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RESEARCH

Impact of Cooking and *Rhizopus oligosporus* Fermentation on Antinutritional Factors and Isoflavones in Soybeans

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Abstract
The aim of this study was to investigate the effect of cooking and fungal fermentation during processing of fermented soybeans on antinutritional factors and isoflavones in soybeans. For this purpose, soybeans were cooked at 95 °C with a seed/water ratio of 1/3 for 10 to 60 min and fermented with the food fungus *Rhizopus microsporus var. oligosporus*. The kinetics of α-galactosides, phytic acid, and isoflavones were measured during cooking and fermentation. Diffusion in cooking water was also characterized and results for fungal fermentation were compared to a non-inoculated treatment. During cooking, α-galactosides, glycosides isoflavones, and in lower extent phytic acid decreased due to diffusion in cooking water and conversion of glycoside isoflavones in aglycone isoflavones. The decrease in α-galactosides was no longer significant or rather small after 30 min of cooking. Fermentation of 30-min cooked soybeans with *R. oligosporus* showed sporulation of the fungus and significantly reduced the total α-galactosides and glycosides isoflavones content of cooked soybeans, but did not affect phytic acid. Overall, the combination of cooking and fungal fermentation of soybeans significantly decreased α-galactosides and glycoside isoflavones by 69% and 80%, respectively, increased aglycone isoflavones up to 26.74 mg/100 g DM, but did not decrease significatively on phytic acid.

Keywords *Rhizopus oligosporus* · Cooking · Diffusion · α-Galactosides · Phytic acid · Legume seeds

Introduction

Soybeans (*Glycine max*) belong to the *Fabaceae* family and is the most widely used legume in the food and feed industry. Soybeans are largely consumed in Asia (China, Japan, and Korea), with a growing trend towards cultivation and food processing in other countries such as the USA and Brazil (Wiederstein, Baumgartner and Lauter, 2023). Soybeans are an important source of plant proteins and isoflavones (Gbedo, Arnaud and Strub, 2024). Isoflavones are bioactive compounds with both antioxidant and estrogenic properties due to their structural similarity to 17β-estradiol (Tham, Gardner and Haskell, 1998). Isoflavones are divided into four forms: β-glycosides, acetylglycosides, malonylglycosides and aglycones. Aglycone forms have been extensively studied for their beneficial health effects, such as reducing the risk of cardiovascular disease, breast cancer, neuronal and oxidative damage, and many other diseases (Langa et al., 2023; Wada et al., 2013). Moreover, aglycone isoflavones have a higher bioavailability in the human body than other forms (Langa et al., 2023). However, in soybeans, malonyl and glycoside isoflavones account for almost 98%, while the aglycone form is present in trace amounts or absent (Liu et al., 2023). Thus, the development of soy-based products containing more aglycone and less glycoside isoflavones is of great interest.

Furthermore, the use of soybeans is also limited by the presence of anti-nutritional factors (ANFs) such as phytic acid and α-galactosides that can have negative effects on human or animal nutrition (Colletti et al., 2020). The presence of phytic acid in seeds can prevent the bioavailability of certain minerals and lead to a reduction in the in vitro digestibility of proteins (Lajolo et al., 2004). As for α-galactosides, they escape digestion and are metabolized in the intestinal tract into carbon dioxide, hydrogen, methane and short-chain

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fatty acids, which can cause flatulence, diarrhea, bloating, cramps and pain (Pusztai et al., 2004; Martínez-Villaluenga, Frias and Vidal-Valverde, 2008).

Most traditional fermented soybeans products (*tempeh*, *natto*, *soy sauce*, *soyadawa, soya afitin* etc.) are produced by combining several unit operations: dehulling, soaking, cooking and fermentation (Gbedo, Arnaud and Strub, 2024). Among all these unit operations, soaking and dehulling may be optional in several traditional legume seed fermentation processes. This is the case of traditional processes for fermenting *African locust bean* into food condiments, which do not involve the soaking operation (Parkouda et al., 2009), and processes for transforming soybeans into *soy sauce* and fermented *Okara*, which disregard the dehulling operation (Asghar et al., 2023; Devanthi & Gkatzionis, 2019). In contrast, cooking systematically precedes fermentation in all traditional legume seed fermentation processes (Gbedo, Arnaud and Strub, 2024).

The aim of this study was to provide a comprehensive analysis of the effect of cooking and fermentation by *Rhizopus oligosporus* on the reduction of ANFs and changes in isoflavones during the processing of fermented soybeans. Fungal fermentation with *Rhizopus oligosporus* was chosen as a model among the microorganisms used for soybean processing in the world. Kinetics of α-galactosides, phytic acid and isoflavones were determined during cooking and fermentation. The diffusion of these compounds in the cooking water was also determined. The cooking operation was also characterized in terms of water uptake, protein denaturation and its effect on water activity and texture of the seeds. Inoculation with *Rhizopus oligosporus* was compared to a non-inoculated treatment, and fungal growth was quantified by measuring ergosterol content.

Materials and Methods

Soybean

Soybean seeds (*Glycine max*, variety *Sinfonia*) were supplied by the RAGT seed group (Rouergue Auvergne Gévaudan Tarnais, Rodez, France). The seeds were stored at −20 °C until use.

Fungal Spore Suspension Preparation

Rhizopus oligosporus (CBS 112586) from the CBS-KNAW collections (Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands) was grown on Malt Extract Agar (MEA) plates and incubated at 30 °C for 4 to 5 days to obtain for spore formation. Vegetative spores were harvested by adding a sterile saline solution (0.9% w/v NaCl + 1% Tween 80) and scraping the spores with a sterile spreader. The spore suspension was filtered through carded cotton, and the concentration of spores was assessed using a hemocytometer. The spore suspension was then stored at 2 °C until use.

Processing of Soybeans

Seeds (90 g per experiment) were cooked in a beaker containing 270 mL of distilled water (seed/water ratio of 1/3) previously heated to 95 °C placed in a water bath (WNB 29, Memmert GmbH, Schwabach, Germany) at 95 °C with mechanical stirring for 10, 20, 30, 40 and 60 min. The beaker was covered with a lid to prevent water evaporation, which was 22.85 ± 0.64 g, 24.40 ± 1.14 g, 27.38 ± 0.16 g, 26.13 ± 1.60 g and 25.25 ± 0.05 g at 10, 20, 30, 40 and 60 min, respectively. The cooking parameters were selected in order to ensure consistency with traditional practices (cooking seeds is performed in boiling water). Seed ratio water of 1/3 allowed to be in excess of water along cooking. After cooking, the seeds were centrifuged at 7 g for 5 min at 25 °C (3-16KL, Sigma, Osterode am Harz, Germany) to remove residual surface water (Coffigniez et al., 2018) before cooling to room temperature. For fermentation, seeds were cooked for 30 min using the same methodology. Seeds cooked for 30 min were packed in perforated plastic bags (length × width of bag: 13 cm × 10 cm, number of perforations: 25 per surface; spacing between perforations: approximately 1 cm; 180 g of cooked soybeans per bag) after being inoculated at a concentration of 10^5 spores/g of cooked seeds or not with *Rhizopus oligosporus (R. oligosporus)*. They were incubated at 30 °C and 70% relative humidity for 24 and 48 h (KBF 240, BINDER GmbH, Germany). Cooking water and seeds during cooking and fermentation were weighed and collected. All experiments were carried out in triplicate.

Sample Preparation

Raw seeds were ground (Retsch Grindomix GM200, Germany) before analysis. After cooking, some seeds (45 g) were directly used for texture measurements. The other cooked seeds as well as fermented seeds were either ground (Retsch Grindomix GM200, Germany) for the analysis of water content and water activity, pH and ergosterol content or freeze dried after being frozen at −20 °C for 24 to 72 h. Freeze dried seeds were then ground (Retsch Grindomix GM200, Germany) and kept at −20 °C for other analyses (protein content, DSC, α-galactosides, phytic acid and isoflavones contents). Cooking water was kept frozen at −20 °C until analyses of dry matter and α-galactosides, phytic acid and isoflavones contents.

Mass Transfers During Cooking

Weight gain during cooking was determined by weighing the seeds before and after cooking. Weight gain *WG* (t), (g/100 g DM) was calculated as a function of cooking time (t) according to Eq. (1):

$$WG(t) = \frac{m(t) - m(i)}{mDM(i)} \times 100 \quad (1)$$

Where m (i), m (t), and m DM (i) denote the mass of the seeds before cooking (g), the mass of the seeds after cooking (g), and the dry mass of raw seeds (g), respectively.

To determine solid losses, dry matter of cooking water was measured. For this, approximately 10 mL of cooking water was weighed and dried at 105 ± 2 °C for 48 h. The dry matter loss DML (t) (g/100 g DM) in the cooking water was calculated as a function of cooking time (t) according to Eq. (2):

$$DML(t) = \frac{[C_a(t) \times V_f(t)]}{mDM(i)} \times 100 \quad (2)$$

Where C_a (t), V_f (t) and m DM (i) are the concentration of solids (g/g of cooking water), the final mass of cooking water (g) and the dry mass of raw seeds (g), respectively.

Water Content and Water Activity

Water content of soybean samples was measured according to the ISO 712 (2010) standard. The sample (5 g) was dehydrated at 105 ± 2 °C for 48 h, with two replicates per measurement.

Water activity (a_w) was measured in duplicate using an a_w-meter (Aqualab 4TE, USA) at 25 ± 0.2 °C after a control of the calibration with solutions of a_w of 0.920 et 1.000.

pH

The sample (3 g) was mixed with 27 mL of distilled water and vortexed for 30 min. The pH of the solution was then measured using a pH meter (HI2002-02 (edge® pH), France) calibrated with standards of pH 4 and 7. The pH was measured in duplicate.

Texture Analysis

Firmness of seeds after cooking was determined according to the AACCI 56–36.01 method described by Wang et al. (2012) using a TA.XT + C texturometer (Stable Micro Systems, Godalming, UK) fitted with a Kramer Miniature cell (Stable Micro Systems, Godalming, UK) with six replicates per time of cooking. At the end of cooking, the seeds were stored at 50 °C (for about 15 min) until measurement. For each measurement, approximately 7.5 g of seeds were placed in the Kramer cell. The program was set as follows: Pre-test speed: 1 mm/sec; test speed: 1.5 mm/sec, post-test speed: 10 mm/sec; target mode: Distance; Distance: 30 mm; Trigger Type: Auto (Force); Trigger force: 5 g. The maximum force during the test was recorded.

Protein Denaturation Rate

Protein denaturation was measured by Differential Scanning Calorimetry (DSC) using the method described by Lefèvre et al. (2022) with some modifications. A Perkin DSC 7 (PerkinElmer, Norwalk, CT, USA) was calibrated using indium as standard. Approximatively, 8 mg of sample and 40 µL of water were weighed into stainless steel capsules. After hermetically sealing and stabilizing for 12 h, the capsules were heated from 25 to 160 °C at a heating rate of 10 °C/min. A control thermogram was recorded by placing an empty capsule in the reference oven and in the sample oven. The enthalpy flux of the sample minus the enthalpy flux of the control was recorded and processed using Pyris thermal analysis software (Perkin Elmer, Norwalk, CT, USA). Measurements were duplicated. The protein denaturation rate, (Rd (t), %) for each protein (7S and 11S) was calculated as a function of cooking time (t) according to Eq. (3):

$$Rd(t) = 1 - \frac{\Delta H(t)}{\Delta H(i)} \times 100 \quad (3)$$

where ΔH (t) and ΔH (i) are the enthalpy of seeds after cooking (J/g DM) and the enthalpy of raw seeds (J/g DM), respectively.

Protein Content

The total protein content was measured by the Kjeldahl method in accordance with standard NF EN ISO 20483 (2013). The nitrogen to protein conversion factor was 6.25. The measurements were duplicated.

Ergosterol Content and Metabarcoding Analysis

The method for extracting ergosterol from fermented soybean samples was adapted from the method described by Nout (1987) with some modifications. About 2 g of sample was diluted with 5 mL ethanol and 5 mL NaOH 2 N, homogenized for 2 min and then heated in a water bath at 70 °C for 1 h. After cooling in the dark for 20 min, the samples were centrifuged for 10 min at 1050 g. The supernatant was transferred to a separation funnel containing 1 mL of

distilled water. The pellet was resuspended in 5 mL of petroleum ether (PE) b.p. 40–60 °C (techn. grade), centrifuged for 10 min at 1050 g and the supernatant was added to the mixture in the separating funnel. After stirring, the PE fraction was collected and the aqueous phase was extracted with 3×5 mL PE b.p. 40°–60 °C. The four combined PE fractions were evaporated to dryness in a vacuum rotary evaporator (40 °C). Traces of water were removed by dissolving the residue in 1 mL of acetone, followed by dry evaporation as described above. Finally, the residue was dissolved in 1 mL of ethanol. The resulting supernatant was syringe-filtered through a 0.45 µm filter (Sartorius, Germany) into an HPLC vial. The extraction of samples was performed in duplicate. Ergosterol was determined by chromatographic separation reversed-phase chromatography, using HPLC equipment (Agilent Technologies 1200 series, Santa Clara, USA) with a UV detector set at 282 nm (Thermo Scientific), equipped with an ACE 5 C18 column (250×4.6 mm, Avantor, USA). The sample was eluted through a mobile phase: acetonitrile (solvent A) and a mixture of water/formic acid (98/2 v/v; solvent B) at a flow rate of 1 mL/min at 40 °C. The retention time for ergosterol was approximately 24 min and the injection volume was 20 µL. Standard solutions of ergosterol (Thermo Scientific) in pure ethanol were prepared in the range 0–400 µg/mL from a 500 µg/mL stock solution. Ergosterol purity was greater than or equal to 98%.

The composition and diversity of bacterial and fungal communities in non-inoculated samples during fermentation (0 h and 48 h) were characterized using a protocol based on high-throughput sequencing targeting the PCR-generated amplicon (Supplementary Fig. S1). Metabarcoding analyses were performed by the ADNID laboratory (Montferrier-sur-Lez, France).

α-Galactosides Content

α-Galactosides were extracted using the method described by Coffigniez et al. (2018) with slight modifications. Approximately 80 mg of soybean was weighed and incubated for 1 h at 80 °C in 10 mL of 80% (v/v) ethanol solution. The supernatant was collected and filtered through a 0.45 µm filter into vials as well as samples of cooking waters. The different forms of α-galactosides in the samples were separated by high-performance anion-exchange chromatography (Dionex ICS-5000 + Ion Chromatography System, Thermo Scientific, France). The separation was performed at room temperature on a 4×30 mm Dionex CarboPac PA210-fast-4 µm pre-column and a 4×150 mm Dionex CarboPac PA210-fast-4 µm column (Dionex, Germany). The injection volume was 10 µL. The mobile phase was constant and consisted of Millipore water coupled to a 12 mM potassium hydroxide solution. The flow rate was 0.8 mL/min. The α-galactosides (raffinose, stachyose and verbascose) were quantified using a calibration curve with external standards (Sigma, USA). The purity of the standards was greater than or equal to 98%. The calibration curves ranged from 1 to 10 mg/L for raffinose, from 1 to 30 mg/L for stachyose and from 1 to 5 mg/L for verbascose. The extraction of samples was performed in duplicate.

Phytic Acid Content

The phytic acid content was determined in duplicate using the Phytic Acid Assay Kit (Megazyme International Ltd., Wicklow, Ireland) based on the method described by McKie and McCleary (2016). Approximately 1 g of soybean was weighed and stirred overnight at room temperature in 20 mL of 0.66 M hydrochloric acid solution. For the cooking water, approximately 5 mL was stirred overnight at room temperature in 5 mL of 0.66 M hydrochloric acid solution. The method involved the addition of phytase and alkaline phosphatase, which ensure the release of phosphate from phytic acid and partially phosphorylated inositol, that reacts with ammonium molybdate to form 12-molybdophosphoric acid. The latter was then reduced to molybdenum blue under acidic conditions. The amount of molybdenum blue formed was proportional to the amount of inorganic phosphate (Pi) in the sample. The absorbance of the reaction was read at 655 nm using a UV–visible spectrophotometer (JENWAY 7205, UK). Pi was quantified using a phosphorus calibration curve. Phytic acid content was determined by calculating the difference between total and free phosphorus using a conversion factor of 28.2% (molar mass fraction of phosphorus in phytic acid).

Isoflavones Content

The method used for isoflavone quantification was adapted from that described by Wang et al. (2023). Approximately 0.3 g of soybean samples was weighed and ultrasonicated at 480 W for 40 min in 10 mL of 80% (v/v) methanol solution. After extraction, the samples were centrifuged (Sigma, Germany) at 7000 g for 10 min and the supernatants were filtered at 0.45 µm into vials as well as cooking waters were. Extracts and cooking waters were analyzed by chromatographic separation using high-performance liquid chromatography, using HPLC equipment (Agilent Technologies 1260 Infinity II, Santa Clara, USA), equipped with a Uptisphere C_{18}—HDO column (250×4.6 mm, Interchim, France). The isoflavones were eluted by a mobile phase composed of: acetonitrile (solvent A) and a mixture of water/formic acid (98/2 v/v; solvent B) at a flow rate of 1 mL/min at 30 °C. The separation of the isoflavones was achieved using the following elution gradient: 0–12.5 min, A: 10%; 12.5–17.5 min, A: 30%; 17.5–18.5 min, A: 40%; 18.5–26 min, A: 95%; 26–30 min, A: 95%; and finally,

30–35 min, A: 10% (B being the complement to reach 100%). The injection volume was 20 µL. Malonylglycosides, β-glycosides and aglycones peaks were detected at 260 nm (UV Detector, Thermo Scientific). β-glycosides and aglycones were quantified using a calibration curve with external standards (Extrasynthèse, France). Standard solutions were prepared in the range 0–20 mg/L from stock solutions of 200 mg/L. The purity of the standards was greater than or equal to 98%. Malonylglycosides peaks were identified by mass spectrometry, using the molecular weight reported by Liu et al. (2023). Extraction was performed in duplicate.

Statistical Analysis

Analysis of variance was performed using XLSTAT software (version 2022.5.1, Paris, France). Significant differences were assessed using the Tukey-HSD test, with significance defined as $p < 0.05$.

Results and Discussion

Cooking

Mass Transfers

The kinetics of weight gain of soybeans and dry matter loss in cooking water, as well as the evolution of water content and water activity in soybeans during 60 min of cooking at 95 °C with a seed/water ratio of 1/3, are reported in Fig. 1 and Fig. 2a. Soybeans demonstrated accelerated weight gain and water content increase at the onset of the cooking process afterwards, changes proceeded more slowly. Water activity increased concomitantly from 0.665 to 0.981 at 30 min of cooking after which the increase was no more significant. At 30 min, water content of seeds was 125.3 g/100 g DM (55.6 g/100 g wet basis). Weight gain was the result of water uptake and solid loss; the solid loss increased throughout the 60-min cooking to reach approximately 7 g/100 g of the dry mass of raw seeds. Water transfers and its pattern observed in this study were consistent with those reported in other studies on soybean (Koriyama et al., 2018) and various pluses (Avezum et al., 2024; Coffigniez et al., 2019) at temperatures and seed-to-water ratios comparable to those in this study. Additionally, similar patterns of dry matter loss were noted on cowpea and chickpea seeds (Sayar, Turhan and Köksel, 2011; Coffigniez et al., 2019). Solid loss from samples during cooking has been described as an interaction of water with hydrophilic molecules in the seed matrix and the release of some of them (mainly polysaccharides, proteins, vitamins, minerals etc.) into the process water (Sayar, Turhan and Köksel, 2011), favored by high cooking temperatures, pectin degradation, and micropyle opening.

Protein Denaturation and Seed Softening

Based on Differential Scanning Calorimetry (DSC) analysis of the thermal properties of raw and cooked soybeans, data for the combined maximum transition temperatures (T_d), denaturation enthalpies (ΔH), and calculated denaturation rates (Rd) of 7S and 11S globulins are shown in Table 1. The T_d of crude soybeans were 76 °C and 96 °C,

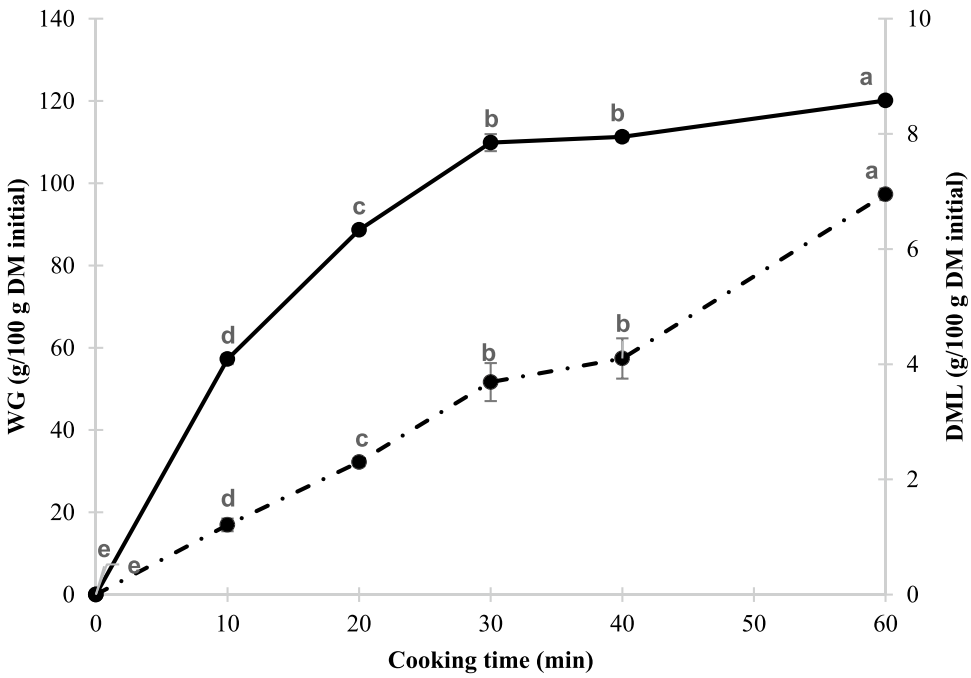

Fig. 1 Weight gain (WG) of soybean and dry matter loss (DML) in cooking water during cooking at 95 °C with a seed/water ratio of 1/3. The lines represent the weight gain of seeds, while the dashed lines represent the dry matter loss in the cooking water. Error bars are standard deviations ($n = 3$). Different letters indicate significant differences between samples ($p < 0.05$) by Tukey's studentized range (HSD) test

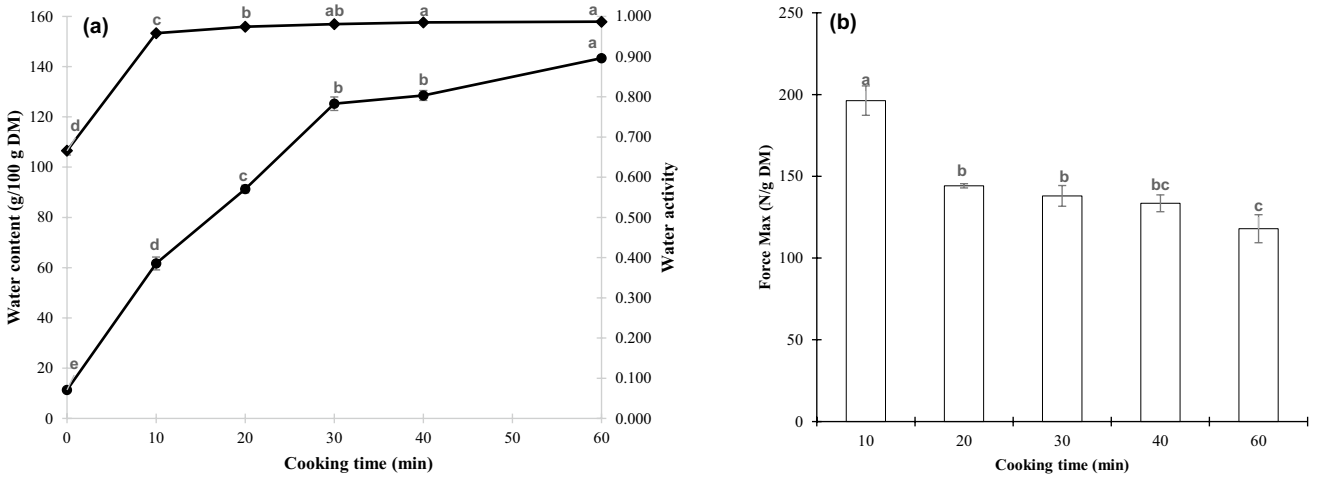

Fig. 2 (**a**) Water content (●), water activity (◆) and (**b**) hardness change of soybean during cooking at 95 °C with a seed/water ratio of 1/3. Error bars are standard deviations (n = 3). Different letters indicate significant differences between samples ($p < 0.05$) by Tukey's studentized range (HSD) test

Table 1 Peak transition temperatures (T_d), enthalpy changes (ΔH), denaturation rates (R_d) and protein content of soybean during cooking at 95 °C with a seed/water ratio of 1/3

Cooking time (min)	T_{d7S} (°C)	ΔH_{7S} (J/g DM)	Rd_{7S} (%)	T_{d11S} (°C)	ΔH_{11S} (J/g DM)	Rd_{11S} (%)	Protein content (g/100 g DM)
0	76.75 ± 0.03	1.33 ± 0.08	na	96.22 ± 1.11	2.88 ± 0.09 a	na	42.12 ± 0.40 d
10	nd	nd	100	96.08 ± 0.06	2.84 ± 0.11 a	1.98 ± 3.41 d	44.79 ± 0.43 ab
20	nd	nd	100	95.77 ± 0.54	2.59 ± 0.06 b	10.01 ± 2.77 c	45.34 ± 0.71 a
30	nd	nd	100	97.49 ± 0.49	2.22 ± 0.02 c	22.98 ± 0.99 b	43.54 ± 0.45 c
40	nd	nd	100	95.56 ± 0.51	2.07 ± 0.08 c	28.30 ± 2.95 b	42.44 ± 0.31 d
60	nd	nd	100	97.54 ± 1.00	1.17 ± 0.04 d	59.56 ± 1.72 a	44.49 ± 0.39 c

T_{d7S}, T_{d11S}, ΔH_{7S}, ΔH_{11S} and Rd_{7S}, Rd_{11S} represent the denaturation temperature, enthalpy changes and denaturation rate of 7S and 11S, respectively. Data are means ± standard deviations (n = 3). nd: no detection of thermal transition. na: not applicable. Different letters in a column indicate significant differences between samples ($p < 0.05$) by Tukey's studentized range (HSD) test

respectively for 7S and 11S. These results are close to those of Zhang et al. (2022), who reported denaturation temperatures of 72–74 °C and 92–93 °C for 7S and 11S, respectively. The 7S protein was completely denatured within the first 10 min of cooking (disappearance of the endothermic peak on the DSC thermogram). Increasing the cooking time from 10 to 60 min led to further denaturation of the 11S protein as shown by the significant decrease in the enthalpy (2.84 ± 0.11 J/g DM at 10 min to. 1.17 ± 0.04 J/g DM at 60 min) and increase of the denaturation rate up to almost 60% at 30 min even taking into account that the protein content (expressed in dry matter) varied slightly from 10 to 60 min. The progressive denaturation of 11S during cooking could be explained by the temperature of cooking (95 °C) which is close to the denaturation temperature of 11S. Furthermore, the lower denaturation of 11S compared to 7S could be due to its structural complexity: 11S is a hexamer (approximately 360 kDa), whereas 7S (180 to 210 kDa) is a trimer (Wu et al., 2021).

Figure 2b presents the change in hardness of soybeans cooked for 10 to 60 min. As expected, seed hardness decreased with increasing cooking time. The hardness of soybeans cooked for 10 min was significantly higher than those cooked for longer times. There was no significant difference between the texture of seeds cooked for 20-, 30- and 40-min. Seeds cooked for 60 min had the lowest hardness, i.e., 117.95 ± 11.22 N/g DM. Rapid initial softening has been showed on several legumes (Anzaldua-Morales, Quintero and Balandran, 1996; Koriyama et al., 2018). By comparing with water content kinetics in Fig. 2, slower decrease in hardness from 20 min happened while water content continued to quickly increase up to 30 min. Koriyama et al. (2018) also showed that beans continue to absorb water even if hardness reaches an equilibrium. Moreover, by measuring kinetics on presoaked and unsoaked soybeans,

they showed that softening occurs in steps. In the earlier one, softening due to water absorption occurs preferentially until a hardness limit which does not depend of the temperature. Then, softening due to heating occurs. Heat induced softening is associated irreversible changes in the structure and physicochemical functions of pectin and medium lamellar proteins, loosening cell walls and producing a chewy texture (Koriyama et al., 2018; Perera et al., 2023).

ANFs Kinetics and Diffusion

Figure 3 presents the evolution of the α-galactosides content (raffinose, stachyose, verbascose, and total) in the soybeans and in the cooking water. The total α-galactosides content (sum of raffinose, stachyose, and verbascose) of raw seeds was 5.45 ± 0.06 g/100 g DM. These values are within the range reported in the literature, which varies between 4.63 and 6.00 g/100 g DM (Egounlety & Aworh, 2003; Wang et al., 2007). In accordance with literature, stachyose was also the main α-galactosides found in soybeans accounting for 81%, followed by raffinose (15%) and verbascose (4%) in the study below. Total α-galactosides content decreased during cooking till 3.39 g/100 g DM (38% reduction) at 60 min of cooking. In fact, increasing the cooking time to 60 min reduced verbascose by 31%, raffinose by 39% and stachyose by 38%, although their decrease was no more significant from 30 and 40 min respectively. This reduction in total α-galactosides content is of the same order of magnitude as that observed on cowpeas (Coffigniez et al., 2018) and on other pulses (Avezum et al., 2024) in similar cooking conditions (90–95 °C; with a seed/water ratio of 1/4). Concomitantly α-galactosides diffused in cooking water. After 60 min of cooking, the total content of raffinose, stachyose, verbascose and total α-galactoside that had diffused into the cooking water was 0.19 g/100 g DM, 1.05 g/100 g DM, 0.05 g/100 g DM and 1.29 ± 0.15 g/100 g DM respectively. These values are lower than what disappears in the seeds. Coffigniez et al. (2018) and Avezum et al. (2024) reported that the sum in the seeds and in the cooking water remained almost constant at similar temperature and seed/

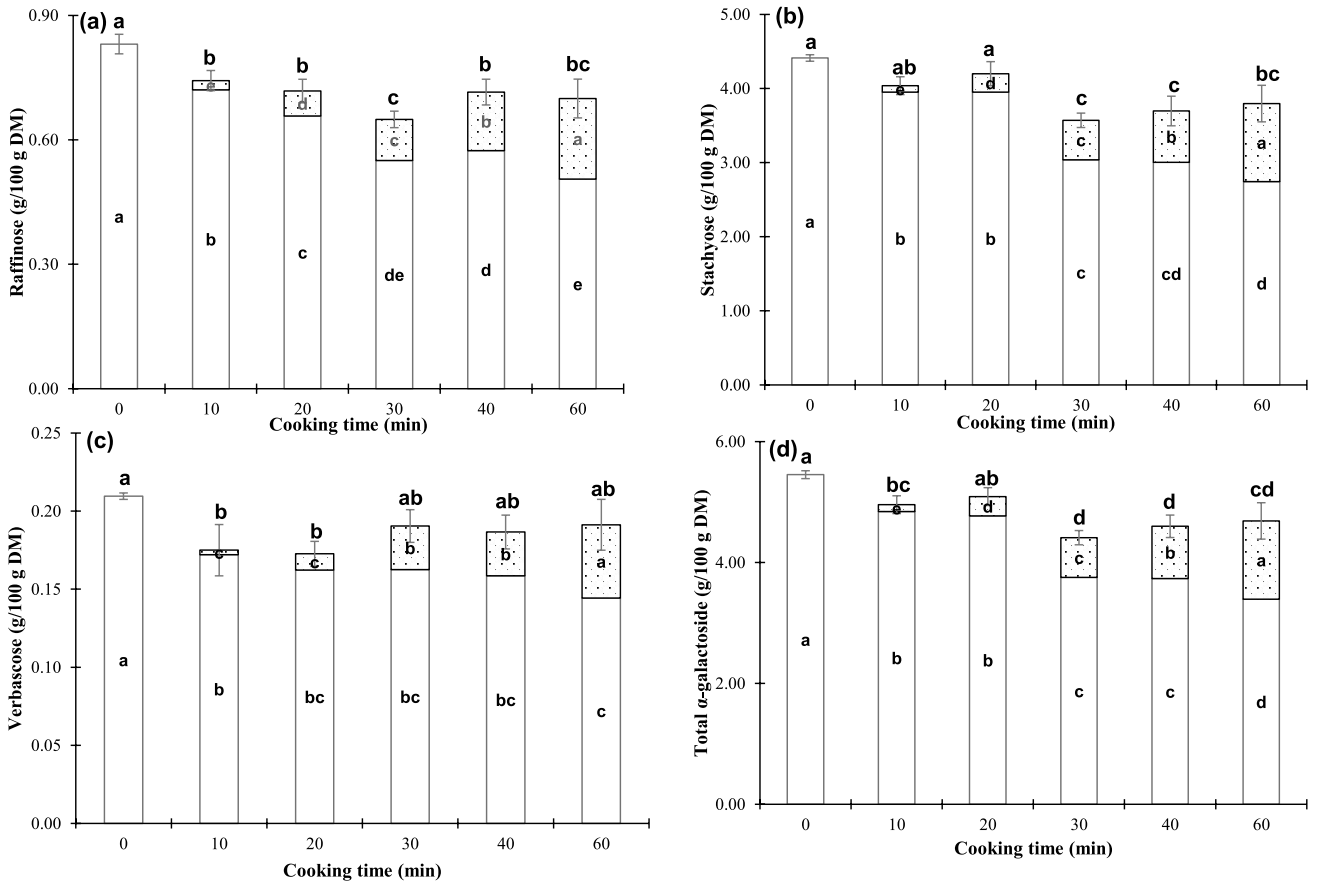

Fig. 3 (**a**) Raffinose, (**b**) stachyose, (**c**) verbascose, and (**d**) total α-galactosides content of soybean and in cooking water during cooking at 95 °C with a seed/water ratio of 1/3. The white and dotted histograms represent the content of compounds in seeds and cooking water, respectively. Error bars are standard deviations (n = 3). Different letters indicate significant differences between samples ($p < 0.05$) by Tukey's studentized range (HSD) test

water ratios. Although this was not the case in our study, diffusion was the major cause of the decrease in α-galactosides content in the seed (at 60 min for example, it accounted for 86% for the total α-galactosides). This indicates that hydrolysis of α-galactosides into compounds such as disaccharides (like sucrose) and simple monosaccharides may occur during cooking as reported by Onigbinde and Akinyele (1983) and Oboh et al. (2000).

Figure 4 shows the evolution of the phytic acid content in the soybeans and in the cooking water. The phytic acid content of raw soybeans was 1.61 ± 0.01 g/100 g DM. About 1.27–3.50 g/100 g DM phytic acid has been reported in different soybean cultivars (Abu-Salem et al., 2014; Egounlety & Aworh, 2003), which is in agreement with our results. Cooking of seeds resulted in a slight reduction in the phytic acid content of soybeans. The decrease was only significant after 40 min and the content reached 1.28 g/100 g DM (20% reduction) at 60 min. This slight reduction could be due to a slower diffusion of phytic acid. The literature reveals conflicting results regarding the impact of cooking on the phytic acid content of legume seeds, which has been observed to increase or decrease even when cooked in excess of water (Gbedo, Arnaud and Strub, 2024). Furthermore, Mohamed et al. (2011) also found a slight decrease in phytic acid content in whole soybeans cooked for 60 min (11%) at 100 °C with a seed/water ratio of 1/10 while Karkle and Beleia (2010) reported no effect of 60 min cooking (seed/water ratio of 1/8) on the phytic acid content of pretreated soybeans (dehulled and/or soaked). The content of phytic acid which diffused in cooking water increased all along cooking although it was significant only from 30 min. To the best of our knowledge, no study has reported a diffusion of phytic acid in cooking water. The amounts in the cooking water are much lower than the amounts that have disappeared in the seeds. For example, at 60 min, phytic acid content in cooking water was only 0.013 g/100 g DM which accounted for 4%. These results indicate that phytic acid diffusion is slow and is not only responsible of the decrease observed in seeds. In fact, the sum of phytic acid content in the cooked soybeans and cooking water obtained after 20, 30, 40, and 60 min were respectively of 4%, 6%, 11%, and 20% lower than the total content initially present in the soybeans. The difference would be due to thermal degradation although larger cooking times/higher temperatures would be required to get higher decrease. Moreover, the formation of insoluble complexes between phytic acid and other compounds (proteins, minerals) that could not be extracted with water or hydrochloric acid (HCl) in cooked legumes has already been reported (Muzquiz et al., 2004; Urbano et al., 2000).

Isoflavones Kinetics and Diffusion

The variations in the isoflavone content of malonylglycosides (Malonyldaidzin, Malonylglycitin and Malonylgenistin), β-glycosides (Daidzin, Glycitin, and Genistin), and aglycones (Daidzein, Glycitein, and Genistein) in soybeans and cooking water are presented in Fig. 5 and Table 2. The total isoflavones content of crude soybeans was 255.91 mg/100 g DM, consisting of 183.80 mg/100 g DM of malonylglycosides, 69.94 mg/100 g DM of β-glycosides and 2.17 mg/100 g DM of aglycones. Thus, the dominant form of isoflavones in soybeans were malonylglycosides,

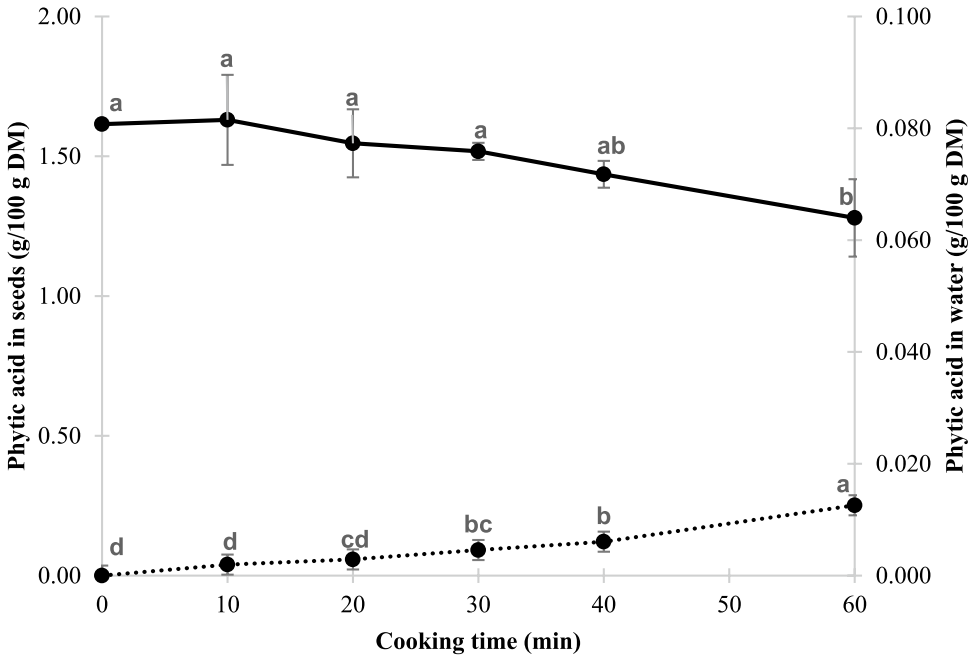

Fig. 4 Phytic acid content of soybean and in cooking water during cooking at 95 °C with a seed/water ratio of 1/3. The lines represent the content of the compounds in seeds, while the dashed lines represent their content in the cooking water. Error bars are standard deviations ($n = 3$). Different letters indicate significant differences between samples ($p < 0.05$) by Tukey's studentized range (HSD) test

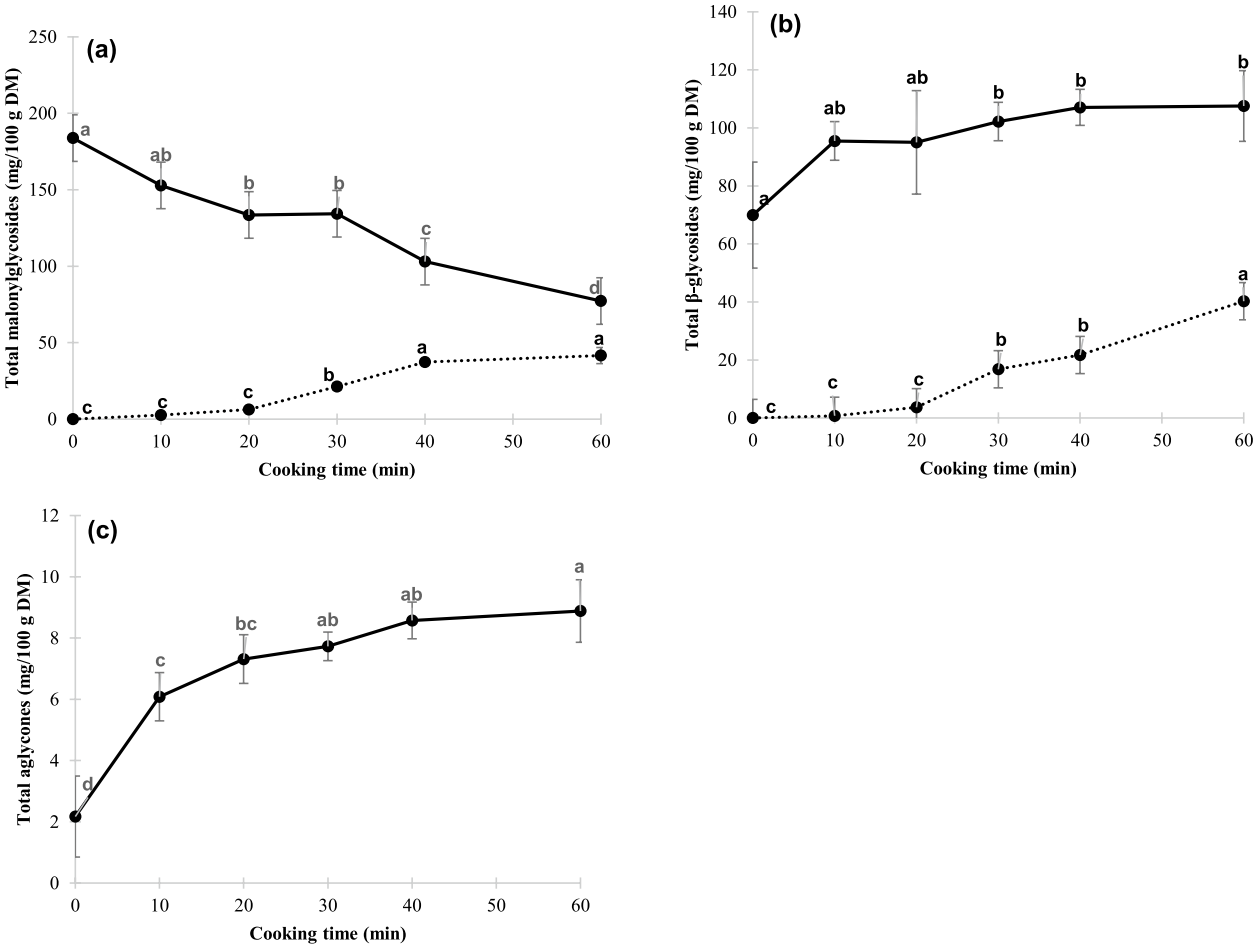

Fig. 5 (**a**) Malonylglycosides, (**b**) β-glycosides, and (**c**) aglycones content of soybean and in cooking water during cooking at 95 °C with a seed/water ratio of 1/3. The lines represent the content of the compounds in seeds, while the dashed lines represent their content in the cooking water. Aglycones is not detected in cooking water. Error bars are standard deviations ($n=3$). Different letters indicate significant differences between samples ($p<0.05$) by Tukey's studentized range (HSD) test

followed by β-glycosides, with small amounts (1%) of aglycones. These results are in agreement with those of Simonne et al. (2000), who reported that the total isoflavones content in mature soybeans ranged from 22.50 to 254.50 mg/100 g DM. In addition, several studies have also reported that isoflavone glycosides (malonylglycosides and β-glycosides) are the predominant forms in immature and mature soybeans (Bavia et al., 2012; Qu et al., 2021; Simonne et al., 2000).

During cooking, the total malonylglycosides content in soybeans decreased all along cooking to reach 77.26 mg/100 g DM (reduction of 58%) at 60 min (Fig. 5a). All the three forms of malonylglycosides (malonyldaidzin, malonylglycitin and malonylgenistin) decreased by more than half after 60 min of cooking (Table 2). The significant decrease in malonylglycosides content during cooking is the main reason for the reduction in total isoflavones content in soybeans since it is the predominant form. In contrast, the total β-glycosides content in soybeans increased during cooking; content at 30 min (46% of increase) was significantly different from the raw material and did not significantly increase up to 60 min (Fig. 5b). Among the 3 forms of β-glycosides (daidzin, genistin and glycitin), glycitin did not vary significantly (Table 2). It has been reported that the decrease in malonylglycosides content is generally accompanied by an increase in β-glycosides content in heat-treated soybeans and soybean products; malonyl glycosides appear to be thermally unstable and convert to β-glycoside under the effect of heat at 95 °C (Aguiar et al., 2012; Huang & Chou, 2008; Kudou et al., 1991; Qu et al., 2021; Toda et al., 2000). Thus, the increase in β-glycoside isoflavones during cooking may be due to the cleavage of malonyl ester groups to form their corresponding β-glycoside derivatives (Jackson et al., 2002). The aglycones content in soybeans increased during cooking. The increase in total aglycones was no longer significant after 30 min of cooking (Fig. 5c), where it reached 7.73

Table 2 Composition of isoflavone isomers (mg/100 g DM) of soybean and in cooking water during cooking at 95 °C with a seed/water ratio of 1/3

			0	10	20	30	40	60
Cooked seeds	Malonyl-glycosides	MDZ	57.46 ± 4.48 a	42.35 ± 2.59 ab	34.74 ± 2.59 bc	31.74 ± 2.58 bcd	25.54 ± 2.59 cd	20.68 ± 2.58 d
		MGL	9.84 ± 2.07 ab	8.15 ± 1.20 ab	6.74 ± 1.20 ab	10.62 ± 1.20 a	5.79 ± 1.20 ab	2.96 ± 1.20 b
		MGN	116.50 ± 6.54 a	102.37 ± 3.77 ab	92.85 ± 3.77 b	91.17 ± 3.77 b	71.69 ± 3.77 c	53.32 ± 3.77 d
	β-glycosides	DZI	17.86 ± 1.62 d	25.58 ± 2.14 c	26.76 ± 1.92 bc	28.60 ± 1.56 abc	31.03 ± 1.92 a	29.76 ± 1.92 ab
		GLI	5.22 ± 1.01 a	5.53 ± 0.58 a	5.38 ± 0.58 a	5.96 ± 0.58 a	5.53 ± 0.58 a	5.22 ± 0.58 a
		GNI	46.86 ± 6.86 b	64.38 ± 4.47 ab	70.05 ± 3.96 ab	60.45 ± 3.96 ab	70.51 ± 3.96 a	72.56 ± 3.96 a
	Aglycones	DZE	0.83 ± 0.20 a	1.08 ± 0.12 a	1.09 ± 0.12 a	1.15 ± 0.12 a	1.10 ± 0.12 a	0.89 ± 0.12 a
		GLE	0.00 ± 0.28 d	2.36 ± 0.16 c	3.98 ± 0.16 b	3.43 ± 0.16 b	4.81 ± 0.16 a	5.38 ± 0.16 a
		GNE	1.35 ± 0.14 b	2.64 ± 0.08 a	2.66 ± 0.08 a	2.73 ± 0.08 a	2.66 ± 0.08 a	2.61 ± 0.08 a
Cooking water	Malonyl-glycosides	MDZ	na	0.83 ± 0.11 c	2.48 ± 0.32 c	8.71 ± 0.88 b	15.93 ± 0.26 a	17.64 ± 2.71 a
		MGL	na	0.21 ± 0.03 c	0.56 ± 0.05 c	2.34 ± 0.24 b	3.45 ± 0.10 a	4.40 ± 1.23 a
		MGN	na	1.56 ± 0.11 cd	3.32 ± 0.33 c	10.28 ± 1.29 b	17.90 ± 0.65 a	19.54 ± 1.88 a
	β-glycosides	DZI	na	0.35 ± 0.16 d	1.73 ± 0.25 d	7.69 ± 0.97 c	10.02 ± 0.70 b	17.72 ± 2.11 a
		GLI	na	0.17 ± 0.04 d	0.59 ± 0.06 d	2.56 ± 0.28 c	3.30 ± 0.18 b	5.81 ± 0.77 a
		GNI	na	0.23 ± 0.22 c	1.38 ± 0.35 c	6.53 ± 1.83 b	8.37 ± 1.32 b	16.71 ± 3.20 a
	Aglycones	DZE	na	nd	nd	nd	nd	nd
		GLE	na	nd	nd	nd	nd	nd
		GNE	na	nd	nd	nd	nd	nd

Data are means ± standard deviations ($n = 3$). *na*: not applicable. *nd:* no detection. Malonyldaidzin (*MDZ*), malonylglycitin (*MGL*), malonylgenistin (*MGN*), Daidzin (*DZI*), glycitin (*GLI*), genistin (*GNI*), Daidzein (*DZE*), glycitein (*GLE*), genistein (*GNE*). Different letters in a row indicate significant differences between samples ($p < 0.05$) by Tukey's studentized range (HSD) test

mg/100 g DM (three times higher than in raw seeds). Glycitein and genistein increased significantly during cooking, ranging from 0 to 5.38 mg/100 g DM and from 1.35 to 2.61 mg/100 g DM, respectively, after 60 min of cooking (Table 2). In contrast, the increase of daidzein content was not significant. The increase of total aglycones in soybeans and soybean products during cooking were also observed by Jackson et al. (2002) and Toda et al. (2000). This increase in aglycones content is due to the deglycosylation of isoflavone glycosides to the aglycone by thermal hydrolysis (Xu, Wu and Godber, 2002).

To our best knowledge, the present study is the first to present isoflavone levels diffused into cooking water. During cooking, the sum of malonylglycosides and β-glycosides contents from soybeans leached into the cooking water were 3.36 mg/100 DM at 10 min, 10.06 mg/100 DM at 20 min, 38.10 mg/100 DM at 30 min, 58.98 mg/100 DM at 40 min, and 81.83 mg/100 DM at 60 min (Fig. 5a and Fig. 5b, Table 2). No aglycone forms were detected in the cooking water (Fig. 5c, Table 2). According to Jackson et al. (2002), aglycones have low solubility in aqueous media, which would explain their absence in cooking water in our study.

Fermentation

Soybeans were cooked 30 min prior fermentation. This cooking time was chosen because water absorption, protein degradation and seed hardness, which may influence fungal fermentation, was no longer significant after 30 min of cooking. Moreover α-galactosides did not change a lot from 30 min and the increase in aglycone isoflavones was no longer significant after 30 min of cooking.

Fungal Growth

Fungal growth was assessed visually (Fig. 6) and quantitatively by measuring ergosterol content (Fig. 7). After cooking, fermentation of soybeans for 48 h by *R. oligosporus* resulted in a visual absence of white mycelium (Fig. 6b), unlike in the production of *tempeh* (Romulo & Surya, 2021). Visual observation showed that the inoculated soybeans were not sufficiently bound together and could be easily detached. Already after 36 h of fermentation, all inoculated soybeans showed sporangiospore production with many black spores around the seeds. This sporulation of *R. oligosporus* observed on soybeans could be due to the

Fig. 6 (**a**) Non-inoculated and (**b**) inoculated soybean with *Rhizopus oligosporus* during fermentation

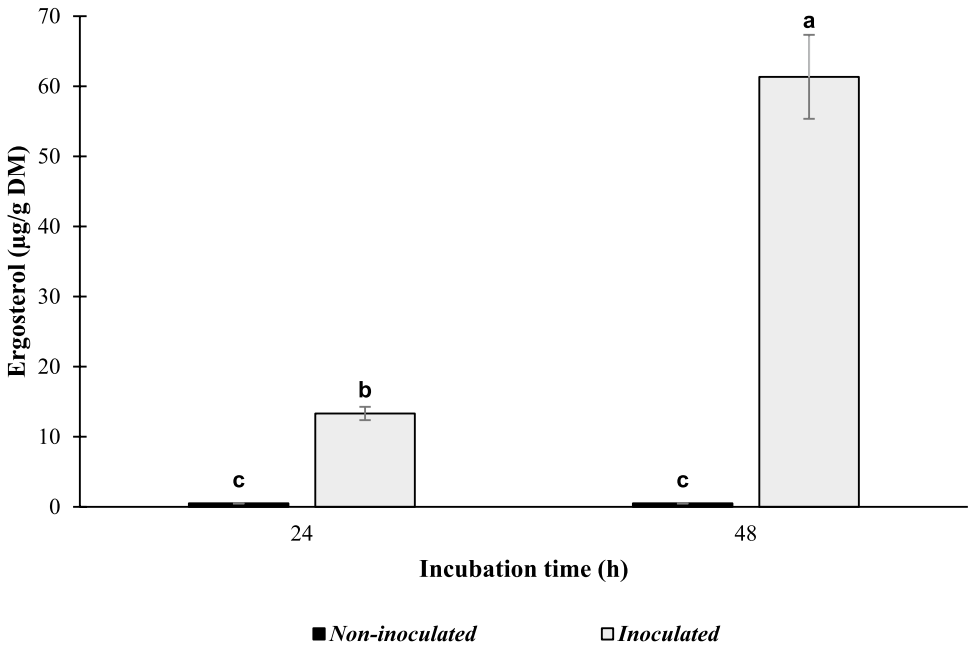

Fig. 7 Ergosterol content of non-inoculated and inoculated soybean with *Rhizopus oligosporus* during fermentation. Error bars are standard deviations ($n=3$). Different letters indicate significant differences between samples ($p<0.05$) by Tukey's studentized range (HSD) test

physiological state of the fungus and/or the presence of the hulls, which act as a barrier and prevent the fungus from accessing the nutrients available in the substrate. In the case of non-inoculated soybeans, no fungal presence was observed.

Ergosterol is the major sterol in fungal membranes and has been proposed as a suitable biomarker for quantifying fungal growth in solid media (Schnürer, 1993; Weete, 1980). Therefore, the ergosterol concentration was determined in non-inoculated soybean and inoculated soybean (Fig. 7). No ergosterol was detected in non-inoculated soybeans. Analysis of the microbial community of non-inoculated soybeans revealed the presence of several dominant microorganisms such as *Bacillus*, *Aspergillus*, *Rhizopus*, and *Penicillium* (Supplementary Fig. S1). In contrast, the average ergosterol concentration in samples inoculated with *R. oligosporus* was 13.32 ± 0.95 µg/g DM and 61.35 ± 5.99 µg/g DM at 24 and 48 h, respectively. Nout (1987) reported ergosterol content of 79 µg/g DM and 1940 µg/g DM at 24 h and 48 h fermentation, respectively, on soybeans that have undergone a dehulling step prior to cooking and fermentation. The differences with the content in inoculated soybeans could be due to the sporulation phenomenon of *R. oligosporus* that occurred during soybean fermentation in our study. Considering the sporulation of the fungus on soybeans, we can assume that the detected ergosterol may be the one initially present in the spore suspension and the result of colonization of the seeds by *R. oligosporus*.

Table 3 Seed mass, water content, water activity (a_w) and pH of non-inoculated and inoculated soybean with *Rhizopus oligosporus* during fermentation

Parameters	Conditions	Fermentation time (h)		
		0	**24**	**48**
Seed mass (g)	*Non-inoculated*	181.96 ± 1.53 a	181.97 ± 1.53 a	182.40 ± 1.31 a
	Inoculated	184.30 ± 2.10 a	183.60 ± 2.07 a	182.73 ± 2.05 a
Water content (%)	*Non-inoculated*	55.26 ± 0.26 b	55.24 ± 0.23 b	56.26 ± 0.04 a
	Inoculated	55.60 ± 0.53 b	58.91 ± 0.53 a	58.04 ± 0.78 a
Water activity	*Non-inoculated*	0.985 ± 0.001 a	0.987 ± 0.001 a	0.987 ± 0.002 a
	Inoculated	0.985 ± 0.001 a	0.989 ± 0.001 a	0.981 ± 0.002 b
pH	*Non-inoculated*	6.83 ± 0.02 a	6.81 ± 0.01 a	6.79 ± 0.01 a
	Inoculated	6.81 ± 0.02 a	6.40 ± 0.32 ab	6.17 ± 0.06 b

Data are means ± standard deviations ($n = 3$). Different letters on a line indicate significant differences at $p < 0.05$ by Tukey's studentized range (HSD) test

Physicochemical Parameters

The evolution of seed mass, water content, water activity and pH during fermentation is shown in Table 3. There was no statistical difference between the seed mass for both inoculated and non-inoculated samples. However, a slight increase in water content was observed after 48 h of fermentation (already from 24 h for the inoculated ones) although it did not always reflect significantly on water activity. Davey, Pefialoza and Kell (1991), also reported an increase in water content from 59.2 to 61.4% after 24-h fermentation of soybeans for *tempeh* production. The water activity measured (between 0.98–0.99) falls within the range of 0.98 and 1 for adequate growth of *R. oligosporus* described in the literature (Sparringa et al., 2002; Ahnan-Winarno et al., 2021). These fluctuations in water content may result from a balance between water production during fungal growth and water consumption during sporulation, or from the possible evaporation of water during fermentation. The increase in water content in non-inoculated samples after 48 h of fermentation may be due to the action of *Bacillus* and other microorganisms naturally present in soybeans. The initial mean pH of cooked seeds was 6.82 (Table 3). In inoculated soybeans, the pH decreased significantly from 6.81 to a final value of 6.17 after 48 h of fermentation while it did not vary significantly for non-inoculated soybeans. The decrease in pH during fermentation in our study may be due to fungal growth, substrate utilization or the production of metabolites (such as organic acids).

ANFs Kinetics

The contents of α-galactosides and phytic acid at the beginning and after 48 h of fermentation of non-inoculated and inoculated soybeans is presented in Fig. 8. In non-inoculated soybeans, the raffinose and stachyose contents did not change significantly during fermentation (Fig. 8a and Fig. 8b). In the presence of *R. oligosporus*, the raffinose and stachyose contents decreased significantly, from 0.55 ± 0.03 g/100 g DM to 0.25 ± 0.04 g/100 g DM (54% reduction) and from 3.02 ± 0.12 g/100 g DM to 1.39 ± 0.29 g/100 g DM (54% reduction), respectively. The reduction in raffinose and stachyose resulting from fermentation is in line with the results of Egounlety and Aworh (2003), with a 54% reduction in our own study compared to the 53% observed in the study cited above. For verbascose, a significant reduction was observed in both inoculated and non-inoculated soybeans (Fig. 8c). It decreased from 0.16 ± 0.01 g/100 g DM to 0.13 ± 0.01 g/100 g DM (19% reduction) in non-inoculated soybeans and from 0.16 ± 0.01 g/100 g DM to 0.02 ± 0.01 g/100 g DM (87% reduction) in the presence of *R. oligosporus*. The reduction in verbascose was thus fourfold greater in the presence of the fungal strain, showing the importance of fungal activity in the reduction of verbascose. These differential reductions in α-galactosides in the presence of the fungal strain can be attributed to the α-galactosidase enzyme produced by the fungus during fermentation (Egounlety & Aworh, 2003; Rehms & Barz, 1995). In the non-inoculated samples, a reduction in α-galactosides was observed, but only on verbascose, which is present in smaller quantities in the seeds than the other forms. These results confirmed that *R. oligosporus* is effective against the reduction of α-galactosides. Therefore, cooking soybeans for 30 min combined with 48-h fermentation with *R. oligosporus* resulted in a 69% reduction in the total α-galactosides content of soybeans, comparable to the 80% reported by Egounlety and Aworh (2003), although the reduction they reported includes soaking along with cooking and fermentation.

Phytic acid levels did not change significantly after 48 h of fermentation in both non-inoculated and inoculated seeds (Fig. 8d). This result contradicts the literature, which reports a reduction in phytic acid (10 to 66%) in soybeans and other legumes fermented with *R. oligosporus* (Gbedo, Arnaud and Strub, 2024). This could be related to the physiological state of the microorganism, which may not produce

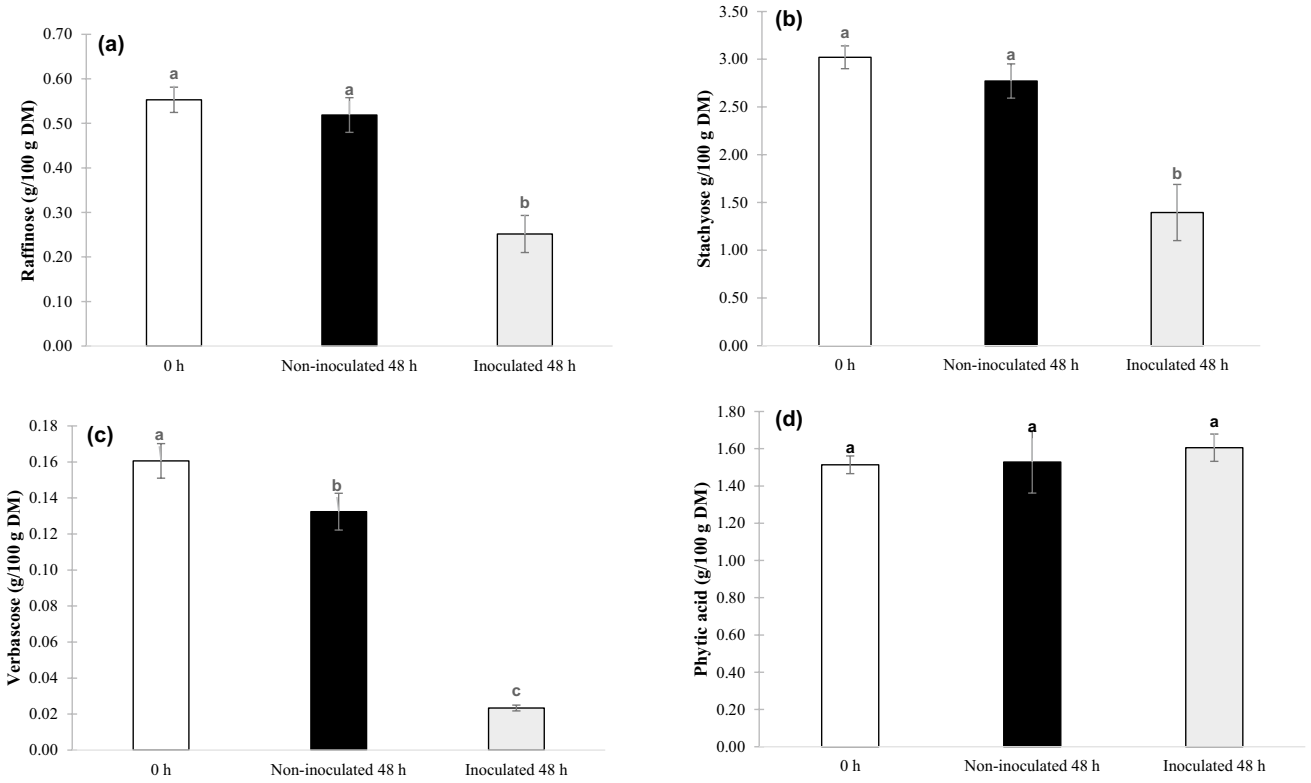

Fig. 8 (**a**) Raffinose, (**b**) stachyose, (**c**) verbascose and (**d**) phytic acid content of non-inoculated and inoculated soybean with *Rhizopus oligosporus* during fermentation. Error bars are standard deviations ($n = 3$). Different letters indicate significant differences between samples ($p < 0.05$) by Tukey's studentized range (HSD) test

sufficient (or any) phytase (Hajos & Osagie, 2004), to the fermentation time, which may be shorter for the enzyme activity (phytase) of the strain, or to the fermentation pH, not optimal for phytase enzyme activity.

Isoflavones Kinetics

Changes in the composition of isoflavone glycosides and aglycones in non-inoculated and inoculated soybeans fermented for 48 h are shown in Fig. 9. In non-inoculated soybeans, the content of malonylglycosides and β-glycosides present after cooking was significantly reduced from 133.52 to 41.08 mg/100 g (69% reduction) and from 101.59 to 39.63 mg/100 g DM (61% reduction), respectively, after 48 h (Fig. 9a and Fig. 9b). These reductions may be attributable to the presence of an endogenous β-glucosidase enzyme within the seeds or produced by microbial community present during the fermentation process. In the presence of *R. oligosporus*, malonylglycosides and β-glycosides isoflavone contents also decreased significantly compared to cooked seeds, from 133.52 ± 7.91 mg/100 g DM to 36.41 ± 4.17 mg/100 g DM (73% reduction) and from 101.59 ± 9.71 mg/100 g DM to 12.86 ± 1.89 mg/100 g DM (87% reduction), respectively. This is in agreement with the results of Chen et al. (2023), who reported an 86% reduction in β-glycosides after 48-h fermentation of soybeans with *Rhizopus oryzae*. Moreover, malonylglycosides isoflavones content did not change significantly during fermentation in both non-inoculated and inoculated seeds (Fig. 9a). The non-significant difference between inoculated and non-inoculated seeds could be due to the limited sporulation/development of the fungus.

The aglycones content of non-inoculated soybeans at 48 h was not statistically different from that of soybeans at 0 h (Fig. 9c), and the aglycones distribution did not change; with approximately 34% of genistein, 15% of daidzein, and 51% of glycitein. In contrast, there was a significant increase in aglycone isoflavones in inoculated soybeans; from 7.75 ± 0.55 mg/100 g DM to 26.74 ± 3.10 mg/100 g DM (245% increase) at 48 h of fermentation (Fig. 9c); with 48% of genistein, 39% of daidzein and 13% of glycitein. The same trend has been reported in other studies, where fermentation of soybeans for 48 h with *Rhizopus* or other microorganisms significantly increased aglycone forms from 0 to 35.4 mg/100 g DM and from 12.06 µg/g DM to 71.3 µg/g DM (Kuligowski et al., 2017; Gbedo, Arnaud and Strub, 2024). Furthermore, Chen et al. (2020). observed similar trends in soybeans subjected to a 15-day fermentation at 28 °C with *Eurotium cristatum*. According

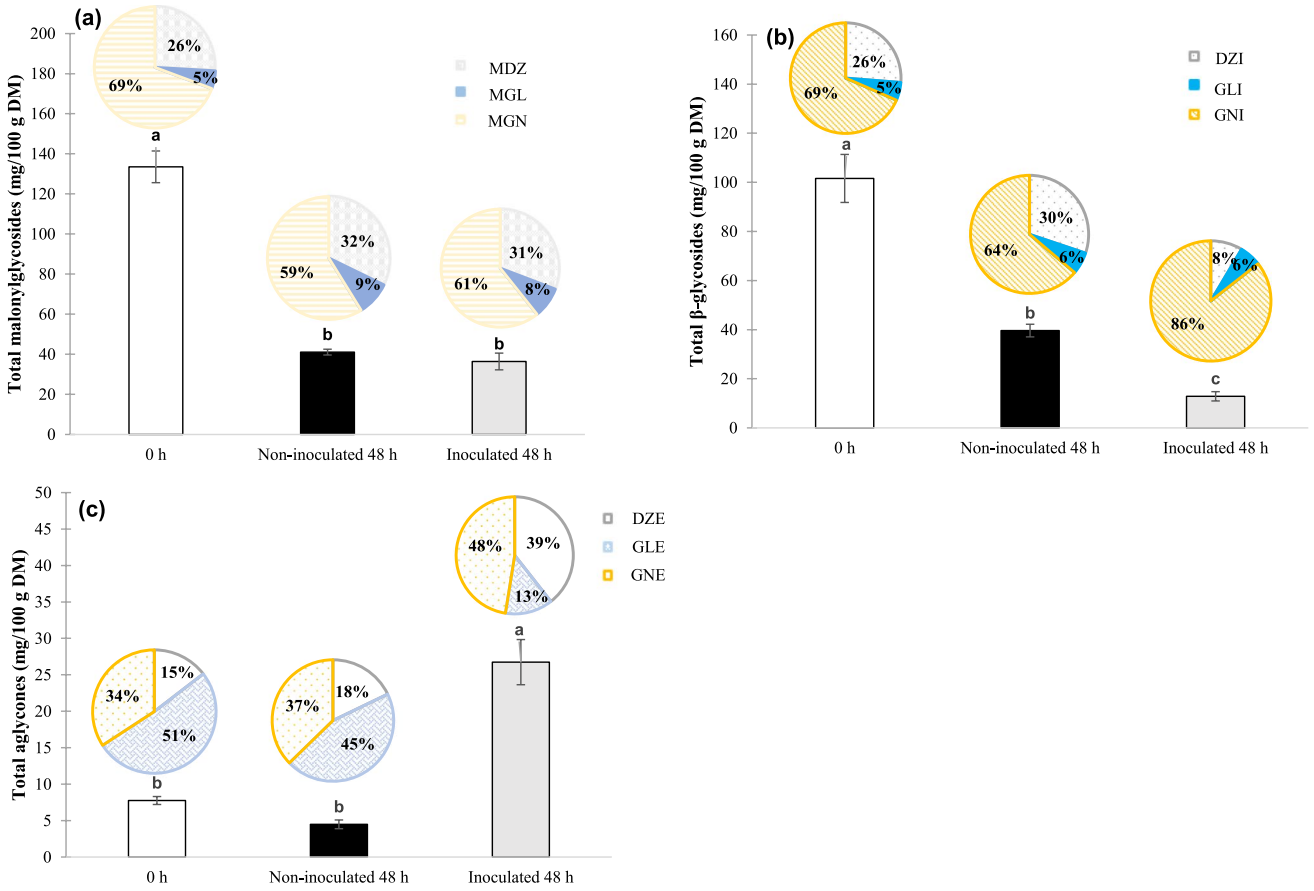

Fig. 9 (**a**) Malonylglycosides, (**b**) β-glycosides and (**c**) aglycones content with repartition of isomers in non-inoculated and inoculated soybean with *Rhizopus oligosporus* during fermentation. *Error bars are standard deviations (n = 3).* Different letters indicate significant differences between samples ($p < 0.05$) by Tukey's studentized range (HSD) test. Pie charts are proportions of isoflavone isomers. Malonyldaidzin (MDZ); malonylglycitin (MGL); malonylgenistin (MGN). Daidzin (DZI); glycitin (GLI); genistin (GNI). Daidzein (DZE); glycitein (GLE); genistein (GNE)

to the literature, the main reason for this variation in soybean isoflavone composition (decrease in isoflavone glycosides and increase in the corresponding aglycone structures) is strongly linked to the activity of β-glucosidase produced by *R. oligosporus* (Puri, Mir and Panda, 2015; Hariyanto et al., 2022). The non-increase in aglycones forms despite the significant decrease in glycosides in non-inoculated soybeans could be due to a potential degradation of glycosides and/or aglycones forms into unidentified compounds during fermentation or to a different type of enzymes due to synthesis pathway by microorganisms naturally present. Furthermore, the difference aglycone distributions between non-inoculated and inoculated soybeans suggests a difference in the type of enzymes present and/or synthesized during fermentation. Finally, cooking soybeans for 30 min combined with a 48-h fermentation with *R. oligosporus* resulted in 80% reduction in glycosides (malonylglycosides and β-glycosides) and increase up to 26.74 mg/100 g DM in aglycones in soybeans.

Conclusion

This study highlighted changes in ANFs (α-galactosides, phytic acid) and isoflavones (glycosides and aglycones) of soybeans during the cooking and fermentation steps involved in the processing of fermented legumes. The results showed that, cooking significantly decreased α-galactosides, phytic acid and glycoside isoflavones in soybeans, while increasing aglycone isoflavones (the forms of isoflavones beneficial to human health). Regarding ANFs and isoflavone glycosides, the longer the cooking time, the higher the reduction. Reduction of α-galactosides and glycoside isoflavones has been shown to be mainly due to diffusion in cooking water. Conversely, isoflavone aglycones demonstrated no diffusion during the cooking process and phytic acid showed little diffusion. Fermentation of 30 min cooked soybeans showed sporulation of the fungus, but with a significant reduction in the total content of α-galactosides and isoflavone glycosides. This reduction in isoflavone glycosides during fermentation

was accompanied by a drastic increase in aglycone isoflavones. However, fermentation had no effect on the phytic acid content. A comparison of non-inoculated and inoculated soybeans revealed that, in the presence of the fungal strain, there was a significant higher reduction of ANFs and increase in aglycones during the fermentation process. This research shows that processing of whole soybeans by cooking and fungal fermentation, even in the spore forms, can largely improve the nutritional quality of soybeans (decrease of α-galactosides by 69% and increase of aglycone isoflavones up to 26.74 mg/100 g DM). It thus offers a new fermented product that could be incorporated into foods, and to define new legume processing strategies by industrial food companies, for dietary diversification and the creation of healthier, tastier and more nutritious foods. Further research is needed to understand fungus physiology and identify the mechanisms underlying *R. oligosporus* sporulation during fermentation of cooked soybeans.

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Author Contribution CG: Writing – original draft, Methodology, Investigation, Formal analysis. EA: Writing – review & editing, Conceptualization, Funding acquisition, Supervision. AS: Methodology, Formal analysis, Writing – review & editing. AF: Methodology, Writing – review & editing. LO: Methodology, Formal analysis. CS: Writing – review & editing, Supervision, Conceptualization, Funding acquisition.

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Data Availability All data generated or analyzed during this study are included in this article (and its supplementary information files).

Declarations

Competing Interests The authors declare no competing interests.

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