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Plant-based polyphenol rich protein supplementation attenuated skeletal muscle loss and lowered the LDL level *via* gut microbiota remodeling in Taiwan's community-dwelling elderly†

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Sarcopenia, characterized by muscle loss, negatively affects the elderly's physical activity and survival. Enhancing protein and polyphenol intake, possibly through the supplementation of fermented black soybean koji product (BSKP), may alleviate sarcopenia by addressing anabolic deficiencies and gut microbiota dysbiosis because of high contents of polyphenols and protein in BSKP. This study aimed to examine the effects of long-term supplementation with BSKP on mitigating sarcopenia in the elderly and the underlying mechanisms. BSKP was given to 46 participants over 65 years old with early sarcopenia daily for 10 weeks. The participants' physical condition, serum biochemistry, inflammatory cytokines, antioxidant activities, microbiota composition, and metabolites in feces were evaluated both before and after the intervention period. BSKP supplementation significantly increased the appendicular skeletal muscle mass index and decreased the low-density lipoprotein level. BSKP did not significantly alter the levels of inflammatory factors, but significantly increased the activity of antioxidant enzymes. BSKP changed the beta diversity of gut microbiota and enhanced the relative abundance of *Ruminococcaceae_UCG_013*, *Lactobacillus_murinus*, *Algibacter*, *Bacillus*, *Gordonibacter*, *Porphyromonas*, and *Prevotella_6*. Moreover, BSKP decreased the abundance of *Akkermansia* and increased the fecal levels of butyric acid. Positive correlations were observed between the relative abundance of BSKP-enriched bacteria and the levels of serum antioxidant enzymes and fecal short chain fatty acids (SCFAs), and *Gordonibacter* correlated negatively with serum low-density lipoprotein. In summary, BSKP attenuated age-related sarcopenia by inducing antioxidant enzymes and SCFAs *via* gut microbiota regulation. Therefore, BSKP holds potential as a high-quality nutrient source for Taiwan's elderly, especially in conditions such as sarcopenia.

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Introduction

The number of older people is predicted to increase dramatically in the coming decades worldwide. The global population of over 65-year-old individuals is expected to increase by approximately 10% to reach 2.1 billion by 2025.[1] Aging-related loss of muscle function is associated with significant alterations to the skeletal muscle structure and function; these processes usually begin in the fourth decade of life and are highly prevalent in the sixth decade of life.[2] Sarcopenia is a multifactorial disease characterized by wasting of muscle fibers, which causes loss of balance, higher morbidity and frailty, limits individuals' ability to remain physically active, and can subsequently lead to disability and dependency on others.[3] Sarcopenia has been related to aging, chronic inflammation, insufficient nutrition, and physical inactivity. Sarcopenia typically develops slowly and the extent of functional loss varies significantly; however, sarcopenia is observed in all aging humans.[4]

A previous study demonstrated that appropriate protein intake and physical activity (PA) may benefit individuals with sarcopenia.[5] A low protein intake can affect muscle mass and lead to a reduction in muscle strength, whereas an appropriate protein intake may prevent or delay sarcopenia. Aging people can especially benefit from the skeletal muscle anabolic effects of dietary protein; therefore, increasing protein intake above the recommended dietary allowance (RDA) of 0.8 g kg^{-1} day^{-1} is considered a valuable strategy to counteract the gradual loss of muscle and increased appetite,[6] which is a major feature of sarcopenic obesity. Thus, development of strategies to help the elderly consume the required amount of dietary protein may lead to a reduction in muscle loss and therefore slow the progression of sarcopenia in the aging population.

Several recent studies reported that polyphenols could reduce the loss of muscle mass in cancer cachexia or due to acute inflammation promoted by a section of the sciatic nerve.[7] The structural diversity of polyphenols leads to varied bioavailability, metabolism, and bioactivities.[8] Phenolic acids, stilbenes, lignans, flavonoids (flavonols, flavones, flavanones, flavonols, isoflavones, and anthocyanidins), and secoiridoids are the key groups of polyphenols.[9] Most dietary polyphenols exist as glycosides (especially flavonoids), *i.e.*, the polyphenols are conjugated to sugars such as glucose, galactose, rhamnose, or rutinose. Moreover, hydroxycinnamic acids are typically esterified to organic acids, sugars, or lipids. However, conjugated or polymeric polyphenols generally have low bioavailability and need to be metabolized to aglycones to be absorbed. This process can be catalyzed by intestinal mucosal enzymes for glucosides; however, the majority of esters and conjugates are hydrolyzed by the microbiota in the colon.[9] Moreover, many studies have reported that dietary polyphenols can modulate the composition of the gut microbiota, and in turn, the gut microbiota can induce the release of bioactive metabolites by metabolizing polyphenols.[10]

The gut microbiota influences human physiology *via* multiple processes, including the regulation of nutrient absorption, inflammatory processes and immune function, oxidative stress, and the anabolic balance.[11] Gut microbiota dysbiosis and sarcopenia commonly occur in the elderly. Gut microbiota dysbiosis is frequently observed in age-related systemic inflammation, which leads to lower muscle function and higher secretion of pro-inflammatory cytokines.[11] Dysregulation of the gut microbiota may induce or promote the progression of sarcopenia and obesity by altering the expression of myostatin and atrogin-1[12] and causing dysfunction in the signaling that occurs between the enteric nervous system and the brain.[13] In turn, these changes negatively impact muscle mass and appetite. Moreover, the gut microbiota was reported to employ SCFAs to benefit the host through several signaling pathways associated with the gut–brain[14] and gut–muscle axes.[15]

Black soybean has long been employed to prepare traditional fermented food products in Eastern Asia such as in-yu and tou si, which are the dried by-products of mashed black soybean sauce. These products provide a plentiful and inexpensive supply of protein and calories. In addition, both the groups of Ribeiro and Salvadori[42] and Takahashi *et al.*[43] reported that black soybean products reduced cyclophosphamide-induced DNA damage and inhibited the oxidation of low-density lipoprotein cholesterol. Our previous findings showed that black soybeans exerted antimutagenic and antioxidant properties. Moreover, fermentation with *Aspergillus awamori* enhanced the functional properties of black soybeans. Fungi-fermented black soybeans (koji) have a higher concentration of the bioactive isoflavone aglycone than unfermented black soybeans.[16] Therefore, fermented black soybean has been proposed as a valuable source of plant protein and polyphenols.

There is some overlap between the biological mechanisms implicated in sarcopenia, which include loss of proteostasis, dysregulation of redox functioning, and chronic low-grade inflammation. Previous studies found that either a higher protein intake or polyphenol supplementation could increase muscle mass in young adults. However, there is a lack of scientific evidence on the combined nutraceutical effects of dietary polyphenols and protein against muscle loss and other pathologies related to sarcopenia in the elderly. Thus, to assess the potential anabolic-induced effects of fermented black soybean products, this study investigated whether supplementation with dietary polyphenol-rich plant-based proteins (BSKPs) could help to sustain skeletal muscle mass in elderly individuals with sarcopenia. Moreover, the relationship between BSKP supplementation and the antioxidant defense system was explored, as well as the possible mechanisms of action of BSKP on the gut microbiota.

Materials and methods

Study design

This was a quasi-experimental intervention, with pre- and post-evaluations, of BSKP supplementation in healthy elderly individuals residing in the community in Taipei, Taiwan.

Intervention

This study was an open-label, single-arm trial with a pre–post study design. In addition to their daily normal diet, all eligible participants were required to consume two packages of BSKP every day for 10 weeks. The participants were suggested to take BSKP supplements as snacks. The supplements were recommended to be taken between meals with 240 mL of water. During the intervention period, 28 packages of BSKP were sent to each participant every two weeks. Each package provided 95 kcal calories, 8.1 g protein, 9.9 g carbohydrate, and 2.5 g fat, as well as isoflavones, aglycones, β-glucosides, and malonyl glucosides (ESI Table 1†). The daily calorie and protein intake of the participants were not restricted.

Outcomes

BSKP supplementation is a good source of plant protein and isoflavones.[16] Therefore, the present study aimed to (1) assess whether BSKP supplementation can improve nutrition status, decrease oxidative stress, and attenuate the progression of muscle loss in elderly individuals and (2) explore the associations between BSKP supplementation and gut microbiome alterations in the elderly.

Participants

Participants over 65 years old suspected to have low muscle mass were recruited from the community in Taipei city, Taiwan. Low muscle mass was defined as calf circumference ≤33 cm for females and ≤34 cm for males based on a previous study.[17] The individuals (1) who were currently under moderate or strong exercise training, (2) with hypothyroidism or TSH > 4.94 μIU mL^{-1}, (3) with an allergy to milk or black soybeans, (4) receiving treatment for or have a history of malignancy within the last year, (5) admitted to a hospital in the past 4 weeks, or (6) with ≥stage 3 chronic kidney disease (GFR < 60 mL min^{-1}/1.73 m^2) were excluded. All participants signed a written informed consent form prior to the start of the study. This research was approved in advance by the National Taiwan University Hospital Institutional Review Board (IRB 201806058RINA) and registered with Clinical Trials.gov (NCT04951843). All participants underwent screening for physical performance, low muscle mass, renal function, and thyroid function and completed questionnaires on their past and current medical histories and daily exercise. The participant flow chart is shown in ESI Fig. 1.†

Physical performance and muscle mass screening

Handgrip strength was evaluated using a hand-held isometric dynamometer (JAMAR® Plus+, Japan) while the participants were seated with a slightly flexed elbow. The participants were asked to use their dominant hand to grasp the dynamometer thrice with maximal strength; the highest value was recorded as the grip strength. Gait speed was measured using the 5-meter walk test. Four markers (at the starting point, 1 m, 6 m, and 7 m) were placed in the ground. The participants were instructed to walk comfortably and the time taken to walk between the 1 m and 6 m markers was recorded as the gait speed. Calf circumference was measured using nonelastic tape to mark the maximal value for both calves.

Assessment of body composition and skeletal muscle mass

Body composition was assessed by bioelectrical impedance analysis (BIA) using an InBody 430 (InBody Co. Ltd, Seoul, Korea). The skeletal muscle mass index (SMI) was calculated as the lean mass (kg) of the four extremities divided by the square of the height (m^2). Sarcopenia was diagnosed according to the consensus statement of the Asian Working Group on Sarcopenia in Older People, which includes low muscle mass (appendicular skeletal muscle index (ASMI) < 7 kg m^{-2} for males and <5.7 kg m^{-2} for females), poor muscle function (handgrip strength < 26 kg for males and <18 kg for females), or walking speed < 0.8 m s^{-1}.

Biochemical and antioxidant analyses

Biochemistry. All participants fasted overnight for at least 8 hours before blood sampling. Blood samples were collected to determine the complete blood cell count, fasting glucose (Glu-AC), insulin, hemoglobin A1c (HbA1c), total cholesterol (T-CHO), triglycerides (TG), low-density lipoprotein (LDL), high-density lipoprotein (HDL), aspartate aminotransferase (AST), creatinine, free-thyroxine (free-T4), thyroid stimulating hormone (TSH), albumin, calcium, high-sensitivity C-reactive protein (Hs-CRP), and uric acid.

Superoxide dismutase activity. The activity of superoxide dismutase (SOD) in blood samples was evaluated using a SOD activity assay (BioVision, cat. #K335-100) at 450 nm following the manufacturers' protocol. This assay is based on the reduction of a water-soluble tetrazolium salt (WST-1) by superoxide anions to produce a water-soluble formazan dye; the rate of reduction correlates linearly with the inhibition of xanthine oxide (XO) by SOD.

Catalase activity. Catalase activity was estimated using a colorimetric/fluorometric catalase assay (BioVision, cat. #K773-100) according to the protocol provided by the manufacturer. Catalase converts H_2O_2 to water and oxygen; unreacted H_2O_2 was detected with a fluorescent probe at 570 nm; catalase activity inversely correlates with the intensity of the signal. Catalase activity was expressed in mU mL^{-1}.

Glutathione peroxidase assay. Glutathione peroxidase (GPx) activity was determined using a GPx activity colorimetric assay (BioVision, cat. #K762-100). GPx reduces cumene hydroperoxide and oxidizes glutathione (GSH) to glutathione disulfide (GSSG) in a reaction coupled to NADPH oxidation *via* glutathione reductase. The levels of NADPH, measured at 340 nm, were inversely proportional to GPx activity. GPx activity was expressed in mU mL^{-1}.

Dietary assessment

All participants completed interview questionnaires on the Three-Day Diet Dairy and Mini Nutritional Assessment (MNA)-Long Form Taiwan revision at the baseline and after the inter-

vention. Total calories and the total amount of protein were calculated in the pre- and post-test.

Stool sample collection and sample preparation

Stool samples were collected from participants who had not taken probiotics or received antibiotic therapy for at least 4 weeks. Fresh stool samples were collected in clean specimen bottles, immediately frozen at −80 °C, and transported to the NGS laboratory. For metabolomic analysis, lyophilized stool samples (1.5 g) were diluted with 3 mL of deionized water, homogenized for 1 min and centrifuged for 30 minutes at 12 000*g* at 4 °C using a 5810R Eppendorf centrifuge, and the supernatants were stored at −80 °C until analysis.

16S rRNA gene sequencing and microbiome data analysis

Bacterial DNA was extracted from 0.5 g of fecal samples using the FastDNA Spin Kit for Feces (MP Biomedicals). The V3–V4 variable region of the 16S rRNA gene was amplified using the 341F/806R bacteria/archaeal primers with barcodes. Fourteen sequencing libraries were generated using the NEB Next® Ultra™ DNA Library Prep Kit for Illumina (NEB). The libraries were sequenced on an Illumina HiSeq 2500 platform (Illumina, Inc.) to generate 250 bp paired-end reads. The analysis was performed following the description of a previous study.[15] Differences in abundance were examined by the MetaStat method with adjustments for multiple comparisons.

Statistical analysis

Continuous variables were reported as means and standard deviations; pre- and post-test values were compared using paired *t*-tests. The independent *t*-test was used to compare the levels of antioxidants between the groups. Categorical variables were reported as numbers and percentages. The linear regression model was employed to assess the relationships between the change in the ASMI and antioxidants after adjusting for gender, age, and the baseline ASMI. Comparisons of baseline gut microbiota characteristics among participants in the pre- and post-test were conducted using univariate parametric and non-parametric tests, including analysis of variance (ANOVA), the Kruskal–Wallis test, MetagenomeSeq, and the Chi-square test. Spearman's correlation analysis was used to determine the correlation coefficients for the relationships between the gut bacterial composition and the antioxidant levels using R software (Lucent Technologies, version 3.3.1). All other statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS Statistics for Windows version 20.0). All tests were two-tailed; $p < 0.05$ was considered significant.

Results

Participants, general characteristics, and dietary intake evaluation

Sixty-eight individuals were recruited and provided written informed consent to participate. Eight participants were excluded due to suspected hypothyroidism, chronic kidney disease, or normal muscle mass or physical function. Fourteen participants dropped out of the intervention due to intolerance to the black soybean koji products and 46 participants completed the intervention.

The basic demographic data, including age, gender distribution, and BMI, of the 46 participants are shown in Table 1. The mean age was 76 ± 5.9 years; 23.9% and 76.1% of participants were males and females, respectively; 37% of participants had a BMI higher than 25 kg m^{-2} and 7% had a BMI lower than 18 kg m^{-2}. Moreover, the calf circumferences of all participants, which serve as surrogate markers of muscle mass for diagnosing sarcopenia, were lower than the cut-off for sarcopenia. The mean calf circumference was 33.4 ± 0.4 cm for males and 31.7 ± 1.0 cm for females (Table 1).

Based on the MNA, there was no significant difference in the nutrition status of the participants between the pre- and post-test, including the total calorie, carbohydrate, protein, and fat intake (Table 2).

Evaluations of nutrition, hematology, biochemistry, and sarcopenia

The clinical data of the participants before and after the intervention are summarized in Table 2. There were no significant differences in most clinical parameters between the pre- and post-test. However, serum LDL significantly decreased between the pre-test and post-test (121 mg dL^{-1} *vs.* 113 mg dL^{-1}, p = 0.048). Moreover, hemoglobulin slightly increased between the pre-test and post-test, although this trend was not significant (13.4 g dL^{-1} *vs.* 13.5 g dL^{-1}, p = 0.093; Table 3).

Both skeletal muscle mass (15.4 kg *vs.* 15.7 kg, p = 0.040) and ASMI (6.2 *vs.* 6.3, p = 0.041) significantly increased between the pre-test and post-test. Borderline significant increases in the basal metabolism rate (1195.2 *vs.* 1209.8 kcal, p = 0.082) and BMI (23.3 kg m^{-2} *vs.* 23.6 kg m^{-2}; p = 0.084) were also observed (Table 4). However, no significant changes in any other metrics of body composition (including waist cir-

Table 1 Demographics of the subjects (N = 46)

	N (%)	Mean ± SD
Demographics		
Age, years		76.0 ± 5.9
Age range, years		
65–75	21 (45.7%)	
76–80	15 (32.6%)	
>80	10 (21.7%)	
Gender		
Male	11 (23.9%)	
Female	35 (76.1%)	
BMI, kg m^{-2}		23.3 ± 3.6
BMI range, kg m^{-2}		
<18.5	7 (15.2%)	
18.5–22.9	12 (26.1%)	
23.0–24.9	10 (21.7%)	
>25.0	17 (37.0%)	
Calf circumference, cm		
Male	11(23.9%)	33.4 ± 0.4
Female	35 (76.1%)	31.7 ± 1.0

Table 2 The nutrition status of the subjects before and after the intervention period of BSKP (*N* = 46)

	Before	After	*p* value
Mini Nutritional Assessment	27.2 ± 1.9	27.1 ± 2.2	0.695
Nutrition intake			
Total calories, kcal	1382.0 ± 328.1	1345.0 ± 281.1	0.504
Carbohydrate, g	177.4 ± 61.5	167.9 ± 41.2	0.379
Protein, g	52.9 ± 17.7	54.4 ± 15.5	0.644
Protein intake per day, g kg^{-1}	1.0 ± 0.4	0.97 ± 0.38	0.721
≥1.2 g per day, *n*	10 (21.7%)	11 (23.9%)	0.763
Fat, g	48.6 ± 16.8	48.9 ± 16.8	0.929

Analysis was done by using the McNemar test method. Values are means ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Table 3 Hematological and biochemical values of the subjects before and after the intervention period of BSKP (*N* = 46)

	Before	After	*p* value
WBC, k μL^{-1}	5.6 ± 1.2	5.7 ± 1.4	0.486
RBC, M/μL^{-1}	4.5 ± 0.6	4.6 ± 0.6	0.253
HB, g dL^{-1}	13.4 ± 1.3	13.5 ± 1.3	0.093
Platelets, k μL^{-1}	225 ± 54.7	226.8 ± 52.2	0.657
Glucose, mg dL^{-1}	105.2 ± 27.2	107.9 ± 27	0.167
HbA1c, mg dL^{-1}	6 ± 0.8	6.1 ± 0.9	0.628
Insulin, mg dL^{-1}	7.1 ± 3.4	9 ± 9.8	0.215
HOMA_IR, mg dL^{-1}	1.8 ± 0.9	2.5 ± 3.6	0.190
TCHO, mg dL^{-1}	196.7 ± 42.7	190.5 ± 38.2	0.153
TG, mg dL^{-1}	117.8 ± 81.2	120 ± 78.6	0.831
LDL, mg dL^{-1}	121.8 ± 41	113 ± 30.2	0.048*
HDL, mg dL^{-1}	59.6 ± 18.9	58.7 ± 19.7	0.482
AST, U L^{-1}	24.7 ± 16.3	22.6 ± 11.2	0.310
ALT, U L^{-1}	22 ± 27.6	19.7 ± 13.6	0.527
r-GT, U L^{-1}	25.4 ± 34.9	24 ± 42.1	0.535
BUN, mg dL^{-1}	16.5 ± 4.4	16.7 ± 5	0.725
Creatinine, mg dL^{-1}	0.8 ± 0.2	0.8 ± 0.2	0.583
e-GFR,	85.7 ± 21.7	86.7 ± 21.9	0.589
Free T4	1 ± 0.1	1 ± 0.1	0.302
TSH, μIU mL^{-1}	1.9 ± 1.3	1.8 ± 1.2	0.891
Albumin, g dL^{-1}	4.3 ± 0.2	4.3 ± 0.2	0.839
Calcium, mg dL^{-1}	2.3 ± 0.1	2.3 ± 0.1	0.598
Hypersensitive CRP, mg L^{-1}	0.2 ± 0.2	0.3 ± 0.6	0.189
Uric acid, mg dL^{-1}	5.3 ± 1.2	5.2 ± 1.3	0.871

Analysis was done by using the *t*-test method. Values are means ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

cumference, hip circumference, and body fat percentage), muscle strength, or physical performance were observed (Table 4).

Table 4 Physical status of the subjects before and after the intervention period of BSKP (*N* = 46)

	Before	After	*p* value
Physical examination			
BMI, kg m^{-2}	23.3 ± 3.6	23.6 ± 3.7	0.084
Waist circumference, cm	88.7 ± 11.3	88.0 ± 10.4	0.568
Arm circumference, cm	27.8 ± 4.3	27.5 ± 3.4	0.598
Hip circumference, cm	96.0 ± 11.0	97.4 ± 7.0	0.332
Calf circumference, cm			
Male	33.4 ± 0.4	33.3 ± 0.6	
Female	31.7 ± 1.0	31.9 ± 1.2	
Body fat percentage, %	32.9 ± 8.1	32.6 ± 7.8	0.756
Basal metabolism rate, kcal	1195.2 ± 149.1	1209.8 ± 154.3	0.082
Appendicular skeletal muscle mass			
Appendicular skeletal muscle mass, kg	15.4 ± 3.5	15.7 ± 3.8	0.040*
Appendicular skeletal muscle mass index	6.2 ± 0.9	6.3 ± 1	0.041*
Low skeletal muscle mass	18 (39.1%)	14 (30.4%)	0.206
Muscle strength			
Handgrip strength, kg	21.0 ± 6.6	21.1 ± 6.1	0.875
Low handgrip strength, *n*	19 (41.3%)	17 (37.0%)	0.593
Physical performance			
5-metre walk, m s^{-1}	1.1 ± 0.3	1.1 ± 0.3	0.728
Low physical performance	5 (10.9%)	7 (15.2%)	0.317
Sarcopenia, *n*	9 (19.6%)	7 (15.2%)	0.480

Analysis was done by using the *t*-test. Values are means ± SD. Appendicular skeletal muscle mass: sum of the muscle mass of the 4 limbs. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Table 5 The change of anti-oxidant activity before and after the intervention period of BSKP in muscle mass (*N* = 35)

	Total (*N* = 35)		
	Before	After	*p* value
TNF-α	5.8 ± 2.7	5.7 ± 2.3	0.601
IL-6	4.4 ± 9.4	3.6 ± 5.7	0.666
Catalase	1.4 ± 0.4	1.9 ± 0.4	<0.001***
GPx	57.2 ± 9.7	81.0 ± 8.8	<0.001***
SOD	25.0 ± 16.0	41.5 ± 12.1	<0.001***

Analysis was done by using the *t*-test method. Values are means ± SD. TNF-α: tumor necrosis factor alpha, IL-6: interleukin-6, GPX: glutathione peroxidase, SOD: superoxide dismutase. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Associations between the markers of inflammation and antioxidant activity and muscle mass

Of the serum antioxidants assessed, significant increases in catalase, GPX, and SOD were observed between the pre- and post-test ($p < 0.01$). These changes were observed in both the groups (Table 5).

Next, linear regression models were employed to investigate the associations between various factors and post-test ASMI. Univariate regression analyses demonstrated that the post-test ASMI was significantly negatively associated with male sex ($\beta = -1.48$, $p < 0.001$) and positively associated with the pre-test baseline ASMI ($\beta = 1.07$, $p < 0.001$). After adjusting for a number of potential confounding factors (such as pre-test ASMI, gender and age), the pre-test ASMI ($\beta = 1.06$, $p < 0.001$) and change in catalase activity between the pre- and post-test ($\beta = 0.32$, $p = 0.011$) remained significantly positively associated with the post-test ASMI (Table 6).

The increases in the serum antioxidant catalase between the pre- and post-test remained significant after adjustment for baseline muscle mass (Table 6). A linear regression model was applied to investigate the relationship between the change in ASMI and the increases of catalase. After adjustment for gender, age, and baseline ASMI, the increase in catalase

Table 6 Bivariate linear regression analysis of factors associated with the final ASMI (N = 35)

	Univariable		Multivariable	
	β (95% CI)	p value	β (95% CI)	p value
Male (*vs.* female)	−1.48 (−2.04–0.93)	<0.001***	0.01 (−0.38–0.40)	0.950
Age	0.01 (−0.04–0.07)	0.578	0.01 (−0.01–0.03)	0.504
Baseline ASMI	1.07 (0.93–1.22)	<0.001***	1.06 (0.87–1.24)	<0.001***
Difference of catalase*	0.51 (−0.06–1.09)	0.08	0.32 (0.08–0.55)	0.011**

Analysis was done by using multiple regression analysis and simple regression analysis. Adjusted R^2 = 0.849. Values are means ± SD. GPX: glutathione peroxidase. ASMI: appendicular skeletal muscle index. *p < 0.05, **p < 0.01, ***p < 0.001.

activity between the pre- and post-test remained significantly associated with the change in ASMI.

Effects of the BSKP intervention on fecal microbiota and metabolite profiles

Subsequently, the potential association between alterations in the gut microbiota and its metabolites and the improving effects of the BSKP intervention on sarcopenia development was further investigated. Fecal samples were collected for 16S sequencing in the pre-test and post-test to evaluate whether BSKPs led to alterations in the gut microbiota composition, and the results of relative abundance are shown in the ESI.† Based on the proportions of reads, *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Fusobacteria*, *Actinobacteria*, and *Verrucomicrobia* were the dominant phyla in both pre- and post-tests (Fig. 1a) and *Bacteroides*, *Prevotella_9*, *Faecalibacterium*, *Escherichia_Shigella*, *Agathobacter*, *Lachnoclostridium*, *Phascolarctobacterium*, *Blautia*, *Megamonas*, and *Roseburia* were the dominant bacterial genera in both pre- and post-tests (Fig. 1b).

Based on the Chao1, Simpson, and Shannon indexes, alpha-diversity was not significantly different between the pre- and post-test (Fig. 1c–e). However, beta-diversity was significantly different between the pre- and post-test based on PLS-DA with weighted UniFrac analysis and the PERMANOVA test (p = 0.01; Fig. 1f).

Thus, the effect of BSKP on specific bacterial genera and species was further investigated. LEfSe analysis revealed that the intervention enriched *Ruminococcaceae_UCG_013* and *Lactobacillus murinus* and reduced the abundance of *Akkermansia*. Moreover, Metagenomeseq analysis showed that *Prevotella_6*, *Bacillus*, *Porphyromonas*, *Gordonibacter*, and *Algibacter* increased after the BSKP intervention (Fig. 2).

Finally, the concentrations of SCFAs in the fecal samples were assessed to examine the associations between fecal microbial metabolites and antioxidant activity. Most SCFAs were enhanced by the BSKP intervention, with a significant increase observed for butyric acid (Fig. 3).

Correlations between BSKP-modulated gut bacteria and the parameters of sarcopenia

Since BSKP supplementation alleviated sarcopenia and altered the gut microbiota, the correlations between the abundance of bacteria and the parameters of sarcopenia were investigated. Spearman's analysis demonstrated that the abundance of the bacterial species *Lactobacillus murinus* correlated positively with serum SOD activity (r = 0.282; p = 0.024) and the fecal levels of propionic acid (r = 0.279; p = 0.037), butyric acid (r = 0.333; p = 0.029), and valeric acid (r = 0.267; p = 0.043). The increase in the abundance of *Bacillus* between the pre- and post-test correlated positively with serum GPx (r = 0.274; p = 0.029) and fecal propionic acid (r = 0.322; p = 0.016) and butyric acid (r = 0.310; p = 0.043). Serum catalase activity correlated positively with the abundance of *Algibacter* (r = 0.243; p = 0.068), *Ruminococcaceae UCG 013* (r = 0.324; p = 0.014), *Porphyromonas* (r = 0.260; p = 0.038), and *Akkermansia* (r = 0.264; p = 0.035). The abundance of another BSKP-induced bacterial genus, *Gordonibacter*, correlated negatively with serum LDL (r = −0.296; p = 0.025). Finally, a positive correlation was observed between the abundance of *Akkermansia* and serum SOD (r = 0.352; p = 0.007) (Table 7).

Discussion

Sarcopenia is a common issue in elderly populations and leads to a poorer quality of life. However, strategies to prevent or alleviate sarcopenia are still limited due to the lack of related clinical studies. In the present study, forty-six participants over 65 years old, who were diagnosed with early sarcopenia and resided in the community in Taiwan, were supplemented with dietary polyphenol-rich plant-based proteins (BSKPs) for 10 weeks. The intervention led to positive effects by decreasing the aging-related ASMI and reducing serum LDL. Moreover, BSKP supplementation increased the activity of the antioxidant enzymes catalase, GPx, and SOD in serum. The fecal microbiota composition and levels of SCFAs in feces were also altered by the 10-week BSKP supplementation. Therefore, long-term supplementation of polyphenol phenol-rich plant-based proteins may represent a new potential strategy to sustain muscle mass by boosting antioxidant activities and beneficially altering the gut microbiota.

BSKP, black soybean fermented by *Aspergillus awamori*, is a polyphenol-rich plant-based protein. Previous studies indicated that higher protein and polyphenol intake had positive effects on muscle mass. Although no previous studies have investigated the effects of BSKP on muscle mass and function

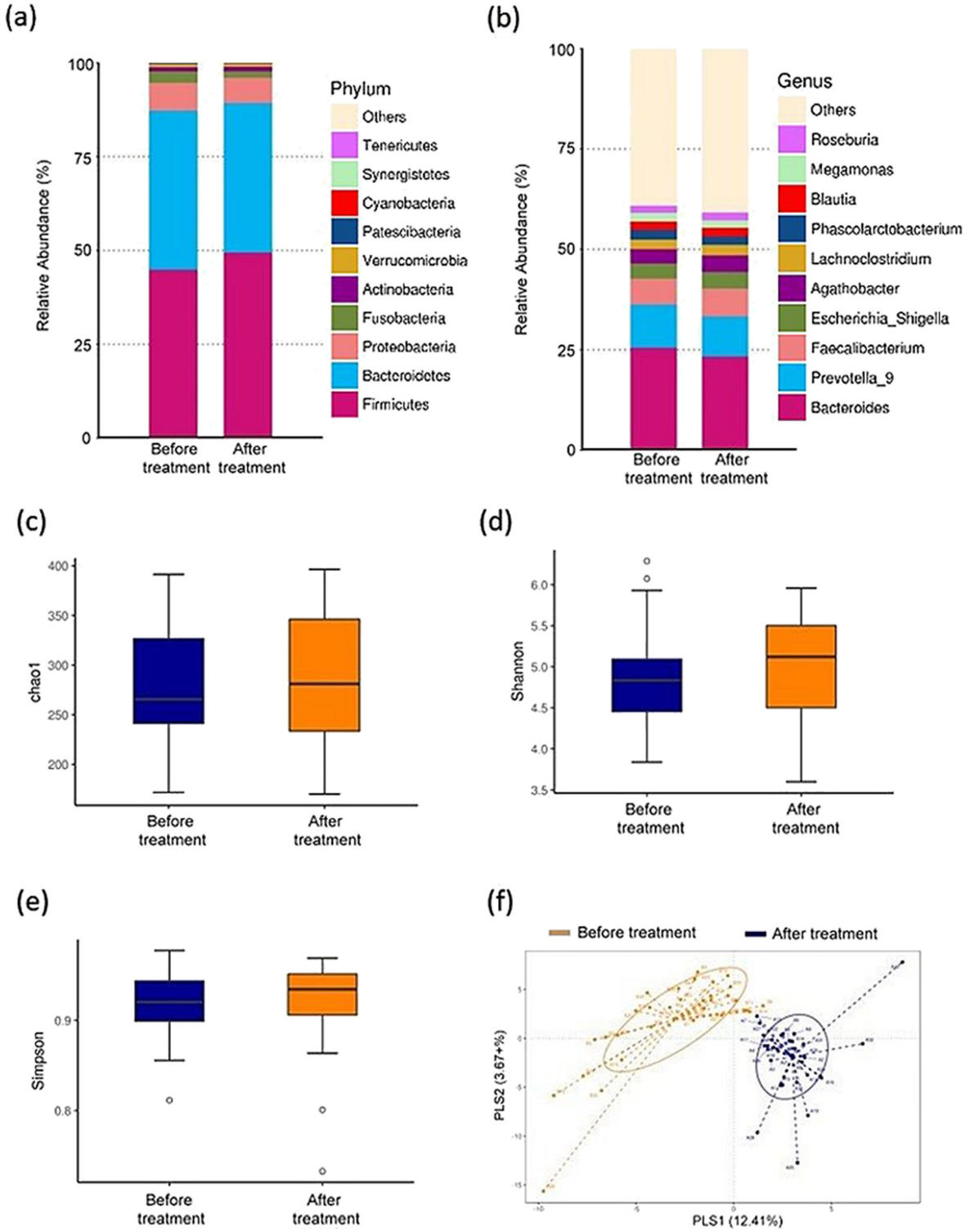

Fig. 1 Alteration of gut bacterial communities between the pre-test and post-test of BSKP supplementation. (a) Abundance at the phylum level; (b) abundance at the genus level; (c–e) alpha diversity: Chao1 (c), Shannon (d), and Simpson (e) indexes; and (f) beta diversity.

in humans, feeding chickens with *Aspergillus awamori* was reported to promote muscle mass and protein metabolism.[18] Administration of *Aspergillus awamori* to rats also reduced the plasma level of LDL in 6-week-old rats.[19] Sarcopenia is associated with high levels of LDL derived from abnormal lipid metabolites.[20] Moreover, LDL was one of the biomarkers for early diagnosis of sarcopenia.[21] Therefore, as BSKP supplementation enhanced the ASMI and reduced LDL in the

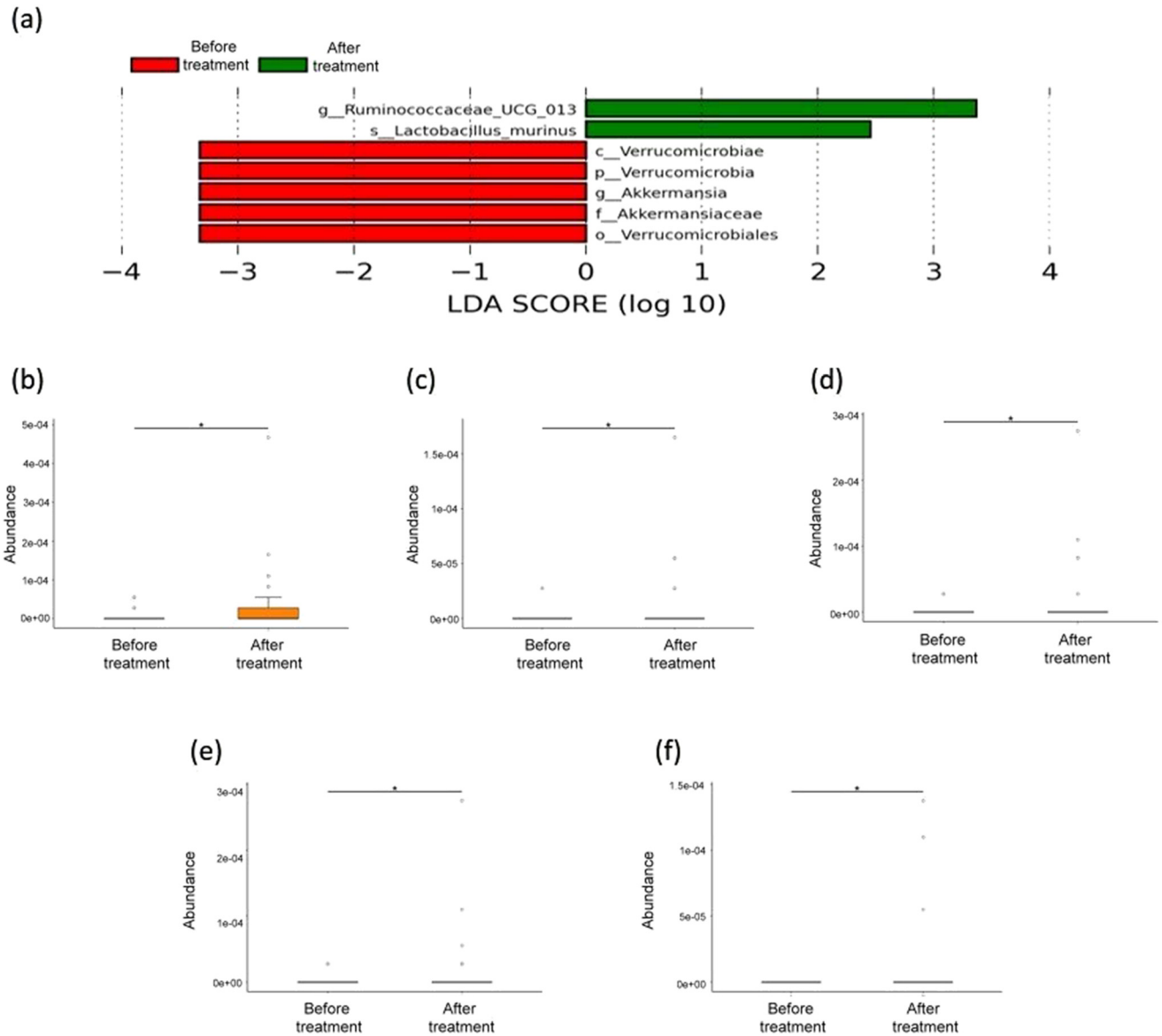

Fig. 2 Changes of microbial profiles in responders' supplementation of BSKP after 10 weeks. (a) The greatest differences of bacteria alteration between pre- and post-BSKP supplementation are shown by linear discriminant analysis effect size (LEfSe; LDA > 2) analysis. (b–f) Changes of the relative abundance of bacteria at the genus level after the BSKP supplementation: *Algibacter* (b), *Bacillus* (c), *Gordonibacter* (d), *Porphyromonas* (e), and *Prevotella_6* (f). $p < 0.05$. $N = 32$.

present study, BSKP supplementation could possibly attenuate the progression of age-related sarcopenia.

Sufficient dietary protein is needed to prevent or attenuate sarcopenia. Although some research has indicated that plants are less effective sources of protein than milk,[22,23] other researchers have reported that plant protein could reduce sarcopenia.[24] Hevia-Larraín *et al.* (2021) reported that soy protein diet supplementation led to similar muscle mass gain in young men as whey protein supplements.[25] Moreover, the abundant branched-chain amino acids in soy protein can promote the synthesis of muscle proteins.[26] Finally, and importantly, soy protein has been reported to exert anti-inflammatory and antioxidant effects that could help to prevent sarcopenia in the elderly.[27] BSKP was derived from fermented black soybeans with a high plant protein content and demonstrated the capacity to mitigate age-related sarcopenia in the present study. Taken together, BSKP could be suggested as a viable protein source warranting consideration for ameliorating sarcopenic conditions.

In addition to its value as a protein source, BSKP also contains high levels of polyphenols. These plant-derived compounds exert a wide variety of antioxidant and inflammatory properties and have been shown to have the ability to reduce muscle atrophy.[41] Several types of polyphenols have been found to positively affect muscle function in aged animals *via* processes linked to antioxidant activity.[28] ROS are an impor-

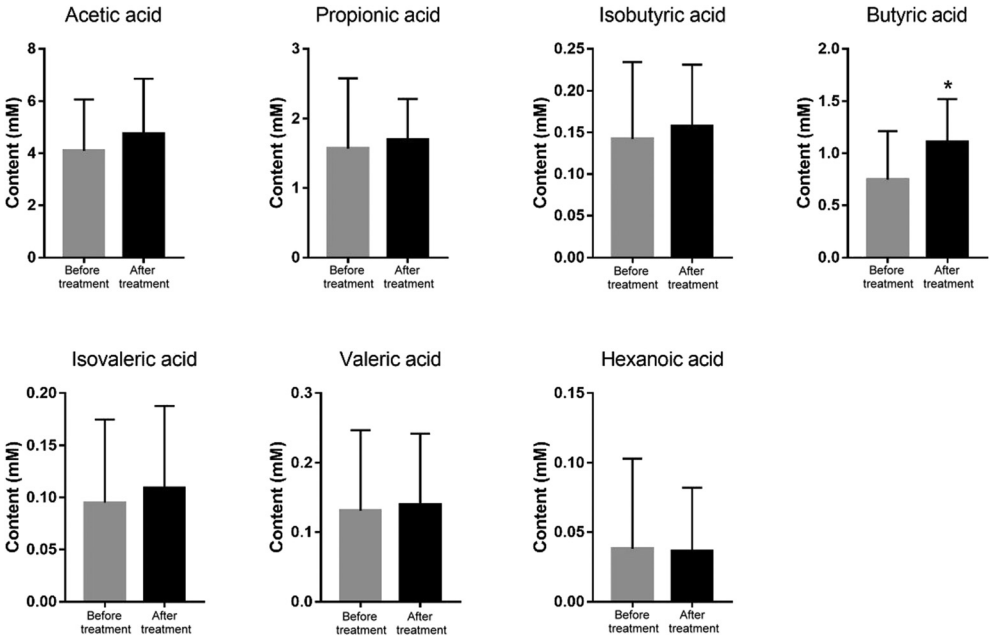

Fig. 3 SCFAs produced in participants by commensal bacteria before and after BSKP supplementation. Data are expressed as mean ± SD. *$p < 0.05$ *vs.* the A group. SCFA: short chain fatty acid. Outcomes were compared using a paired-sample *t* test. $N = 32$.

Table 7 Correlation between the abundance of bacterial taxa and sarcopenia parameters in different genera

Sarcopenia parameters	Correlation	Bacterial taxa
LDL	−	*Gordonibacter* ($r = -0.296$; $p = 0.025$)
Catalase	+	*Algibacter* ($r = 0.243$; $p = 0.048$), *Ruminococcaceae_UCG_013* ($r = 0.324$; $p = 0.014$), *Porphyromonas* ($r = 0.260$; $p = 0.038$), *Akkermansia* ($r = 0.264$; $p = 0.035$)
GPx	+	*Bacillus* ($r = 0.274$; $p = 0.029$)
SOD	+	*Lactobacillus murinus* ($r = 0.282$; $p = 0.024$), *Akkermansia* ($r = 0.352$; $p = 0.007$)
Propionic acid	+	*Bacillus* ($r = 0.322$; $p = 0.016$), *Lactobacillus murinus* ($r = 0.279$; $p = 0.037$)
Butyric acid	+	*Bacillus* ($r = 0.310$; $p = 0.043$), *Lactobacillus murinus* ($r = 0.333$; $p = 0.029$)
Valeric acid	+	*Lactobacillus murinus* ($r = 0.267$; $p = 0.043$)

The significant correlations between the abundance of bacteria taxa and sarcopenia parameters were selected using Pearson's correlation test and then validated with multivariate linear regression. "Plus signs" (+) represent a positive correlation, and "minus signs" (−) represent a negative correlation. Only significant correlations where $p < 0.05$ are represented. LDL: low density cholesterol. GPx: glutathione peroxidase. SOD: superoxide dismutase. SMI: skeletal muscle index.

tant factor associated with sarcopenia; thus, decreasing the levels of ROS should help to prevent or mitigate sarcopenia. Polyphenols are reported to exhibit ROS-scavenging effects by directly reacting with ROS and inducing increased levels of antioxidant enzymes, such as catalase, SOD, and GPx. A previous study reported that *Aspergillus awamori*-fermented soybean and black soybean contained high levels of polyphenols and exerted potent antioxidant ability.[29,30] Similarly, BSKP supplementation upregulated the activities of catalase, SOD, and GPx in our aging participants. Therefore, the polyphenols in BSKP may reduce age-associated sarcopenia by enhancing antioxidant capacity.

BSKP was given *via* the oral route in this study; therefore, the gut–muscle axis may play an important role in the positive effects of BSKP on sarcopenia. The gut microbiota and SCFAs are key players in the gut–muscle axis. SCFAs are metabolites of gut bacteria and exert a range of properties, including antioxidant and immunomodulatory activities and the ability to regulate energy metabolism. An increase in SCFAs correlated positively with better muscle conditions in elderly subjects.[15,31] In this intervention, the fecal levels of butyric acid, heptanoic acid, and total SCFAs were enhanced by BSKP supplementation. Moreover, the abundance of *Bacillus*, a bacterial genus that was enriched by BSKP supplementation, correlated positively with butyric acid and total SCFA levels. Similarly, positive correlations were detected between several *Bacillus* species and butyric acid in previous studies. Furthermore, some studies indicated that *Bacillus* sp. could enhance the levels of butyric acid and total SCFAs in the intestine and that both butyric acid and *Bacillus* could benefit muscle conditions in the elderly.[32] Therefore, BSKP may

exert a positive effect on sarcopenia by altering the levels of butyric acid and *Bacillus*.

Lactobacillus murinus was also identified as a BSKP-enriched bacterium in this intervention. This finding further supports the hypothesis that BSKP modulates sarcopenia *via* the gut–muscle axis, because an increased abundance of *Lactobacillus murinus* was associated with higher levels of SCFAs[33] and SOD,[34] which are key members of the gut–muscle axis. Moreover, *Lactobacillus murinus* has been reported to exert anti-inflammatory effects by inducing IL-10[35] and increasing the number of regulatory T cells.[36] Thus, *Lactobacillus murinus* might contribute to the prevention or mitigation of age-related sarcopenia by modifying the linkage between inflammation and age-related sarcopenia. Similarly, our results revealed a positive correlation between *Lactobacillus murinus* and both serum SOD and fecal SCFAs, and the abundance of *Lactobacillus murinus* increased after BSKP supplementation in the present intervention. Therefore, BSKP may also improve muscle mass *via* increasing the abundance of *Lactobacillus murinus* in the gut microbiota.

The bacterial genus *Akkermansia* was reduced by BSKP supplementation in this study. Margiotta (2021) and colleagues reported that a higher abundance of *Akkermansia* was associated with sarcopenia in advanced chronic kidney disease.[37] However, in other studies, *Akkermansia* was considered as a bacterial genus that exerts anti-inflammatory and gut barrier-protecting properties[38] and was present at a lower abundance in individuals with sarcopenia.[39] These inconsistent results indicate that the role of *Akkermansia* in the beneficial effects of BSKP on sarcopenia remains uncertain; thus, further research is necessary.

This study has limitations. Firstly, it employed a single-arm trial methodology instead of the more robust randomized controlled trial approach. Therefore, despite incorporating several selection criteria, it might have been unable to avoid a non-objective comparison due to variations in patients' baseline conditions and the influence of other factors on prognosis. Thus, although the present study demonstrated a significant improvement in the parameters of sarcopenia, such as the skeletal muscle mass index and the LDL level, post-test in comparison with pre-test, large-scale randomized controlled trials are needed to confirm the findings of this study. Furthermore, while the sample size was consistent with previous studies,[40] it is conceivable that a larger participant cohort could have enhanced the ability to detect significant patterns, given the subtle distinctions between pre-test and post-test measurements. In addition, a systematic review article underscores that nutrition-based interventions for sarcopenia management involve durations ranging from 4 weeks to 18 months.[41] Thus, extending the duration of the intervention may help to observe stronger effects of BSKP on sarcopenia.

In conclusion, consumption of polyphenol-rich plant-based proteins such as BSKP may promote anabolic effects that enhance the maintenance of muscle mass and could provide a potential strategy to slow down the progression of sarcopenia in elderly individuals.

Data availability

The raw data concerning the gut microbiota have been successfully submitted along with the manuscript. The additional raw data referenced in the manuscript will be made accessible upon request. Interested individuals can initiate the process by applying to the corresponding author. Moreover, to ensure ethical considerations, all requests will be subject to approval by the Institutional Review Board of the National Taiwan University Hospital.

Author contributions

SSC and LHC designed the study, performed the experiments, and drafted the article. KCH and SWH drafted the article. CCC, KWL, ECH, YPC, and PCH performed the data analysis and edited the article. YWC performed the experiments and contributed to the interpretation of the data. HYH supervised the project and drafted the article. All authors discussed the results and contributed to the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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