated for 15 min at 50  $^{\circ}\text{C}$ . The reaction was quenched by adding 400  $\mu\text{l}$  of glycine buffer, and the amount of p-nitrophenol released during the reaction was determined with a spectrophotometer at 430 nm. A p-nitrophenol calibration curve was previously prepared to calculate the enzyme activity. One unit of  $\beta$ -glucosidase activity was defined as the amount of enzyme equivalent to the release of 1  $\mu\text{mol}$  of p-nitrophenol per minute.

#### 2.8. HPLC analysis of isoflavone.

The ethanol extracts from *R. oligosporus* NRRL 2710 fermentation of SB + BR (0, 24, 48, and 72 h) were filtered with a Spritzen syringe filter of 0.22  $\mu\text{m}$  (Millipore, Bedford, MA). Isoflavones content was analyzed using an Agilent Technology 1200 Series system, coupled to a diode array detector (DAD) at wavelengths 235, 260, 275, 280, 290, 311, 357, 370 nm and scanning from 190 nm to 600 nm. A Zorbax Elipse XDB – C 18 (4.6 x 150 mm, 5 – micron) column was used at 0.7 mL  $\text{min}^{-1}$  flow. The mobile phase utilized as methanol and acetic acid 2.5% (50:50 v/v). Genistein and daidzein (SIGMA Aldrich, EUA) were used as standards. To

obtain the calibration curve, all standard reagents were solved in the mobile phase and used at 1, 2, 5, 8, and 10 ppm. The injection volume was 10  $\mu\text{L}$ , and the run time was 36 min. The resulting chromatograms values were graphed, and the linear equation was used to calculate the isoflavones content of the samples.

#### 2.9. Identification and quantification of antioxidant compounds by RP-HPLC-DAD/UV.

The ethanol extracts from *R. oligosporus* NRRL 2710 fermentation of SB + BR (0, 48, and 72 h) were filtered with a Spritzen syringe filter of 0.22  $\mu\text{m}$  (Millipore, Bedford, MA). The filtrate was used to quantify the polyphenolic compounds by High-performance liquid chromatography with UV-vis detection (HPLC-DAD/UV-vis). HPLC analysis was performed according to [37], with a reversed-phase Acclaim® 120 column, C18 5 $\mu\text{m}$  120 Å (4.6 mm $\times$ 250 mm). The column was maintained at 40°C, and the detection was recorded at 280, 300, and 320 nm. Polyphenolics and flavonols were detected in the range of 210 and 320 nm. The mobile phase was acidified water with phosphoric acid 1 % and methanol. The solvent gradient was as follows: 0–15 % B for 2 min, 15–25 % B for 5 min, 25–30 % B for 10 min, 30–35 % B for 15 min, 35–50 % B for 25 min, 50–60 % B for 30 min, 60–80 % B for 35 min, 80–100 % B for 45 min and 100–5 % B for 60 min. Flow rate: 1 mL min<sup>-1</sup>. Standards used: for phenolic acids (gallic acid, chlorogenic acid, trans-cinnamic acid, caffeic acid, p-coumaric acid, ferulic acid) and flavonols (rutin, myricetin, quercetin, and kaempferol). Stock solutions of all standards were prepared in methanol. The calibration curves were obtained from triplicate injections of at least five concentrations and identification based on the retention times of the standards [38] in chromatogram at 280 nm. Retention times (min) for the standards at the conditions used were: gallic acid: 6.83 min; caffeic acid: 32 min; p-cumaric acid: 18.54 min; ferulic acid: 20.07 min; rutin: 25.03 min; myricetin: 28.22 min; trans-cinnamic acid: 32.40 min; kaempferol: 37.08 min.

#### 2.10. In vitro evaluation of the anti-inflammatory activity.

The anti-inflammatory activity of the substrate obtained by *R. oligosporus* NRRL 2710 fermentation of SB + BR was evaluated by the *in vitro* approach, according to [39], [40] and [41]. 50  $\mu\text{L}$  of the extracted ethanolic extracts were prepared at different concentrations (1, 3, 10, 30, 100, 300  $\mu\text{g mL}^{-1}$ ) added to 0.5 mL of the potassium salt of hyaluronic acid (Sigma-Aldrich, St.Louis, MO, USA) (1.2 mg hyaluronic acid per mL of 0.1 M acetate buffer, pH 3.6, containing 0.15 M NaCl). The control tube consisted of the same reagent of test tubes without ExPP. The reactions were incubated at 37°C for 5 min. Then, 50  $\mu\text{L}$  of the hyaluronidase enzyme (Sigma-Aldrich, St.Louis, MO, USA) was added and incubated at 37 °C for 40 min. The reaction was stopped by adding 10  $\mu\text{L}$  of sodium hydroxide (4 N) and immediately placing 0.1 mL of 0.8 M potassium tetraborate and incubating in a boiling bath for 30 min. After the incubation time, 3 mL of 4-dimethylaminobenzaldehyde (DMAB) (10% solution in glacial acetic acid containing 12.5% 10 N hydrochloric acid) was added and incubated at 37 °C for 20 min. Next, the absorbance of the solutions was measured in a spectrophotometer (SP 2000 UV Spectrum) at 585 nm. DMSO was used as a positive control due to its ability to inhibit de hyaluronidase enzyme completely. Propolis, a natural anti-inflammatory agent, was also included as a positive control. The results were expressed as the ability to inhibit the hyaluronidase enzyme, in percentage.

2.11. Cytotoxicity assessment for tumor (CaCo-2) and normal (MCF-10) cells by colorimetric assay MTT (1- (4,5-dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium).

The cell lines CaCo-2, which is Colorectal Cancer, and MCF-10 A, which is a non-tumoral breast epithelial cell, were used to perform the assays with ethanol extracts from *R. oligosporus* NRRL 2710 fermentation of SB + BR. The ethanol extracts were first lyophilized for subsequent resuspension. Both cell lines were purchased from the Cell Bank of Rio de Janeiro, Brazil, passage 33. The lines were cultured in a 96-well plate in DMEM F12 medium with 1% penicillin/streptomycin for 24 h for cell adhesion. CaCo-2 cells were plated at  $5 \times 10^4$  cells per well and MCF 10A cells at the concentration of  $3 \times 10^4$  cells per well. After the 24 h incubation period, the medium was monitored by DMEM F12 with 10% penicillin/streptomycin and supplemented with 10% fetal bovine for both species. Plates were maintained in an incubator at 37 °C, 95% air atmosphere and 5% CO<sub>2</sub> for 48 h.

Cell viability was analyzed by colorimetric assay MTT (1- (4,5-dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium), described by [42]. The MTT solution (Sigma-Aldrich) was prepared at the concentration of 5 mg ml<sup>-1</sup> and added to the culture medium, where the final concentration in the well was 0.5 mg ml<sup>-1</sup>. After 3 h of incubation at 37°C, 95% air atmosphere, and 5% CO<sub>2</sub>, the blue crystals formed were solubilized in DMSO (Panreac®), considered the most effective solvent to be capable of dissolving the formazan crystals easily. The reading was performed in a spectrophotometer (Biotek) at 595 nm.

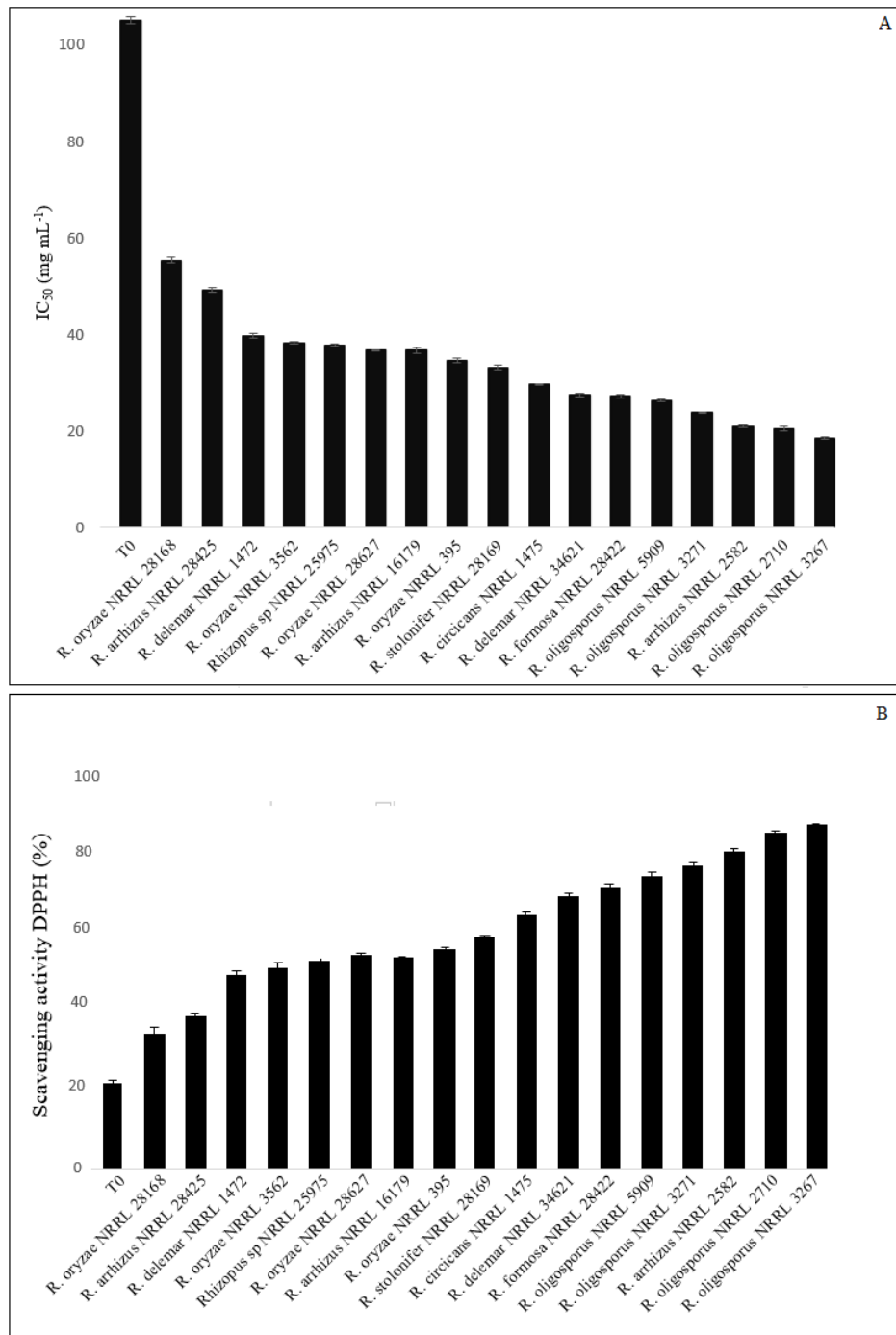
2.12. Statistical analysis.

Analysis results were submitted to the statistical variance, and the post-test was chosen according to the normality of the data obtained, such as Tukey Post-Test for data which follow normality. The program PRISMA® (GraphPad Prism 5 for Windows, version 5.4) was used. The averages of data are presented in graphs and the standard deviation shown by the error bars.

### 3. Results and Discussion

3.1. Screening of *Rhizopus* strains.

In Asiatic countries, *Rhizopus* spp. have been known as one of the economically important molds because of their role as an inoculum source for making tempeh, a traditional soybean-based fermented food [11, 13, 43]. In this study, a preliminary screening was carried out with 17 fungi strains of the genus *Rhizopus* based on their DPPH scavenging activity after 72 h of soybean under SSF. All *Rhizopus* strains presented positive results for antioxidant activity, *i.e.*, they decreased IC<sub>50</sub> values, which is a concentration of sample required to scavenge 50% of the DPPH (Figure 1a). Three strains, namely *R. oligosporus* NRRL 3267 (18.53 mg mL<sup>-1</sup> and 88.1%±0.38 for IC<sub>50</sub> and scavenging DPPH %, respectively), *R. oligosporus* NRRL 2710 (20.49 mg mL<sup>-1</sup> and 85.7%±0.27 for IC<sub>50</sub> and scavenging DPPH %, respectively), and *R. arrhizus* NRRL 2582 (20.91 mg mL<sup>-1</sup> and 81.2%±0.31 for IC<sub>50</sub> and scavenging DPPH %, respectively), were pre-selected for possessing the best scavenging activities (Figure 1b). Other authors also reported good results of radical scavenging activity in fermented beans of *Canavalia* using *Rhizopus microspores* var. *oligosporus* (MTCC#556) for 1 week [44] and *Bacillus subtilis* SB-MYP-1 for 72 h [14].



**Figure 1** DPPH activity (IC<sub>50</sub> mg mL<sup>-1</sup>) (A) and scavenging DPPH % (B) of the ethanolic extracts of different *Rhizopus* strains cultivated in soybeans by 72 h.

### 3.2. Substrate selection for antioxidant biosynthesis.

The three pre-selected *Rhizopus* strains were cultured in soybeans supplemented with different cereal grains (brown rice, wheat, corn, and oat) aiming to achieve a higher antioxidant activity. All supplementation processes were efficient in increasing the antioxidant power of the substrate, as determined by the decrease of IC<sub>50</sub> values after 72 h of fermentation (Table 1). The best results ( $p < 0.05$ ) were achieved when SB was supplemented with BR by all strains analyzed. There is a lack of data in the literature on the real increase in scavenging activities due to the fermentation process. In general, existing studies do not assess scavenging activity at initial fermentation time, dedicating only to resulting products. As an example, [45] found

19.1 mg mL<sup>-1</sup> scavenging activity of fermented soybean + rice by *Aspergillus oryzae* after 16 days for sufu production. However, the increment of the values during fermentation was not reported. The data of the present study clearly show the real increment in scavenging activities promoted by the fermentation process. SB supplementation with brown rice resulted in the greatest antioxidant potential for *R. oligosporus* NRRL 2710 (IC<sub>50</sub> 14.3 mg mL<sup>-1</sup>), followed by *R. arrhizus* NRRL 2582 (IC<sub>50</sub> 19.2 mg mL<sup>-1</sup>) and *R. oligosporus* NRRL 3267 (IC<sub>50</sub> 33.7 mg mL<sup>-1</sup>) (Table 1). For this reason, the SB supplemented with BR and fermented by *R. oligosporus* NRRL 2710 and *R. arrhizus* NRRL 2582 were selected for further studies. The choice of using *R. oligosporus* NRRL 2710 was also based on its wide use as a fermenting agent for Tempeh production [46].

**Table 1.** IC<sub>50</sub> values (mg mL<sup>-1</sup>) during fermentation in SB ethanol extracts and grain mixtures at the started point (T<sub>0</sub>) and after 72 hours of fermentation with the selected strains.

		<i>R. oligosporus</i> NRRL 2710	<i>R. oligosporus</i> NRRL 3267	<i>R. arrhizus</i> NRRL 2582
<b>SB</b>	T <sub>0</sub>	105.01±0.62 <sup>aA</sup>	105.01±0.62 <sup>aA</sup>	105.01±0.62 <sup>aA</sup>
	72 h	20.69±0.27 <sup>aA</sup>	18.53±0.38 <sup>aB</sup>	20.90±0.31 <sup>aA</sup>
<b>SB + BR</b>	T <sub>0</sub>	<b>374.5±0.61<sup>dA</sup></b>	<b>374.5±0.61<sup>eA</sup></b>	<b>374.5±0.61<sup>eA</sup></b>
	72 h	<b>14.3±0.13<sup>aA</sup></b>	<b>33.7±0.51<sup>bB</sup></b>	<b>19.2±0.31<sup>aA</sup></b>
<b>SB + OAT</b>	T <sub>0</sub>	180.8±0.39 <sup>bA</sup>	180.8±0.39 <sup>cA</sup>	180.8±0.39 <sup>cA</sup>
	72 h	69.5±0.84 <sup>bA</sup>	131.9±1.15 <sup>dB</sup>	63.3±0.16 <sup>cA</sup>
<b>SB + C</b>	T <sub>0</sub>	346.2±0.32 <sup>cA</sup>	346.2±0.32 <sup>dA</sup>	346.2±0.32 <sup>dA</sup>
	72 h	23.3±0.50 <sup>aA</sup>	32.93±0.59 <sup>bB</sup>	45.7±0.50 <sup>bC</sup>
<b>SB + W</b>	T <sub>0</sub>	166.1±0.97 <sup>abA</sup>	166.1±0.97 <sup>bA</sup>	166.1±0.97 <sup>bA</sup>
	72 h	54.0±0.93 <sup>bA</sup>	76.6±0.31 <sup>cB</sup>	52.2±0.50 <sup>cA</sup>

\*Means followed by the same letter did not differ statistically (p > 0.05). Lowercase letters refer to the statistical test between the lines and capital letters between the columns.

### 3.3. Kinetic of fermentation with *R. oligosporus* NRRL 2710 and *R. arrhizus* NRRL 2582 in SB + BR.

Kinetics of scavenging activity (IC<sub>50</sub>) and phenolic content were performed through 72h of SB+BR fermentation by *R. oligosporus* NRRL 2710 and *R. arrhizus* NRRL 2582 (Figure 2). For both strains, scavenging activity had a sharp decrease from 0 to 72 h, followed by a stabilization until the end of the fermentation process (Figure 2a). The best antioxidant activity (indicated by the small IC<sub>50</sub> value) was reached by *R. oligosporus* NRRL 2710 (14.1 mg mL<sup>-1</sup>) in relation to *R. arrhizus* NRRL 2582 (23.15 mg mL<sup>-1</sup>). The content of phenolic compounds showed a progressive increase in the course of the fermentation (Figure 2b). Similarly, *R. oligosporus* NRRL 2710 reached the highest phenolic content (6.44 mg and 3.81 mg GAEg<sup>-1</sup> for *R. oligosporus* NRRL 2710 and *R. arrhizus* NRRL 2582, respectively). Thus, *R. oligosporus* NRRL 2710 was selected for further characterization.

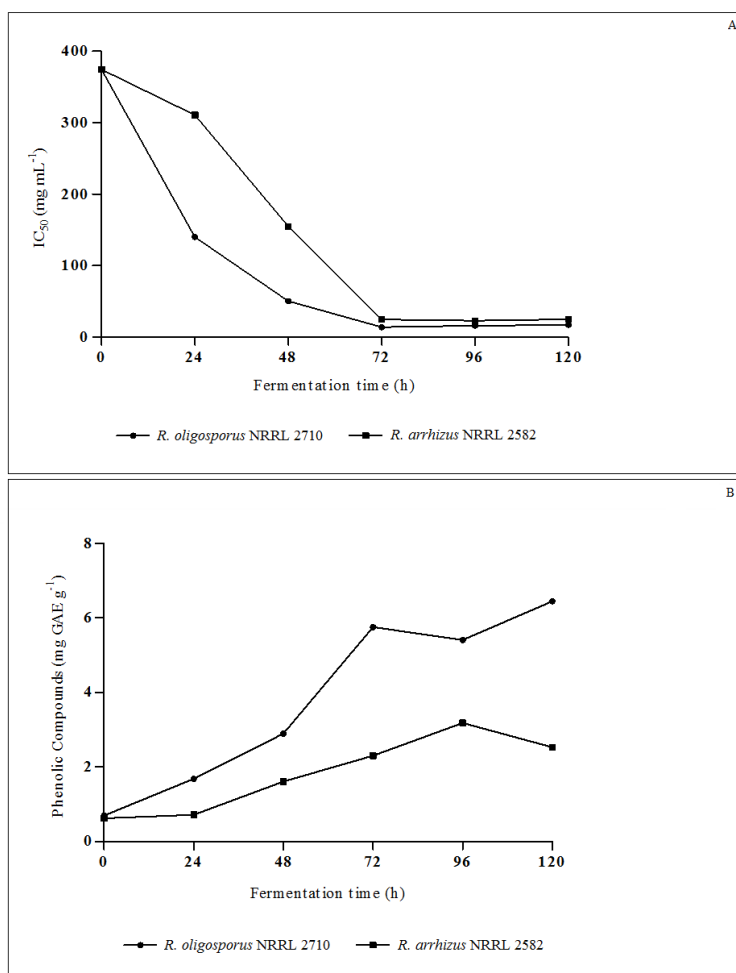
### 3.4. Identification and quantification of polyphenolic compounds.

Table 2 shows the identification and respective concentrations of polyphenolic compounds synthesized by *R. oligosporus* NRRL 2710 under SSF of SB and SB+BR, as quantified by RP- HPLC-DAD/UV. Different polyphenolic compounds were detected, such as gallic acid, ferulic acid, rutin, myricetin, caffeic acid, trans-cinnamic acid, quercetin, kaempferol, syringic acid, and p-cumaric acid. These compounds have been previously found in SSF by different fungi species [47–50][47,48].

The data showed that trans-cinnamic acid was the compound present in higher amounts, reaching 124.7 µg mL<sup>-1</sup> (3,197 mg gdb<sup>-1</sup>) and 103.3 µg mL<sup>-1</sup> (2,649 mg gdb<sup>-1</sup>) in SB and SB+BR



fermented after 72 h, respectively. The cinnamic acid or *trans*-3-phenylacrylic acid is an important precursor of the biosynthesis of other important groups of polyphenolic compounds in the microorganism metabolism pathway. Cinnamic acid is biosynthesized by the enzyme phenylalanine ammonia-lyase (PAL), which converts the amino acid phenylalanine in cinnamic acid. It is also a precursor to the sweetener aspartame via enzyme-catalyzed amination to phenylalanine.



**Figure 2.** Kinetics of DPPH scavenging activity (A) and phenolics content (B) of the ethanolic extracts of *R. oligosporus* NRRL 2710 and *R. arrhizus* NRRL 2582 cultivated in soybean supplemented with brown rice by 72h.

Cinnamic acid has been studied as an antitumoral compound, which induces tumor cell differentiation by modulating the expression of genes implicated in tumor metastasis and immunogenicity in cultured human melanoma cells. There are few reports on cinnamic acid production by fermentation process [51]. This work indicates a great potential for producing this compound due to the high accumulation in the fermented substrate. The separation and concentration of cinnamic acid from SB +BR fermented by *R. oligosporus* NRRL 2710 represent a great perspective for application in food and supplement products.

Soy is a known source of isoflavones—a subclass of flavonoids with powerful antioxidant activity and linked to cancer prevention [52-53]. Soybeans contain more isoflavones in the glycoside form (daidzin and genistin) than aglycone form (daidzein and genistein). Fermentation of soybeans promotes the conversion of glycoside into aglycone isoflavones through the action of  $\beta$ -glucosidase produced by microorganisms [54, 55]. While

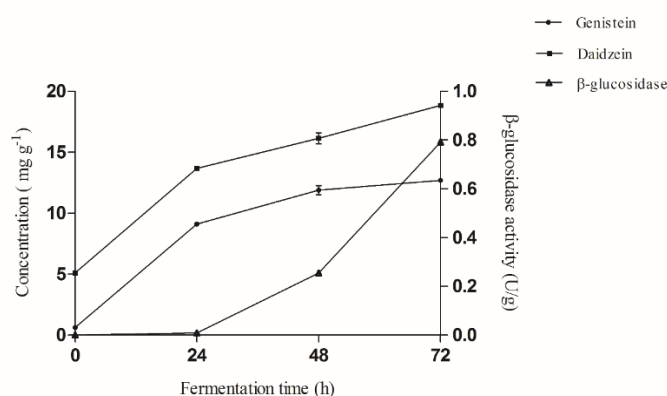
all isoflavones are absorbed into the mucosa of the small intestine, the aglycone form is absorbed in a greater rate and also has higher antioxidant activity than the glycoside form [56].

**Table 2.** Identification and quantification (mg gdb<sup>-1</sup>) of polyphenolic at the ethanolic extracts during SSF of SB mixture with BR synthesized by *R. oligosporus* NRRL 2710.

Substrates	Concentrations (µg mL <sup>-1</sup> )	Fermentation time (h)		
		0	48	72
SB	Gallic acid	0.2	0.0	0.0
	Ferrulic acid	0.3	0.3	0.3
	Rutin	29.3	6.1	8.7
	Miricetin	9.8	6.9	7.4
	Caffeic acid	0.0	0.0	0.0
	Trans-cinnamic acid	0.0	93.4	124.7
	Quercetin	0.0	0.0	0.2
	Kaempferol	0.0	0.1	0.1
SB + BR	Gallic acid	0.0	0.0	0.1
	Ferrulic acid	0.1	0.2	0.2
	Rutin	11.8	9.3	2.9
	Miricetin	6.5	7.0	7.4
	Caffeic acid	0.0	0.0	0.0
	Trans-cinnamic acid	19.8	57.3	103.3
	Quercetin	0.0	0.0	0.0
	Kaempferol	0.0	0.0	0.1
	Siringic acid	4.0	0.0	0.0
p-cumaric acid	0.1	0.0	0.0	

3.5. Changes in the isoflavone profile during fermentation of SB+BR by *Rhizopus oligosporus* NRRL 2710.

Thus, it is more effective to obtain the benefits of ingesting isoflavones when in the aglycone than glycoside form [57]. In this study, the biotransformation of isoflavones during fermentation of SB+BR by *Rhizopus oligosporus* NRRL 2710 was studied using high-performance liquid chromatography (HPLC) and β-glucosidase activity using spectrophotometry (Figure 3). The concentrations of daidzein and genistein (aglycone form) in the final fermented product (18.65 ± 0.08 mg g<sup>-1</sup> and 12.68 ± 0.05 mg g<sup>-1</sup>, respectively) were 3.75 and 20.10 fold, respectively, compared to non-fermented soybeans.



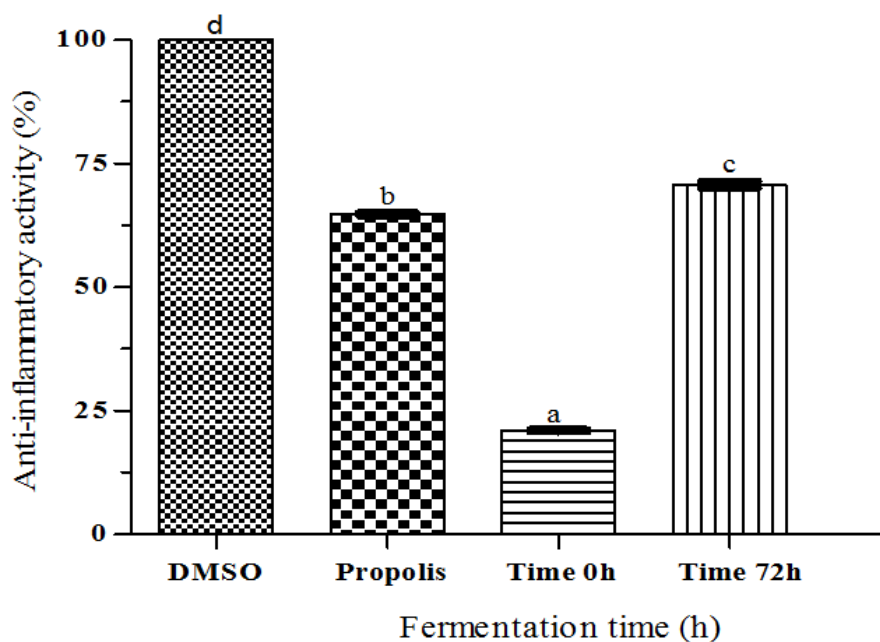
**Figure 3.** β-glucosidase activity and isoflavones aglycone content (daidzein and genistein) during soybeans plus brown rice fermentation by selected *R. oligosporus* NRRL 2710.

Simultaneously with the accumulation of aglycone isoflavones, β-glucosidase increased significantly after 24 h of fermentation (ranging from 0.01 to 0.81 U/g at the end of the process), is thus responsible for catalyzing aglycone isoflavones from glycine form. The values found in this study are significantly higher than those observed by [58] (0.24 mg g<sup>-1</sup> and

0.043 mg g<sup>-1</sup> for daidzein and genistein, respectively) after 72 h of soybean fermentation using *Tricholoma matsutake*, and by [24] (1.084 mg g<sup>-1</sup> and 1.810 mg g<sup>-1</sup> of daidzein and genistein, respectively) after 72 h of okara fermentation with *Saccharomyces cerevisiae*.

### 3.6. Anti-inflammatory activity.

The anti-inflammatory activity of the *R. oligosporus* NRRL 2710 ethanolic extract was assessed by hyaluronidase inhibitory activity. The hyaluronidase enzyme acts on the extracellular matrix of the tissues, increasing the permeability, and facilitating the access of pathogens involved in inflammatory processes [59–61]. The results obtained are shown in Figure 4. The fermentation process increased hyaluronidase inhibitory activity 3.36 times when compared to unfermented soybeans (70.75% and 21.03% inhibition capacity for fermented and unfermented soybeans, respectively). The fermented ethanolic extract also showed significantly higher activity than propolis, a commercial anti-inflammatory product [41] tested a group of 31 flavonoids against the bovine testicular hyaluronidase enzyme. The presence of double bond between carbons 2 and 3 of the fundamental nucleus of flavonoids was a fundamental condition for the compound to present hyaluronidase inhibitory activity; a keto group at position 4 and the introduction of 5, 7 and 4' hydroxyls showed to increase the effect of flavonoids [62]. However, the presence of glycosidic substituents decreased the inhibitory effect on the bovine testicular enzyme. The author concluded that aglycones forms of flavonoids are better inhibitors of hyaluronidase than their glycosylated derivatives. Thus, the improved fermentation process of *R. oligosporus* NRRL 2710 in SB + BR can have changed levels and forms of the isoflavones, promoting an increase in the aglycone content [63–65]. A dose-response analysis demonstrated that the ideal concentration value for inhibition to occur was 0.62 mg mL<sup>-1</sup>, in a 95% confidence interval (data not shown).

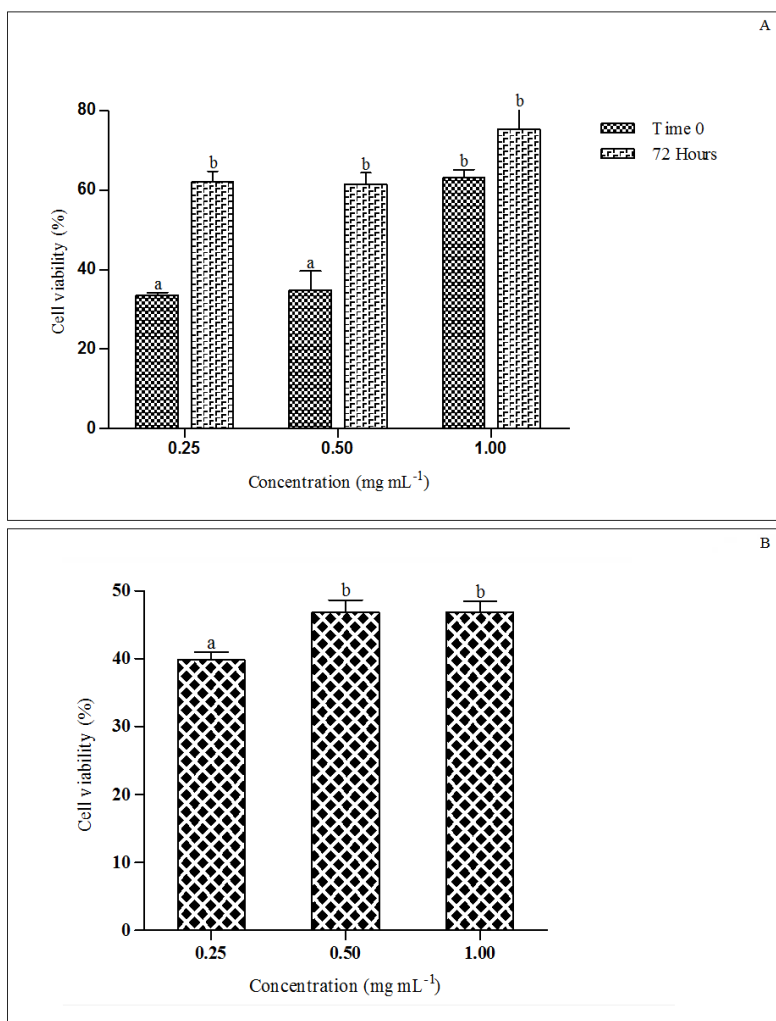


**Figure 4.** Anti-inflammatory activity of alcoholic extract of the *R. oligosporus* NRRL 2710 cultivated in soybean supplemented with brown rice by 72 h.

\*DMSO: positive control capable of completely inhibiting the enzyme hyaluronidase (HIA); Propolis: commercial anti-inflammatory

3.7. Cytotoxicity assessment for tumor (CaCo-2) and normal (MCF-10) cells.

The values of cytotoxicity assessment for both unfermented soybeans (0 h) and fermented substrate (72 h) showed cytotoxicity for normal cells (MCF-10), as demonstrated by the decrease of viable cells at levels below 50% (Figure 5A). However, ethanolic extracts from 48 and 72 h were considered moderately cytotoxic, with viable cells percentage values between 61.07 and 66.49%, respectively. According to statistical analysis, these extracts presented no significant difference at the 95% level ( $p < 0.05$ ). Considering these results, a cell viability study was carried out with CaCo-2 cancer cells to evaluate the cytotoxicity of the 72-h *R. oligosporus* NRRL 2710 fermented ethanolic extract at different concentrations (Figure 5B). The extracts presented a percentage of cellular viability between 39.53 and 46.89%. The inhibitory concentration values ( $IC_{50}$ ) were  $0.27 \text{ mg mL}^{-1}$  for CaCo-2 cells and  $0.79 \text{ mg mL}^{-1}$  for normal MCF-10 cells, indicating a higher cytotoxic effect against the carcinogenic cell.



**Figure 5.** Cytotoxicity assessment of alcoholic extract of the *R. oligosporus* NRRL 2710 cultivated in soybean supplemented with brown rice against (A) normal cells (MCF-10) and (B) CaCo-2 tumor cells.

\*The data represent the mean values  $\pm$  standard deviation of three independent experiments.

A lower percentage of cancer cell viability was observed at lower concentrations, showing that high concentrations are not required to attain cancer cell death. According to the statistical analysis, the extracts at different processing times showed a significant difference at the 95% level ( $p < 0.05$ ), suggesting that the *R. oligosporus* NRRL 2710 SB+BR fermentation process was efficient; the lower value of cell viability of the cancer cells was obtained after 72 h of fermentation (39.53%). According to [66] the variability of the results can be explained

due to the presence of isoflavones and other phenolic compounds. Several studies show the inverse association between the consumption of soy products and the risk of degenerative diseases, such as cancer [68, 68]. The chemopreventive effects of soy are attributed to its bioactive molecules, such as isoflavones, which interact with the metabolic pathways responsible for controlling cell growth, proliferation, and differentiation [69–72].

#### 4. Conclusions

This study reported that the selection of alternative substrates and fungal strains is an important strategy to improve the biosynthesis of antioxidant compounds by SSF. Soybean supplementation with brown rice increased the scanning activity significantly compared to the exclusive use of soybean, by *R. oligosporus* NRRL 2710 and *R. arrhizus* NRRL 2582 after 72 h of fermentation. This study demonstrated that the fermentation process was effective in the accumulation of myricetin, gallic acid, ferulic acid, kaempferol, and increased accumulation of trans-cinnamic acid. The fermented substrate could be processed as a new flour to be applied as a nutritional complement or as a food ingredient and dietary supplement with high antioxidant properties. In addition, this novel fermentation process points out to accumulation of cinnamic acid, which can be used as a novel, alternative way for the production of this antitumor compound. The actions are shown with *in vitro* anti-inflammatory, and antitumor models were effective. Further *in vivo* studies should be performed for the specification of these biologically active principles.

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#### Conflicts of Interest

The authors declare no conflict of interest.

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