RESEARCH ARTICLE

Effects of Fermentation Process and in Vitro Digestion on the Content of Total Phenols and Isoflavones and Antioxidant Properties in Natto

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Abstract

To investigate the changes of bioactive compounds during natto fermentation, this study focused on the variations in the levels of phenolic compounds, isoflavones, and antioxidant capabilities at various stages of the natto fermentation. Additionally, the impact of simulated in vitro digestion process of natto on phenolic compounds, isoflavones, and antioxidant capabilities was evaluated. The results indicated that fermentation process increased the phenolic content of 60.56%, while the isoflavone content decreased in 63.30%. Following in vitro digestion, the total phenolics content exhibited a release rate of 70.64%, while the isoflavones content had a residual rate of 21.79%. Antioxidant activity was assessed through DPPH, ABTS, and ORAC assays. After in vitro digestion at different fermentation stages, the ability of scavenging DPPH radicals decreased, whereas the ABTS and ORAC scavenging capabilities exhibited an upward trend. Specifically, during in vitro gastric digestion, the ABTS and ORAC radical scavenging abilities of after-ripening soybeans (24h) were 1.80 and 2.22 folder than those of dried soybeans respectively. Similarly, during in vitro intestinal digestion phase, these scavenging abilities were 1.70 and 1.14 folder than those of dried soybeans respectively.

Keywords: natto, total phenolics, isoflavone, in vitro digestion, antioxidant activity.

1 Introduction

Owing to a rich presence of vital fatty acids, protein,and mineral composition, soybeans are favored by many consumers [1]. Recent research has identified that the health benefits of soybeans are associated with their phenolic compounds [2, 3]. Furthermore, studies have demonstrated that fermentation can enhance both the content and activity of these phenolic compounds [4, 5]. By utilizing microorganisms such as *Bacillus*, *Streptococcus*, and Lactobacillus, for soybean fermentation, it is possible to obtain high-quality fermentation products while minimizing the occurrence of unpleasant odors and anti-nutritional properties [6, 7].

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Typical fermented soy products encompass black bean paste, tofu, natto, soy sauce and so on. Among soybean-based products, natto is a distinctive fermented food that primarily uses soybeans as its main ingredient. The preparation of natto involves soaking or steaming the soybeans, inoculating them with *Bacillus subtilis natto*, and fermenting the mixture in a sterile environment. Temperatures between 37°C and 42°C are maintained for 15 to 24 hours. After fermentation, natto is transferred to a 4°C environment for an additional 24 hours before consumption [8]. This process preserves the active substances found in soybeans, such as isoflavones and polyphenols, while also producing new bioactive substances, including nattokinase, antioxidant peptides, and antimicrobial peptides. Nattokinase exhibits potent fibrinolytic activity, which keeps cardiovascular health, while the antioxidant and antimicrobial peptides help combat oxidative stress and microbial infections [8–11]. Additionally, natto aids in mitigating obesity and diabetes by inhibiting lipid peroxidation, regulating lipid metabolism, and reducing oxidative damage. These properties make natto a valuable dietary intervention for improving overall health and preventing chronic diseases [12, 13].

In vitro simulated digestion is a simple and effective method for mimicking the digestive process, primarily involving the simulation of gastric and intestinal digestion stages. During simulated digestion, salivary, pepsin, and pancreatic enzymes are sequentially added to samples, followed by incubation to mimic *in vivo* digestion, thereby simplifying and accelerating the investigation into structural alterations, ease of digestion and bioactive release during food digestion [14]. Within biological organisms, reactive oxygen species (ROS) and antioxidants maintain a delicate balance. Once this balance is disrupted, highly reactive ROS can react with cell membranes, leading to cell death. However, antioxidants protect the organism from ROS damage [15].

Bacillus subtilis was chosen for natto fermentation because it is the traditional and main microorganism used in the process. *Bacillus subtilis* can produce specific enzyme systems, such as nattokinase and protease, which are essential for the flavor and texture of natto. In addition, this bacterium is able to withstand the high temperatures and pH changes during the fermentation process, which helps to maintain its activity and stability. *Bacillus subtilis* also produces a variety of bioactive compounds, including antioxidant peptides. Thence, using *Bacillus subtilis* for natto fermentation can not only preserve the traditional characteristics of natto, but also make full use of its unique bioactive potential.

2 Methods

2.1 Materials and Reagents

The *Bacillus subtilis natto* strain was isolated from a commercial natto product and further purified in our laboratory. The medium was prepared with peptone of 10 g/L, yeast extract of 5 g/L, sodium chloride 10 g/L, and pH was adjusted to 7.0. The strain was stored at 4°C with regular subculturing. All media components were obtained from Beijing Huikai Sci-Tech Co., Ltd.

The soybeans produced in Northeast China in the current year. Diphenyl-2-picrylhydrazyl (DPPH), the ABTS diammonium salt, Trolox, sodium fluorescein (FL), and 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) were acquired from Beijing Baierdi Technology Co., Ltd. Folin-Ciocalteu reagent, α-amylase, pepsin, pancreatin, and sodium taurocholate were purchased from Sigma-Aldrich, All of reagents were analytical grade standards.

2.2 Sample Preparation

The fermentation process of natto followed the method described in reference [16]: The strain stored at 4°C was activated in a nutrient agar medium at 37°C. Then, it was inoculated into a culture medium composed of peptone (10 g/L), yeast extract (5 g/L), and sodium chloride (10 g/L) at 37°C with agitation at 180 rpm for 15 hours. High-quality soybeans were soaked in water until the moisture content reached approximately 65%, and then steamed at high temperature and high pressure until the soybeans were soft. Soft soybeans were cooled to 50°C, then Bacillus subtilis natto were inoculated at a 3% inoculation rate. After inoculation, the soybeans were covered with gauze and fermented at 37.5°C for 24 hours, after which it was subjected to a ripening phase at 4°C for an additional 24 hours.

During the soybean steaming and fermentation processes, samples were taken at the following time points: dried soybeans, steamed soybeans, soybeans fermented for 12 h/18 h/24 h, and soybeans post-ripened for 12 h/24 h. The samples were prepared by freeze-drying, crushing, and then sieving through a 60-mesh screen before being kept at 4°C.

2.3 Simulated in Vitro Digestion Process

The simulated saliva, gastric fluid and intestinal fluid were prepared as follows:

1. Simulated saliva: Prepare a solution by dissolving 2.38 g of disodium hydrogen phosphate, 0.19 g of potassium dihydrogen phosphate, and 8 g of sodium chloride in 1 L of deionized water. Adjusted the pH to reach 6.75 and subsequently added α-amylase to achieve a concentration of 200 U/mL.
2. Simulated gastric juice: Formulate a solution of sodium chloride with a concentration of 0.03 mol/L, adjusted to a pH of 1.2. Added pepsin to a final concentration of 300 U/mL.
3. Simulated intestinal juice: Prepare a solution containing 0.1 M sodium bicarbonate. Withdraw 35 mL of this solution and added trypsin and bile salts to concentrations of 200 U/mL and 10 mg/mL.

The *in vitro* simulated digestion process was divided into two stages: salivary digestion stage and gastrointestinal digestion stage.

Firstly, a blend of 0.9 g natto powder was combined with 3 mL of simulated saliva and 3 mL of deionized water, then incubated in a shaking water bath at 37°C for 10 minutes. Post-incubation, the pH was modified to 1.2 with a 5 M HCl solution, followed by the addition of 9 mL of simulated gastric fluid and thorough mixing. This mixture was further incubated at 37°C for 1 hour. Eventually, the pH was raised to 6 using a 0.1 M solution of sodium bicarbonate, followed by the addition of 9 mL of simulated intestinal fluid. The pH was fine-tuned to 7 with a 1 M NaOH solution, and 3 mL of saline solution was incorporated and mixed. The resulting mixture was incubated at 37°C for 2 hours, rapidly transferred to an ice water bath for rapid cooling, and then stored at -20°C.

2.4 Determination of Total Phenols

The extraction of total phenols was performed as follows. A 0.3g sample of natto from different preparation stages was dissolved in 9 mL of 60% ethanol. The blend was agitated for 2 hours and then subjected to extraction in a dark environment overnight. Then the blend was centrifuged at a speed of 5000 rpm for 20 minutes, and the supernatant was retrieved. This extraction process was repeated three times, and combined supernatants and stored at 4°C for no more than 2 days. The amount of total phenols was evaluated through the application of the Folin-Ciocalteu procedure. The initial step in the test tube involved the mixture of 0.15 mL deionized water and 0.20 mL sample solution. Afterwards, 0.25 mL of Folin-Ciocalteu reagent and 0.75 mL of 7% sodium carbonate solution were added to the tube. The volume of the reaction mixture was made up to 5 mL with deionized water and left to equilibrate at ambient temperature for a period of 2 hours. Subsequently, the absorbance was recorded at a wavelength of 765 nm employing a microplate reader. A calibration curve was constructed utilizing gallic acid solutions spanning concentrations from 0 to 20 μg/mL. Triplicate measurements were conducted for each sample, and the mean values were determined.

Before measuring the total phenol content in the simulated digestive fluid. To eliminate protein interference, the solution was supplemented with anhydrous ethanol to achieve a concentration of 60%. The total phenol content was then determined using the Folin-Ciocalteu procedure, and the starting amount of total phenols in the simulated digestive fluid was determined.

2.5 Determination of Isoflavone Content

Isoflavone extraction was carried out following a method of Chaiyasut [**?**]. A 0.3g sample of natto was dissolved in 9mL of 80% ethanol and shaken at 70°C for 2 hours. The mixture was allowed to extract in the dark for an overnight period, after which it was centrifuged at 5000 rpm for 20 minutes. The supernatant was gathered. This extraction procedure was repeated thrice, amalgamating the collected supernatants and stored at 4°C for no more than 2 days.

The content of isoflavones in natto was determined using a three-wavelength UV spectrophotometric method. An aliquot of 0.2 mL from the sample was extracted, with absorbance readings taken at wavelengths of 240, 260, and 280 nm. The delta absorbance ΔA was then computed using the subsequent formula:

$$\Delta A = A_{260} - \frac{A_{240} + A_{280}}{2} \tag{1}$$

A calibration curve was constructed with genistein as the reference compound, covering concentrations from 0 to 10 μg/mL. Triplicate measurements were taken for each assay, and the mean values were determined.

For eliminating protein interference in the simulated digestive fluid, supernatants were adjusted by adding anhydrous ethanol to achieve a final concentration of 80%. The isoflavone content was then determined, and the initial total isoflavone content in the simulated digestive fluid was calculated.

2.6 Assessment of Antioxidant Potential

2.6.1 DPPH Procedure

The DPPH radical scavenging ability was evaluated according to the defined process described in reference [17] with some modifications. The experiment was designed with sample, blank, and control groups. A 0.05 mL sample aliquot was added to 0.15 mL of 0.2 mmol/L DPPH ethanol solution. The blend was vigorously stirred and allowed to react in darkness at room temperature for 30 minutes, after which the absorbance was recorded at 517 nm. Anhydrous ethanol was used as the blank, and 60% ethanol was used as the control. Each measurement was conducted three times, and the mean values were determined. The DPPH radical scavenging activity was then calculated using the subsequent equation:

$$\text{Clearance}(\%) = \left(1 - \frac{A_x - A_1}{A_2}\right) \times 100 \qquad (2)$$

where A_x is the absorbance of the sample, A_1 is the optical density of the control, and A_2 is the optical density of the reference sample.

2.6.2 ABTS Method

The antioxidant activity of ABTS was determined according to the method of Gawlik et al. [18] with some modifications. Equal volumes of 7 mmol/L ABTS and 2.45 mmol/L potassium persulfate were combined and left to react in the dark at room temperature for 12-16 hours. The resulting $ABTS^+$ solution was then diluted with 0.2 mol/L phosphate-buffered saline (PBS) at pH 7.4 until an absorbance of 0.7 ± 0.05 at 734 nm was reached, forming the working solution. A 50 μL sample was mixed with 0.15 mL of the $ABTS^+$ working solution, and the reaction mixture was incubated in darkness at room temperature for 6 minutes. After the incubation, absorbance was measured at 734 nm. Each sample was measured in triplicate.

Trolox concentrations ranging from 0 to 80 μmol/L served as a reference to create a standard curve following the identical protocol. The ABTS radical scavenging capacity of samples was quantified in terms of Trolox equivalent concentration. Triplicate measurements were conducted for each sample, and the mean values were determined.

2.6.3 ORAC Method

The ORAC test was executed following the protocol outlined in the cited literature [17]. Within a 96-well assay plate, 20 μL sample was combined with 120 μL of 70 nmol/L fluorescein sodium solution. The mixture was incubated for 15 minutes in a dark chamber at 37°C. Then, 80 μL of 200 mmol/L AAPH solution was introduced, followed by a 30 seconds mixing. The fluorescence intensity was monitored every 2 minutes over a 150 minute duration, with fluorescence measurements taken at an excitation of 485 nm and an emission of 520 nm. A PBS buffer was employed as a baseline for comparison, and a standard curve was generated using Trolox standards at concentrations from 0 to 20 μmol/L. The AUC of the fluorescence decay curve was calculated according to the formula provided:

$$AUC = 1 + \sum_{i=0}^{90} \frac{f_i}{f_0} \qquad (3)$$

where, f_0 is the initial fluorescence, f_i is the fluorescence after i minutes, and AUC_{net} is the AUC_{sample} minus the AUC_{blank}.

2.6.4 Statistical Analysis

Each experiment was conducted three times, and the average values was determined. The data analysis was performed using IBM SPSS Statistics version 20.0 from SPSS Inc., Chicago, IL. The results were recorded as the mean value $\pm$ standard deviation (SD). Statistical significance was set at a p-value<0.05.

3 Findings and Interpretation

3.1 Changes in Bioactive Compounds During Fermentation

3.1.1 Changes in Total Phenolic Content

Figure 1 illustrates the variations in total phenolic content throughout the various stages of natto fermentation.

The concentration of phenolic compounds in dry soybeans, steamed soybeans, and soybeans fermented for 12, 18, and 24 hours, as well as those matured for 12 and 24 hours, were 8.26×10^{-2}, 9.38×10^{-2}, 8.48×10^{-2}, 10.51×10^{-2}, 12.04×10^{-2}, 11.99×10^{-2}, and 13.64×10^{-2}g/g, respectively. The total phenolic content increased slightly after steaming, decreased slightly at the initial phase of the fermentation process, and then increased gradually. It further increased during the maturation stage. Overall, the total phenolic content significantly increased from 8.26×10^{-2} g/g in dry soybeans to 13.64×10^{-2} g/g in natto ($\rho < 0.05$). Chen et al. [17] also demonstrated that the total phenolic content increases during the fermentation process.

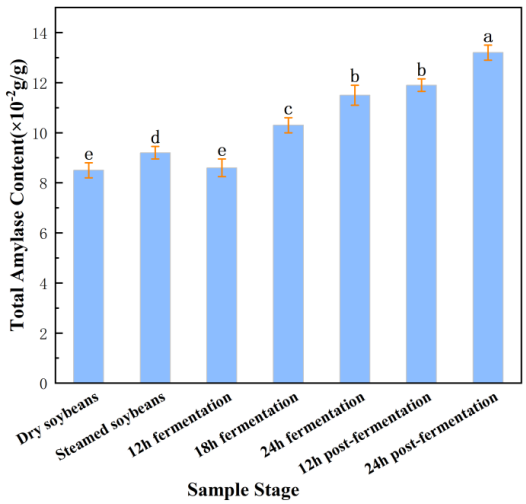

Figure 1. Influence of the natto fermentation process on the levels of total phenolics.
Notes: Different letters in the numerical values indicate significant differences between corresponding groups ($\rho < 0.05$) conversely, groups marked with identical letters are not statistically different from one another ($\rho \geq 0.05$).

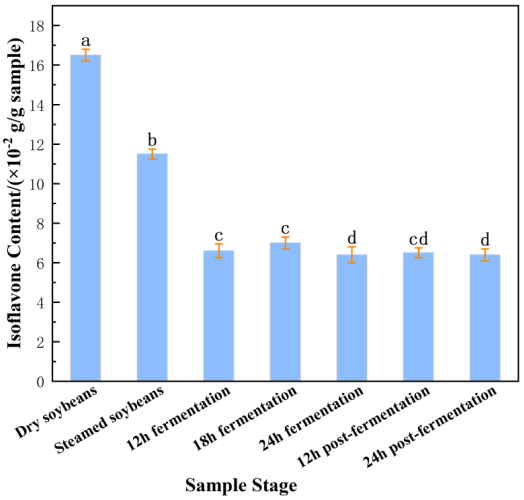

Figure 2. Effect of natto fermentation on isoflavone content.
Notes:Values marked with distinct letters signify statistically significant disparities among the respective groups ($\rho < 0.05$); conversely, groups labeled with identical letters exhibit no statistically significant differences ($\rho \geq 0.05$).

The rise in total phenolic levels throughout the fermentation process is due to the action of β-glucosidase and esterase enzymes secreted by the fermenting microorganisms, which liberate bound phenolics, making them available as free phenolics. These bound phenolics were originally conjugated with sugars and glycosides. The 13.62% increase in total phenolic content in steamed soybeans is considered to be due to the high-temperature treatment, which disrupted the soybean cell wall, accelerating the release of free phenolics [19].

3.1.2 Changes in Isoflavone Content

The enhancement of total phenolic release rate throughout the course of the simulated digestion procedure as shown in Figure 2.This reason was attributed to the digestive enzymes (amylase, pepsin, and pancreatic enzymes) in the simulated digestive fluid progressively breaking down the cellular structures that constitute the food matrix, thereby promoting the gradual release of free phenolics [20]. As for the concentration of phenolic compounds during the intestinal digestion phase still being lower than that of the untreated, it may be due to some phenolics being sensitive to digestive enzymes or gastrointestinal environment, leading to the destruction of phenolics themselves [21]. The preparation process of natto significantly influences the levels of isoflavones ($\rho < 0.05$). The isoflavone contents of dried soybeans, boiled soybeans, fermented for 12/18/24 hours, and post-ripened for 12/24 hours are shown in Figure 2.

As shown in the Figure 2, the isoflavone contents of dried soybeans, boiled soybeans, fermented for 12/18/24 hours, and post-ripened for 12/24 hours are 16.81×10^2, 11.65×10^2, 6.78×10^2, 6.63×10^2, 6.17×10^2, 6.46×10^2, and 6.17×10^2 g/g of sample, respectively. Throughout the entire fermentation process, the isoflavone content was reduced by 63.30%, with a 30.70% loss during the boiling process and a 59.67% loss during the early fermentation process. The high-temperature treatment during the boiling process caused destruction of isoflavones content. Chalida et al. [22] conducted research on soybeans and confirmed that higher processing temperatures (130–170°C) lead to a greater degradation of isoflavones. The decrease in isoflavone content during the early fermentation process may be due to the physiological activities of the fermenting bacteria. Nirat et al. [23] investigated the changes in flavonoid content in Kinema after 24 to 70 hours of fermentation and found that the flavonoid content gradually decreased as fermentation time increased, which is consistent with our research findings.

3.2 Bioactive Compound Variations in Simulated Digestion

The digestive system serves as the nutritional uptake apparatus of organisms, where the interplay between food and digestive fluids is intricately complex. While digestive fluids facilitate the breakdown of cellular

Table 1. Impact of simulated digestion on total phenolic contents ($\times 10^{-2}$ g/g sample).

Processing	Dry	Cooked	Fermentation			After-ripening	
			12h	18h	24h	12h	24h
Untreated	8.26 ± 0.46^{d}	9.38 ± 0.38^{c}	8.48 ± 0.29^{d}	10.51 ± 0.51^{c}	12.04 ± 0.43^{b}	11.99 ± 0.48^{b}	13.64 ± 0.64^{a}
Gastric Digestion	3.60 ± 0.18^{d}	3.38 ± 0.13^{d}	3.62 ± 0.15^{d}	4.16 ± 0.18^{c}	4.43 ± 0.16^{b}	4.43 ± 0.23^{b}	4.93 ± 0.21^{a}
Intestinal Digestion	5.85 ± 0.34^{cd}	6.32 ± 0.30^{c}	6.05 ± 0.26^{c}	7.88 ± 0.28^{b}	8.23 ± 0.32^{b}	8.33 ± 0.38^{b}	9.11 ± 0.33^{a}

Notes: Table entries denote the average $\pm$ standard deviation (n=3). Distinct letters within the same row signify a statistically significant variation ($\rho < 0.05$).

Table 2. Effect of simulated digestion on isoflavone content ($\times 10^{-2}$ g/g sample).

Processing	Dry	Cooked	Fermentation			After-ripening	
			12h	18h	24h	12h	24h
Untreated	16.81 ± 0.93^{a}	11.65 ± 0.55^{b}	6.78 ± 0.42^{c}	6.63 ± 0.37^{c}	6.17 ± 0.32^{d}	6.46 ± 0.31^{c}	6.17 ± 0.34^{d}
Gastric Digestion	9.35 ± 0.32^{a}	8.44 ± 0.28^{b}	6.28 ± 0.23^{c}	4.84 ± 0.15^{d}	4.46 ± 0.19^{d}	4.46 ± 0.23^{d}	4.86 ± 0.16^{d}
Intestinal Digestion	5.69 ± 0.33^{a}	4.05 ± 0.16^{b}	3.37 ± 0.15^{c}	1.04 ± 0.03^{d}	0.47 ± 0.02^{e}	0.47 ± 0.02^{e}	0.47 ± 0.03^{e}

structures, thereby promoting the release of bioactive compounds from food, they can concurrently cause a degree of degradation to these bioactive substances.

3.2.1 *Variations in Phenolic Levels*

This contents of phenolic compounds in natto at different fermentation stages during simulated gastrointestinal digestion. The results were presented in Table 1. The release rates of total phenols in the gastric digestion and intestinal digestion stages were 67.71% and 71.19% respectively. The total phenolic content of natto cooked for 24 h after in vitro simulated digestion was significantly higher than that of dried soybeans ($\rho < 0.05$). The overall phenolic content of the sample decreased after both gastric and intestinal simulated digestion. A further comparison of the variation in phenolic levels during the digestion stages revealed that the release rate of total phenolics increased throughout the entire simulated digestion process.

3.2.2 *Changes in Isoflavone Content*

Table 2 illustrates the impact of such simulated digestion on the isoflavone levels in natto.

As Table 2 shown that the isoflavone content had a significant decrease trend ($\rho < 0.05$) in the simulated digestion process. For dry soybeans, the isoflavone content increases from 16.81×10^{-2} g/g sample dropped to 5.69×10^{-2} g/g sample in the intestinal digestion stage. The decrease of isoflavones in dry soybeans was smaller than that of isoflavones in 24 h post-ripened process of natto, and the decrease proportions were 66.15% and 92.38% respectively. The isoflavone content in the simulated intestinal digestive fluid was lower than that of in the untreated and simulated gastric digestive fluid samples. Several investigations have reported that the low-pH conditions present in the stomach during digestion can result in a reduction of isoflavone levels [24]. In examinations of how an in vitro simulated digestion process affects isoflavone levels in soy milk, researchers observed that the concentration of total flavonoids in the intestinal digesta was significantly lower than that in the gastric digesta [25].

3.3 Antioxidant Activity of Simulated Digestive Fluid

Foods with antioxidant activity have a greater protective effect on the health of organisms. This view has become a consensus among many scientists in previous studies. This paper uses DPPH, ABTS and ORAC assays to assess the capacity of natto to quench free radicals at various stages of fermentation, and to conduct a thorough evaluation of the shifts in the antioxidant properties of natto throughout different

Table 3. Fermentation and digestion's impact on natto's DPPH scavenging activity (%).

Processing	Dry	Cooked	Fermentation			After-ripening	
			12h	18h	24h	12h	24h
Gastric Digestion	47.12 ± 3.14^{a}	44.69 ± 1.65^{b}	43.96 ± 1.91^{b}	36.04 ± 1.47^{c}	37.77 ± 2.26^{c}	36.58 ± 1.48^{c}	35.03 ± 1.69^{d}
Intestinal Digestion	37.89 ± 1.31^{a}	35.39 ± 1.61^{b}	33.46 ± 2.10^{c}	34.64 ± 1.78^{b}	35.54 ± 2.31^{b}	34.99 ± 1.16^{b}	33.49 ± 1.12^{c}

phases of fermentation when subjected to *in vitro* simulated digestion.

3.3.1 DPPH Radical Scavenging Capacity

DPPH exists as a stable radical in organic media. When an antioxidant is present, the single electron of DPPH is captured, causing the light absorption to disappear or weaken, thereby obtaining the scavenging effect of the antioxidant on the DPPH free radical. This technique is extensively applied to measure the antioxidant potential of naturally occurring antioxidants [24]. The changes in the scavenging capacity of DPPH free radicals at different stages of natto fermentation were shown in Table 3.

From the Table 3, it was evident that the DPPH free radical scavenging ability of natto at various fermentation stages was significantly reduced ($\rho < 0.05$), with an average decrease of 16% after undergoing gastric and intestinal digestion in a simulated digest environment. The DPPH free radical scavenging rates for dry soybeans during the simulated gastric and intestinal digestion stages were 47.12% and 37.89%, respectively. In contrast, the DPPH free radical scavenging rates for soybeans 24 h post-ripening during the simulated gastric and intestinal digestion stages were 35.03% and 33.49%, respectively. Furthermore, during the simulated digestion process of natto at different fermentation stages, the sample exhibited a higher capacity for neutralizing DPPH radicals during the simulated gastric digestion stage than during the intestinal digestion stage.

3.3.2 The Capacity to Quench ABTS Radicals

The presence of an antioxidant leads to the conversion of ABTS into the blue-green $ABTS^{+}$ radical. With the addition of an antioxidant, the levels of $ABTS^{+}$ were reduced. As depicted in Table 4, the capacity of natto to neutralize ABTS radicals after 24 h of fermentation was considerably greater than that of unfermented soybeans ($\rho < 0.05$). Throughout the process of simulated digestion in the stomach and intestine, natto that had been ripened for 24 hours demonstrated an ABTS radical scavenging capacity which was 1.86-fold and 1.70-fold of unfermented soybeans, respectively. Cumulatively, across various stages of fermentation and simulated digestion phases, the quench $ABTS^{+}$ radicals ability of natto increased on average by 78%. During the simulated digestion process, the stage of gastric digestion was more effective at scavenging $ABTS^{+}$ radicals compared to that of the intestinal digestion stage.

Examining the fluctuations in phenolic levels throughout fermentation and simulated digestion revealed certain trends. Throughout fermentation, the phenolic content exhibited a notable ($\rho < 0.05$) increasing trend, and the ABTS radical-neutralizing capacity displayed a strong positive correlation. There are other antioxidant components in the sample, such as antioxidant peptides. Factors like pH and temperature can influence the antioxidant properties of antioxidant peptides. Since the DPPH and ABST methods have different sample processing methods, this may result in the changing trends of antioxidant activity were different. Recent studies have demonstrated a significant link between the metabolic byproducts and the antioxidant potency of fermented soybeans following simulated digestion. The antioxidant potential of soybeans post-fermentation is significantly higher than that of both raw soybeans and unfermented soybeans. Furthermore, the study indicates that the choice of raw materials significantly affects the final product's antioxidant characteristics [26].

3.3.3 ORAC Experiment for Free Radical Defense

ORAC evaluates antioxidant capacity by measuring hydrogen atom donation to neutralize free radicals [27]. The changes in the ORAC value of natto at different fermentation stages are presented in Table 5.

Table 5 indicated that there was an overall enhancement in the ORAC value, and the antioxidant activity of soybeans was significantly enhanced during the fermentation process into natto ($\rho < 0.05$).

Table 4. Influence of simulated digestion and fermentation on natto's ABTS radical scavenging capacity (TE, μM).

Processing	Dry	Cooked	Fermentation			After-ripening	
			12h	18h	24h	12h	24h
Gastric Digestion	15.39±0.61^{e}	17.67±0.91^{d}	21.40±0.79^{c}	22.73±1.35^{a}	25.00±0.89^{b}	27.33±1.39^{a}	28.67±1.01^{a}
Intestinal Digestion	14.91±0.85^{d}	15.67±0.58^{c}	14.67±0.51^{d}	14.00±0.59^{d}	16.21±0.66^{c}	22.48±1.09^{b}	25.33±1.17^{a}

Table 5. Effect of simulated digestion and fermentation on the ORAC value of natto (TE, μM).

Processing	Dry	Cooked	Fermentation			After-ripening	
			12h	18h	24h	12h	24h
Gastric Digestion	27.09±1.42^{d}	28.24±1.05^{d}	34.82±1.39^{c}	15.59±0.73^{e}	71.82±2.62^{a}	57.65±2.11^{b}	59.49±1.98^{a}
Intestinal Digestion	35.88±1.31^{e}	42.35±2.24^{d}	47.35±1.94^{c}	24.24±1.45^{f}	61.65±2.17^{a}	51.42±2.63^{b}	50.35±2.37^{b}

However, there were large fluctuations at different stages of fermentation. During simulate gastric and intestinal digestion stages, the time of 18 h and 24 h reached minimum and maximum values, respectively; The ORAC value of soybeans that have been matured for 24 hours in the mid-section is about 2.22 and 1.40 times that of dry soybeans. In the simulated digestion process, the capacity for oxygen radical scavenging during the gastric digestion phase exceeds that of during the intestinal phase.

Antioxidants mainly play an antioxidant role by inhibiting lipid oxidative degradation [28], scavenging free radicals, inhibiting pro-oxidants and reducing capacity. Free radicals are intermediate metabolites of many biochemical reactions in human tissues. When the clearance and formation of endogenous radicals and oxidative species are out of balance, it can cause diseases such as aging and cancer. This study uses DPPH free radical scavenging ability, ABST antioxidant capacity for neutralizing free radicals and ORAC process to evaluate the antioxidant capacity for neutralizing free radicals of samples at different stages in the natto preparation process. The results showed that for samples of natto at different fermentation stages that underwent *in vitro* simulated digestion, the ABTS antioxidant capacity for neutralizing free radicals and ORAC value increased significantly with the fermentation process, while the DPPH antioxidant capacity for neutralizing free radicals decreased. Besides, polyphenols and isoflavones affecting the antioxidant properties of natto in the *in vitro* simulated digestion process at different fermentation stages, it may also be due to other antioxidant active ingredients. For example, the protein in natto will be hydrolyzed through *in vitro* simulated digestion , resulting in products with antioxidant activity [29]. Polypeptides, the presence of these antioxidant peptides will also cause changes in the antioxidant activity of natto. In summary, samples of *in vitro* simulated digestion of natto at different fermentation stages can scavenge DPPH free radicals, indicating that natto has an effective concentration of reducing hydroxyl free radicals, alkyl free radicals or peroxy free radicals; and the samples can act on free radical cations and remove oxygen free radicals from the body.

During the fermentation of natto by Bacillus subtilis natto, the bioactive compounds in natto undergo significant changes. This study investigates the changes in total phenols and isoflavones content at different stages of fermentation and their antioxidant activity during *in vitro* simulated digestion, providing a basis for the processing and consumption of fermented soybean products.

4 Conclusion

This investigation meticulously examined the variations in phenolic compounds and isoflavone concentrations in natto as influenced by fermentation and simulated digestive processes, establishing a foundation for the development of industrially produced, health-enhancing natto that contains components designed for superior digestibility and bioavailability. The findings revealed that throughout the fermentation process, the total phenol content increased by 65.12%, however, the isoflavone content decreased by 63.28%. After the simulated

digestion process, the liberation percentage of total phenols amounted to 70.64%, whereas the remaining percentage of isoflavones was 21.79%. The assessment of antioxidant potency was conducted via the DPPH, ABTS, and ORAC assays, yielding intricate outcomes. Broadly speaking, the capacity of natto to neutralize DPPH radicals diminished post-fermentation, yet its ability to quench ABTS radicals and its ORAC values were elevated. Throughout the simulated digestion, natto exhibited more robust antioxidant properties during the gastric phase compared to the intestinal phase.

Conflicts of Interest

The authors declare no conflicts of interest.

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