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Comparative analysis of nutritional and functional properties of soybean meal, wheat germ, and their mixtures fermented by *Rhizopus oligosporus*

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ABSTRACT: The effect of solid-state fermentation by *Rhizopus oligosporus* RT-3 on the nutritional and functional properties of soybean meal (SBM), wheat germ (WG), and their mixtures (2SBM-1WG, 1SBM-1WG, and 1SBM-2WG) was investigated. During fermentation, the contents of protease, α-galactosidase activity, soluble protein, peptide (< 10 kDa), glucose, galactose, antioxidants, phenolic acids, and aglycones isoflavones increased in all samples. In contrast, anti-nutritional factors such as stachyose, raffinose, trypsin inhibitor activity, and glucoside isoflavones decreased significantly. The volatile compound analysis identified 53 compounds, including 12 alcohols, 7 aldehydes, 4 esters, 6 ketones, 15 acids, and 9 other compounds in the fermented and non-fermented samples, the addition of wheat germ improved the taste and flavor by increasing the ester content, especially ethyl decanoate, ethyl octanoate, and hexamethyl methyl, Meanwhile, fermentation decreased the 1-hexanol level from soybean meal. The fermented 1SBM-1WG blend showed the most favorable flavor and a well-balanced nutritional value. Metabolic profiling of fermented (1SBM-1WG) and non-fermented (NF-1SBM-1WG) samples revealed 1506 metabolic peaks. Among these, 20 differential metabolites were significantly affected by fermentation (P-value < 0.05; VIP = 1.165). This comprehensive analysis reveals that the 1SBM-1WG ratio represents the optimal substrate formulation for fermentation, providing a balanced nutritional profile and improved functional properties.

Keywords: Soybean meal, Wheat germ, Solid-state fermentation, *Rhizopus oligosporus,* Anti-nutritional factor

1. Introduction

Soybean meal and wheat germ are widely recognized for their high nutritional value and functional components, including plant-based proteins, dietary fiber, vitamins, and bioactive compounds. Soybean meal, a by-product of soybean milling for oil extraction, is rich in isoflavones, essential amino acids, and high-quality protein[1]. Wheat germ, a by-product of wheat milling, is an abundant source of vitamins E and B as well as polyunsaturated fatty acids[2]. However, both raw soybean meal and wheat germ have limitations that hinder their full nutritional potential. Unfermented soybean meal contains anti-nutritional factors such as trypsin inhibitors, stachyose, and raffinose, which impair protein digestibility and cause flatulence[3].

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Similarly, the high amount of fatty acids in wheat germ affects its shelf life and the bioavailability of nutrients, making it susceptible to oxidative rancidity[4]. Moreover, both ingredients in their raw form have poor sensory properties[5- 6].

Solid-state fermentation (SSF) is a widely used technique to improve the nutritional, functional, and sensory qualities of plant-based materials by promoting microbial activity under low-moisture conditions[7-8]. *Rhizopus oligosporus* (RT-3) has gained attention for use through SSF due to its strong enzymatic potential, effectively degrading anti-nutritional compounds, enhancing protein solubility, promoting the release of free phenolics, and increasing antioxidant activity, improving flavor and aroma, making it a suitable strain for enriching plant-based ingredients through SSF[9-10].

Blending SBM with WG offers complementary nutritional profiles, potentially enhancing the benefits of fermentation through synergistic interactions. Despite the individual health-promoting properties of these ingredients, research on the metabolic and nutritional transformations of their combinations during fermentation remains limited. In particular, little is known about how different blending ratios affect antioxidant capacity, enzyme activity, flavor development, and metabolite profiles.

Therefore, this study aims to evaluate the metabolic and nutritional characteristics of fermented SBM and WG blends in varying ratios using *Rhizopus oligosporus* (RT-3). Specifically, the research focuses on antioxidant activities (ABTS, DPPH, FRAP, reducing power), soluble protein and peptide content, enzyme activities (protease, α-galactosidase, trypsin inhibitor), soluble sugars, phenolic compounds, and volatile flavor compounds. In addition, a non-targeted metabolomic analysis was conducted exclusively on the 1SBM-1WG blend, selected for its balanced and complementary nutritional characteristics, to further explore the biochemical transformations induced by fermentation. The findings from this study may support the development of functional fermented foods with enhanced nutritional and sensory qualities and guide future optimization of fermentation strategies using mixed plant-based substrates.

2. Materials and methods

2.1. Chemicals

SBM and WG were sourced from Jiangsu Gangwan Agricultural Science and Technology Co., Ltd. (Yangzhou, China). The RT-3 strain, investigated for the fermentation of SBM and WG, was isolated from solid-state fermented foods and is deposited in the Food Microbiology Laboratory, Nanjing Agricultural University.

The following compounds were acquired from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA): 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), and 2,4,6-tris (2-pyridyl)-S-triazine (TPTZ), vanillic acid, daidzin, glycitin, genistin, daidzein, glycitein, genistein, quercetin, syringic acid, ferulic acid, along with phosphate buffer, potassium ferricyanide solution, trichloroacetic acid solution, ferrous chloride solution, and phenanthroline solution, lactic acid, acetic acid and methanol. All other chemicals and reagents utilized were of analytical grade.

2.2. Experimental design and sample collection

RT-3 was cultured on potato-dextrose agar at 32℃ for 72 h. Under sterile conditions, physiological saline was added, and spores were collected by scraping the plate and filtering through sterile cotton. The spore suspension was diluted and counted using a hemocytometer, yielding approximately 10^6 spores/mL. Fermentation followed the method of[11]. Briefly, SBM was mixed with water (1:2 ratio), steamed for 20 minutes, cooled, then supplemented with 1% lactic acid and 1.5% spore suspension (v/w). The mixture was placed in a fermentation vessel and sealed with perforated plastic wrap (~2 cm holes). WG followed the same procedure and was combined with SBM in 1SBM-1WG, 1SBM-2WG, and 2SBM-1WG ratios, as shown in Table 1. Fermentation was conducted at 32℃, with samples collected every 4 to 28 hours. Fermented samples were freeze-dried and ground for subsequent analysis.

Table 1. An overview of the different mixing ratios for SBM and WG

	SBM	WG
SBM	1	0
WG	0	1
1SBM-1WG	1	1
1SBM-2WG	1	2
2SBM-1WG	2	1

2.3. Determination of enzyme activities

The crude enzyme was extracted using the method provided by Huang et al.[12]. Briefly, samples were homogenized in 0.2 M phosphate buffer (pH 7.0) under constant agitation (60 min, 25℃), followed by centrifugation (4,000 × g, 10 min, 4℃). The supernatant was assayed for protease, α-galactosidase, and trypsin inhibitor activity using established protocols[13-14].

2.4. Soluble protein and peptide content

Soluble protein content was determined as Kruger described[15]. First, 1 g of freeze-dried sample was dissolved in 10 mL of distilled water, shaken for 1 hour, then centrifuged at 12,000 r/min and 4 ℃ for 20 minutes. 1 mL of the supernatant was mixed with 5 mL Coomassie Brilliant Blue G250 solution and left at room temperature for 5 minutes. Absorbance was measured at 595 nm. Distilled water served as the blank, and bovine serum albumin was used to create a standard curve for calculating soluble protein content (mg/g). The peptide content was measured according to a protocol reported by[16] with casein tryptone used as a standard.

2.5. Determination of soluble sugars

An Agilent 1100 HPLC system with a COSMOSIL Sugar-D column (Tosoh, Tokyo, Japan) was used to monitor soluble sugar changes during fermentation of SBM, WG, 1SBM-1WG, 1SBM-2WG, and 2SBM-1WG at various time points. Sugars were extracted from dried samples following Huang et al.[12] by using 70% ethanol, eluted with 75% aqueous acetonitrile at 0.8 mL/min, and detected by an evaporative light-scattering detector. Standard solutions of glucose, sucrose, raffinose, stachyose, and galactose (0.125–2 mg/mL) were used to identify and quantify sugars based on retention time and concentration.

2.6. Analysis of phenolics

Phenolic compounds were extracted using the method described by Yin et al.[17]. Briefly, 1g of sample powder was mixed with 80 mL of 80% methanol (v/v), incubated at 45℃ for 1 h, then centrifuged at 10,380×g for 15 min; residues were re-extracted twice. The combined supernatants were concentrated, defatted with hexane, and extracted three times with ethyl acetate. Ethyl acetate fractions were evaporated to dryness and reconstituted in 5 mL of 80% methanol to analyze soluble phenolic content.

Phenolic compounds were detected using a Waters 2695 HPLC system with a ZORBAX Eclipse Plus C18 column. Samples were filtered through a 0.22 μm syringe filter, and 10 μL was injected. Separation was done at 0.8 mL/min using a gradient elution of solvent A (0.4% acetic acid in water) and solvent B (acetonitrile). The gradient ranged from 5% to 50% B over 55 minutes. Detection was performed at 280 nm to identify phenolic compounds in the samples.

2.7. Total antioxidant activities

The extracts of fermented SBM, WG, and their mixtures were collected and assessed for antioxidant activity using four standard assays: $ABTS^{\bullet+}$ radical scavenging, ferric reducing antioxidant power (FRAP), reducing power (RP), and DPPH radical scavenging following a method provided by Xiao et al.[18]. Antioxidant capacity was evaluated across a concentration range of 0–20 mg/mL.

2.8. Analysis of volatile compounds

Volatile compounds were analyzed using GC–MS with headspace solid-phase microextraction (HS-SPME) as described by Dai et al.[19]. 2 g of sample was placed in a 20 mL SPME vial, and a 70 μm polydimethylsiloxane/divinylbenzene (PDMS/DVB) fiber (Supelco, Bellefonte, PA, USA) was inserted to extract volatiles at 60 ℃ for 30 minutes. Separation was performed using a ZB-WAX capillary column (30 m × 0.25 mm × 0.25 μm; Agilent) on a TSQ 8000 EVO GC-MS system (Thermo Fisher, USA). The oven temperature was held at 40 ℃ for 3 min, then increased to 200 ℃ at 5 ℃/min, and to 230 ℃ at 10 ℃/min (held for 3 min). Injector and interface temperatures were 250 ℃ and 280 ℃, respectively, with a splitless GC inlet. Helium was used as the carrier gas at 1.2 mL/min. Mass spectra were recorded at 70 eV (electron ionization), scanning from 35 to 350 amu at 4.5 scans/s. Compounds were identified using NIST 105 and Wiley 7.0 libraries, and relative concentrations were calculated by peak area normalization.

2.9. Sensory evaluation

The sensory evaluation was performed as described by Jeong et al. and Ng'ong'ola et al. [20-21]. Twenty panelists (10 males, 10 females, aged 25–35), who were regular consumers of fermented plant-based products, were selected for this study. The panelists were trained over two sessions, which involved familiarization with the sensory characteristics of the specific commercial fermented samples being tested (WG, SBM, 1SBM-1WG, 2SBM-1WG, and 1SBM-2WG). This training was designed to anchor the descriptive scales used for evaluation, ensuring panelists understood the scope of each scale and could use them consistently. Fermented samples were presented at a uniform thickness of 7 mm. Panelists evaluated flavor, texture, and overall acceptability using a 10-point descriptive scale, flavor (1 = extremely inadequate, 10 = extremely

adequate), texture (1 = extremely tough, 10 = extremely tender), tenderness (1 = extremely hard, 10 = extremely soft), and overall acceptability (1 = extremely unacceptable, 10 = extremely acceptable).

2.9. Metabolite extraction and derivatization

Sample extraction followed a modified protocol from Lin et al.[22]. Freeze-dried 1SBM-1WG and NF-1SBM-1WG (0.50 g) were homogenized in 10 mL of methanol: acetonitrile: water (2:2:1, v/v) with deuterated internal standards. After vertexing, bead homogenization, and sonication (three cycles), samples were incubated at -40 ℃ for protein precipitation, then centrifuged at 13,800 ×g for 15 min at 4 ℃. A 400 μL supernatant was vacuum filtered through a protein precipitation plate, and the filtrate was collected for analysis. A pooled QC sample was made from all supernatants.

Metabolomic profiling was conducted using LC-MS/MS (Thermo Vanquish UHPLC; Sciex X500R QTOF-MS). A 3 μL sample was injected into a Waters ACQUITY UPLC BEH Amide column (50 mm × 2.1 mm, 1.7 μm). Chromatography was performed at 40 ℃ with a 0.300 mL/min flow rate, using 0.1% formic acid (A) and acetonitrile (B) as mobile phases. The gradient ranged from 2% to 99% B over 20 minutes. Mass spectrometry employed electrospray ionization in both positive (+5.5 kV) and negative (−4.5 kV) modes. Data were acquired in IDA mode, capturing full-scan MS and MS/MS spectra over an m/z range of 50–1000.

Raw data were converted to mzXML via ProteoWizard and processed with an R-based pipeline using XCMS for peak detection, alignment, and integration. Features without confirmed annotations or with poor spectral similarity were removed. Compounds missing in >50% of control samples were excluded; k-nearest neighbors imputed those with ≤50% missing values.

2.10. Statistical analysis

All the experiments were measured in triplicate, and the results were expressed as mean ± standard deviation. Statistical analysis was performed using IBM SPSS (v.24) with one-way ANOVA and Duncan's test for significance ($P < 0.05$). Data visualization was performed using Origin Pro 2024 B software. The Analysis of volatile compounds data was conducted using MetaboAnalyst (v.6.0) (https://www.metaboanalyst.ca).

Metabolomics analysis was conducted using R software (v.3.6.2). Multivariate statistical analyses, including principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA), were performed using the ropls package. Score scatter plots were generated, and model validity was assessed via a 200-iteration permutation test. Variable importance in projection (VIP) scores were calculated to identify key metabolites contributing to group separation. Univariate analysis was performed using a custom Perl script, including Student's t-test and fold change (FC) calculation. Differential metabolites were screened based on P-value < 0.05 and VIP > 1. Metabolites with FC > 1 were defined as upregulated, and those with FC < 1 as downregulated. Volcano plots were constructed using the ggplot2 package in R. Identified differential metabolites were mapped to metabolic pathways using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (https://www.kegg.jp/kegg/pathway.html), to assess their biological relevance.

3. Results and discussion

3.1. Enzyme Activity

3.1.1. Protease enzyme

Protease activity was analyzed due to its critical role in protein hydrolysis during fermentation. As shown in Fig.1A, protease activity increased notably throughout the fermentation process. A time-dependent rise in protease activity was observed across all fermented samples. After fermentation, the highest protease activity was recorded in the 2SBM-1WG sample (94.06 U/g^{-1}), followed by SBM (91.70 U/g^{-1}), 1SBM-1WG (55.46 U/g^{-1}), 1SBM-2WG (49.64 U/g^{-1}), and WG (13.89 U/g^{-1}). These differences likely reflect the varying protein compositions and availabilities of the substrates. The higher protease activity observed in samples with greater proportions of soybean meal can be attributed to its higher protein content, which provides more substrate for enzymatic hydrolysis.

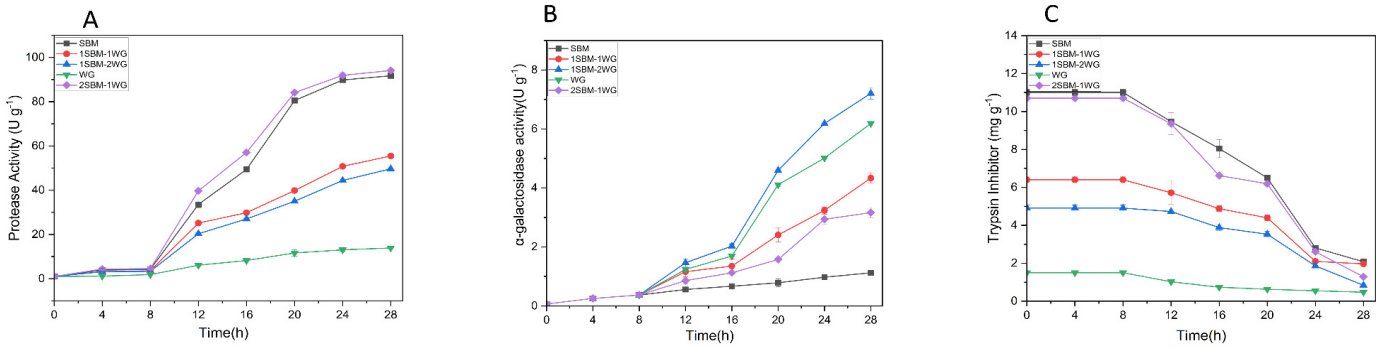

Figure 1. Enzyme activation in fermented samples of SBM, WG, 1SBM-1WG, 2SBM-1WG, and 1SBM-2WG. (A)Protease activity, (B)α-galactosidase activity, (C)Trypsin inhibitor activity, values are expressed as mean ± SD (n = 3). SBM, soybean meal; WG, wheat germ;1SBM-1WG,1soybean meal-1wheatgerm;2SBM-1WG,2soybean meal-1wheatgerm;1SBM-2WG,1soybean meal-2wheatgerm.

Several studies have reported that the use of high-protein substrates, particularly soybean meal, enhances microbial protease production. This is likely due to the increased availability of nitrogenous compounds that support enzyme synthesis and secretion. Soy protein meal has been shown to significantly improve extracellular protease output compared to other nitrogen sources[23]. Elevated protease activity is particularly beneficial, as it is linked to improved protein digestibility and a reduction in potential allergens, thereby enhancing the nutritional value and safety of the fermented products[24].

3.1.2. α-galactosidase activity

Initial α-galactosidase activity was low in all unfermented substrates but showed a gradual increase after 8 h. As shown in Fig. 1B, the fermented 1SBM-2WG exhibited the highest enzyme activity at 7.20 U/g^{-1}, followed by WG at 6.18 U/g^{-1}, 1SBM-1WG at 4.33 U/g^{-1}, 2SBM-1WG at 3.16 U/g^{-1}, and SBM at 1.12 U/g^{-1}. This enzyme activity variation highlights the substrate composition's influence on α-galactosidase production. Notably, while WG alone demonstrated relatively high activity, its combination with SBM (as in 1SBM-2WG) resulted in a significant increase compared to WG alone. This suggests a synergistic effect between SBM and WG, where the nutritional and biochemical properties of both substrates together stimulate enhanced enzyme production by RT-3.

α-galactosidase plays a key role in breaking down raffinose and stachyose oligosaccharides[25] that are not digested in the human small intestine. These compounds act as prebiotics, serving as substrates for beneficial gut microbiota and thus supporting probiotic growth and activity[27 ,26]. However, when present in excessive amounts, they can lead to intestinal flatulence due to fermentation by colonic bacteria[28]. Therefore, the increased α-galactosidase activity observed in fermented samples contributes to the reduction of these antinutritional factors, enhancing the digestibility, nutritional quality, and functional value of the final product[29].

3.1.3. Trypsin inhibitor activity

Trypsin inhibitors are well-established anti-nutritional factors known to impair protein and amino acid digestibility, thereby reducing overall protein quality[30]. Trypsin inhibitor activity serves as a key indicator of protein quality in foods and feeds, particularly those derived from soy products[31]. These inhibitors function by binding to trypsin, impairing its enzymatic activity and thereby hindering protein digestion[32]. As shown in Fig. 1C, trypsin inhibitor levels decreased significantly after fermentation. SBM had the highest initial level, 11.01 mg/g^{-1}, dropping to 2.079 mg/g^{-1} after fermentation. The 2SBM-1WG mixture decreased from 10.70 mg/g^{-1} to 1.289 mg/g^{-1}, 1SBM-1WG from 6.40 mg/g^{-1} to 1.97 mg/g^{-1}, 1SBM-2WG from 4.91 mg/g^{-1} to 0.84 mg/g^{-1}, and WG from 1.50 to 0.47 mg/g^{-1}. Fermented WG had the lowest residual trypsin inhibitor content, followed by the mixtures, then soybean meal. This reduction is likely due to proteolytic enzymes secreted by RT-3[11], which degrade trypsin inhibitors and improve nutritional value.

3.2. Soluble protein and peptide content

Soluble protein and peptide contents increased significantly in all samples during fermentation, with SBM consistently showing the highest levels, followed by 2SBM-1WG and 1SBM-1WG. Samples with higher proportions of wheat germ (1SBM-2WG and WG) exhibited the lowest increases, likely due to their lower initial protein content and weaker proteolytic activity. As shown in Table 2, before fermentation, the soluble protein and peptide contents were as follows: SBM (18.14 mg/g^{-1}and 99.02 mg/g^{-1}), 2SBM-1WG (17.76 mg/g^{-1} and 86.10 mg/g^{-1}), 1SBM-1WG (15.94 mg/g^{-1} and 61.05 mg/g^{-1}), 1SBM-2WG (9.12 mg/g^{-1} and 27.95 mg/g^{-1}), and WG (7.05 mg/g^{-1} and 9.25 mg/g^{-1}), respectively. After fermentation, all SBM-containing samples demonstrated notable increases. SBM reached 31.19 mg/g^{-1} soluble protein and 180.80 mg/g^{-1} peptides, followed by 2SBM-1WG (27.23 mg/g^{-1}, 142.83 mg/g^{-1}), 1SBM-1WG (24.38 mg/g^{-1}, 113.62 mg/g^{-1}), 1SBM-2WG (16.78 mg/g^{-1}, 51.90 mg/g^{-1}), and WG (14.25 mg/g^{-1}, 21.32 mg/g^{-1}). These results highlight SBM's superior proteolytic potential and its key role in enhancing soluble protein and peptide levels during fermentation.

Table 2. Changes in soluble protein and peptide content of SBM, WG, 2SBM-1WG, 1SBM-1WG, and 1SBM-2WG during fermentation with *Rhizopus oligosporus.*

Fermentation time(h)	0		4		8		12		16		20		24		28	
	Soluble protein content (mg/g^{-1})	Peptide content (mg/g-1)	Soluble protein content (mg/g-1)	Peptide content (mg/g-1)	Soluble protein content (mg/g-1)	Peptide content (mg/g-1)	Soluble protein content (mg/g-1)	Peptide content (mg/g-1)	Soluble protein content (mg/g-1)	Peptide content (mg/g-1)	Soluble protein content (mg/g-1)	Peptide content (mg/g-1)	Soluble protein content (mg/g-1)	Peptide content (mg/g-1)	Soluble protein content (mg/g-1)	Peptide content (mg/g-1)
SBM	18.14± 0.44^{f}	99.02± 1.12f	18.92± 0.22^{ef}	100.18± 0.11^{f}	19.57± 0.11^{ef}	103.23± 0.56^{e}	20.29± 0.22^{e}	147,76± 0.33^{d}	22.95± 2.02^{d}	148,34± 0.00^{d}	26.45± 0.00^{c}	165.67± 1.34^{c}	29.24± 1.12^{b}	170.61± 0.22^{b}	31.19± 1.12^{a}	180.80± 0.56^{a}
WG	7.05± 0.11^{f}	9.25± 0.44^{f}	8.41± 1.23^{e}	10.03± 0.22^{e}	10.88± 1.34^{d}	10.62± 0.11^{e}	11.65± 0.33^{cd}	14.70± 0.11^{d}	12.17± 0.11^{bc}	15.22± 0.22^{d}	12.95± 0.22^{b}	18.14± 0.11^{c}	13.34± 0.40^{ab}	19.83± 0.33^{b}	14.25± 0.11^{a}	21.32± 0.89^{a}
2SBM-1WG	17.76 ± 2.04^{d}	86.10± 1.79^{e}	18.40± 2.95^{d}	87.27± 1.1.91^{e}	19.31± 2.32^{d}	89.60± 1.12^{d}	20.48± 1.84^{cd}	116.67± 1.12^{c}	23.08± 1.07^{bc}	118.168± 1.68^{c}	25.80± 1.12^{ab}	138.35± 0.56^{b}	27.88± 1.23^{a}	141.14± 0.33^{a}	27.23± 1.36^{a}	142.83± 1.12^{a}
1SBM-1WG	15.94± 0.00^{e}	61.05± 1.46^{g}	16.33± 033^{e}	61.89± 1.75^{g}	16.52± 0.19^{e}	66.04± 1.29^{f}	17.63± 0.59^{d}	81.43± 1.67^{e}	18.27± 0.38^{d}	85.58± 1.23^{d}	22.10± 0.40^{c}	93.56± 0.29^{c}	23.60± 0.22^{b}	100.64± 1.94^{b}	24.38± 0.56^{a}	113.62± 1.21^{a}
1SBM-2WG	9.12± 0.00^{h}	27.95± 1.46^{g}	9.97± 0.44^{g}	31.51± 0.19^{f}	11.20± 0.11^{f}	33.14± 0.11^{e}	13.15± 0.22^{e}	35.86± 0.56^{d}	13.60± 0.19^{d}	37.03± 0.22^{d}	14.38± 0.33^{c}	40.54± 0.29^{c}	15.48± 0.29^{b}	45.08± 0.62^{b}	16.78± 0.11^{a}	51.90± 0.78^{a}

Each value was expressed as the mean ± standard deviation (n = 3). Within each row, means followed by different lowercase letters (a, b, c, d) indicate statistically significant differences ($P < 0.05$). SBM, soybean meal; WG, wheat germ;1SBM-1WG,1soybean meal-1wheatgerm;2SBM-1WG,2soybean meal-1wheatgerm;1SBM-2WG,1soybean meal-2wheatgerm.

The importance of blending SBM with WG lies in the ability of such combinations to produce distinct profiles of soluble proteins and bioactive peptides compared to the fermentation of individual substrates[33]. This highlights substrate composition's critical role in shaping fermented products' nutritional and functional properties. Although the 2SBM-1WG sample exhibited the highest protease activity (Fig. 1A), the highest soluble protein and peptide content was observed in SBM, followed by 2SBM-1WG, 1SBM-1WG, 1SBM-2WG, and WG. This pattern suggests that while protease activity is a major factor driving protein hydrolysis, the resulting soluble protein and peptide levels are also significantly influenced by the initial protein content of the substrate.

SBM, being inherently rich in protein, yielded more soluble protein and peptides after fermentation[34], although its protease activity was slightly lower than that of the 2SBM-1WG mixture(Fig.1A). In the 2SBM-1WG blend, the protein diversity of soybean meal and wheat germ provides strong protease induction by *Rhizopus oligosporus*, resulting in a slightly higher protease content than that of SBM. However, the lower protein content of WG may not support equivalent peptide and protein solubilization[35-36]. As a result, this blend exhibits high protease activity but does not achieve a proportional accumulation of soluble proteins and peptides higher than SBM. This observation suggests that enzyme activity alone is not sufficient to ensure maximum production of soluble nitrogenous compounds, as efficient recovery of peptides and soluble protein also depends on substrate availability. The moderate levels in 1SBM-1WG suggest a balanced synergistic effect, where SBM contributes protein and WG adds complementary nutrients or cofactors, enhancing enzymatic activity. In contrast, mixtures with higher proportions of WG, such as 1SBM-2WG, displayed lower soluble protein and peptides levels, likely due to the lower total protein content of WG and its reduced susceptibility to enzymatic degradation. Thus, the higher soluble protein and peptide content in SBM reflects a synergistic effect between substrate composition and enzyme activity, emphasizing their combined influence on the efficiency of protein solubilization during fermentation[37-38].

3.3. Soluble sugar

Changes in the raffinose, stachyose, glucose, and galactose in SBM, WG, 1SBM-1WG, 2SBM-1WG, and 1SBM-2WG during fermentation are shown in Fig. 2. The results showed a decrease in stachyose and raffinose content. As illustrated in Fig. 2A, WG, and 1SBM-2WG showed complete hydrolysis of stachyose after fermentation, while SBM showed the highest stachyose content compared to all samples. The initial content of stachyose in SBM, WG, 1SBM-1WG, 1SBM-2WG, 2SBM-1WG, were 10.60 mg/mL^{-1}, 1.09 mg/mL^{-1}, 5.80 mg/mL^{-1}, 1.65 mg/mL^{-1}, 7.30 mg/mL^{-1} respectively, and after fermentation decreased to 6.22 mg/mL^{-1} in SBM, 3.21 mg/mL^{-1} in 1SBM-1WG, 5.11 mg/mL^{-1} in 2SBM-1WG, while stachyose content in WG and 1SBM-2WG consume.

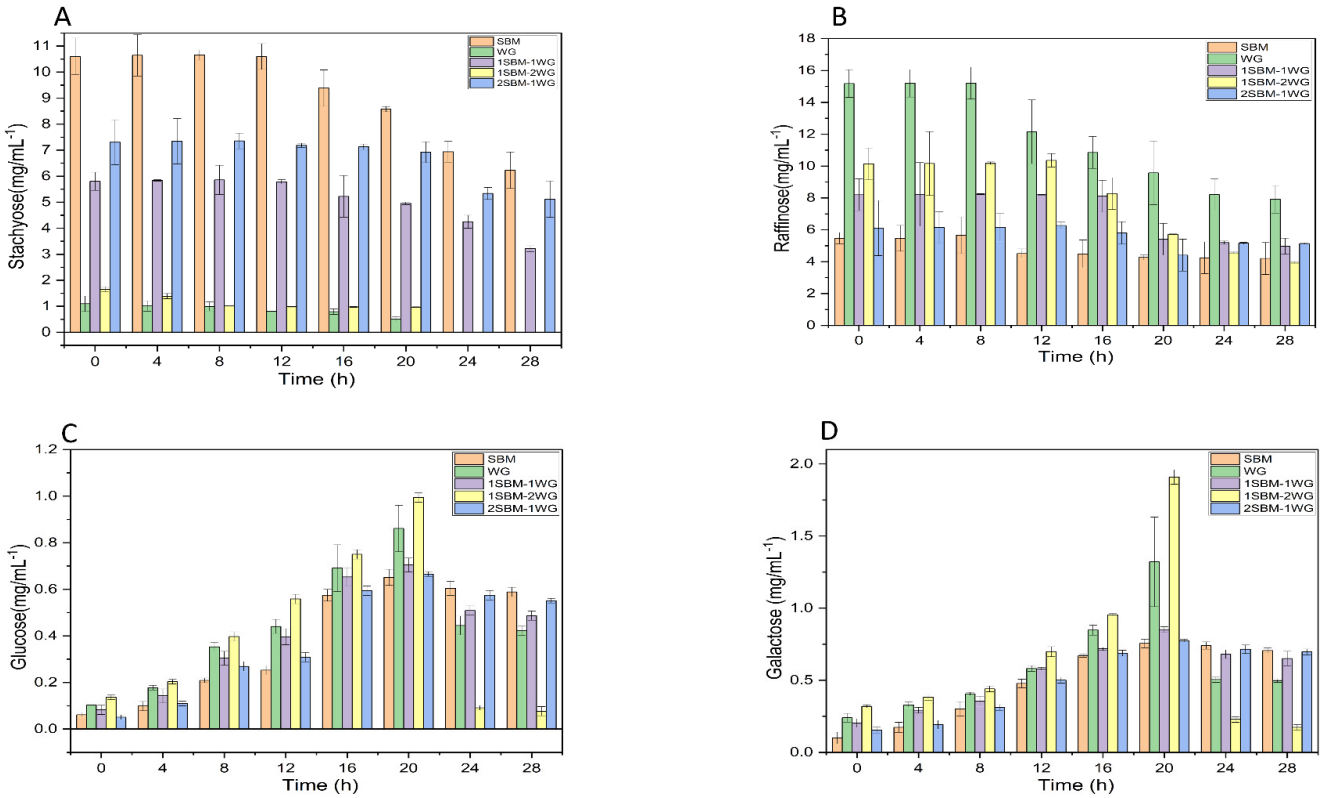

Figure 2. Changes in glucose, galactose, raffinose, and stachyose in SBM, WG, 1SBM-1WG, 2SBM-1WG, and 1SBM-2WG, during fermentation by *Rhizopus oligosporus*, values are expressed as mean ± SD (n = 3). SBM, soybean meal; WG, wheat germ;1SBM-1WG,1soybean meal-1wheatgerm;2SBM-1WG,2soybean meal-1wheatgerm;1SBM-2WG,1soybean meal-2wheatgerm.

As shown in Fig. 2B, the raffinose content decreased with increasing fermentation time. After fermentation, WG exhibited the highest raffinose content, while 1SBM-2WG had the lowest content. Raffinose levels were reduced from 5.50 to 4.20 mg/mL^{-1} in SBM, from 15.17 to 7.91 mg/mL^{-1} in WG, from 8.81 to 4.97 mg/mL^{-1} in 1SBM-1WG, from 10.13 to 3.98 mg/mL^{-1} in 1SBM-2WG, and from 6.11 to 5.14 mg/mL^{-1} in 2SBM-1WG, WG naturally high in raffinose content[39] which may be explained the highest amount of raffinose in wheat germ. For different samples, 1SBM-2WG showed the highest α-galactosidase enzyme activity, resulting in the most effective degradation of stachyose and raffinose. As a result, after fermentation levels of raffinose and stachyose were decreased, with raffinose concentrations reaching 3.98 mg/mL^{-1} (Fig. 2B) while stachyose was completely degraded (Fig. 2A). This effective degradation is attributed to the strong secretion of α-galactosidase by *Rhizopus oligosporus* RT-3 in the 1SBM-2WG ratio (Fig. 1B). This is because the high proportion of wheat germ, rich in raffinose and containing stachyose, provided an essential substrate. The complete degradation of stachyose and the persistence of enzymatic activity against raffinose led to a significant decrease in the residual levels observed.

A substantial amount of glucose and galactose was released during fermentation (Fig. 2C and D), with both sugars showed a first increase and then a decrease during fermentation. The increase is attributed to α-galactosidase activity, which hydrolyzed stachyose and raffinose, while the subsequent decrease likely resulted from sugar consumption by RT-3 for growth. As shown in (Fig. 2C), after fermentation, glucose reached 0.58 mg/mL^{-1} in SBM, 0.42 mg/mL^{-1} in WG, 0.48 mg/mL^{-1} in 1SBM-1WG, 0.07 mg/mL^{-1} in

1SBM-2WG, and 0.55 mg/mL^{-1} in 2SBM-1WG. Galactose levels reached 0.70 mg/mL^{-1} in SBM, 0.49 mg/mL^{-1} in WG, 0.65 mg/mL^{-1} in 1SBM-1WG, 0.17 mg/mL^{-1} in 1SBM-2WG, and 0.69 mg/mL^{-1} in 2SBM-1WG. Notably, 1SBM-2WG showed the lowest levels of both sugars, possibly due to their rapid utilization driven by the highest α-galactosidase activity (Fig. 1B).

Humans' digestive system lacks α-galactosidase, causing raffinose and stachyose to ferment in the intestines and produce gas[40]. Therefore, reducing these compounds is essential for improving the nutritional quality and digestibility of fermented soybean products. α-Galactosidase is widely recognized as the principal enzyme responsible for hydrolyzing raffinose and stachyose[41-42]. Complete degradation of these sugars often requires the cooperative action of both α-galactosidase and β-fructosidase[43]. While α-galactosidase cleaves terminal galactose residues from raffinose and stachyose, resulting in the production of galactose and sucrose units. Galactose can be utilized directly, and sucrose may be hydrolyzed by β-fructosidase to yield glucose and fructose. This explains the increasing production of glucose and galactose, and then consumption by microbes for growth[44].

Previous studies have similarly reported a reduction in raffinose and stachyose during the fermentation of soybean products, attributing this to enzymatic activity from fermenting microbes[45- 46]. The accumulation of glucose was probably attributed to the hydrolysis of raffinose and stachyose by the action of enzymes[29]. Consistent with these findings, the present results suggest extensive hydrolysis of raffinose and stachyose in all samples, especially in 1SBM-2WG, due to higher activation of α-galactosidase by RT-3, supporting the enzyme's critical role in enhancing the nutritional profile of fermented products.

3.4. Analysis of soluble phenolic compounds

As shown in Table 3, fermentation led to a significant reduction in the content of glucoside isoflavones, specifically glycitin, daidzin, and genistin, alongside an increase in their corresponding aglycones, of genistein, daidzein, and glycitein, in all samples except WG. SBM showed the highest aglycones content, while WG didn't show any glycogen content. After fermentation, SBM showed aglycones content, with 93.43 μg/g genistein, 87.49 μg/g daidzein, and 25.47 μg/g glycitein. Followed by 2SBM-1WG, which showed aglycones content, with 76.12 μg/g genistein, 54.82 μg/g daidzein, and 22.74 μg/g glycitein. Then 1SBM-1WG showed aglycones content, with 73.50 μg/g genistein, 48.22 μg/g daidzein, and 18.51 μg/g glycitein. While 1SBM-2WG showed aglycones content with 31.18 μg/g genistein, 47.62 μg/g daidzein, and 17.15 μg/g glycitein. Either there was no aglycone content detected in WG.

Table 3. Content of the identified phenolics in SBM, WG,1SBM-1WG,2SBM-1WG, and 1SBM-2WG during fermentation.

Phenolic compound (μg/g)		Fermentation time (h)							
		0	4	8	12	16	20	24	28
Genistein	SBM	24.20±3.63^{e}	37.26±8.81^{d}	40.36±2.60^{d}	54.60±4.35^{c}	60.08±5.08^{c}	69.73±3.08^{b}	93.20±3.46^{a}	93.43±0.96^{a}
	WG	ND	ND	ND	ND	ND	ND	ND	ND
	1SBM-1WG	14.26±6.42^{f}	17.33±4.16^{ef}	20.45±2.65^{ef}	24.43±4.38^{e}	34.04±1.97^{d}	41.38±5.34^{c}	57.04±4.36^{b}	73.50±2.64^{a}
	1SBM-2WG	10.19±3.61^{e}	12.66±4.93^{de}	17.16±6.08^{de}	19.46±1.05^{bc}	24.74±4.58^{ab}	29.44±3.55^{a}	29.73±1.17^{a}	31.18±3.05^{a}
	2SBM-1WG	18.65±3.06^{f}	22.75±3.48^{ef}	28.96±7.20^{de}	31.65±2.87^{d}	32.73±6.09^{d}	52.20±3.46^{c}	70.28±4.35^{b}	76.12±1.73^{a}
Genistin	SBM	229.66±3.99^{a}	217.04±2.65^{b}	211.21±5.98^{b}	194.82±7.81^{c}	176.56±4.40^{d}	169.43±6.01^{d}	149.81±2.98^{e}	147.62±3.01^{e}
	WG	ND	ND	ND	ND	ND	ND	ND	ND
	1SBM-1WG	199.02±3.21^{a}	198.04±9.17^{a}	187.32±8.90^{b}	178.13±3.46^{b}	161.41±2.66^{c}	140.72±5.05^{d}	122.06±1.99^{e}	118.76±2.05^{e}
	1SBM-2WG	110.93±5.02^{b}	109.61±6.49^{bc}	105.83±6.66^{bc}	106.23±3.60^{bc}	99.22±4.02^{cd}	93.45±6.94^{c}	90.74±5.04^{c}	88.21±8.10^{c}
	2SBM-1WG	225.43±1.97^{a}	216.63±1.99^{a}	203.17±2.61^{b}	188.11±2.01^{b}	164.01±8.71^{c}	159.52±6.02^{c}	133.98±4.72^{d}	123.27±5.03^{e}
Daidzein	SBM	55.25±3.97^{b}	56.84±3.08^{b}	60.72±5.99^{b}	62.14±2.41^{b}	64.12±3.62^{b}	80.37±10.22^{a}	84.32±3.99^{a}	87.49±1.65^{a}
	WG	ND	ND	ND	ND	ND	ND	ND	ND
	1SBM-1WG	27.03±1.63^{e}	31.22±1.95^{de}	31.48±5.13^{de}	35.84±4.35^{cd}	38.12±1.95^{bc}	41.33±2.66^{b}	46.41±1.10^{a}	48.22±2.04^{a}
	1SBM-2WG	20.26±4.08^{e}	22.20±2.10^{e}	26.93±1.02^{d}	32.05±1.98^{c}	35.26±2.59^{c}	35.82±3.85^{c}	42.15±1.99^{b}	47.62±1.96^{a}
	2SBM-1WG	30.78±9.30^{e}	43.54±4.80^{cd}	44.22±5.01^{bcd}	44.52±2.95^{bcd}	40.01±6.99^{d}	48.03±1.99^{abc}	51.42±2.41^{ab}	54.82±3.63^{a}
Daidzin	SBM	121.58±4.51^{a}	117.89±2.07^{a}	113.54±1.38^{ab}	92.34±7.20^{c}	82.53±6.03^{d}	67.08±2.522^{e}	60.52±2.99^{e}	51.05±2.04^{f}
	WG	ND	ND	ND	ND	ND	ND	ND	ND
	1SBM-1WG	84.22±2.99^{a}	70.67±7.13^{b}	58.27±1.80^{c}	49.31±7.80^{d}	35.92±5.60^{e}	33.64±3.46^{e}	29.06±3.63^{e}	27.60±4.01^{e}
	1SBM-2WG	77.22±2.62^{b}	66.71±3.47^{c}	51.15±1.99^{d}	39.43±1.95^{e}	35.74±2.03^{e}	29.44±2.66^{f}	26.26±2.68^{f}	21.44±1.90^{g}
	2SBM-1WG	95.31±6.90^{a}	87.63±2.00^{b}	80.32±5.01^{c}	73.44±3.03^{c}	60.91±4.90^{d}	52.13±1.99^{e}	45.73±3.05^{e}	52.12±1.98^{e}
Glycitein	SBM	12.42±1.80^{e}	14.03±2.025^{de}	14.35±3.08^{de}	17.04±1.99^{cde}	18.57±2.15^{cd}	20.94±2.05^{bc}	23.86±2.01^{ab}	25.47±4.05^{a}
	WG	ND	ND	ND	ND	ND	ND	ND	ND
	1SBM-1WG	7.43±1.70^{d}	8.52±1.05^{cd}	9.01±1.53^{cd}	12.20±3.20^{bc}	13.44±1.03^{b}	14.84±1.60^{ab}	18.54±2.18^{a}	18.51±3.17^{a}
	1SBM-2WG	6.04±1.98^{e}	8.58±2.40^{de}	9.01±0.99^{de}	10.86±1.02^{cd}	13.59±1.42^{bc}	12.73±1.53^{c}	16.71±2.99^{ab}	17.15±1.98^{a}
	2SBM-1WG	10.19±2.06^{e}	10.47±2.02^{e}	12.34±1.85^{e}	13.69±1.78^{de}	16.04±1.98^{cd}	19.35±1.08^{c}	19.18±3.20^{c}	22.74±0.20^{b}
Glycitin	SBM	50.24±9.23^{a}	47.21±3.09^{ab}	47.24±3.63^{ab}	43.50±3.70^{abc}	40.56±1.98^{bcd}	38.61±2.05^{cd}	38.09±4.05^{cd}	34.64±3.35^{d}
	WG	ND	ND	ND	ND	ND	ND	ND	ND
	1SBM-1WG	39.96±4.02^{a}	37.33±2.98^{ab}	34.31±2.99^{abc}	37.64±9.38^{abc}	29.58±3.61^{bc}	27.12±4.34^{c}	26.43±3.99^{c}	25.51±1.06^{c}
	1SBM-2WG	30.17±9.92^{a}	29.24±6.54^{a}	28.54±7.96^{a}	25.39±5.40^{a}	23.54±3.65^{a}	21.82±3.01^{a}	21.44±3.96^{a}	20.43±3.58^{a}
	2SBM-1WG	42.94±3.05^{a}	42.93±7.02^{a}	39.58±6.24^{ab}	35.97±5.09^{abc}	35.41±3.23^{abc}	32.54±2.51^{bc}	30.04±3.62^{c}	29.04±1.96^{c}
Sinapinic acid	SBM	4.90±0.99^{c}	5.41±1.10^{bc}	6.04±1.02^{abc}	6.45±1.02^{ab}	6.65±0.48^{ab}	7.04±0.04^{a}	7.06±0.01^{a}	7.41±0.09^{a}
	WG	92.18±2.01^{f}	93.48±1.10^{f}	96.76±1.05^{d}	99.98±1.74^{d}	110.97±1.01^{c}	118.32±1.74^{b}	120.29±2.05^{b}	124.32±1.02^{a}
	1SBM-1WG	79.57±0.80^{g}	81.51±1.05^{f}	86.48±1.01^{e}	89.74±1.72^{d}	92.38±0.62^{c}	95.26±0.05^{b}	97.07±0.02^{a}	98.34±0.98^{a}

	1SBM-2WG	85.15±1.00^{f}	86.11±0.99^{f}	91.13±1.01^{e}	94.19±0.99^{d}	101.60±1.64^{c}	104.98±2.00^{b}	110.50±0.30^{a}	111.38±0.03^{a}
	2SBM-1WG	9.06±0.99^{b}	9.25±0.05^{b}	9.67±1.74^{ab}	10.44±0.98^{ab}	10.92±1.01^{a}	11.06±0.01^{a}	11.16±0.11^{a}	11.27±0.07^{a}
Ferulic acid	SBM	33.67±4.07^{d}	34.12±2.66^{d}	36.63±3.19^{d}	35.95±3.76^{d}	42.16±1.72^{c}	52.67±2.89^{b}	60.53±0.76^{a}	64.76±3.45^{a}
	WG	87.34±1.98^{f}	88.27±6.92^{ef}	90.37±1.13^{ef}	93.35±0.85^{de}	97.52±2.17^{cd}	100.78±1.04^{c}	113.14±1.03^{b}	119.34±2.65^{a}
	1SBM-1WG	85.50±0.77^{f}	89.25±2.99^{ef}	92.24±2.01^{de}	95.12±4.99^{cd}	98.84±3.01^{bc}	101.35±1.72^{ab}	102.67±2.01^{ab}	105.64±5.17^{a}
	1SBM-2WG	80.76±1.70^{d}	83.15±2.99^{d}	83.87±1.01^{d}	89.70±2.23^{c}	91.20±2.05^{c}	93.31±2.15^{c}	111.63±2.96^{b}	115.25±4.03^{b}
	2SBM-1WG	73.43±2.72^{f}	75.34±2.98^{ef}	78.02±0.99^{def}	80.67±4.12^{de}	84.63±4.05^{cd}	89.72±2.01^{bc}	94.39±3.20^{ab}	97.07±7.05^{a}
Vanillic acid	SBM	5.41±1.01^{b}	5.73±1.01^{b}	5.55±1.09^{b}	6.29±1.12^{ab}	7.23±1.03^{ab}	7.63±1.96^{ab}	7.62±0.72^{ab}	8.04±1.17^{a}
	WG	7.03±2.65^{d}	9.16±0.025^{d}	10.93±2.00^{c}	10.94±1.40^{c}	13.28±2.63^{bc}	15.69±2.24^{ab}	17.93±2.05^{a}	18.28±2.01^{a}
	1SBM-1WG	6.14±2.06^{d}	6.57±1.38^{d}	6.49±0.92^{d}	8.68±0.66^{cd}	9.47±3.45^{bcd}	10.25±1.71^{bc}	12.17±1.74^{b}	16.34±1.01^{a}
	1SBM-2WG	6.44±1.06f	6.41±0.98f	7.20±1.10ef	9.19±0.96de	10.12±1.01cd	11.92±2.06bc	14.39±1.79b	17.05±1.99a
	2SBM-1WG	6.83±0.035c	6.13±1.97c	7.79±1.58bc	7.72±1.97bc	9.31±2.01abc	10.76±2.02ab	11.63±1.46a	11.95±1.03a
Quercetin	SBM	10.30±2.07^{e}	10.76±1.02^{e}	12.41±2.55^{de}	15.79±0.80^{cd}	17.65±2.09^{bc}	20.42±1.03^{abc}	22.16±4.02^{ab}	23.82±5.03^{a}
	WG	36.22±5.20^{e}	37.73±1.05^{e}	41.05±1.02^{de}	45.64±4.33^{cd}	49.14±4.98^{c}	59.34±3.01^{b}	62.73±1.98^{ab}	68.44±4.20^{a}
	1SBM-1WG	28.22±3.20^{f}	30.25±1.97^{ef}	36.15±1.96^{e}	44.02±2.65^{d}	52.47±2.76^{c}	56.12±5.01^{bc}	61.54±5.22^{ab}	63.54±3.03^{a}
	1SBM-2WG	42.05±1.70^{e}	43.63±2.67^{de}	43.58±4.91^{de}	48.24±1.73^{d}	55.88±3.08^{c}	58.55±1.74^{bc}	62.25±2.99^{ab}	64.10±3.50^{a}
	2SBM-1WG	17.15±2.02^{e}	18.96±1.51^{e}	26.83±3.00^{d}	30.55±1.84^{cd}	35.74±3.38^{bc}	39.57±2.15^{b}	48.23±6.91^{a}	48.34±3.04^{a}
Protocatechuic acid	SBM	8.81±0.10^{e}	9.25±0.19^{e}	9.71±0.19^{e}	11.46±0.06^{d}	13.21±0.87^{c}	14.52±0.30^{b}	15.74±0.29^{a}	15.83±0.02^{a}
	WG	251.30±0.97^{h}	256.12±1.02^{g}	259.63±0.10^{f}	261.82±1.10^{e}	278.48±1.69^{d}	286.81±1.64^{c}	296.60±1.04^{b}	300.41±0.26^{a}
	1SBM-1WG	201.75±1.00^{e}	210.51±1.95^{d}	213.16±3.01^{d}	219.29±1.02^{c}	225.85±0.95^{b}	228.53±2.98^{b}	230.72±3.10^{a}	233.04±3.02^{a}
	1SBM-2WG	244.72±1.05^{h}	246.91±1.00^{g}	249.54±1.01^{f}	256.55±1.74^{e}	267.52±1.01^{d}	271.57±0.57^{c}	279.35±1.74^{b}	287.25±0.95^{a}
	2SBM-1WG	9.71±1.90^{e}	10.58±0.80^{e}	11.46±0.88^{e}	13.65±1.02^{d}	15.41±1.15^{c}	16.28±1.02^{c}	17.16±1.01^{bc}	18.81±1.32^{b}

Each value was presented as the mean ± standard deviation (n = 3). Within each row, means followed by different lowercase letters (a, b, c, d) indicate statistically significant differences (P < 0.05). SBM, soybean meal; WG, wheat germ;1SBM-1WG,1soybean meal-1wheatgerm;2SBM-1WG,2soybean meal-1wheatgerm;1SBM-2WG,1soybean meal-2wheatgerm.

Fermentation enhanced other phenolic profiles, as shown in Table 3, by increasing the levels of ferulic acid, vanillic acid, quercetin, Protocatechuic acid, and Sinapinic acid across all samples. Among the samples, WG exhibited the highest concentrations of these phenolic compounds after fermentation, whereas SBM consistently showed the lowest. Specifically, WG contained 119.34 μg/g ferulic acid, 18.28 μg/g vanillic acid, 68.44 μg/g quercetin, 300.41μg/g protocatechuic acid, and 124.32 μg/g sinapinic acid. The 1SBM-2WG followed closely with 115.25 μg/g ferulic acid, 17.05 μg/g vanillic acid, 64.10 μg/g quercetin, 287.25 μg/g protocatechuic acid, and 111.38 μg/g sinapinic acid. The 1SBM-1WG yielded 105.64 μg/g ferulic acid, 16.34 μg/g vanillic acid, 63.54 μg/g quercetin, 233.04 μg/g protocatechuic acid, and 98.34 μg/g sinapinic acid. while 2SBM-1WG showed a further decline to 97.07 μg/g ferulic acid, 11.95 μg/g vanillic acid, 48.34 μg/g quercetin, 18.81 μg/g protocatechuic acid, and 11.27 μg/g sinapinic acid. In contrast, fermented SBM alone contained the lowest levels, with 64.76 μg/g ferulic acid, 8.04 μg/g vanillic acid, 23.82 μg/g quercetin, 15.83μg/g protocatechuic acid, and 7.41 μg/g sinapinic acid.

Several studies also reported that fermentation of soybean products leads to bioconversion of isoflavone glucosides to their corresponding aglycones [44, 47]. This transformation is primarily attributed to the activity of microbial β-glucosidases, including those produced by RT-3[17, 47]. This enzymatic activity explains the elevated concentrations of aglycones observed in the fermented SBM samples. WG is naturally rich in phenolic compounds, particularly ferulic acid, protocatechuic acid, and sinapinic acid[48], while[49] noted that WG also contains considerable levels of quercetin. Therefore, the combination of fermentation-induced enzymatic conversion in SBM and the inherent phenolic richness of WG may synergistically contribute to the enhanced phenolic and aglycone profiles observed in the mixed samples, particularly in the 1SBM-2WG.

3.5. Antioxidant capacity

Different antioxidant compounds can act through diverse mechanisms to neutralize oxidative stress, resulting in varying levels of antioxidant activity[17, 50]. To comprehensively assess the antioxidant potential of RT-3 fermented SBM, WG, and their mixtures, four complementary antioxidant assays were employed: $ABTS^{\bullet+}$ radical scavenging activity, DPPH radical scavenging activity, reducing power (RP), and ferric reducing antioxidant power (FRAP). As illustrated in Fig.3, all antioxidant assays, $ABTS^{\bullet+}$ scavenging activity, DPPH radical scavenging activity, ferric reducing antioxidant power (FRAP), and reducing power (RP) demonstrated a significant increase during the fermentation in all samples.

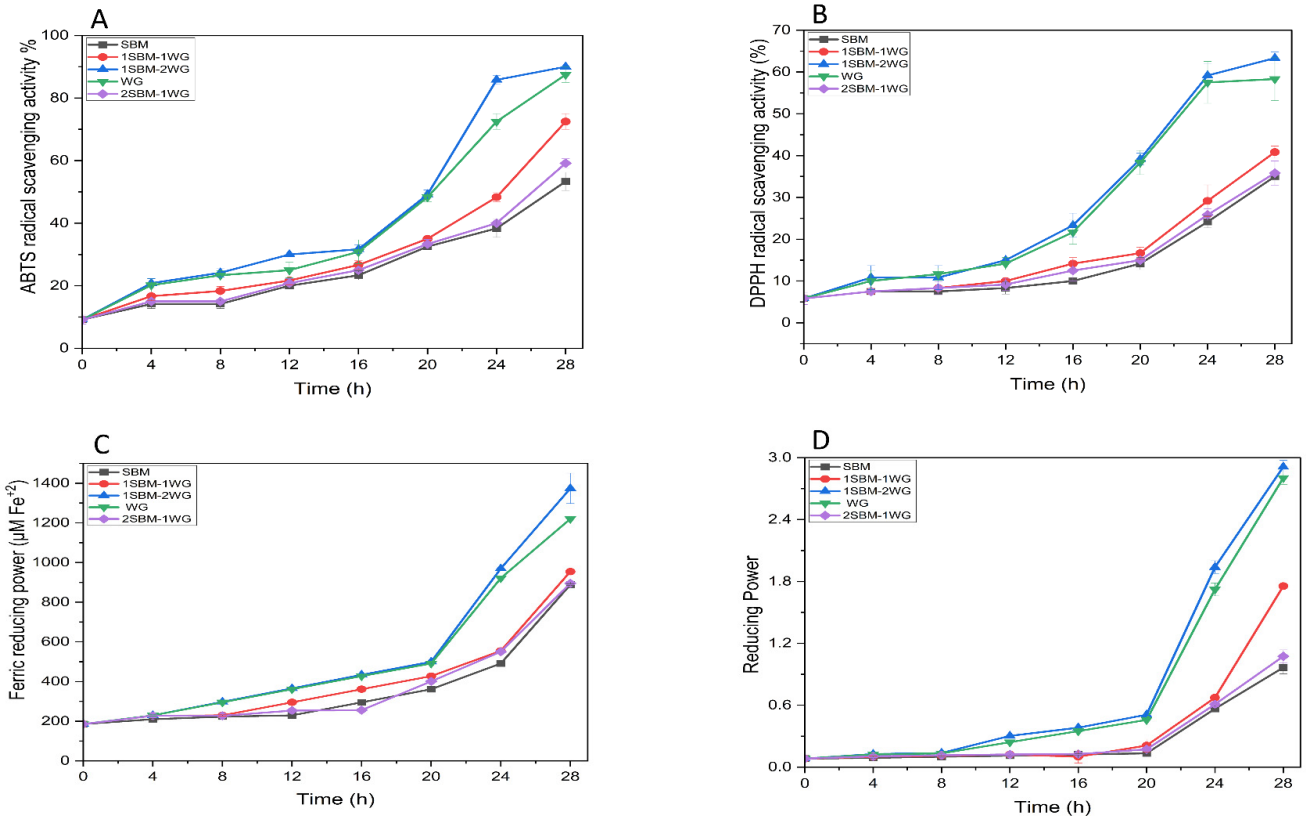

Figure 3. Antioxidant activities of fermented SBM, WG, 1SBM-1WG, 2SBM-1WG, and 1SBM-2WG, during fermentation by *Rhizopus oligosporus*. (A)ABTS cation radical scavenging ability, (B)DPPH radical scavenging activity, (C) ferric reducing antioxidant power (FRAP), and (D) reducing power, each value was expressed as mean ± SD (n = 3). SBM, soybean meal; WG, wheat germ;1SBM-1WG,1soybean meal-1wheatgerm;2SBM-1WG,2soybean meal-1wheatgerm;1SBM-2WG,1soybean meal-2wheatgerm.

In addition, the antioxidant activity, including SBM, WG, and their mixtures, was in the following order: 1SBM-2WG > WG > 1SBM-1WG > 2SBM-1WG > SBM. Moreover, the EC_{50} values (Table S1) reflected this same trend, with antioxidant strength in the order: 1SBM-2WG < WG < 1SBM-WG < 2SBM-1WG < SBM, confirming the superior antioxidant efficiency of WG-containing formulations.

These results demonstrate that fermentation enhances antioxidant activity across all formulations, with WG-containing samples consistently outperforming others. The superior antioxidant performance of 1SBM-2WG can be attributed to its high baseline levels of phenolic acids such as ferulic acid, vanillic acid, quercetin, Protocatechuic acid, and Sinapinic acid, which are further liberated or transformed into more bioavailable forms during fermentation[48] also, the SBM contributed to raising the antioxidants in 1SBM-2WG from the bioconversion of isoflavone glycosides into aglycones, a transformation facilitated by the β-glucosidase activity of RT-3[51- 52]. These findings align with previous studies showing that microbial fermentation can both release bound phenolics and convert inactive phenolic precursors into more potent antioxidant[53-54-55]. The synergistic interaction between these two substrates provides a promising strategy for the development of functional foods rich in antioxidants, which is demonstrated in the mixtures 1SBM-2WG,1SBM-1WG, and 2SBM-1WG.

3.6. Analysis of volatile compounds

Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) effectively distinguished between fermentation stages, revealing key volatile compounds contributing to flavor development (Fig.4A).

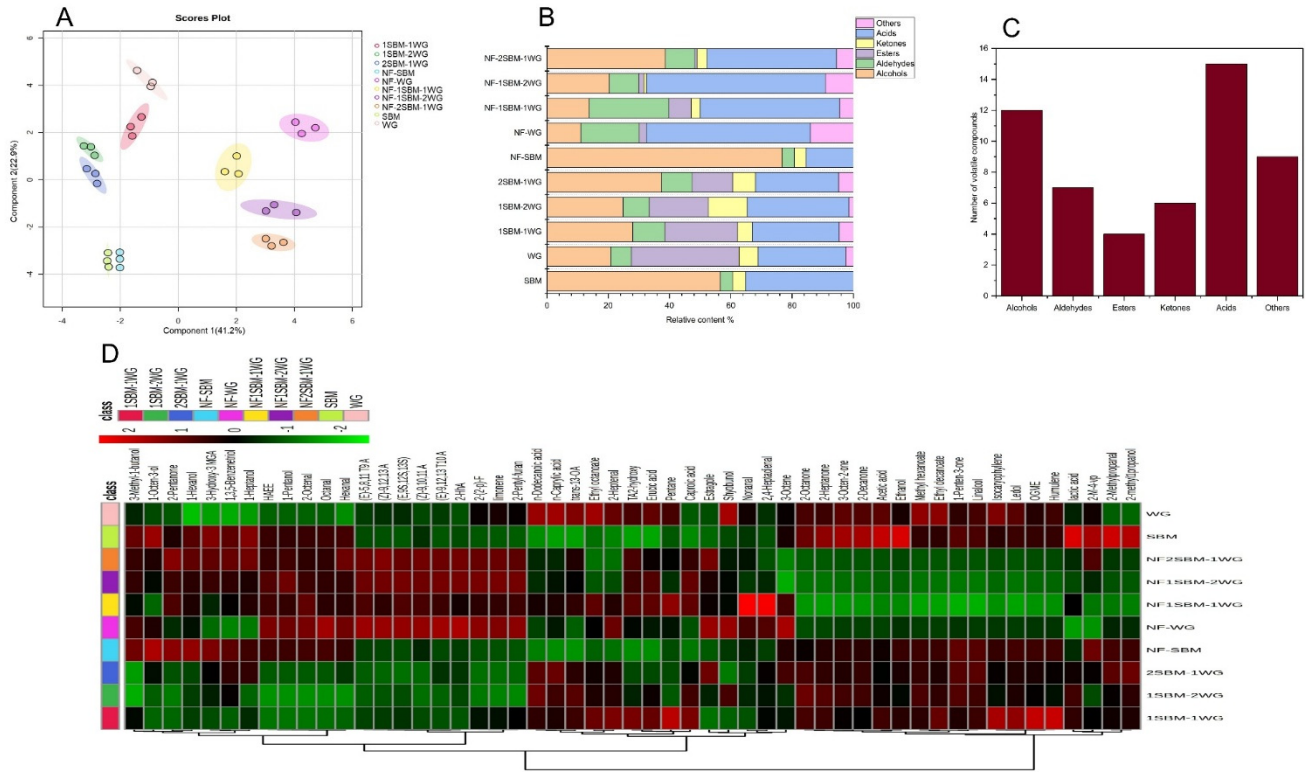

Figure 4. Classification of SBM, WG,1SBM-1WG,1SBM-2WG,2SBM-1WG, and 1SBM-2WG collected at different fermentation time points using orthogonal projection on latent structure-discriminant analysis (OPLS-DA) (A). The relative contents of volatile flavor compounds (B) and classification of volatile flavor compounds (C). Hierarchical cluster analysis of differential metabolites identified in fermented and unfermented SBM, WG, 1SBM-1WG, 2SBM-1WG, and 1SBM-2WG. Samples are represented in columns, metabolites are represented in rows, and color indicates the relative concentration of metabolites expressed in the cluster. Red indicates a higher expression level, and green indicates a lower expression level. SBM, soybean meal; WG, wheat germ;1SBM-1WG,1soybean meal-1wheatgerm;2SBM-1WG,2soybean meal-1wheatgerm;1SBM-2WG,1soybean meal-2wheatgerm. 2-(2-p)-F=2-(2-propenyl)-Furan, (Z)-9,12,13 A=(Z)-9,12,13-trihydroxyoctadec-15-enoic acid, (Z)-9,10,11 A=(Z)-9,10,11-trihydroxyoctadec-12-enoic acid, (E)-9,12,13 T10 A=(E)-9,12,13-trihydroxyoctadec-10-enoicacid,(E)-5,8,11T9A=(Z)-5,8,11-trihydroxyoctadec-9-enoic acid,(E,9S,12S,13S)=(E,9S,12S,13S)-9,12,13-trihydroxyoctadec-10-enoicacid,2-HhA=2-Hydroxyhexanedioic acid ,3-Hydroxy-3MGA=3-Hydroxy-3-methylglutaricacid,2-M-4-vp=2-Methoxy-4 vinylphenol,TA2,hydroxy=Tetradecanoic acid, 2-hydroxy-,OGME=Octaethylene glycol monododecyl ether,trans-13-OA=trans-13-Octadecenoic acid,HAEE=Hexanoic acid ethyl ester.

Volatile compounds primarily originated from the metabolic conversion of carbohydrates, lipids, and proteins. Fermentation significantly altered the volatile profiles; the number of compounds increased from 9-37 in non-fermented (NF) samples to 17-48 after fermentation. Specifically, NF-SBM, NF-WG, NF-1SBM-1WG, NF-1SBM-2WG, and NF-2SBM-1WG had 9, 32, 37, 36, and 36 volatiles, respectively, which increased to 17 in SBM, 42 in WG, 48 in 1SBM-1WG, 48 in 1SBM-2WG, and 48 in 2SBM-1WG after fermentation (Table S2).

Alcohols, aldehydes, ketones, esters, and acids accounted for the majority of the volatiles, 99.98% in SBM, 97.43% in WG, 95.38% in 1SBM-1WG, 98.56% in 1SBM-2WG, and 95.13% in 2SBM-1WG (Fig. 4B). In total, 53 volatile compounds were identified, comprising 12 alcohols, 7 aldehydes, 6 ketones, 14 acids, 4 esters, and 9 miscellaneous (Fig. 4C).

Flavor development was linked to metabolic pathways including shikimate, phenylpropanoid, fatty acid oxidation, terpenoid synthesis, microbial fermentation, and Maillard reactions. Alcohols formed via carbohydrate and protein fermentation were prominent. Notable volatiles included 2-methoxy-4-phenol contributes spicy, sour, and fruity notes[56], 3-Methyl-1-butanol adds a malty flavor[57], and ethanol were identified. SBM had the highest alcohol contents: 13.11% (2-methoxy-4-phenol), 2.91% (3-methyl-1-butanol), and 22.7% (ethanol). WG had lower levels: 5.57%, 0.48%, and 4.20%, respectively. Alcohol levels in mixtures varied with WG proportion, 2SBM-1WG (7.14%, 0.04%, 6.25%), 1SBM-1WG (5.66%, 1.09%, 6.85%), and 1SBM-2WG (2.64%, 0.03%, 4.84%). The 1SBM-1WG sample had the highest Ledol content, associated with sweet, green aromas[58]. Also, the content of Linalool increased in WG, 2SBM-1WG, 1SBM-2WG, and 1SBM-1WG, which imparts floral, sweet, rose, and fruity odors[59]. NF samples contained high levels of 1-hexanol, known for its green and flowery aroma[60], and commonly found in unfermented soybeans[61- 62], particularly in NF-SBM (35.04%), followed by NF-2SBM-1WG (12.81%), NF-1SBM-1WG (6.78%), NF-1SBM-2WG (6.60%), and NF-WG (1.32%).

Fermentation significantly reduces aldehyde-related flavors and aromas by degrading these volatile compounds[63]. Hexanal, a lipid oxidation product[64], was abundant in NF samples, NF-SBM (3.96%), NF-WG (6.70%), NF-1SBM-1WG (6.06%), NF-1SBM-2WG (5.29%), and NF-2SBM-1WG (6.98%). After fermentation, levels dropped, SBM (1.91%), WG (0.67%), 1SBM-2WG (1.19%), 2SBM-1WG (2.78%), and 1SBM-1WG (1.38%). Similarly, 2-Octenal, responsible for a green, nutty, fatty, oily odor[64], was found in NF-WG (3.62%), NF-1SBM-1WG (4.16%), NF-1SBM-2WG (1.78%), and NF-2SBM-1WG (0.92%) but disappeared after fermentation.

In contrast, 2-heptenal, which contributes bean and fruity notes[65], increased after fermentation, WG (4.50%), 1SBM-2WG (4.42%), 2SBM-1WG (3.44%), and peaked in 1SBM-1WG (7.16%). This shift highlights fermentation's role in reducing off-flavors and enhancing pleasant aromas. Among mixtures, 1SBM-1WG showed the most balanced aldehyde profile.

Key esters contributed to the distinctive aroma profiles. Ethyl decanoate, with a grape-like odor[66], was highest in WG (15.12%) and 1SBM-1WG (11.11%), followed by 1SBM-2WG (7.40%) and 2SBM-1WG (4.70%). Ethyl octanoate imparts a fruity, fresh, and apricot odor[67], appearing at 11.00% in WG, 8.60% in 1SBM-1WG, 5.64% in 1SBM-2WG, and 4.30% in 2SBM-1WG. Methyl hexanoate, with fresh, sweet, and fruity aromas [68], was detected at 8.96% in WG, 3.94% in 1SBM-1WG, 6.25% in 1SBM-2WG, and 4.21% in 2SBM-1WG. These esters enhance overall aroma quality, with 1SBM-1WG showing the most favorable ester balance.

During fermentation, enzyme secretion breaks down amino acids and fats, forming ketones. Six ketones were identified with total contents of 4.25% in SBM, 6.22% in WG, 4.72% in 1SBM-1WG, 12.65% in 1SBM-2WG, and 7.45% in 2SBM-1WG, respectively. Among them, 2-heptanone, with a pungent, Sulfur, green odor[69], 2-octanone, with a floral odor[70],2-decanone, with an intense citrusy-orange and a floral scent[71], and 3-octen-2-one contributes a flowery odor[72] were highest in 1SBM-2WG, while 1SBM-1WG

showed the lowest total ketone content compared to 1SBM-2WG, which has the highest content, suggesting it delivers the most distinctive and appealing aroma.

Fifteen acids were found, with total contents of 35.1% (SBM), 28.54% (WG), 28.4% (1SBM-1WG), 33.22% (1SBM-2WG), and 27.07% (2SBM-1WG). NF samples showed higher acid contents: NF-WG (53.55%), NF-1SBM-2WG (58.39%), NF-1SBM-1WG (45.54%), NF-2SBM-1WG (42.32%), and NF-SBM (15.49%). Fermentation reduced levels of oxidized derivatives of long-chain unsaturated fatty acids, such as (Z)-5,8,11-trihydroxyoctadec-9-enoic acid and (Z)-9,12,13-trihydroxyoctadec-15-enoic acid, likely due to enzymatic hydrolysis. 3-Hydroxy-3-methylglutaric acid was present in several NF samples but decreased after fermentation. Lactic acid was consistently found in all samples, while acetic acid, known for its sour, acidic, fruity, pungent, vinegar taste[73], was abundant in NF-SBM, NF-WG, and other NF mixtures, contributing noticeably to their flavor profiles before fermentation[19].

Among all formulations, 1SBM-1WG demonstrated the most balanced volatile compound profile, combining moderate alcohols, rich esters, favorable aldehydes, and lower ketones, indicating superior aroma complexity and sensory quality.

3.7. Sensory evaluation

Figure 5 presents the sensory evaluation of SBM, WG, and their mixtures. The 1SBM-1WG blend exhibited the highest scores in flavor (9.26), appearance (7.42), texture (7.99), aroma (8.49), and overall acceptability (9.43), whereas SBM alone scored lowest in all mentioned categories. The sensory ranking followed the order: 1SBM-1WG > 1SBM-2WG > WG > 2SBM-1WG > SBM. These results align with our volatile compound analysis (Table S2), which revealed that the 1SBM-1WG blend produced the most diverse flavor profile with 48 compounds, including balanced levels of key alcohols 5.66% 2-methoxy-4-phenol and 1.09% 3-methyl-1-butanol. Additionally, the significantly higher ester content, particularly 11.15% ethyl decanoate, imparted a grape-like odor[66], and 8.60% ethyl octanoate imparted a desirable, fruity, fresh odor[67], explaining the superior flavor of 1SBM-1WG. The balanced 1SBM-1WG substrate ratio also enhanced proteolytic efficiency by RT-3, improving texture. Several studies have reported the effect of proteolytic enzymes on the texture[20, 74]. The direct correlation between volatile chemistry (reduced hexanal 0.67%, enhanced 2-heptenal 7.16%) and sensory scores further confirms the 1SBM-1WG ratio's superiority over pure ingredients. In contrast, the 2SBM-1WG blend showed alcohol dominance (7.14% 2-methoxy-4-phenol, 0.04% 3-methyl-1-butanol) with limited esters (7.40% ethyl decanoate, 5.64% ethyl octanoate) and elevated hexanal (2.78%), while 1SBM-2WG displayed reduced alcohols (2.64% 2-methoxy-4-phenol, 0.03% 3-methyl-1-butanol) but higher ketones (3.09% 2-heptanone, 3.32% 2-octanone) and high total acids 33.15% (Table S2). These biochemical imbalances directly explained their poorer sensory performance compared to 1SBM-1WG, confirming the 1SBM-1WG ratio's optimal balance of flavor compounds and fermentation efficiency in plant-based products.

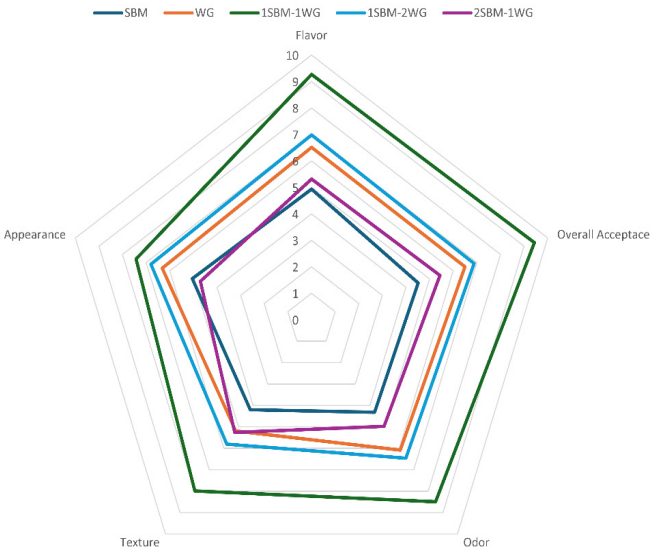

Figure 5. Sensory evaluation of fermented SBM, WG, 2SBM-1WG, 1SBM-1WG, and 1SBM-2WG using *Rhizopus oligosporus*.

3.8. Metabolite Extraction and Derivatization

To gain deeper insight into the biochemical transformations and bioactive compound production during fermentation, a comprehensive metabolomics analysis was conducted. The 1SBM-1WG formulation was selected for further analysis due to its balanced performance, showing enhanced antioxidant activity, improved enzymatic activity, and favorable sensory attributes, making it a strong candidate for functional food development. The LC-MS analysis revealed 1506 metabolic peaks in the 1SBM-1WG and NF-1SBM-1WG samples. Further analysis of PCA revealed a clear separation between the two samples, indicating the metabolic profiles were mainly affected by fermentation (Fig. 6A)

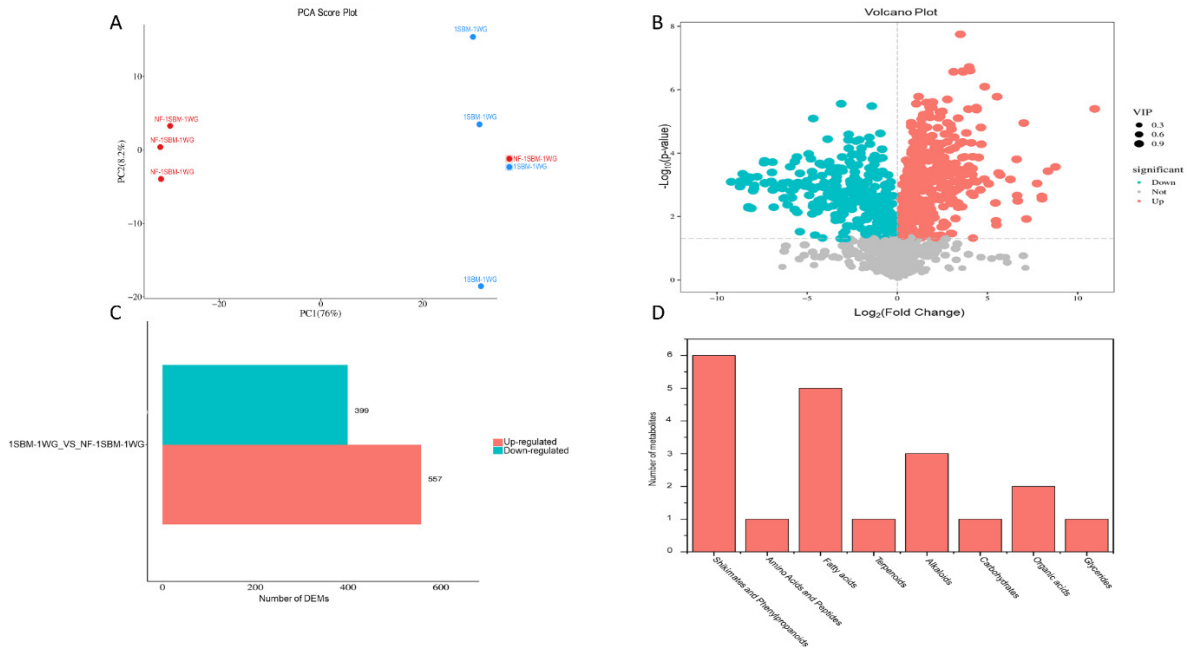

Figure 6. The PCA score plot (A), Volcano plot showing the differential metabolites between 1SBM-1WG and NF-1SBM-1WG the red dots represent upregulated metabolites, the blue dots represent downregulated metabolites, and the black dots represent not significant metabolites(B), Number of DEMs between 1SBM-1WG and NF-1SBM-1WG the red dots represent 557 upregulated metabolites, the blue dots represent 399 downregulated metabolites(C), Classification of differential metabolites(D). 1SBM-1WG,1soybean meal-1wheatgerm; NF-1SBM-1WG, non-fermented 1soybean meal-1wheatgerm.

In total, 956 metabolites showed significant changes between the two groups ($P < 0.05$), with 557 upregulated and 399 downregulated in the 1SBM-1WG compared to NF-1SBM-1WG (Fig. 6 B and C). Among these, 20 differential metabolites were highly impacted (*P-value* < 0.05, VIP > 1) between the groups (Table 4 and Fig. S1), indicating that these metabolites were the most influential contributors to the separation between the two groups. These 20 metabolites were subsequently categorized by their chemical classes (Table 4 and Fig. 5D), and were mainly distributed among shikimates and phenylpropanoids (6 metabolites), glycerides (1), amino acids and peptides (1), fatty acids (5), terpenoids (1), alkaloids (3), carbohydrates (1), and organic acids (2).

Table 4. Top 20 differential metabolites in the 1SBM-1WG, and NF-1SBM-1WG

Compounds	VIP	Fold change	*P.value*	significant
Shikimates and Phenylpropanoids				
Nortriptyline	1.165	0.0002	0.005	Up
Laricitrin	1.165	0.744	0.010	Down
threo-Syringoylglycerol	1.165	11.28	< 0.001	Down
2-(4-hydroxyphenyl)-7-[(2S,3R,4S,5S,6R)-3 ,4,5-trihydroxy-6-(hydroxymethyl) tetrahydropyran-2-yl] oxy-chromen-4-one	1.165	15.37	< 0.001	Down
2-(3,4-dihydroxyphenyl)-7-hydroxy-2,3-dih ydrochromen-4-one	1.165	12.30	< 0.001	Down
(9R,10S)-rel-(-)-9,10-bis (Acetyloxy)-9,10-dihydro-5-methoxy-8,8-di methyl-2-phenyl-4H,8H-benzo[1,2-b:3,4-b'] dipyran-4-one	1.165	0.1157	< 0.001	Up
Glycerides				
Glyceryl monooleate	1.165	0.120	< 0.001	Up
Amino Acids and Peptides				
Goralatide (acetate)	1.165	0.0082	< 0.001	Up
Fatty acids				
(Z)-9,12,13-trihydroxyoctadec-15-enoic acid	1.165	17.19	< 0.001	Down
(Z)-9,10,11-trihydroxyoctadec-12-enoic acid	1.165	17.191	< 0.001	Down
(E)-9,12,13-trihydroxyoctadec-10-enoic acid	1.165	17.191	< 0.001	Down
(Z)-5,8,11-trihydroxyoctadec-9-enoic acid	1.165	17.191	< 0.001	Down
(E,9S,12S,13S)-9,12,13-trihydroxyoctadec-10-enoic acid	1.165	17.191	< 0.001	Down
Terpenoids				
Abscisic acid	1.1625	2.969	< 0.001	Down
Alkaloids				
Cadiamine	1.165	0.021	< 0.001	Up
Oxoglaucine	1.165	128.95	< 0.001	Down
ISOFEBRIFUGINE (B604866K055)	1.165	1967.142	< 0.001	Down
Carbohydrates				
1,2-Anhydro-myo-inositol	1.165	11.091	< 0.001	Down
Organic Acids				
3-Hydroxy-3-methylglutaric acid	1.165	11.091	< 0.001	Down
2-Hydroxyhexanedioic acid	1.165	11.09	< 0.001	Down

Figure 7 shows the relationship among metabolic pathways involved in the RT-3 fermentation of 1SBM-1WG. RT-3 fermentation substantially impacted carbohydrate composition (Fig. 7 and Table 4). Metabolomics analysis revealed a marked reduction in oligosaccharide levels, indicating enzymatic breakdown by RT-3. Enzymes such as α-amylase, β-glucosidase, cellulases, and xylanase likely depolymerize complex carbohydrates into monosaccharides, facilitating fungal growth and potentially increasing glucose

levels[75]. In addition, a decrease in myo-inositol, the precursor for 1,2-Anhydro-myo-inositol synthesis, alongside an increase in glycerol, a precursor for glyceryl monooleate, was detected in 1SBM-1WG samples (Table 4). The reduction in myo-inositol suggests its diversion into glycolytic metabolism during fermentation[76]. Conversely, the elevated glycerol levels may result from glycerophospholipid hydrolysis, aligning with the concurrent rise in free fatty acid content.

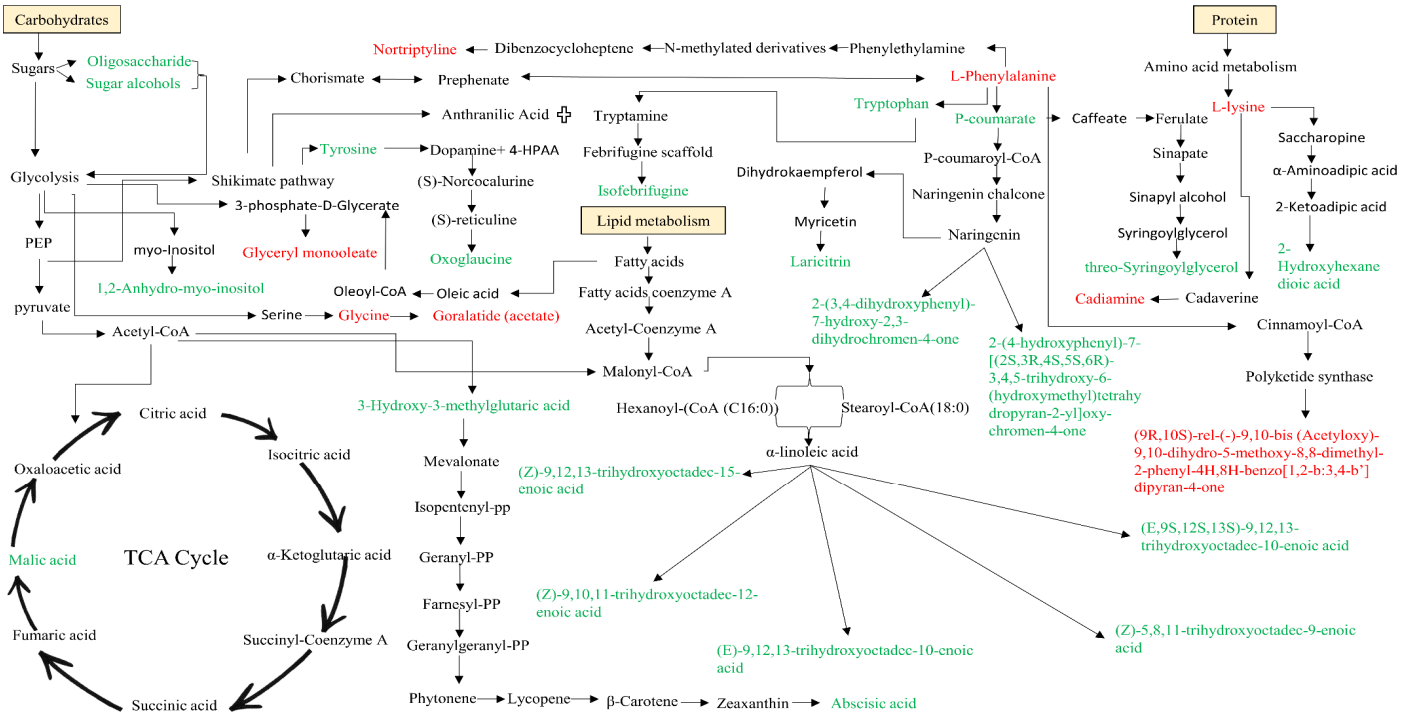

Figure 7. The connection of the metabolic pathways occurring during fermentation with *Rhizopus oligosporus*, the red color means upregulated metabolites; the green color means downregulated metabolites; the black color means undetected metabolites. 4-HPAA = 4-Hydroxyphenylacetaldehyde.

RT-3 fermentation significantly affected lipid levels (Fig. 7 and Table 4). Precursors of oxidized derivatives of long-chain unsaturated free fatty acids, including linoleic acid, α-linolenic acid, and oleic acids, were identified in 1SBM-1WG and NF-1SBM-1WG. WG oil naturally contains high levels of linoleic acid, α-linolenic acid, and oleic acid[77]. During fermentation, RT-3 secretes lipases that degrade triglycerides into free fatty acids[78], making them available for microbial metabolism. RT-3 likely uses these fatty acids as an energy source via β-oxidation or incorporates them into cytoskeletons, reducing their availability for oxidation, this explains the decrease in these precursors after fermentation. These liberated fatty acids may also enhance food quality as flavor components or precursors[79-80].

During fermentation, RT-3 secretes proteases that hydrolyze proteins into free amino acids (fig. 1A), notably increasing L-phenylalanine, this aromatic amino acid may be enter multiple biosynthetic pathways, it can serve as a precursor for (9R,10S)-rel-(-)-9,10-bis(acetyloxy)-9,10-dihydro-5-methoxy-8,8-dimethyl-2-phenyl-4H,8H-benzo[1,2-b:3,4-b']dipyran-4-one the flavone[81] production via the polyketide synthase pathway, which increased in 1SBM-1WG. Alternatively, it may be converted into naringenin through the phenylpropanoid and flavonoid pathway, producing 2-(4-hydroxyphenyl)-7-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)

tetrahydropyran-2-yl] oxy-chromen-4-one, 2-(3,4-dihydroxyphenyl)-7-hydroxy-2,3-dihydrochromen-4-one, and Laricitrin, which decreased in 1SBM-1WG compared to NF-1SBM-1WG.

L-phenylalanine may also contribute to the synthesis of nortriptyline, which increased in 1SBM-1WG. nortriptyline is a tricyclic secondary amine with antidepressant, β-adrenergic reuptake inhibitory, analgesic, antitumor, and apoptosis-inducing properties[82]. Supporting these findings, Blake et al.[83]reported that soy-based diets may have antidepressant effects. L-phenylalanine may also be channeled into the p-coumarate via monolignol biosynthesis pathway to produce threo-Syringoylglycerol, which decreased in 1SBM-1WG compared to NF-1SBM-1WG. Moreover, L-phenylalanine might be transformed into L-tryptophan, which then decreases due to its conversion into isofebrifugine through anthranilic acid-dependent, isofebrifugine also decreased in 1SBM-1WG compared to NF-1SBM-1WG. Simultaneously, the observed decline in L-tyrosine could indicate its utilization in the benzylisoquinoline alkaloid pathway for the production of Oxoglaucine, which decreased after fermentation.

Elevated L-lysine levels suggest its involvement in the biosynthesis of cadiamine, which increased in 1SBM-1WG compared to NF-1SBM-1WG, and may have antioxidant properties[84]. L-lysine may also contribute to 2-hydroxyhexanedioic acid formation via oxidative deamination and reduction; levels of this acid decreased after fermentation. Glycine increased in 1SBM-1WG compared to NF-1SBM-1WG, and may contribute to Goralatide (acetate) production, which may regulate hematopoiesis and protect tissues[85].

4. Conclusion

Solid-state fermentation using RT-3 improved the nutritional and functional properties of SBM, WG, and their mixtures (1SBM-1WG, 1SBM-2WG, and 2SBM-1WG). Compared to single-substrate fermentation of SBM, WG, these mixtures demonstrated enhanced enzyme activity, resulting in increased and varied protein and peptide contents, degradation of antinutritional factors, and elevated levels of phenolics and isoflavones, leading to improved antioxidant activity. These mixtures also improved food flavor, particularly through increased ester production. Among the mixtures, 1SBM-1WG demonstrated the most balanced profile, combining strong antioxidant capacity, moderate enzymatic function, reduced ANFs, and enhanced sensory and metabolic characteristics. These findings support 1SBM-1WG as a promising candidate for the development of high-quality, soybean-based functional foods.

Declaration of competing interest

The authors declare that they have no competing interests.

Acknowledgement

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