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# Breeding of 'DND358': A new soybean cultivar for processing soy protein isolate with a hypocholesterolemic effect similar to that of fenofibrate

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ABSTRACT

Soy proteins have hypocholesterolemic effects (HCE). Here, we developed a soybean cultivar 'DND358', which is superior for processing hypocholesterolemic soy protein isolate beverage powder (DND358-SPI). 'DND358' carries four recessive null alleles for the α subunit of 7S globulin and the G1, G2, and G4 subunits of 11S globulin (tetra null), was selected from a population derived from a three-way cross. The tetra null mutation resulted in a compensatory increase in the α′ subunit level and arginine content. The oral administration (300 mg/kg/day) of DND358-SPI processed from 'DND358' significantly decreased the fatty liver symptoms and hepatic lipid accumulation; reduced the hepatic total cholesterol (TC), triacylglycerol (TG) levels and atherogenic index (AI) value, in rats fed a high-cholesterol diet. Conversely, DND358-SPI administration markedly elevated high-density lipoprotein cholesterol levels, to values significantly higher to those fed on fenofibrate (FF, 30 mg/kg/day), a hypocholesterolemic drug. The HCE of DND358-SPI were similar to that of FF.

## 1. Introduction

The hypocholesterolemic effects (HCE) of dietary soybean has been experimentally established in both animal and human subjects (Knopp, 1999; Lovati et al., 1987; Poli et al., 2008; Sirtoriet al., 1984), and the mechanisms responsible for the HCE have been hypothesized (Anthony, Clarkson, & Williams, 1999; Potter, 1998; Sirtori et al., 1998). Soy protein amino acids, specific soy peptides and globulins, and the isoflavones and saponins associated with soy protein have all been suggested as factors influencing the hypocholesterolemic response (Ramdath, Padhi, Sarfaraz, Renwick, & Duncan, 2017).

Soy protein components in particular are considered to be crucial for the hypocholesterolemic effect. Soy protein consists of two main components, namely β-conglycinin (7S globulin) and glycinin (11S globulin). There is a strong negative correlation between 11S and 7S concentrations, but neither 11S nor 7S has a significant correlation with the total seed protein content (Fehr et al., 2003; Ogawa, Tayama, Kitamura, & Kaizuma, 1989). The genetic modification of the 11S and 7S subunit composition may improve the nutritional value and functional properties of soybean seeds. However, soybean can undergo proteome rebalancing if one or both storage proteins are missing, resulting in seeds with the standard protein content, but an altered proteome resulting from the additional accumulation of compensating proteins (Kinney, Jung, & Herman, 2001; Schmidt et al., 2011).

Lovati et al. (1992), Lovati et al. (1996) and Lovati, Manzoni, Gianazza, and Sirtori (1998) demonstrated that 7S is considerably more

*Abbreviations:* HCE, hypocholesterolemic effect; SPI, soy protein isolate; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; FF, fenofibrate; LDL, low-density lipoprotein; HCD, high-cholesterol diet; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; STD, standard diet; TG, triacylglycerol; HDL-C, high-density lipoprotein cholesterol.

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effective than 11S at enhancing low-density lipoprotein (LDL) uptake and metabolism in human liver cells (Duranti et al., 2004). In previous human and animal studies, 7S-enriched diets led to decreased serum levels of cholesterol, triglycerides, and insulin (Aoyama et al., 2001; Moriyama et al., 2004). The native 7S globulin is a randomly assorted heterotrimer comprising α′ (71 kDa), α (67 kDa), and β (50 kDa) subunits, which are the products of a multigene family (Harada, Barker, & Goldberg, 1989). Different 7S subunits have diverse HCE. Earlier research revealed that the α′-free 'Keburi' variant does not affect LDL receptor activation (Kitamura & Kaizuma, 1981; Manzoni, Lovati, Gianazza, Morita, & Sirtori, 1998). Manzoni et al. (2003) proved that the α′ subunit, and not the β subunit, is responsible for activating LDL receptors.

Soy protein isolate (SPI) is the most refined soy protein product, containing more than 90% protein on a dry weight basis. Because SPI is highly nutritious and possesses desirable functional properties, it has been widely used as an important ingredient in the food industry. Furthermore, SPI may affect the plasma cholesterol levels (Anderson, Johnstone, & Cook-Newell, 1995; Nagata, Ishiwaki, & Sugano, 1982). Processed foods made from SPI can also decrease human plasma cholesterol levels (Aoyama et al., 2000; Kito, Moriyama, Kimura, & Kambara, 1993; Liu et al., 2017).

In the present study, we developed a new hypocholesterolemic soybean cultivar, 'DND358', which has mutations eliminating the accumulation of the α subunit of 7S globulin and the G1 ($A_{1a}B_2$), G2 ($A_2B_{1a}$), and G4 ($A_5A_4B_3$) subunits of 11S globulin (i.e., Tetra Null). We have also examined the agronomic traits, amino acid quality, and isoflavone content of 'DND358' seeds. Additionally, we produced SPI beverage powder (DND358-SPI) from defatted 'DND358' soy flour, and investigated the HCE of orally administered DND358-SPI (300 mg/kg/day) in high-cholesterol diet (HCD)-induced hypercholesterolemic rats. Furthermore, fenofibrate (FF), which is a hypolipidemic and hypocholesterolemic drug, was used at 30 mg/kg/day as a reference control in the same model.

## 2. Materials and methods

### 2.1. Breeding materials

Three parents were used to develop the 'DND358' breeding population. Parent 1(female parent) was the Chinese cultivar 'Dongnong 47' ('DN47'), which is a high-oil elite soybean cultivar that contains all 7S and 11S subunits. Parent 2 (null trait donor male parent) was 'HS99B', which lacks the 7S α and α′ subunits as well as all of the 11S subunits. Parent 3 (male parent) was the 'Suinong 10' ('SN10') Chinese phytophthora blight-resistant cultivar. The genotypes of the three parents are presented in Table 1.

### 2.2. Breeding scheme

The steps involved in developing 'DND358' are depicted in Fig. S1. In this study, the initial cross involved 'DN47' as the female parent and 'HS99B' as the male parent (DN47 × HS99B). The $F_1$ generation was selfed to produce $F_2$ seeds, all of which were analyzed to select seeds with the *cgy-2/gy1/gy2/gy4* genotype (free of the 7S α and11S G1, G2, and G4 subunits; tetra null seeds). The confirmed $F_2$ tetra null seeds were used to produce the female parent for the cross with 'SN10' [(DN47 × HS99B) × SN10]. The three-way cross $F_1$ population was obtained in 2009 and then selfed to generate the three-way cross $F_2$ seeds. All hybridizations were completed in the greenhouse to minimize the possibility of contamination. To identify tetra null seeds (lacking α, G1, G2, and G4), the proteins from all $F_2$ seeds were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) according to the method described by Laemmli (1970).

A total of 33 three-way cross $F_2$ tetra null seeds were sown to produce $F_3$ strains for advanced breeding. Eighteen $F_3$ and $F_4$ lines with acceptable agronomic traits, such as flowering date, maturity date, growth habit, plant type, lodging resistance, and seed quality, were selected in 2011 and 2012. A pedigree selection method was used to identify plants that produced seeds with a yellow coat and a tetra null subunit phenotype in the $F_3$–$F_6$ generations. Selected lines from the $F_7$ and $F_8$ generations were examined regarding their agronomic trait performance, yield potential, seed protein composition, seed protein content, oil content, amino acid composition, and response to soybean disease and insect infestation. The homozygous *cgy-2/gy1/gy2/gy4* genotype for the tetra null phenotype (free of the 7S α and the11S G1, G2, and G4 subunits) was verified by an SDS-PAGE analysis of the $F_3$–$F_8$ generations.

### 2.3. Analysis of the seed protein subunit composition

A small section of the dry seed that was carefully removed manually to avoid the embryonic axis was used as the soy flour sample. Total seed proteins were extracted from the soy flour of the three parents and 'DND358' as well as from DND358-SPI using SDS sample buffer (2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 5 M urea, and 62.5 mMTris) and then centrifuged at 15,000*g*. A 10-μL aliquot of the supernatant was separated on 4.5% stacking and 12.5% separating polyacrylamide gels and stained with Coomassie Brilliant Blue R 250. Gels were scanned using the SHARP JX-330 scanner (Amersham Biosciences, Quebec, Canada). The quantitative estimation of each subunit of 7S and 11S proteins was calculated as the percentage of the area of the subunit with respect to the total 7S or 11S area.

### 2.4. Amino acid assays

The amino acid compositions of finely ground 'DN47' and'DND358' seeds as well as DND358-SPI were analyzed. The protein content was determined by calculating the nitrogen content and then multiplying by

**Table 1**
Genotypes and phenotypes for the 7S α subunit and the 11S G1 ($A_{1a}B_2$), G2 ($A_2B_{1a}$), and G4 ($A_5A_4B_3$) subunits of the three parents, 'DND358', and DND358-SPI as well as the protein contents and ratios of α′ to (7S + 11S).

| Cultivars/SPI | Genotype | | | | Phenotype[a] | | | | α'/(7S + 11S) | Protein (% ) |
|---|---|---|---|---|---|---|---|---|---|---|
| | 7S | | 11S | | Absent subunit | | | | | |
| | α *(Cgy-2/cgy-2)* | $G_1$*(Gy1/gy1)* | $G_2$ *(Gy2/gy2)* | $G_4$ *(Gy4/gy4)* | 7S | 11S | | | | |
| | | | | | α | $G_1$ | $G_2$ | $G_4$ | | |
| DN47(P1) | *Cgy-2* | *Gy1* | *Gy2* | *Gy4* | + | + | + | + | 18.33 ± 3.14[b] | 39.02 ± 0.16[c] |
| HS99B(P2) | *cgy-2* | *gy1* | *gy2* | *gy4* | – | – | – | – | 0.00 ± 0.00[c] | 44.69 ± 0.03[b] |
| SN10(P3) | *Cgy-2* | *Gy1* | *Gy2* | *Gy4* | + | + | + | + | 13.61 ± 5.47[b] | 39.17 ± 0.08[c] |
| DND358(P1/P2//P3) | *cgy-2* | *gy1* | *gy2* | *gy4* | – | – | – | – | 28.58 ± 0.85[a] | 44.53 ± 0.18[b] |
| DND358-SPI | *cgy-2* | *gy2* | *gy2* | *gy4* | – | – | – | – | 29.00 ± 0.70[a] | 83.01 ± 0.04[a] |

P1, parent 1 ('DN47'); P2, parent 2 ('HS99B'); P3, parent 3 ('SN10'); P1/P2//P3, three-way cross [(DN47 × HS99B) × SN10].
[a] + and − indicate the presence and absence of the subunits, respectively.

a conversion factor of 6.25. Total amino acids were obtained from seed meal hydrolyzed in 6 M HCl for 22 h insealed evacuated tubes in boiling water maintained at 110 °C. The L-8800 amino acid analyzer (Hitachi, Tokyo, Japan) was used to determine the amino acid composition of the hydrolysates.

Free amino acids were extracted from 5.00 g seed meal. Seed and SPI meals (seeds were sampled according to a sample quartile method, fully dried, ground using a mill grinder, filtered through a 0.25-mm sieve, and thoroughly mixed) were finely homogenized in 30 mL sulfosalicylic acid (10 g per 100 mL) and disrupted ultrasonically for 30 min. Samples were centrifuged at 5,000*g* for 5 min. The supernatant was filtered using a 22-μm GD/X sterile disposable syringe filter. The L-8800 amino acid analyzer (Hitachi) was then used to analyze the filtrate. The amino acid concentration was calculated as follows: g/16 g N in the test protein divided by g/16 g N in the scoring pattern.

### 2.5. Isoflavone analysis

The soybean sample powder was weighed and then 0.25 g was dissolved in 10 mL of 80% HPLC grade methanol in water. After oscillatingina 65 °C water bath for 2 h and cooling to room temperature, 0.3 mL 2 M sodium hydroxide solution was added. The solution was oscillated at room temperature for 10 min, after which 0.1 mL of glacial acetic acid was added as well as methanol for a final volume of 5 mL. A 0.5-mL aliquot of the supernatant was mixed with 0.4 mL water. Methanol was added again for a final volume of 1 mL. The solution was centrifuged at 1,500 rpm for 10 min. The supernatant was filtered through a 0.2-nm membrane and then transferred to liquid chromatography bottles. Each sample was prepared in triplicates. The chromatographic analysis was performed using the Inertsil ODS4 column (4.6 mm × 250 mm, 5 μm). The column temperature was 40 °C. Mobile phase A comprised water:methanol:acetic acid (44:5:1, v/v/v) and mobile phase B comprised methanol:acetic acid (49:1, v/v). The wave length was set at 260 nm. The flow rate was 1.0 mL/min and the sample volume was 10 μL.

### 2.6. Defatted soy flour preparation

DND358 seeds (harvested in Harbin, China in 2019) were heated in microwave oven 90 °C, for 2 min, and then extruded with a co-rotating twin-screw extruder. The extruded defatted soybean flakes were then further crushed into particles that passed through a 60-mesh sieve. The extruded-defatted soybean flour was kept at 4 °C in plastic bags until subsequent DND358-SPI preparation.

### 2.7. Preparation of DND358-SPI

The DND358-SPI was prepared from the extruded-defatted soybean flour via alkaline extraction (pH 8.0) followed by precipitation at pH 4.5. The precipitate was redissolved in distilled water and then neutralized to pH 7.5 using 2 M NaOH. Subsequently, the protein solution was dialyzed against distilled water at 4 °C for 48 h and lyophilized. The SPI protein content was also determined according to the Kjeldahl method (N × 6.25) using an autoanalyzer (Foss, 2300 Kjeltec Analyzer Unit; Foss Tecator AB, Höganas, Sweden) according to the manufacturer's instructions.

### 2.8. Animals, diets, and experimental protocol

Forty male Sprague-Dawley rats (5 weeks old, 220–250 g body weight) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, China. Rats were individually housed in wire-bottomed cages at 23 °C, humidity (50–60%), with a 12-h light–dark cycle. Food and water were freely accessible. After acclimatization for 1 week, one group (n = 10 per group) continued to consume the standard diet (STD); the other rats were fed a high cholesterol induced diet (Table S1) for 4 weeks, to induce hypercholesterolemic model rats. High cholesterol rats were then randomly divided into three different groups. Next, DND358-SPI and FF were administered daily to two groups by gavage at every morning at 9:00o'clock. The groups were as follows: STD, HCD, HCD + 300 mg/kg/day DND358-SPI, and HCD + 30 mg/kg/day FF (Abbott Laboratories). The DND358-SPI (300 mg/kg/day) and FF (30 mg/kg/day) solutions were dissolved in 5 mL purified water (40 mg/mL). The experiment lasted 28 days, after which the rats were immediately sacrificed (Fig. S2). The animal experiments were conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals. The experimental protocol was approved by the Laboratory Animal Ethics Committee.

### 2.9. Blood collection and analysis

At the end of weeks 1, 5, and 9, the rats were fasted overnight and then 0.5 mL blood from the postcaval vein was collected in a heparinized capillary tube containing an anticoagulant. Blood samples were immediately centrifuged at 1,900*g* for 15 min. The serum was collected and stored at −80 °C for biochemical analysis.

The serum total cholesterol (TC), triacylglycerol (TG), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) levels were measured using commercial enzymatic kits (Nanjing Jiancheng Bioengineering Institute, China). The Arteriosclerosis index (AI) was calculated using the formula: AI = (TC-HDL-C)/HDL-C.

### 2.10. Liver and adipose tissue analysis

Following the last blood sample collection at the end of week 9, all rats were sacrificed. The liver and the perirenal and epididymal white adipose tissue were excised, rinsed with cold saline, weighed, frozen, and stored at −80 °C for less than a month before the comparative analysis.

The liver and adipose tissue were embedded in paraffin and dissected into 5-μm thick sections. Samples were stained with hematoxylin and eosin and then examined using the BX53 light microscope (×200 magnification) (Olympus, Japan).

The concentrations of TC and TG in the liver were analyzed using commercial assay kits (Nanjing Jiancheng Bioengineering Institute, China).

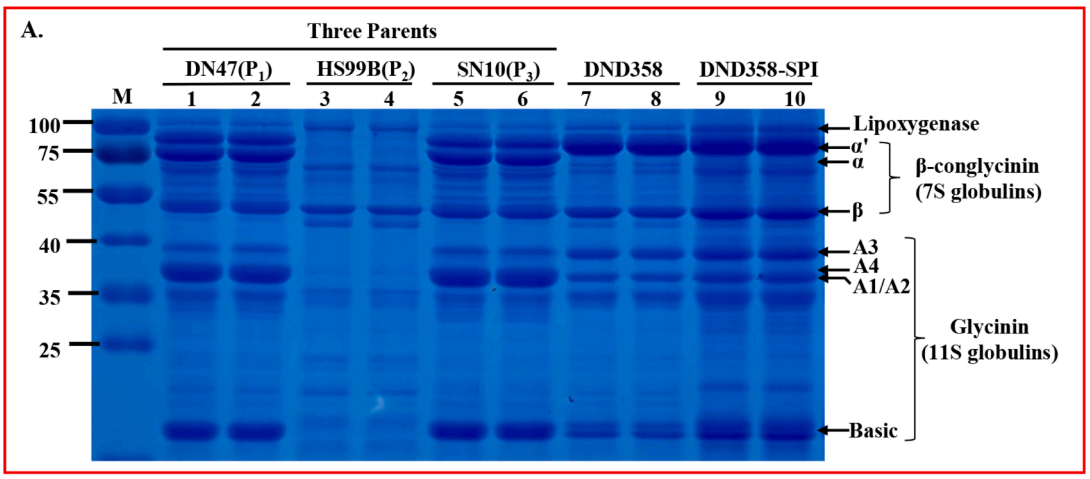
### 2.11. Statistical analysis

Data were expressed as the mean ± standard deviation (SD). Following a one-way ANOVA analysis of variance, a post hoc Least Significant Difference (LSD) test or Duncan multiple-range test was performed to assess the differences among the group means using the IBM SPSS Statistics program (version 19.0), with $p < 0.05$ set as the threshold for determining significance.

## 3. Results

### 3.1. Protein subunit composition of 'DND358' seeds and DND358-SPI

The gel electrophoretic patterns of the three parents, 'DND358', and DND358-SPI are illustrated in Fig. 1A. Both'DN47'and 'SN10'contained all 7S and 11S subunits (Fig. 1A; lanes1, 2, 5, and 6); the protein patterns were consistent with those reported earlier for soybean cultivars (Pesic, Vucelic-Radovic, Barac, & Stanojevic, 2005). 'HS99B' is a mutant line lacking the α′ and α subunits of 7S and all 11S subunits (Fig. 1A; lane 3 and 4). 'DND358' lacking the α subunit of 7S and the G1 ($A_{1a}B_2$), G2 ($A_2B_{1a}$), and G4 ($A_5A_4B_3$) subunits of 11S (Fig. 1A; lanes 7 and 8) was developed by transferring the null trait of 'HS99B' to the 'DN47' genetic background through a three-way cross (Fig. S1). The absence of the α, G1, G2, and G4 subunits (tetra null) in the'DND358' seeds resulted in the




**B. Agronomic performance of the three parents and 'DND358' under field conditions in 2019**

| Cultivars | Planting date | Maturing date | Stem height (cm) | NP/P | NS/P | SW | Yield (t/ha) |
|---|---|---|---|---|---|---|---|
| DN47 (P1) | May.6 | Oct. 9 | 80.00±3.24$^{c}$ | 37.20±3.11$^{c}$ | 94.00±7.91$^{c}$ | 21.01±0.56$^{a}$ | 2.31 |
| HS99B (P2) | May.6 | Nov. 30 | 135.00±7.91$^{a}$ | 103.60±8.79$^{a}$ | 220.80±20.39$^{a}$ | 14.00±0.30$^{d}$ | ※ |
| SN10 (P3) | May.6 | Oct. 9 | 99.20±3.90$^{b}$ | 45.60±7.09$^{b}$ | 116.60±15.14$^{b}$ | 18.30±0.47$^{b}$ | 2. 58 |
| DND358 (P1/P2//P3) | May.6 | Oct. 9 | 93.40±2.97$^{b}$ | 43.60±2.30$^{bc}$ | 104.60±3.21$^{bc}$ | 16.30±0.19$^{c}$ | 2.63 |

**Different letters in columns indicate significant differences at the 5% probability level.**


**Fig. 1.** Development of 'DND358', a new soybean cultivar which is superior for processing hypocholesterolemic soy protein isolate (DND358-SPI). A. Results of the SDS-PAGE analysis of the subunit compositions of the 7S and 11S globulins in three parents [P1, parent 1 ('DN47'); P2, parent 2 ('HS99B'); P3, parent 3 ('SN10')], 'DND358', and DND358-SPI. M: marker protein. Lanes 1 and 2: 'DN47' (normal); Lanes 3 and 4: 'HS99B' (null 7S α′ and α subunits and all 11S subunits); Lanes 5 and 6: 'SN10' (normal); Lanes 7 and 8: 'DND358' (null α, G1, G2, and G4); Lanes 9 and 10: DND358-SPI (null α, G1, G2, and G4). B. Agronomic performance of the three parents and 'DND358' under field conditions in 2019. ※ The growth period for 'HS99B' was too long to enable a field harvest. Thus, samples were harvested in a greenhouse in Heilongjiang province, China. NP/P, number of pods per plant; NS/P, number of seeds per plant; SW, 100-seed weight; P1/P2//P3, three-way cross [(DN47 × HS99B) × SN10]. C. Appearance of 'DND358' plants, seeds and its soybean protein isolate powder (DND358-SPI).

compensatory accumulation of the α′ subunit of 7S (Table1). The analysis of the progeny seeds by SDS-PAGE throughout the breeding process revealed that the 'DND358' tetra null (lacking α, G1, G2, and G4) and α′-enriched phenotypes are inherited traits and are independent of the environment (Fig. S1).

The DND358-SPI beverage powder was prepared from defatted 'DND358' soy flour. The protein profile of 'DND358' was similar to that of DND358-SPI (Fig. 1.A; lanes 7 and 8 compared with lanes 9 and 10). Thus, there were no major changes to the DND358-SPI protein subunit components. However, the protein subunit band area and density on the gels indicated that the α′ subunit was enriched in DND358-SPI (Table 1 and Fig. 1A; lanes 9 and 10). Furthermore, despite the absence of these abundant seed proteins, the overall seed protein content of 'DND358' (44.53%) and DND358-SPI (83.01%) were unaffected (Table 1).

### 3.2. Agronomic performance of 'DND358'

The agronomic performance of 'DND358' is summarized in Fig. 1B and C. Despite the lack of α, G1, G2, and G4 subunits, 'DND358' seeds germinated, and the resulting seedlings grew, flowered, and reproduced normally under field conditions. Additionally, the marked changes to the seed protein composition did not adversely affect the total protein content, seed yield, maturity, and oil concentration. These results indicate that the tetra null mutation does not affect these traits.

The 'DND358'plants (Fig. 1C) had white flowers, an indeterminate growth habit, oval leaves, a yellow seed coat, a yellow hilum, and tan pods at maturity. The cotyledon of mature seeds was yellow. The 'DND358' stem was 93.40 cm long. The 'DND358' 100-seed weight and yield were 16.30 g and 2.63 t/ha, respectively. The number of pods and the number of seeds per 'DND358'plant were 43.60 and 104.60, respectively (Fig. 1B). Furthermore, 'DND358' has been registered as a new soybean cultivar (registration number: Heishendou20210046; registration date: June 11, 2021) by the Department of Agriculture and Rural Affairs of Heilongjiang Province, China.

### 3.3. Amino acid analysis

A comparison of the amino acid content of the three parents, 'DND358', and DND358-SPI revealed the crude protein content (Table 1), total essential amino acid content, total sulfur-containing (methionine and cysteine) amino acid content, and total amino acid content (Table 2) were respectively 14.12%, 7.91%, 8.51%, and 9.05% higherin 'DND358' than in 'DN47'. Additionally, the arginine concent ration increased by 51.28% in 'DND358' seeds. Significant increases in the valine (10.27%), lysine (8.45%), phenylalanine (9.20%), and threonine (10.00%) concentration of 'DND358' seeds resulted in a significant increase in the total essential amino acid content. The total sulfur-containing (methionine and cysteine) amino acid concentration increased significantly in 'DND358' because of a significant increase in cysteine production. The total amino acid concentration also increased in 'DND358' seeds because of a general increase in the abundance of most amino acids (Table 2). These changes in the total amino acid composition in 'DND358' appear to be related to the maintenance of the nitrogen content.

Because DND358-SPI was prepared from 'DND358', its nutritional quality and functionality depend on the original 'DND358' traits. The total amino acid composition of DND358-SPI was similar to that of"DND358'. All amino acids were significantly enriched in DND358-SPI (Table 2).

We next analyzed the free amino acid content of the three parents and 'DND358' seeds (Table S2). The free amino acid content was 2.17-times higher in the 'DND358' seeds than in the 'DN47' seeds. Free arginine represented more than two-thirds of the total free amino acid content of 'DND358', and was 6.47-times higher in 'DND358' seeds than in the 'DN47' seeds. Thus, the tetra null mutation in 'DND358' significantly affected the seed free amino acid composition, especially the free arginine content (TableS2).

**Table 2**
Amino acid (A.A.) compositions of mature seeds of the three parents [P1, parent 1 ('DN47'); P2, parent 2 ('HS99B'); P3, parent 3 ('SN10')],'DND358' grown in field trial (2019) and DND358-SPI processed from 'DND358'.

| A.A. (%) | Cultivars and DND358-SPI | | | | |
|---|---|---|---|---|---|
| | DN47 | HS99B | SN10 | DND358 | DND358-SPI |
| Essential amino acid | | | | | |
| Met | 0.44 ± 0.01$^{c}$ | 0.65 ± 0.04$^{b}$ | 0.45 ± 0.03$^{c}$ | 0.46 ± 0.01$^{c}$ | 1.02 ± 0.02$^{a}$ |
| Val | 1.46 ± 0.04$^{d}$ | 1.85 ± 0.09$^{b}$ | 1.49 ± 0.05$^{cd}$ | 1.61 ± 0.04$^{c}$ | 3.24 ± 0.09$^{a}$ |
| Lys | 2.13 ± 0.06$^{d}$ | 2.47 ± 0.10$^{b}$ | 2.22 ± 0.05$^{cd}$ | 2.31 ± 0.08$^{c}$ | 4.81 ± 0.12$^{a}$ |
| Ile | 1.38 ± 0.04$^{c}$ | 1.59 ± 0.11$^{b}$ | 1.43 ± 0.03$^{c}$ | 1.45 ± 0.05$^{c}$ | 3.46 ± 0.08$^{a}$ |
| Phe | 1.63 ± 0.05$^{c}$ | 1.93 ± 0.12$^{b}$ | 1.68 ± 0.04$^{c}$ | 1.78 ± 0.05$^{bc}$ | 4.07 ± 0.11$^{a}$ |
| Leu | 2.53 ± 0.08$^{c}$ | 2.94 ± 0.08$^{b}$ | 2.70 ± 0.10$^{c}$ | 2.69 ± 0.09$^{c}$ | 6.46 ± 0.19$^{a}$ |
| Thr | 1.30 ± 0.05$^{d}$ | 1.78 ± 0.02$^{b}$ | 1.30 ± 0.05$^{d}$ | 1.43 ± 0.04$^{c}$ | 2.82 ± 0.09$^{a}$ |
| Total EAA | 10.87 ± 0.33$^{d}$ | 13.21 ± 0.41$^{b}$ | 11.28 ± 0.32$^{cd}$ | 11.73 ± 0.36$^{c}$ | 25.89 ± 0.70$^{a}$ |
| Non-essential amino acid | | | | | |
| Asp | 3.56 ± 0.11$^{c}$ | 4.45 ± 0.11$^{b}$ | 3.74 ± 0.08$^{c}$ | 3.77 ± 0.12$^{c}$ | 8.31 ± 0.22$^{a}$ |
| Ser | 1.72 ± 0.05$^{c}$ | 1.96 ± 0.07$^{b}$ | 1.81 ± 0.08$^{c}$ | 1.84 ± 0.05$^{bc}$ | 4.12 ± 0.09$^{a}$ |
| Glu | 6.15 ± 0.19$^{bc}$ | 5.37 ± 0.05$^{d}$ | 6.54 ± 0.26$^{b}$ | 6.01 ± 0.18$^{c}$ | 14.74 ± 0.40$^{a}$ |
| Gly | 1.33 ± 0.04$^{d}$ | 1.68 ± 0.03$^{b}$ | 1.36 ± 0.02$^{cd}$ | 1.43 ± 0.05$^{c}$ | 2.97 ± 0.08$^{a}$ |
| Ala | 1.39 ± 0.04$^{c}$ | 1.81 ± 0.02$^{b}$ | 1.43 ± 0.07$^{c}$ | 1.48 ± 0.04$^{c}$ | 2.89 ± 0.08$^{a}$ |
| Cys | 0.49 ± 0.01$^{b}$ | 0.81 ± 0.11$^{a}$ | 0.47 ± 0.02$^{b}$ | 0.55 ± 0.01$^{b}$ | 0.86 ± 0.02$^{a}$ |
| Tyr | 0.94 ± 0.03$^{c}$ | 1.21 ± 0.07$^{b}$ | 1.00 ± 0.02$^{c}$ | 1.02 ± 0.03$^{c}$ | 2.85 ± 0.09$^{a}$ |
| His | 0.83 ± 0.03$^{d}$ | 1.15 ± 0.02$^{b}$ | 0.89 ± 0.04$^{d}$ | 1.05 ± 0.03$^{c}$ | 2.35 ± 0.06$^{a}$ |
| Arg | 2.34 ± 0.08$^{d}$ | 5.06 ± 0.40$^{b}$ | 2.54 ± 0.07$^{d}$ | 3.54 ± 0.11$^{c}$ | 5.99 ± 0.17$^{a}$ |
| Pro | 1.67 ± 0.04$^{b}$ | 1.47 ± 0.06$^{c}$ | 1.77 ± 0.04$^{b}$ | 1.69 ± 0.04$^{b}$ | 3.87 ± 0.10$^{a}$ |
| Total SAA | 0.94 ± 0.02$^{c}$ | 1.46 ± 0.15$^{b}$ | 0.92 ± 0.04$^{c}$ | 1.02 ± 0.02$^{c}$ | 1.88 ± 0.03$^{a}$ |
| Total AA | 31.28 ± 0.93$^{d}$ | 38.19 ± 0.67$^{b}$ | 32.83 ± 0.92$^{cd}$ | 34.11 ± 1.03$^{c}$ | 74.84 ± 1.96$^{a}$ |

Letters indicate statically significant ($p < 0.05$) differences among average values within a row (Duncan multiple-range test). AA, amino acids; EAA, essential amino acid; SAA, sulfur-containing amino acid (Met + Cys).

### 3.4. Isoflavone analysis

To investigate whether the loss of abundant seed proteins and SPI-processing altered the isoflavone content, we compared the isoflavones of 'DN47', 'DND358', and DND358-SPI. As shown in Table 3, genistein (48.78% and 46.83% in 'DN47' and 'DND358', respectively) accounted for most of the isoflavone content. In contrast, glyciteinaccounted for only 11.63% and 11.21% of the isoflavone content of 'DN47' and 'DND358' mature seeds, respectively. The daidzein, glycitein, genistein, and total isoflavone contents were respectively16.03%, 5.48%, 5.09%, and 9.47% higher in 'DND358' seeds, which lacked the α, G1, G2, and G4 subunits, than in'DN47' seeds.

To prepare DND358-SPI, soluble proteins were extracted from defatted 'DND358' soy flour via dilute alkali extraction, which was followed by precipitation at the isoelectric point, neutralization, and drying of the protein fraction. This processing resulted in a significant increase in the glycitein, genistein, and total isoflavone content in DND358-SPI, but decrease in the daidzein contents (Table 3).

### 3.5. Effects of the ingestion of DND358-SPI on serum TC, TG, LDL-C, HDL-C levels and AI value

To examine the HCE of DND358-SPI, we first constructed hypercholesterol model rats (Fig. S2). High cholesterol rats were randomly divided into HCD, HCD + FF, and HCD + DND358-SPI three different groups. There were no statistical differences in body weight among groups. As shown in Fig. 2, compared to the control STD group, high-cholesterol-induced rats showed significantly higher TC, TG, and LDL-C levels, and significantly lower HDL-C level in the serum ($p < 0.05$). Before treatment, no significant difference in levels of TC, TG, LDL-C and HDL-C were found among hypercholesterol model rats (Fig. 2).

The hypercholesterolemic model rats were daily administered by gavage with DND358-SPI (300 mg/kg/day) or FF, a hypocholesterolemic drug (30 mg/kg/day). DND358-SPI and FF treatments showed the similar trends of upward and downward of serum parameters (Fig. 2). Both in DND358-SPI and FF treatments, significant decrease in serum TC, TG, and LDL-C levels, but significant elevated HDL-C levels

**Table 3**
Isoflavone contents in 'DN47' and 'DND358' seeds and DND358-SPI in 2019.

| Cultivar/ SPI | Daidzein (μg/g) | Glycitein (μg/g) | Genistein (μg/g) | Total isoflavone (μg/g) |
|---|---|---|---|---|
| DN47 | 677.03 ± 1.53$^{c}$ | 198.90 ± 0.62$^{c}$ | 834.27 ± 2.60$^{c}$ | 1710.27 ± 3.96$^{c}$ |
| DND358 | 785.53 ± 0.32$^{a}$ | 209.80 ± 0.17$^{b}$ | 876.77 ± 1.66$^{b}$ | 1872.17 ± 2.02$^{b}$ |
| DND358-SPI | 760.37 ± 0.97$^{b}$ | 228.10 ± 0.61$^{a}$ | 1511.70 ± 0.44$^{a}$ | 2500.13 ± 1.62$^{a}$ |

Different letters in columns indicate significant differences at the 5% probability level as determined by Duncan multiple-range test.

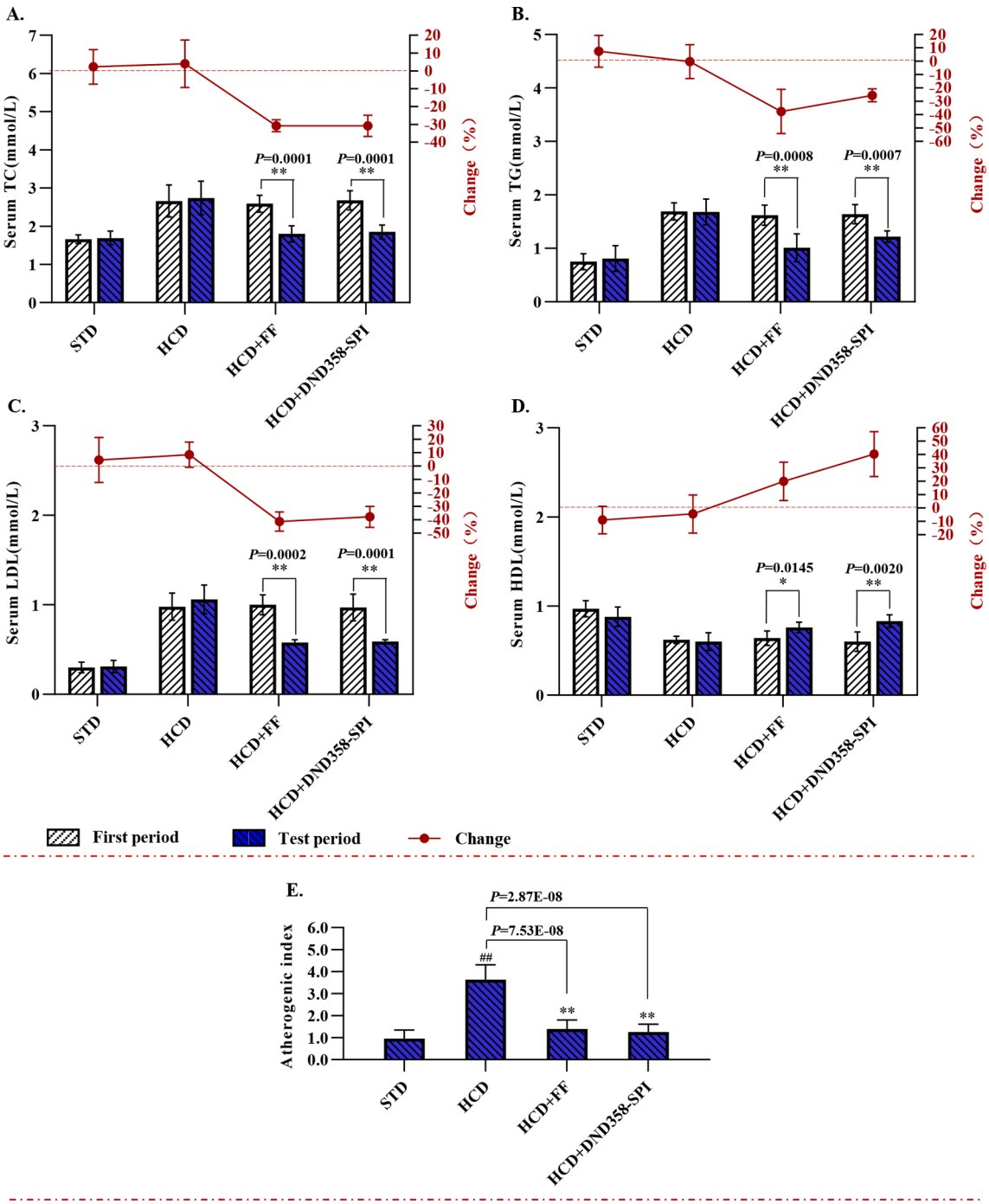



**Fig. 2.** Changes in the serum total cholesterol (TC) (A), triacylglycerol (TG) (B), low-density lipoprotein cholesterol (LDL-C) (C), high-density lipoprotein cholesterol (HDL-C) (D) levels, and atherogenic index (AI) (E) during the fenofibrate (FF) and DND358-SPI administered by gavage for 28 days to moderately hypercholesterolemic rats. STD: standard diet; HCD: high-cholesterol diet; FF: fenofibrate; DND358-SPI: soy protein isolate prepared from 'DND358' seeds (null 7S α and 11S G1, G2, and G4 subunits). Each bars represents the mean ± SD of 6 rats. Mean values were analyzed using Least Significant Difference (LSD) at a significance level of 0.05. #P < 0.05 versus STD group, ##P < 0.01 versus STD group; *P < 0.05 versus HCD group, **P < 0.01 versus HCD group;

were observed by the end of the 28-day treatment (Fig. 2).

The serum TC, TG and LDL-C levels respectively decreased by 30.97%, 25.61% and 39.18% in rats treated with DND358-SPI, but by 30.50%, 37.65% and 42.05% in the rats treated with FF, respectively (Fig. 2A, B and C). Thus, DND358-SPI and FF had similar effects on the serum TC and LDL-C levels (Fig. 2A and C). Additionally, a substantial increase (38.33%) in the serum HDL-C level was detected in the DND358-SPI-fed group, which was almost 2-times of the corresponding increase (18.75%) induced by FF (Fig. 2D). Hence, a diet that includes DND358-SPI may be useful for significantly increasing the serum HDL-C content in hypercholesterolemic rats.

To better elucidate the access accuracy, we further examined the atherogenic index (AI) of serum, a possible indicator of coronary heart disease risk (Buzzetti, Pinzani, & Tsochatzis, 2016), which is calculated by subtracting HDL-C from TC and dividing the product by HDL-C in serum. As shown in Fig. 2E, both treatment with FF or DND358-SPI seemed to have positive impact on AI, and the reduction of AI reached statistical significance by 61.81% and 65.66%, respectively (Fig. 2E, P <

0.01).

### 3.6. Effects of the ingestion of DND358-SPI on the histological characteristics of the liver and adipose tissue

The STD rats had normal liver and adipose tissue structures (Fig. 3A and E). In contrast, the hepatocytes in the HCD group had white fat granules and large fat vacuoles that partly infiltrated the inflammatory cells (Fig. 3B compared with A), reflecting extensive steatosis induced by the HCD. Moreover, compared with the STD group, the HCD group had clearly hypertrophic adipocytes (Fig. 3E compared with F).

Histological changes were observed after a 28-day treatment with DND358-SPI or FF. The administration of DND358-SPI markedly decreased the number of fat granules and fatvacuoles in hepatocytes (Fig. 3C) as well as the size of adipocytes (Fig. 3G). Our data indicate that DND358-SPI inhibited hepatic lipid accumulation (Fig. 3B compared with C) and suppressed the growth of white adipose tissue (Fig. 3F compared with G), similar to the effects of FF in hypercholesterolemic rats (Fig. 3D and H).

### 3.7. Effects of the ingestion of DND358-SPI on liver TC, TG levels

The liver TC, TG levels and AI value of tested rats are shown in Fig. 4. In this study, the liver TC, TG levels in the HCD group were significantly higher than those in the STD group (Fig. 4A and B, $P < 0.01$), showing that the rat experimental hypercholesterolemic models was successful. Compared to the HCD group, the liver levels for FF and DND358-SPI treatment groups were significantly decreased by 42.44% and 22.93% for TC (Fig. 4A), by 34.25% and 30.82% for TG (Fig. 4B), respectively, showing that FF and DND358-SPI administration effectively decreased liver TC and TG levels in hypercholesterolemic model rats.

## 4. Discussion

### 4.1. Subunit compositions and their association with the cholesterol-lowering effect

Previous studies confirmed that 7S is much more effective than 11S at increasing LDL uptake and metabolism in human liver cells, but the 7S subunits vary in terms of their cholesterol-lowering effects (Adams et al., 2004; Aoyama et al., 2001; Knopp, 1999; Lovati et al., 1992; Moriyama et al., 2004). Duranti et al. (2004) demonstrated that the α′ subunit of the soybean 7S globulin is critical for the cholesterol homeostasis of Hep G2 cells (i.e., a human hepatoma cell line). They proved for the first time that a dietary α′ subunit may be as effective as the hypolipidemic drug clofibrate. The α′ subunit lowered plasma lipid levels and activated liver β-VLDL receptors in rats fed a hypercholesterolemic diet. Because the α′ subunit is responsible for the lipid-lowering effects of soybean proteins, there may be an increasing market demand for α′-rich soybean cultivars.

Many studies revealed the remarkable plasticity in the soybean seed proteome (Jenkinson & Fehr, 2010; Kinney et al., 2001; Schmidt et al., 2011; Takahashi et al., 2003). The 11S globulin accounts for about 40% of the total seed protein content (Nielsen et al., 1989) and is composed of the following five subunits: G1($A_{1a}B_2$), G2($A_2B_{1a}$), G3($A_{1b}B_{1b}$), G4 ($A_5A_4B_3$), and G5($A_3B_4$), which are encoded by five major genes (*Gy1* to *Gy5*) (Nielsen et al., 1989). The 7S globulin accounts for 25% of the total seed protein content and is composed of the following three subunits: α′, α, and β. Despite the considerable abundance of these proteins in seeds, all five 11S and all three major 7S subunits are dispensable; null lines lacking 11S and 7Ssubunitscan grow and reproduce relatively normally (Fehr et al., 2003; Ogawa et al., 1989; Takahashi et al., 2003). Manipulating the variant storage protein subunit null alleles identified to date has enabled the breeding of soybean varieties with a markedly modified protein composition (Ogawa et al., 1989; Song et al., 2014, 2016; Song, Oehrle, Liu, & Krishnan, 2018).

In the present study, we have developed a new soybean cultivar, 'DND358', with recessive null alleles (*cgy-2/gy1/gy2/gy4*) for the 7S α subunit and the 11S G1 ($A_{1a}B_2$), G2 ($A_2B_{1a}$), and G4 ($A_5A_4B_3$) subunits (i.e., Tetra Null). The 7S α subunit and the 11S acidic subunits $A_{1a}$, $A_{1b}$, $A_2$, $A_3$, and $A_4$ are allergenic (Djurtoft, Pedersen, Aabin, & Barkholt, 1991; Krishnan, Kim, Jang, & Kerley, 2009; Zeece, Beardslee, Markwell, & Sarath, 1999). The elimination of undesirable allergenic subunits via genetic manipulation may improve soy protein safety and increase the nutritional value of mutant seeds. In our study, stacking recessive null alleles for the α, G1, G2, and G4 subunits resulted in enhanced accumulation of the α′α′ subunits of 7S (Table 1 and Fig. 1). The removal of

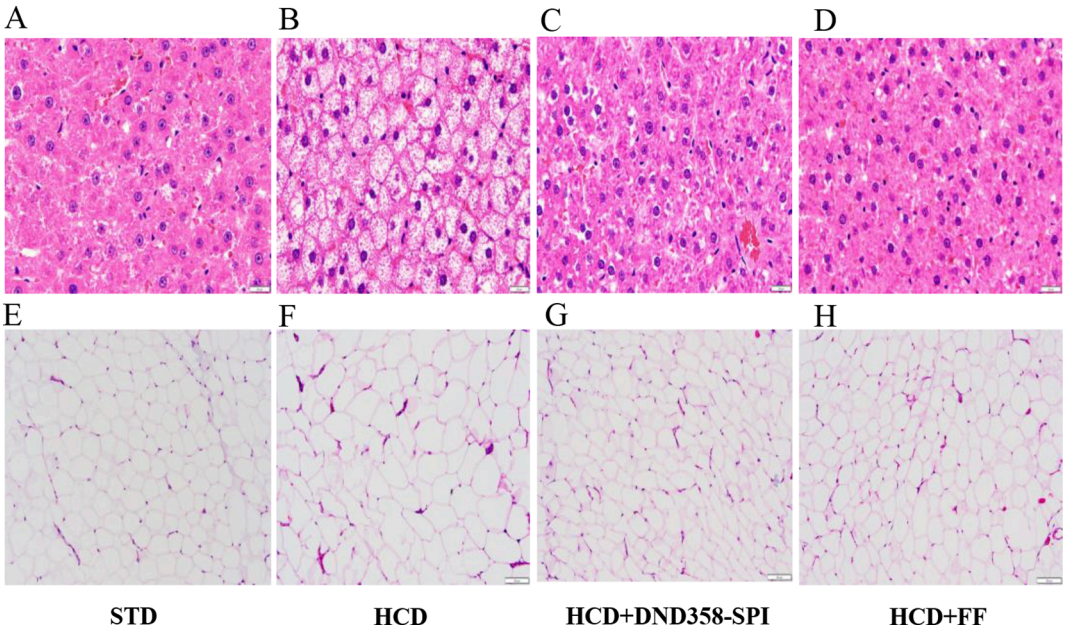



**Fig. 3.** Hematoxylin and eosin (HE) staining for the histological examination of the liver and adipose tissue from four groups of hypercholesterolemic rats (×200 magnification) after a 4-week treatment. Liver histological changes were observed after a 28-day fenofibrate or DND358-SPI treatment (A–D). White adipose tissue histological changes (E–H). STD, standard diet; HCD, high-cholesterol diet; DND358-SPI, soy protein isolate prepared from 'DND358' soybean (null 7S α and 11S G1, G2, and G4 subunits); FF, fenofibrate.

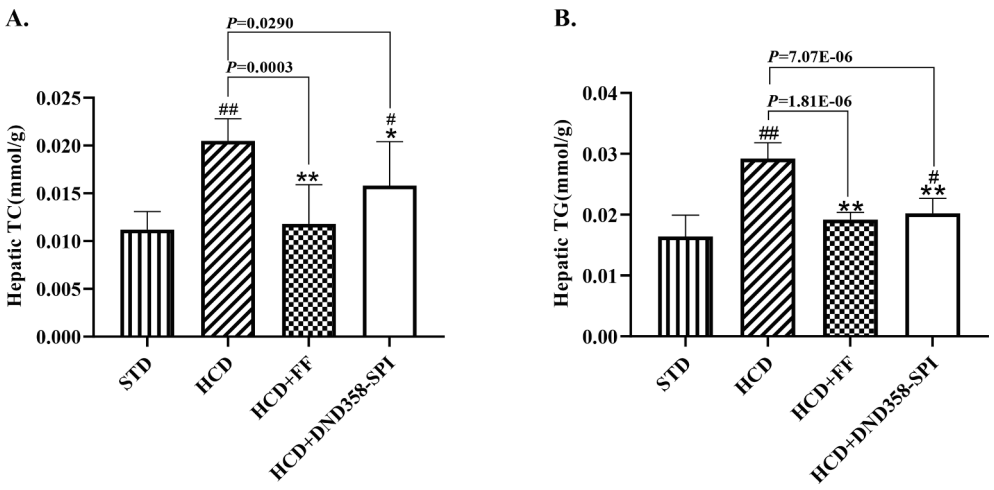



**Fig. 4.** Effects of daily administration of DND358-SPI (300 mg/kg/day) or Fenofibrate (FF) (30 mg/kg/day) for 28 days on hepatic total cholesterol, TC (A), triacylglycerol, TG (B). Each bars represents the mean ± SD of 6 rats. Mean values were analyzed using Least Significant Difference (LSD) at a significance level of 0.05. $^{\#}P < 0.05$ versus STD group, $^{\#\#}P < 0.01$ versus STD group; $^{*}P < 0.05$ versus HCD group, $^{**}P < 0.01$ versus HCD group; STD = standard diet; HCD = high-cholesterol diet; FF = fenofibrate; DND358-SPI = soy protein isolate prepared from 'DND358' soybean (null 7S α and 11S G1, G2, and G4 subunits).

the α, G1, G2, and G4 subunits through the use of the respective null alleles and the resulting protein subunit compositions are important determinants of the hypocholesterolemic effect of 'DND358'.We speculated that unique bioactive peptide derived from DND358-SPI associated with its peculiar tetra null characteristic may be partly responsible for the HCE found in DND358-SPI (Kim, Yang, & Kim, 2021).

### 4.2. Amino acid compositions and their cholesterol-lowering effects

Soy amino acids have a high nutritive value, but they also have several health benefits. For example, they can decrease the blood cholesterol level (Potter, 1995) and minimize the risk of coronary heart disease (Anderson et al., 1995), while also contributing to anti-obesity treatments (Allison et al., 2003). Kurowska and Carroll (1992, 1994) reported that arginine can decrease the plasma TC level in rabbits, whereas methionine and lysine have the opposite effects.

Soybean seeds usually contain relatively low levels of free amino acids. Instead, they accumulate amino acids in the form of storage proteins. However, some recently reported results indicate that a decrease in the amount of storage proteins is compensated by the activation of an alternative nitrogen assimilation pathway, resulting in the accumulation of large amounts of free amino acids in soybean seeds. More specifically, overproducing free arginine seems to be a common strategy for addressing the decreased nitrogen content due to changes in the soybean 7S and11S globulins (Schmidt et al., 2011; Song et al., 2014, 2016, 2018; Takahashi et al., 2003).

Arginine is an important amino acid in plants. It serves as an important nitrogen resource and participates in nitrogen recycling. The amino acid analysis in the present study revealed that more than two-thirds of the total free amino acid content of the 'DND358' seeds was free arginine. Moreover, the free arginine content was 6.74-times greater in the 'DND358' seeds than in the 'DN47' seeds (Table 3). Compared with the 'DND358' raw soy flour, DND358-SPI refined from 'DND358' had a similar amino acid composition profile, with a 155.98% and 143.16% increase in the seed arginine and total amino acid content, respectively (Table 3). Hence, the tetra null mutation (absence of the α, G1, G2, and G4 subunits) in 'DND358' was compensated by large adjustments in the amino acid composition, especially anincrease in thearginine content. The high arginine content likely contributes to the serum cholesterol lowering activity of DND358-SPI.

### 4.3. Isoflavone contents and their cholesterol-lowering effects

Soy flour contains several components with hypocholesterolemic effects, including proteins, isoflavones, dietary fiber, and lecithin (Kirk et al., 1998). Although the mechanism underlying the decrease in serum cholesterol levels has not been determined, atleast two hypotheses have been proposed. Specifically, the protein components (mainly 7S and 11S globulins or fragments thereof) may directly mediate the decrease or the isoflavones in soybean (daidzein and genistein) may be involved. Messina and Barnes (1991) proved that SPI is a rich source of isoflavones. Several reports suggest that the isoflavones in SPI may have cholesterol-lowering effects (Anthony, Clarkson, Hughes, Morgan, & Burke, 1996; Tovar-Palacio, Potter, Hafermann, & Shay, 1998).

The major soybean isoflavones glycitein, genistein, and daidzeinwere originally identified several decades ago (Walter, 1941). Genistein and daidzein may be responsible for the hypocholesterolemic activity of dietary soy protein. In the present study, the traditional SPI processing resulted in a significant increaseinthe genistin and total isoflavone contents (Table 3). In summary, our data imply that the enriched α′, arginine, and isoflavone contents in DND358-SPI cooperatively contribute to the hypocholesterolemic effect.

### 4.4. Inclusion of DND358-SPI in the diet maybe a feasible way to achieve cholesterol homeostasis

Traditionally, the atherogenic lipid profile is made of increase of TC, TG, LDL-C, and decreased HDL-C. HDL is the major lipoprotein responsible for transporting cholesterol from the periphery back to the liver for clearance (Toth, 2003). The association of high HDL-C concentrations with decreased risk of coronary heart disease (CHD) was first reported more than 40 years ago, and has since been confirmed in numerous population studies (Castelli et al., 1977; Gordon et al., 1989; Ouimet, Barrett, & Fisher, 2019; Colombo et al., 2021). The HDL-C concentration is regarded as an index of the cardio-protective roles of HDL, and clinical trials reported negative correlations between CHD risk and HDL-C (Rosenson et al., 2013; Karathanasis, Freeman, Gordon, & Remaley, 2017). Moreover, recent studies have suggested that the atherogenic index (AI) value can reflect the balance between atherosclerosis and anti-atherosclerosis lipoprotein and the status of cardiovascular events, and may be more appropriateto assess the relative contribution of lipids to the coronary heart disease risk (Kinosian, Glick, & Garland, 1994; Elshazly et al., 2015;Nam et al., 2020; Won et al., 2021).

In the present study, compared with HCD group, the daily administration of DND358-SPI resulted in a significant reduction in the serum levels of TC, TG and LDL-C, attaining the similar levels obtained with FF therapy (Fig. 2). Moreover, the oral administration of DND358-SPI decreased hepatic lipid accumulation and suppressed the growth of white adipose tissues to similar degree to those fed on FF in hypercholesterolemic rats (Fig. 3). Furthermore, DND358-SPI produced a considerable increase of serum HDL-C (increased by 38.33%), the

changes in HDL-C levels were found to be significantly higher than that of FF drug treatment (increased by 18.75%) (Fig. 2D), which consequently resulted in the value of AI in DND358-SPI group was even lower than that measured in the FF drug therapy (Fig. 4C) ($p < 0.05$). Taken together, all these data suggested that DND358-SPI exerts strong cholesterol-lowering effect across the lipid profile, therefore can be used for cholesterol-lowering dietary therapy. Additional investigation is needed to explain the mechanism of the cholesterol-lowering effect of DND358-SPI.

Soybean contains proteins and peptides with biological activity on plasma cholesterol levels and this property makes soy proteins a functional food (Caponio, Wang, Di Ciaula, De Angelis, & Portincasa, 2020; Kim et al., 2021). The US Food and Drug Administration had approved the labeling of food products with the health benefits of the daily consumption of 25 g soy protein, including 5 g 7S globulin (Samoto et al., 2007). However, the long-term consumption of large amounts of unpalatable soybean proteins is unlikely for most people. On the basis of the findings presented herein, we suggest that including DND358-SPI in the diet may be a more realistic way for people to ingest substantial amounts of soy proteins. The DND358-SPI used in this study was obtained by directly processing 'DND358' soybean flour to produce a soymilk-like beverage powder (Fig. 2D). Advantages of DND358-SPI include the fact that (1) it concentrated cholesterol-lowering functional components α′ subunit, arginine, and isoflavone content, (2) it possesses a hypocholesterolemic effect similar to FF, and (3) it can be handled and incorporated in diets relatively easily (Fig. 2C).

Thus, DND358-SPI may become a widely consumed concentrated, convenient, and functional soybean dietary nutraceutical that induces cholesterol homeostasis. We are currently focused on examining the effect of HCE of DND358-SPI on cardiovascular patients and committed to improving its taste so that flavored DND358-SPI can be used as a commercially available hypocholesterolemic dietary therapeutic SPI beverage, particularly in patients who are nonresponsive or intolerant to drug therapy.

## Ethical statement

The animal experiments were conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals. The experimental protocol was approved by the Laboratory Animal Ethics Committee.

### *CRediT authorship contribution statement*

**Bo Song:** Project administration. **Zhendong Qiu:** Data curation. **Mingxue Li:** Investigation. **Tingting Luo:** Formal analysis. **Qi Wu:** Formal analysis. **Hari B. Krishnan:** Writing – review & editing. **Junjiang Wu:** Data curation. **Pengfei Xu:** Data curation. **Shuzhen Zhang:** Funding acquisition. **Shanshan Liu:** Conceptualization, Writing – original draft.

## 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.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2022.104979.

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