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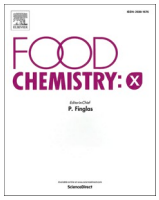

Effects of *Eurotium Cristatum* on soybean (*Glycine* max L.) polyphenols and the inhibitory ability of soybean polyphenols on acetylcholinesterase under different conditions

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ABSTRACT

Most phenolic compounds in beans exist in complex, insoluble binding forms that bind to cell wall components *via* ether, ester, or glucoside bonds. In the process of solid-state fermentation, *Eurotium Cristatum* can produce many hydrolase enzymes, such as α-amylase, pectinase, cellulase and β-glucosidase, which can effectively hydrolyze ether, ester or glucoside bond, release bound polyphenols, and increase polyphenol content in soybeans. When the fermentation conditions of soybean were fermentation time 12 days, inoculation amount 15% and initial pH 2, the content of free polyphenols in fermented soybean was 2.79 mg GAE/g d.w, which was 4.98 times that of unfermented soybean. The contents of bound polyphenols and total phenols in fermented soybean were 0.62 mg GAE/g d.w and 3.41 mg GAE/g d.w, respectively, which were 2.38 times and 4.16 times of those in unfermented soybean. At the same time, the inhibitory effect of free polyphenols in fermented soybean on acetylcholinesterase reached 91.51%. Thus, our results demonstrated that solid state fermentation and *Eurotium Cristatum* can be used as an effective way to increase soybean polyphenol content and combat Alzheimer's disease.

1. Introduction

A rapidly evolving food processing technique that has the potential to lower production costs, boost productivity, and lessen environmental pollution is the use of solid state fermentation technology to enhance the nutritional value of natural resources and bioactive ingredients (Singh, Chauhan, Kaur, & Pandey, 2020). Cellulase, protease, glycosylase, pectinase, and other hydrolase enzymes are released by microorganisms during fermentation. These hydrolase enzymes can break down plant cell walls (Leite, Salgado, Venâncio, Domínguez, & Belo, 2016; Leite, Silva, Salgado, & Belo, 2019; Nguyen, Wikee, & Lumyong, 2018) and release active ingredients by releasing the chemical bonds that hold them to cellulose, lignin, and other compounds.

As one of the most important crops, soybean is rich in primary and secondary metabolites, such as proteins, amino acids, carbohydrates, phenolic acids, isoflavones, saponins and other substances (Wang, Chen, & Zhang, 2014; Kim, Kim, & Yang, 2021). Studies have found that isoflavones, phenolic acids, saponins and other substances in soybean have strong biological activities such as antioxidant, anti-obesity, hypoglycemic, cholesterol-lowering and immunomodulatory (Zhang et al., 2003; Jayachandran & Xu, 2019; Ramdath, Padhi, Sarfaraz, Renwick, & Duncan, 2017), and pectin and dietary fiber in soybean epiderma have certain effects in preventing cardiovascular disease, coronary heart disease and improving intestinal osmotic pressure (Claudia, Elizabeth, & René, B. Q., 2018; Yang et al., 2019). The bioavailability of soybean was improved after microbial fermentation. At the same time, under the action of microorganisms, bioactive ingredients are accumulated, thus improving the biological activity of soybeans and improving the quality of soybean products. In addition, anti-nutritional factors in soybeans, such as inositol hexaphosphate, trypsin inhibitors and lectins, are very low in fermented soybeans (Licandro et al., 2020; Anderson & Wolf, 1995).

Eurotium Cristatum is a kind of non-toxic and harmless probiotic bacteria with golden granules isolated from Fu brick tea, which gives Fu brick tea special aroma, color and taste. At the same time, studies have shown that *E. cristatum* has good effects in regulating blood sugar, lipid balance and cholesterol metabolism, enhancing immunity, alleviating obesity, regulating intestinal flora and inhibiting bacterial growth

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Table 1
Orthogonal optimization test factor level table.

Horizontal	Factor		
	A fermentation time (d)	B inoculation amount(%)	C initial pH
1	8	10	2
2	10	15	4
3	12	20	6

(Kang, Su, Duan, & Huang, 2019; Du, Wang, & Yang, 2019; Du, Li, Li, Zhu, & Wang, 2017). The biological activity of the fermented substance was significantly improved through the fermentation of *E. cristatum*. For example, the theabrownins of dark tea increased and the lipid-lowering activity of dark tea was significantly improved after it was fermented by *E. cristatum* (Xiao, Li, Wu, Zhong, & Gao, 2020). The contents of crude protein, amino acids, isoflavones, and total phenols in black beans fermented by *E. cristatum* have significantly increased, which also has great potential in oxidative disease, prevention of atherosclerosis, arthritis and other diseases (Xiao et al., 2021). After wheat bran fermentation, soluble dietary cellulose, total phenol, anthocyanin and phenylethanol were significantly increased. In addition, under the action of hydrolase enzyme secreted by *E. cristatum*, the content of ferulic acid was increased to 12.06 times, which further enhanced the antioxidant and anti-obesity effects of wheat bran (Lu, Jing, Li, Zhang, & Cao, 2022).

The purpose of this study was to investigate the effects of fermentation conditions on the content of soybean polyphenols and the changes of bioactivity of soybean polyphenols. The effects of fermentation time, inoculation amount and initial pH on the free and bound polyphenols of soybean were studied. The changes of main components in soybean during fermentation were analyzed, and the key factors that may affect the changes of lipid-lowering activity and anti-aging activity in soybean bioprocessing were speculated. This study provides a theoretical basis for the increase of polyphenol content in soybean substrate during solid state fermentation, and provides a new research direction for the development and utilization of soybean resources.

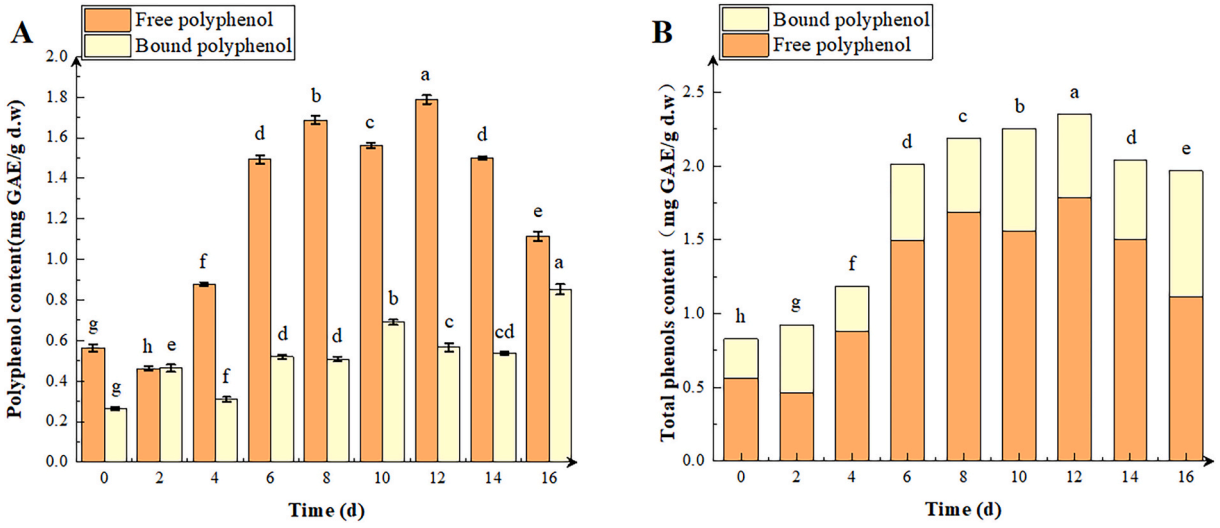

Fig. 1. Effects of fermentation time on contents of free polyphenols, bound polyphenols and total phenols in soybean.
Note: Values in the figure are mean ± standard deviation ($n = 3$), and different letters in the same column indicate statistical differences between components, ANOVA, $p < 0.05$.

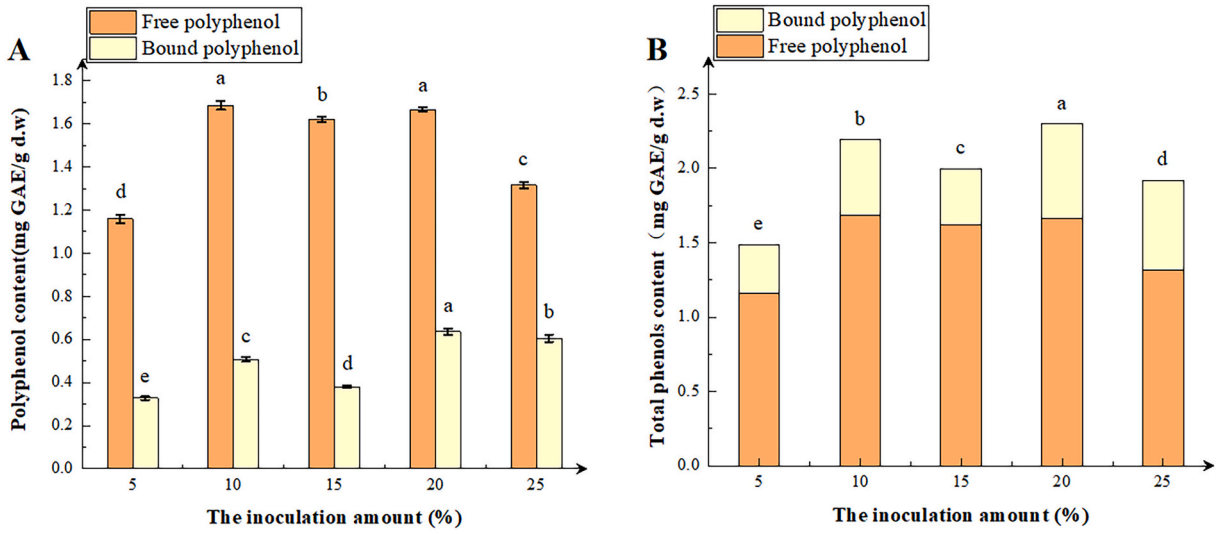

Fig. 2. Effects of the inoculum amount of *E. cristatum* on the contents of free polyphenols, bound polyphenols and total phenols in soybean.
Note: Values in the figure are mean ± standard deviation ($n = 3$), and different letters in the same column indicate statistical differences between components, ANOVA, $p < 0.05$.

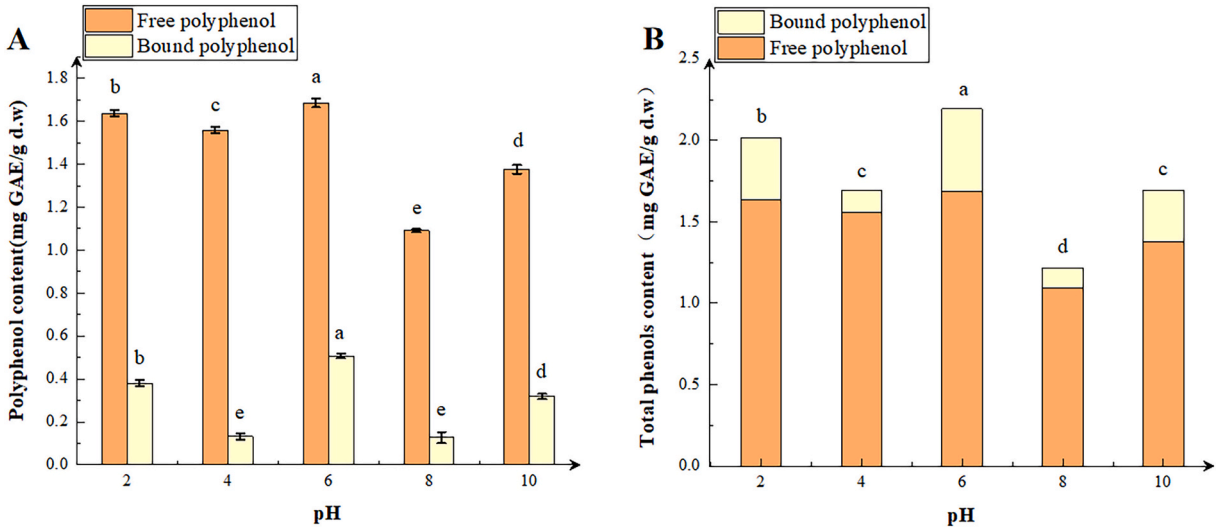

Fig. 3. Effects of initial pH on the contents of free polyphenols, bound polyphenols and total phenols in soybean.
Note: Values in the figure are mean ± standard deviation (n = 3), and different letters in the same column indicate statistical differences between components, ANOVA, $p < 0.05$.

Table 2
Experimental results of orthogonal optimization.

Test No.	A fermentation time(d)	B inoculation amount(%)	C initial pH	Free polyphenols (mg GAE/g d.w)
1	8	10	2	1.64
2	8	15	4	1.62
3	8	20	6	1.67
4	10	10	4	2.50
5	10	15	6	2.19
6	10	20	2	2.06
7	12	10	6	1.81
8	12	15	2	2.79
9	12	20	4	2.22
K1	1.643	1.983	2.163	
K2	2.250	2.200	2.113	
K3	2.273	1.983	1.890	
Range	0.630	0.217	0.273	
Factor		A > C > B		
Optimal		$A_3B_2C_1$		

2. Materials and methods

2.1. Materials

Anhydrous sodium carbonate, gallic acid, anhydrous methanol, sodium hydroxide, anhydrous sodium sulfite, glucose, potassium chloride, potassium dihydrogen phosphate, fish peptone, ferrous sulfate heptahydrate, hydrochloric acid, potassium phosphate dibasic, Folin-Ciocalteu reagent, were obtained from Sinopharm Chemical Reagent Co., Ltd. (China). Methanol, acetonitrile and formic acid are HPLC grade and purchased from Merck (USA).

AGAR powder, LP0021 yeast extract, acetylthiocholine iodide, acetylcholinesterase, 5,5′-Dithiobis(2-nitrobenzoic acid) were obtained from Shanghai yuanye Bio-Technology Co., Ltd. (Shanghai, China), Shanghai Macklin Biochemical Technology Co., Ltd. (Shanghai, China), OXOID Company of Britain (US), respectively. Soybeans purchased from Zaozhuang Guocheng Agricultural Products Co., Ltd. (Shandong, China). The strain *E. cristatum* J4 was isolated from the Fu-brick tea of Anhua (Hunan, China) for the first time in our research group. It was cultured on an AGAR slope every month for 6 days at 30 °C and stored at 4 °C.

2.2. Preparation of seed solution

1.20 g glucose, 0.15 g yeast extract, 0.6 g fish meal peptone, 0.3 g KH_2PO_4, 0.025 g KCl and 0.0005 g $FeSO_4 \bullet 7H_2O$ were dissolved in a conical bottle containing 50 mL distilled water. The seed solution was sterilized at 121 °C for 20 min and placed at room temperature on the aseptic operating table. A piece of bacteria with diameter of 8 mm was extracted from the potato dextrose agar culture medium of *E. cristatum* cultured for 6 d using a sterile hole punch and inserted into the sterilized seed solution. Shaker incubator at 30 °C, 180 r/min for 2 d.

2.3. Extraction and content determination of polyphenols

The extraction of free polyphenols was performed as follows: 1.00 g of fermented soybean powder was added to a solution containing 20 mL of 80% methanol. The mixture was subjected to a water bath and maintained at a temperature of 40 °C for 1 h. Subsequently, centrifugation was carried out at a speed of 4000 r/min for 10 min, and the resulting supernatant was collected. To remove methanol, the supernatant underwent rotary evaporation at 40 °C (RE-2000 A rotary evaporator Yarong biochemical instrument factory, Shanghai,China) until its volume reached 20 mL, which was adjusted with distilled water. N-hexane (20 mL) is added to the mixture for degreasing, followed by separation from the organic phase. Further extraction with ethyl acetate (20 mL) was conducted twice, and the combined organic phases were collected. The obtained organic phase underwent evaporation at a temperature of 40 °C until complete evaporation of ethyl acetate occurred. It was then redissolved in a solution consisting of 5 mL of 80% methanol (*v*/v) solvent to obtain fermented soybean free polyphenol (FS-FP) and unfermented soybean free polyphenol (US-FP), respectively. Finally, these solutions were stored at −20 °C (Bei, Liu, Wang, Chen, & Wu, 2017).

Extraction of bound polyphenols was performed by adding 20 mL of a 4 M NaOH solution to the filter residue after the extraction of free polyphenols. The resulting mixture was hydrolyzed at room temperature for 4 h, followed by pH adjustment to 2.0 using 6 M HCl. After centrifugation and filtration, the filtrate was degreased with 20 mL of n-hexane, and the organic phase was separated. The aqueous phase was then subjected to two extractions with 20 mL of ethyl acetate, and the organic phases were combined. Subsequently, the obtained organic phase underwent evaporation at a temperature of 40 °C until complete evaporation of ethyl acetate occurred. It was then redissolved in a solution

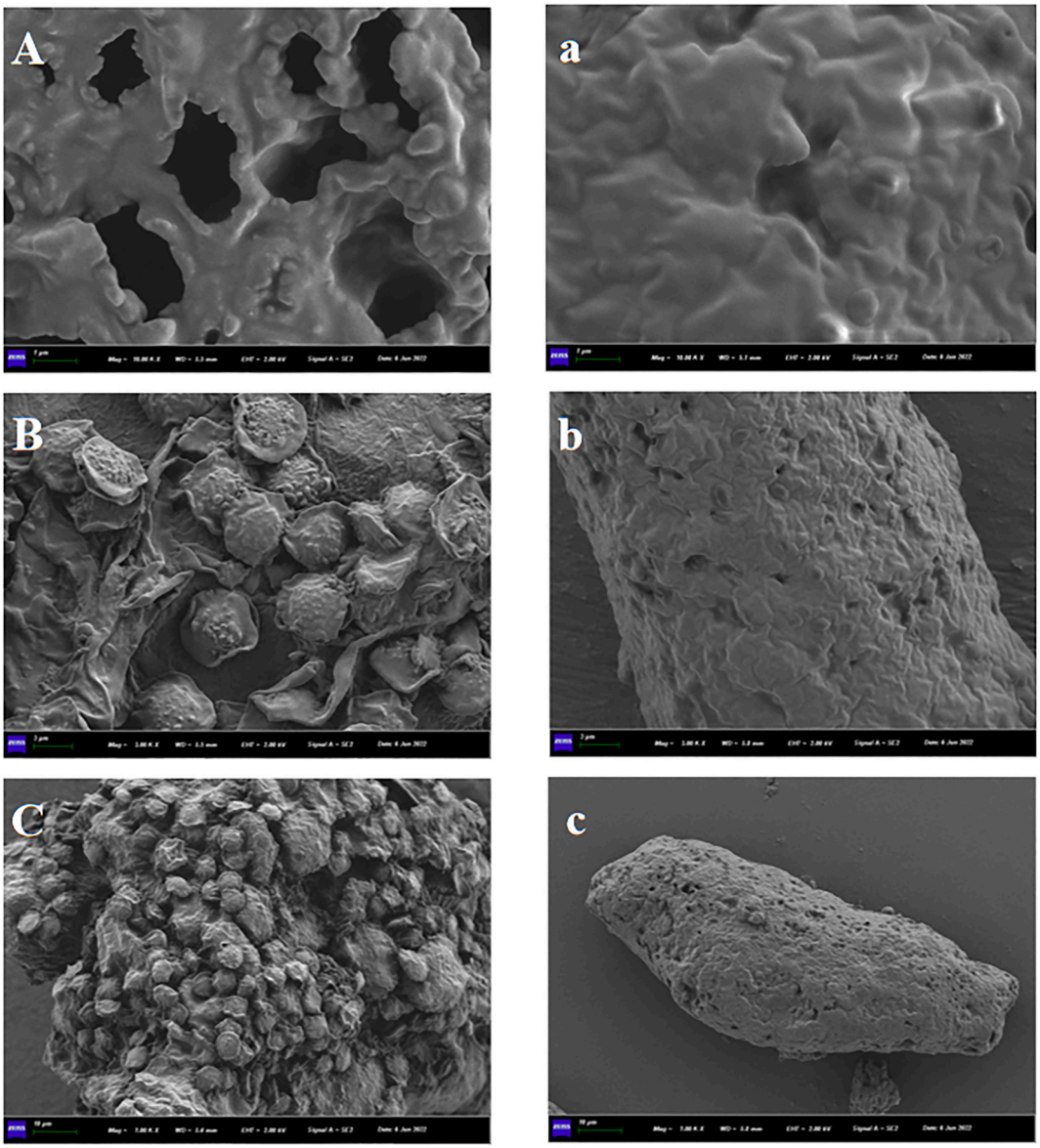

Fig. 4. SEM image of fermented soybean and unfermented soybean.
A: Local map of 10.00 K× fermented soybean; B: Local map of 3.00 K× fermented soybean; C: Local map of 1.00 K× fermented soybean; a: Local map of 10.00 K× unfermented soybean; b: Local map of 3.00 K× unfermented soybean; c: Local map of 1.00 K× unfermented soybean.

consisting of 5 mL of 80% methanol (*v*/v) solvent to obtain fermented soybean bound polyphenol (FS-BP) and unfermented soybean bound polyphenol (US-BP). The resulting solutions were stored in a − 20 °C refrigerator (Bei et al., 2017).

Determination of polyphenol content: The phenolic content of the samples was determined using the Folin-Ciocalteu method. A mixture of 1 mL polyphenol extract and 1 mL Folin-Cteu reagent was vigorously shaken and allowed to stand for 5 min. Subsequently, 6 mL deionized water and 2 mL of 15%(m/v) Na_2CO_3 solution were added sequentially. The reaction mixture was protected from light and incubated for 1 h, measure absorbance at 760 nm. The results were expressed as mg gallic acid equivalent (mg GAE/g d.w) (Bei et al., 2017). The obtained standard curve equation is $y = 8.88\times + 0.0401(R^2 = 0.9996)$.

2.4. Single factor experiment on solid state fermentation conditions of E. cristatum

Effect of fermentation time on polyphenol content. 10.00 g soybeans were soaked in 25 mL pH 6 solution in a conical bottle overnight, and excess water was poured off. After sterilization at 121 °C for 20 min, the samples were placed on a sterile operating table and cooled to room temperature. Each sample was added with 10% seed solution (v/m) and fermented in an incubator at 30 °C and 60% humidity for 2, 4, 6, 8, 10, 12, 14 and 16 d, respectively.

Effect of inoculation amount on polyphenol content. 10.00 g soybeans were soaked in 25 mL pH 6 solution in a conical bottle overnight, and excess water was poured off. The samples were sterilized at 121 °C for 20 min, placed on a sterile operating table, and cooled to room temperature. Each sample was added with 5%, 10%, 15%, 20% and 25% seed liquid (v/m), respectively, and fermented in an incubator at 30 °C and 60% humidity for 8 d.

Effect of initial pH on polyphenol content. The pH of distilled water was adjusted by hydrochloric acid and sodium hydroxide solution to obtain pH 2, 4, 6, 8, 10 solutions. 10.00 g is soaked in 25 mL of different pH solution overnight, then excess water was poured away. After sterilization at 121 °C for 20 min, the samples were placed on a sterile operating table and cooled to room temperature. Each sample was added with 10% seed solution (v/m) and fermented in an incubator at 30 °C

Table 3
The relative content of the top 8 flavonoids in the FS-FP and UF-FP sample.

Sample	Compounds	Relative amount (10^6)	Sample	Compounds	Relative amount (10^6)
FS-FP	6-Hydroxydaidzein	59.88 ± 0.62	UF-FP	Sophoricoside	161.21 ± 12.4
	Luteolin-4'-O-glucoside	39.07 ± 5.48		Genistein-7-O-galactoside	161.21 ± 12.4
	Luteolin-3'-O-glucoside	34.61 ± 6.98		Apigenin-7-O-glucoside	157.28 ± 16.41
	Kaempferol-3-O-neohesperidoside	44.95 ± 9.84		Galangin-7-O-glucoside	125.92 ± 20.98
	7,4'-Dihydroxyflavone	38.19 ± 0.52		Daidzin	95.88 ± 6.04
	6,7,8-Tetrahydroxy-5-methoxyflavone	38.07 ± 1.18		Apigenin-5-O-glucoside	106.58 ± 25.35
	Cynaroside	33.84 ± 0.05		Daidzein-7-O-Glucoside-4'-O-Apioside	86.87 ± 1.04
	Lonicerin	34.80 ± 1.44		Daidzein-7-O-apiosyl(1 → 6)glucoside	85.39 ± 7.85

and 60% humidity for 8 d.

Fermented soybeans were dried in the oven at 45 °C to constant weight, and the fermented soybeans (FS) are crushed into powder for backup. The unfermented soybeans with the same treatment as the experimental group are fermented as the control group, named unfermented soybeans (US). fermented soybean free polyphenol (FS-FP), fermented soybean free polyphenol (US-FP), fermented soybean bound polyphenol (FS-BP) and unfermented soybean bound polyphenol (US-BP) were fermented.

2.5. Orthogonal optimization experiment

The optimal fermentation conditions were selected, and the L9 (3^3) orthogonal experiment was carried out according to orthogonal Table 1. The fermentation time (A), inoculation amount (B) and initial pH (C) were adjusted for the experiment, and the free polyphenol content was selected as the index to optimize the experiment.

2.6. Analysis of free polyphenols by UPLC-MS/MS

Biological samples are freeze-dried by vacuum freeze-dryer (Scientz-100F). The freeze-dried sample was crushed using a mixer mill (MM 400, Retsch) with a zirconia bead for 1.5 min at 30 Hz. Dissolve 50 mg of lyophilized powder with 1.2 mL 70% methanol solution, vortex 30 s every 30 min for 6 times in total. Following centrifugation at 12000*g* for 3 min, the extracts were filtrated (SCAA-104, 0.22 μm pore size; ANPEL, Shanghai, China) before UPLC-MS/MS analysis.

The detection method and data comparison results refer to the method of Chen et al., Fraga et al. (Chen et al., 2013; Fraga, Clowers, Moore, & Zink, 2010).

2.7. Soybean morphological change

The morphology of soybean before and after fermentation was observed. The soybean was crushed before and after fermentation and observed under electron microscope at different multiples.

2.8. Determination of acetylcholinesterase activity

At present, acetylcholinesterase is an attractive target for the treatment of Alzheimer's disease, and acetylcholinesterase inhibitors are the main approved drugs for the treatment of this neurodegenerative disease (Lee, Lee, & Ju, 2019).

In 96-well plates, 20 μL polyphenol samples and 120 μL 0.1 mol/L phosphate buffer (pH 8.0) were added, and 15 μL 0.5 U/mL acetylcholinesterase (phosphate buffer dissolved) was mixed. After the reaction at 25 °C for 10 min, 10 μL of 5 '5-dithiobibis (2-nitrobenzoic acid) (DTBN) at 10 mmol/L and 10 μL of 7.5 mmol/L acetylthiocholine iodide, were added and mixed evenly. The absorbance was measured at 405 nm after 30 min reaction at 37 °C. The calculation formula is as follows:

$$\text{Inhibition rate } (\%) == \left(1 - \frac{A_1 - A_2}{A_0}\right) \times 100$$

where A_1: the absorbance of the sample group; A_2: the absorbance of the sample control group, buffer solution was used instead of enzyme solution; A_0: refers to the absorbance of the blank group and replaces the sample with buffer solution.

2.9. Data analysis

All the experiments were used out in triplicate and the data were presented as the mean value standard deviation and treated with one-way analysis of variance (ANOVA). Significant differences ($p < 0.05$) between the means were determined using the Voeller-Duncan 's multiple range test which conducted by SPSS version 26 (SPSS Inc., Chicago, USA). Origin 2019b statistical software (OriginLab Co., USA) was carried out to prepare the data graphs. Orthogonal software assistant is used for orthogonal optimization analysis.

The types and relative contents of phenols in the samples were analyzed by using the compounds in the database, and two parallel experiments were conducted. The quantification of the compound is based on the MRM model to screen the precursor ion (Q1) of the target substance and remove the corresponding ion to remove interference. The chromatographic peaks were integrated and corrected with Multia Quant 3.0.2 edition, and the relative content of corresponding substances was calculated according to the peak area. Rstudio software was used to draw a heatmap of phenols.

3. Experimental results

3.1. Effect of fermentation time on polyphenol content

Fig. 1 shows the effects of different fermentation time on the contents of free polyphenols and bound polyphenols in soybeans. Fig. 1A shows

Table 4
The relative content of the top 8 phenolic acids in the FS-FP and UF-FP sample.

Sample	Compounds	Relative amount(10^6)	Sample	Compounds	Relative amount(10^6)
FS-FP	Dimethyl phthalate	59.88 ± 0.62	UF-FP	6-O-Caffeoylarbutin	161.21 ± 12.4
	Syringic acid	39.07 ± 5.48		5′-Glucosyloxyjasmanic acid	161.21 ± 12.4
	Benzoylmalic acid	34.61 ± 6.98		Syringic acid	157.28 ± 16.41
	Ferulic acid	44.95 ± 9.84		Hydrangeifolin I	125.92 ± 20.98
	Vanillic acid	38.19 ± 0.52		Dimethyl phthalate	95.88 ± 6.04
	2-(Formylamino)benzoic acid	38.07 ± 1.18		4-Nitrophenol	106.58 ± 25.35
	3,4-Dimethoxyphenyl acetic acid	33.84 ± 0.05		Ferulic acid	86.87 ± 1.04
	4-Nitrophenol	34.80 ± 1.44		Anthranilate-1-O-Sophoroside	85.39 ± 7.85

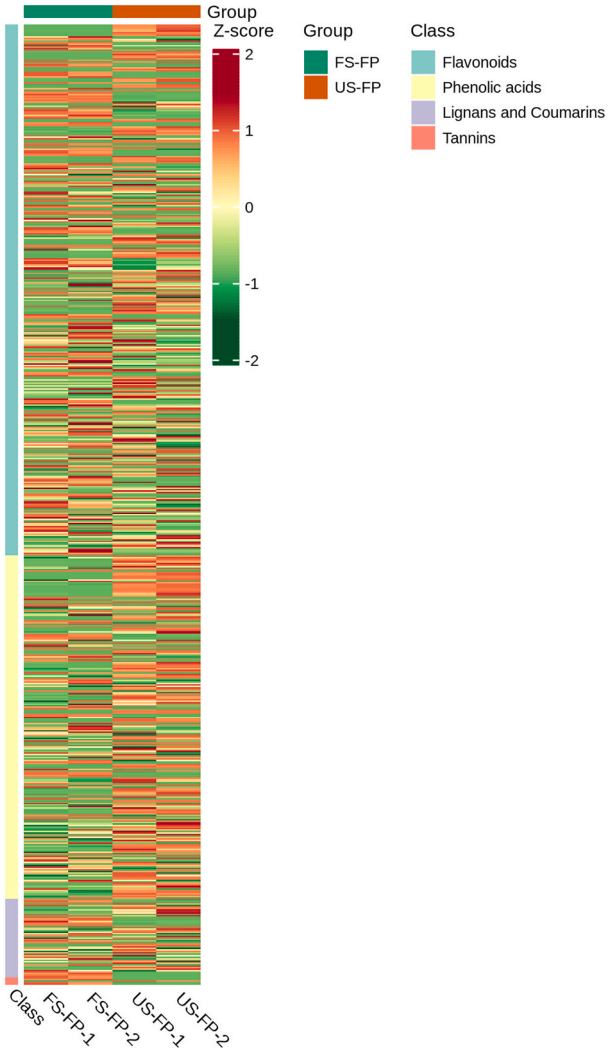

Fig. 5. Cluster heat map analysis of two polyphenol samples.
FS-FP: fermented soybean free polyphenol; US-FP: unfermented soybean free polyphenol.

the changes of free polyphenols and bound polyphenols in soybeans with the increase of fermentation time, and Fig. 1B shows the changes of the proportion of total phenols and two different forms of polyphenols in soybeans with the increase of fermentation time.The results are depicted in Fig. 1, with the increase of fermentation time, the free polyphenol content and total phenol content in soybean showed a trend of first increasing and then decreasing. The contents of free polyphenols, bound polyphenols and total phenols in unfermented soybeans were 0.56 mg GAE/g d.w, 0.26 mg GAE/g d.w and 0.82 mg GAE/g d.w, respectively. After 12 days of fermentation, the content of free polyphenols in soybean reached 1.79 mg GAE/g d.w, which was 3.20 times that of unfermented soybean, and the content of free polyphenols was significantly increased. After 16 days of fermentation, the content of bound polyphenols reached 0.85 mg GAE/g d.w, which was 3.30 times that of unfermented soybean. After 12 days of fermentation, the total phenol content reached 2.35 mg GAE/g d.w, which was 2.87 times that of unfermented soybean, and the free polyphenol content was relatively large. The above results indicated that fermentation could significantly increase the polyphenol content in soybean.

After 2 days of fermentation, the content of free polyphenols in soybean decreased slightly, while the content of bound polyphenols increased, which may be due to the fact that the growth of *E. cristatum* requires nutrients from soybean, and nutrients such as cellulose and

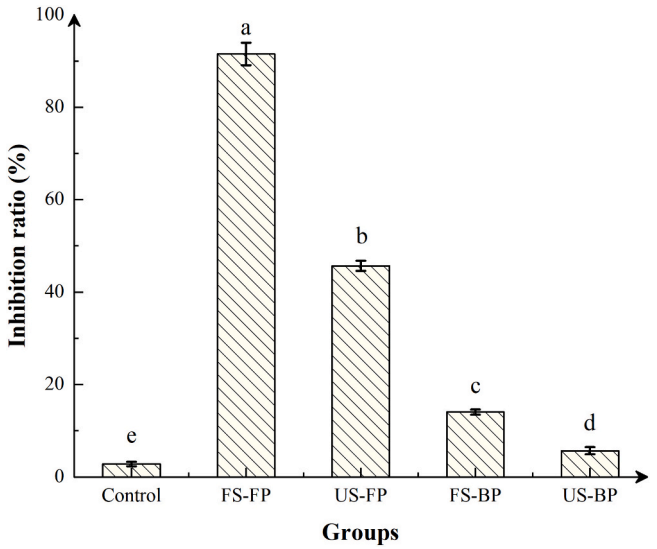

Fig. 6. Inhibition effect of four polyphenol samples on acetylcholinesterase.
Note: Values in the figure are mean ± standard deviation ($n = 3$), and different letters in the same column indicate statistical differences between components, ANOVA, $p < 0.05$.

protein combine with polyphenols (Dey, Chakraborty, Jain, Sharma, & Kuhad, 2016). The content of free polyphenols decreased slightly after 10 days of fermentation, which may be due to the fact that the bacteria reached a certain number at this time, the nutrient was insufficient, and the enzyme activity of the secreted hydrolase enzyme decreased, resulting in the reduction of the transformation and release of polyphenols.

Studies have shown that most phenolic compounds in legumes, grains and fruits bind to lignin, cellulose and polysaccharides in cell walls through ether, ester and glucoside bonds to form complex insoluble binding substances (Acosta-Estrada, Gutiérrez-Uribe, & Serna-Saldívar, 2014; Hur, Lee, Kim, Choi, & Kim, 2014). In the process of fermentation, *E. cristatum* produces hydrolase enzyme such as α-amylase, β-glucosidase, cellulase and protease. Under the action of hydrolase enzyme, the covalent bonds between binding phenols and proteins, starch, amines and other substances are hydrolyzed, resulting in free phenolic substances (Zou et al., 2019). At the same time, with the proliferation of *E. cristatum* and the secretion of hydrolase enzyme enzyme, the hydrolase enzyme enzyme can hydrolyze the protein, cellulose or starch in soybean into small molecular peptides, amino acids and oligosaccharides as energy, so that *E. cristatum* can grow well in soybean, thus improving the biosynthesis of phenolic compounds (Chen et al., 2020; Chen et al., 2020) and changing the content of free and bound phenols in soybean. Thus, the solid-state bioprocessed soybeans using *E. cristatum* enhances polyphenols, which may be applied in health care products and functional foods.

3.2. *Effect of inoculation amount on polyphenol content*

Fig. 2 shows the effects of different amount of inoculation on the contents of free polyphenols and bound polyphenols in fermented soybeans. Fig. 2A shows the changes of free polyphenols and bound polyphenols in soybeans with the increase of inoculation amount, and Fig. 2B shows the changes of the proportion of total phenols and two different forms of polyphenols in soybeans with the increase of inoculation amount.

As can be seen from the results in the Fig. 2, the free polyphenol content in soybean was 1.69 mg GAE/g d.w, which was 3.02 times that of unfermented soybean when the bacterial dosage was 10%. The content of bound polyphenols in soybean was 0.64 mg GAE/g d.w, which

was 2.46 times that of unfermented soybean, and the total phenol content was 2.30 mg GAE/g d.w, which was 2.80 times that of unfermented soybean. The content of polyphenols in soybean was significantly changed with the change of the amount of *E. cristatum.* With the increase of the amount of *E. cristatum*, the activity of hydrolase enzyme was enhanced, and the polyphenols in soybean were easily dissociated. Cellulase produced by filamentous fungi breaks down plant cell walls, leading to the release of phenolic compounds.(Dey et al., 2016;) Moreover, protease can effectively transform bound polyphenols in protein into free phenols. Therefore, proteases may be one of the contributors to the enhancement of phenols during fermentation(Chen, Liu, et al., 2020; Chen, Wang, et al., 2020). At the same time, due to the growth of *E. cristatum*, the bacterial base increases, and the consumption of nutrients increases, which affects the biosynthesis of phenolic compounds.

3.3. Effect of initial pH on polyphenol content

The effects of different initial pH on the contents of free polyphenols and bound polyphenols in fermented soybeans, as Fig. 3 shows. Fig. 3A shows the changes in the contents of free polyphenols and bound polyphenols in fermented soybeans with the change of initial pH, and Fig. 3B shows the changes in the proportion of total phenols and two different forms of polyphenols in fermented soybeans with the increase of initial pH.

As can be seen from the Fig. 3, when the pH of the solution is 6, the free polyphenol content in fermented soybeans can reach 1.69 mg GAE/g d.w, the bound polyphenol content is 0.51 mg GAE/g d.w, and the total phenol content is 2.20 mg GAE/g d.w. They were 3.02, 1.96 and 2.68 times that of unfermented soybean. Studies have shown that the activity of *E. cristatum* is higher in acidic environment and it secretes more types of hydrolase enzyme, while the activity of *E. cristatum* is lower in alkaline condition, which affects the activity of hydrolase enzyme and thus the content of polyphenols (Jiang et al., 2022). In addition, different initial pH may also have certain effects on the structure and content of phenolic substances in fermented soybeans (Gulsunoglu-Konuskan, Karbancıoğlu-Güler, & Kılıç-Akyılmaz, 2021).

3.4. Experimental results of orthogonal optimization

According to the results of the previous three single factor experiments, the content of free polyphenols in soybeans after fermentation by *E. cristatum* accounted for a relatively large proportion, and was greatly affected by single factor conditions, and there were significant differences in the data within the groups. Therefore, in the orthogonal optimization experiment, the suitable fermentation conditions were selected, and the free polyphenol content was taken as the detection index according to the orthogonal Table 1, and the optimization experiment results were shown in Table 2.

The optimized range $R_A > R_C > R_B$ is obtained from Table 2, fermentation time (A), was the most influential factor on free polyphenols, followed by initial pH (C), followed by inoculation amount (B). According to K value (mean value), the optimal condition of soybean fermentation was determined as $A_3B_2C_1$, which was consistent with the data obtained by orthogonal optimization experiment. Therefore, the optimal experimental conditions for soybean fermentation were fermentation time 12 days, bacterial dosage 15% and initial pH 2. Under these conditions, the content of free polyphenols in fermented soybean was 2.79 mg GAE/ G.D·W, which was 4.98 times that of unfermented soybean. The contents of bound polyphenols and total phenols in fermented soybean were 0.62 mg GAE/g d.w and 3.41 mg GAE/g d.w, respectively, which were 2.38 times and 4.16 times of those in unfermented soybean. The above experimental results showed that the polyphenol content in soybean could be significantly increased by the fermentation of *E. cristatum*.

3.5. Soybean morphology was observed by scanning electron microscopy

Scanning electron microscopy was used to observe the microscopic morphology of soybeans before and after fermentation and the attachment of *E. cristatum*. The experimental results are shown in Fig. 4. As can be seen in Fig. 4A, large pores appeared on soybeans after fermentation by *E. cristatum*. This phenomenon may be caused by the action of hydrolase enzyme secreted by *E. cristatum* on soybeans and the penetration of mycelium through soybean matrix. Fig. 4a, b, and c are electron microscopic images of unfermented soybeans at different magnifications. Pores on soybeans are small or non-porous, which may be related to mechanical failure. Fig. 4B and C can obviously observe the adhesion of *E. cristatum*. A large number of *E. cristatum* attached to soybean released a variety of hydrolase to hydrolyze cellulose, polysaccharide, protein and other macromolecular substances in soybean, further affecting the change of polyphenol content (Gebru & Sbhatu, 2020).

3.6. UPLC-MS/MS analysis

According to the analysis of UPLC-MS/MS, as shown in Table 3 and Table 4, the free polyphenol in fermented soybean was mainly 6-Hydroxydaidzein, and its content increased significantly, which was similar to the results of Chen *et al* (Chen, Liu, et al., 2020; Chen, Wang, et al., 2020). Meanwhile, as shown in Fig. 5, the cluster heat map analysis of the two polyphenol samples showed significant differences in the polyphenol components of FS-FP and US-FP. Among them, flavonoids, tannins, lignans and coumarins in FS-FP had relatively high abundance, while the abundance of phenolic acids was lower than that of unfermented soybeans. This may be due to the combination of phenolic acids with cellulose, lignin, proteins, polysaccharides and other macromolecular substances in plants or conversion to other aromatic substances (Adaškevičiūtė, Kaškonienė, Barčauskaitė, Kaškonas, & Maruška, 2022; Chen, Wang, Yang, Wang, & Xu, 2023; Ma et al., 2022). As the main components of fermented soybean, flavonoids have potential research significance.

3.7. Acetylcholinesterase inhibition effect

The inhibitory effects of four soybean polyphenols on acetylcholinesterase are shown in Fig. 6. The inhibitory rate of FS-FP (91.51 ± 0.43%) on acetylcholinesterase was higher than that of US-FP (45.69 ± 1.08%), and that of FS-BP (14.34 ± 0.36%) on acetylcholinesterase was higher than that of US-BP (4.67 ± 0.78%). The inhibitory effect of soybean polyphenols on acetylcholinesterase will be enhanced.

Other relevant studies have shown that the inhibitory effect of Perilla leaves on acetylcholinesterase was enhanced after fermentation with six kinds of probiotics, which may be mainly related to the changes of rutin, rorosemary acid, luteolin and flavonoids in the fermentation process (Wang et al., 2022). According to the results, the content, structure and types of polyphenols in the fermented soybean were changed. There was a significant increase in 6-Hydroxydaidzein, which may be the main reason for the decrease of acetylcholinesterase activity.

4. Conclusion

The experimental results show that when the fermentation conditions of soybean were fermentation time 12 days, inoculation amount 15% and initial pH 2, the content of free polyphenols in fermented soybean was 2.79 mg GAE/g d.w, which was 4.98 times that of unfermented soybean. The contents of bound polyphenols and total phenols in fermented soybean were 0.62 mg GAE/g d.w and 3.41 mg GAE/g d.w, respectively, which were 2.38 times and 4.16 times of those in unfermented soybean. At the same time, the inhibitory effect of free polyphenols in fermented soybean on acetylcholinesterase reached 91.51%. The contents and types of polyphenols in soybean were significantly increased by the fermentation of *E.crustatum*. By secreting a variety of

hydrolase enzyme, the *E.crustatum* can metabolize and transform the bioactive ingredients in soybean and increase the abundance of various active ingredients. At the same time, after fermentation, the inhibition effect of free polyphenols on acetylcholinesterase was significantly improved compared with that without fermentation. This depends on the characteristics of the metabolites formed during the fermentation process and the type of conversion products that form polyphenol compounds, especially flavonoids. The results of this study are expected to be used in the production of new functional food or health care products. Meanwhile, further studies are needed to evaluate the safety of *E.crustatum* bioprocessed soybeans and their advantages in functional food development.

CRediT authorship contribution statement

Shuo-shuo Shi: Writing – review & editing, Writing – original draft, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Ting Hu:** Validation, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

References

Acosta-Estrada, B. A., Gutiérrez-Uribe, J. A., & Serna-Saldívar, S. O. (2014). Bound phenolics in foods, a review. *Food Chemistry, 152*, 46–55. https://doi.org/10.1016/j.foodchem.2013.11.093

Adaškevičiūtė, V., Kaškonienė, V., Barčauskaitė, K., Kaškonas, P., & Maruška, A. (2022). The impact of fermentation on bee pollen polyphenolic compounds composition. *Antioxidants, 11*(4), 645. https://doi.org/10.3390/antiox11040645

Anderson, R. L., & Wolf, W. J. (1995). Compositional changes in trypsin-inhibitors, phytic acid, saponins and isoflavones related to soybean processing. *Journal of Nutrition, 125*(3), 581S–588S. https://doi.org/10.1093/jn/125.3_suppl.581

Bei, Q., Liu, Y., Wang, L., Chen, G., & Wu, Z. Q. (2017). Improving free, conjugated, and bound phenolic fractions in fermented oats (*Avena sativa* L.) with *Monascus anka* and their antioxidant activity. *Journal of Functional Foods, 32*, 185–194. https://doi.org/10.1016/j.jff.2017.02.028

Chen, G., Liu, Y., Zeng, J. R., Tian, X. F., Bei, Q., & Wu, Z. Q. (2020). Enhancing three phenolic fractions of oats (*Avena sativa* L.) and their antioxidant activities by solid-state fermentation with *Monascus anka* and *Bacillus subtilis*. *Journal of Cereal Science, 93*, Article 102940. https://doi.org/10.1016/j.jcs.2020.102940

Chen, L., Wang, H. J., Yang, Y., Wang, Y. F., & Xu, P. (2023). Structural insight into polyphenol oxidation during black tea fermentation. *Food Chemistry: X, 17*, Article 100615. https://doi.org/10.1016/j.fochx.2023.100615

Chen, W., Gong, L., Guo, Z. L., Wang, W. S., Zhang, H. Y., Liu, X. Q., … Xiong, L. Z. (2013). A novel integrated method for large-scale detection, identification, and quantification of widely targeted metabolites: Application in the study of Rice metabolomics. *Molecular Plant, 6*(6), 1769–1780. https://doi.org/10.1093/mp/sst080

Chen, Y. L., Wang, Y. L., Chen, J. X., Tang, H., Wang, C. H., Li, Z. J., & Xiao, Y. (2020). Bioprocessing of soybeans (*Glycine max* L.) by solid-state fermentation with *Eurotium cristatum* YL-1 improves total phenolic content, isoflavone aglycones, and antioxidant activity. *RSC Advances, 10*, Article 16928. https://doi.org/10.1039/c9ra10344a

Claudia, L. E., Elizabeth, C. M., & Rene, B. Q.. (2018). Pectin and pectin-based composite materials: Beyond food texture. *Molecules, 23*(4), 942. https://doi.org/10.3390/molecules23040942

Dey, T. B., Chakraborty, S., Jain, K. K., Sharma, A., & Kuhad, R. C. (2016). Antioxidant phenolics and their microbial production by submerged and solid state fermentation process: A review. *Trends in Food Science and Technology, 53*, 60–74. https://doi.org/10.1016/j.tifs.2016.04.007

Du, F. Y., Li, X., Li, X. M., Zhu, L. W., & Wang, B. G. (2017). Indolediketopiperazine alkaloids from *Eurotium cristatum* EN-220, an endophytic fungus isolated from the marine alga Sargassum thunbergii. *Marine Drugs, 15*(2), 24. https://doi.org/10.3390/md15020024

Du, H. P., Wang, Q., & Yang, X. B. (2019). Fu brick tea alleviates chronic kidney disease of rats with high fat diet consumption through attenuating insulin resistance in skeletal muscle. *Journal of Agricultural and Food Chemistry, 67*(10), 2839–2847. https://doi.org/10.1021/acs.jafc.8b06927

Fraga, C. G., Clowers, B. H., Moore, R. J., & Zink, E. M. (2010). Signature-discovery approach for sample matching of a nerve-agent precursor using liquid chromatography-mass spectrometry, XCMS, and chemometrics. *Analytical Chemistry, 82*(10), 4165–4173. https://doi.org/10.1021/ac1003568

Gebru, Y. A., & Sbhatu, D. B. (2020). Effects of Fungi-mediated solid-state fermentation on phenolic contents and antioxidant activity of Brown and White Teff (*Eragrostis tef* (Zucc.) trotter) grains. *Journal of Food Quality, 2020*, Article 8819555. https://doi.org/10.1155/2020/8819555

Gulsunoglu-Konuskan, Z., Karbancıoğlu-Güler, F., & Kılıç-Akyılmaz, M. (2021). Development of a bioprocess for production of ellagic acid from chestnut (*Castanea sativa* mill.) waste by fermentation with *aspergillus spp*. *Food Bioscience, 42*, Article 101058. https://doi.org/10.1016/j.fbio.2021.101058

Hur, S. J., Lee, S. Y., Kim, Y. C., Choi, I., & Kim, G. B. (2014). Effect of fermentation on the antioxidant activity in plant-based foods. *Food Chemistry, 160*, 346–356. https://doi.org/10.1016/j.foodchem.2014.03.112

Jayachandran, M., & Xu, B. J. (2019). An insight into the health benefts of fermented soy products. *Food Chemistry, 271*, 362–371. https://doi.org/10.1016/j.foodchem.2018.07.158

Jiang, L. Z., Wang, L., Yang, T., Zheng, H. N., Zhao, J. L., Xiang, D. B., … Wan, Y. (2022). Research progress on *Eurotium cristatum* and its fermentation application. *Science and Technology of Food Industry, 43*(4), 454–462. https://doi.org/10.13386/j.issn1002-0306.2021030124

Kang, D. D., Su, M., Duan, Y. W., & Huang, Y. (2019). *Eurotium cristatum*, a potential probiotic fungus from Fuzhuan brick tea, alleviated obesity in mice by modulating gut microbiota. *Food & Function, 10*(8), 5032–5045. https://doi.org/10.1039/c9fo00604

Kim, I. S., Kim, C. H., & Yang, W. S. (2021). Physiologically active molecules and functional properties of soybeans in human health-a current perspective. *International Journal of Molecular Sciences, 22*, 4054. https://doi.org/10.3390/ijms22084054

Lee, Y., Lee, J., & Ju, J. (2019). *Perilla frutescens* Britton Var. *Frutescens* leaves attenuate dextran sulfate sodium-induced acute colitis in mice and lipopolysaccharide-stimulated angiogenic processes in human umbilical vein endothelial cells. *Food Science and Biotechnology, 29*, 131–140. https://doi.org/10.1007/s10068-019-00711-8

Leite, P., Salgado, J. M., Venâncio, A., Domínguez, J. M., & Belo, I. (2016). Ultrasounds pretreatment of olive pomace to improve xylanase and cellulase production by solid-state fermentation. *Bioresource Technology, 214*, 737–746. https://doi.org/10.1016/j.biortech.2016.05.028

Leite, P., Silva, C., Salgado, J. M., & Belo, I. (2019). Simultaneous production of lignocellulolytic enzymes and extraction of antioxidant compounds by solid-state fermentation of agro-industrial wastes. *Industrial Crops and Products, 137*, 315–322. https://doi.org/10.1016/j.indcrop.2019.04.044

Licandro, H., Ho, P. H., Nguyen, T. K. C., Petchkongkaew, A., Nguyen, H. V., Chu-Ky, S., … Waché, Y. (2020). How fermentation by *lactic acid bacteria* can address safety issues in legumes food products? *Food Control, 110*, Article 106957. https://doi.org/10.1016/j.foodcont.2019.106957

Lu, X. J., Jing, Y., Li, Y. Y., Zhang, N. S., & Cao, Y. J. (2022). *Eurotium cristatum* produced β-hydroxy acid metabolite of monacolin K and improved bioactive compound contents as well as functional properties in fermented wheat bran. *Lebensmittel-Wissenschaft & Technologie, 158*, Article 113088. https://doi.org/10.1016/j.lwt.2022.113088

Ma, Y., Jiang, B., Liu, K., Li, R. Y., Chen, L. J., Liu, Z. W., … Zhao, M. (2022). Multi-omics analysis of the metabolism of phenolic compounds in tea leaves by *aspergillus luchuensis* during fermentation of pu-erh tea. *Food Research International, 162*, Article 111981. https://doi.org/10.1016/j.foodres.2022.111981

Nguyen, K. A., Wikee, S., & Lumyong, S. (2018). Brief review: Lignocellulolytic enzymes from polypores for efficient utilization of biomass. *Mycosphere, 9*(6), 1073–1088. https://doi.org/10.5943/mycosphere/9/6/2

Ramdath, D. D., Padhi, E. M., Sarfaraz, S., Renwick, S., & Duncan, A. (2017). Beyond the cholesterol-lowering effect of soy protein: A review of the effects of dietary soy and its constituents on risk factors for cardiovascular disease. *Nutrients, 9*(4), 324. https://doi.org/10.3390/nu9040324

Singh, R. S., Chauhan, K., Kaur, K., & Pandey, A. (2020). Statistical optimization of solid-state fermentation for the production of fungal inulinase from apple pomace. *Bioresource Technology Reports, 9*, Article 100364. https://doi.org/10.1016/j.biteb.2019.100364

Wang, Y. Q., Chen, P. Y., & Zhang, B. (2014). Quantitative trait loci analysis of soluble sugar contents in soybean. *Plant Breeding, 133*(4), 493–498. https://doi.org/10.1111/pbr.12178

Wang, Z. X., Jin, X. M., Zhang, X. C., Xie, X., Tu, Z. C., & He, X. (2022). From function to metabolome: Metabolomic analysis reveals the effect of probiotic fermentation on the chemical compositions and biological activities of *Perilla frutescens* leaves. *Frontiers in Nutrition, 9*, Article 933193. https://doi.org/10.3389/fnut.2022.933193

Xiao, Y., Huang, Y. X., Chen, Y. L., Fan, Z. Y., Chen, R. Y., He, C., … Wang, Y. L. (2021). Effects of solid-state fermentation with *Eurotium cristatum* YL-1 on the nutritional value, total phenolics, isoflflavones, antioxidant activity, and volatile organic compounds of black soybeans. *Agronomy, 11*(6), 1029. https://doi.org/10.3390/agronomy11061029

Xiao, Y., Li, M. Y., Wu, Y. P., Zhong, K., & Gao, H. (2020). Structural characteristics and hypolipidemic activity of theabrownins from dark tea fermented by single species *Eurotium cristatum* PW-1. *Biomolecules, 10*(2), 204. https://doi.org/10.3390/biom10020204

Yang, J., Wu, X. B., Chen, H. L., Sun-waterhouse, D. X., Zhong, H. B., & Cui, C. (2019). A value-added approach to improve the nutritional quality of soybean meal byproduct: Enhancing its antioxidant activity through fermentation by *bacillus amyloliquefaciens* SWJS22. *Food Chemistry, 272*, 396–403. https://doi.org/10.1016/j.foodchem.2018.08.037

Zhang, X. L., Shu, X. O., Gao, Y. T., Yang, G., Li, Q., Li, H. L., … Wang, Z. (2003). Soy food consumption is associated with lower risk of coronary heart disease in chinese women. *Journal of Nutrition, 133*(9), 2874–2878. https://doi.org/10.1093/jn/133.9.2874

Zou, M. M., Guo, X. H., Huang, Y., Cao, F. L., Su, E. Z., & Wang, J. H. (2019). Improvement of the quality of *Ginkgo biloba* leaves fermented by *Eurotium cristatum* as high value-added feed. *Processes, 7*(9), 627. https://doi.org/10.3390/pr7090627