Original Article

Nutritional evaluation of different bacterial douchi

Hua Li MEng, Feng-Qin Feng PhD, Li-Rong Shen PhD, Yun Xie BEng and Duo Li PhD

Department of Food Science and Nutrition, Zhejiang University, Hangzhou, China

The aim of this study was to determine the content and/or composition of protein, peptide, amino acid, lipid and fatty acid in bacterial douchi (BD) made by different pure starter fermentation. Protein content of BD3, BD5, BD7 and BD8 was significantly higher than that of autoclaved soybean (AS). Lipid content of BD1, BD5 and BD6 was also significantly higher than that of AS. Predominant amino acids were glutamic acid (11.3-15.2%), proline (11.2-14.5%), aspartic acid (8.7-10.0%), leucine (9.2-10.0%) and alanine (7.4-8.9%). BD had EAA_7 and EAA_9 values of 34.4-36.4% and 40.5-41.7% respectively. Threonine with the amino acid score of 61-85 was the limiting amino acid. Triacylglycerol (82.4-88.2%) was the most abundant lipid in BD, followed by phospholipid (9.6-16.4%) and phytosterol (1.2-2.9%). Major fatty acids were palmitic (10.6-11.3%), oleic (20.5-21.9%), linoleic (54.2-55.6%) and α-linolenic acid (8.2-9.1%). The ratio of n-6 polyunsaturated fatty acids (PUFA) to n-3 PUFA ranged from 6.1 to 6.7. Amino acid nitrogen and trichloroacetic acid soluble protein increased from 0.2% (AS) to 1.8% (BD8) and from 1.3% (AS) to 4.0% (BD7) at the highest level respectively. Peptides with molecular weight ≤ 2000Da accounted for at least 75% of total peptide. Bacterial fermentation of soybean increased amino acid nitrogen and trichloroacetic acid soluble protein except BBDC6, decreased molecular weight of 100-500 and increased 500-1000 and 2000-5000 peptides. Composition of lipids, fatty acids, and amino acids were no significant change after soybean bacterial fermentation.

Key Words: fermented soybean, bacterial douchi, nutritional evaluation, fatty acid, amino acid, peptides, China

Introduction

Douchi, a traditional Chinese fermented soybean product, has been used as a medicinal food and seasoning for millennia in China.[1] According to the dominant microorganisms playing roles in fermentation, douchi can be divided into two large groups: bacterial douchi (BD) and mould douchi.[2] Currently, BD is made not by the use of pure microbial cultures, but by natural fermentation.[3] This kind of spontaneous and uncontrolled fermentation may lead to a potential problem of hygienic safety, unstable sensory and nutritional properties.[4,5] Therefore application of pure microbial cultures for BD production is essential to accelerate the development of douchi.

The raw material of BD is soybean, which is abundant in protein and lipid. Soybean is a good source of all essential amino acids (EAA) except methionine, and soybean protein has been proven to have cholesterol-lowering effects both in humans and in experimental animals.[6] Additionally soybean is rich in essential fatty acids (EFA): linoleic and α-linolenic acid with cardiovascular advantage. The high percentage of oleic acid can decrease low density lipoprotein-cholesterol (LDL-c) to high density lipoprotein-cholesterol ratio and so reduce the risk of cardiovascular disease[7] and several cancers.[8] Furthermore, the preferred ratio of n-6 to n-3 polyunsaturated fatty acids (PUFA) in soybean[9-11] may reduce the risk of cardiovascular disease[9] and some cancers[8,12] and the low percentage of saturated fatty acids (SFA) can prevent LDL-c level in blood from increasing to reduce the risk of cardiovascular disease.[13]

During soybean fermentation, protein will be hydrolyzed to low molecular weight components such as peptides and amino acids due to the action of enzymes produced by bacteria.[14] These outcomes may be favorable to the flavor, absorbability, digestibility and functionality of BD. For example, glutamic acid is the most important flavour-enhancing amino acid[15] and glycine and alanine give the sweet flavour.[16] Several bioactive peptides derived from soybean protein have been found to be angiotensin I-converting enzyme inhibitory peptides, antioxidant peptides and antithrombotic peptides.[1, 17, 18]

Whether there are some differences in the nutrient content between soybean and BD, the content and molecular weight distribution of peptide as well as the free amino acid content in BD has yet to be studied. Thus the aim of this study is to produce BD and to determine the content and/or composition of protein, peptide, amino acid, lipid and fatty acid in BD made by different pure starter fermentation.

Materials and methods

Materials and reagents

Soybean used in BD preparation was purchased from local market in Hangzhou, Zhejiang, China.

Corresponding Author: Professor Duo Li, Dept of Food Science and Nutrition, Zhejiang University, 268 Kaixuan Rd, Hangzhou, Zhejiang, China 310029
Tel: 86 571 86971024; Fax: 86 571 86971024
Email: duoli@zju.edu.cn

Acetonitrile and n-hexane were chromatographic grade, all the other chemicals were analytical grade. Water used in all experiments except the preparation of BD was doubly-distilled and deionised. The fatty acid methyl ester standard, the amino acid standard and the peptide standard were purchased from Nu-Chek-Prep, Elysian, MN, BDH Ltd., Poole, Dorset, U.K. and Sigma-Aldrich Trading Co. Ltd., Shanghai, China, respectively. Six types of bacteria, namely BBDC1, BBDC2, BBDC3, BBDC4, BBDC5 and BBDC6, were isolated and purified from Babao douchi, a typical BD made in Linyi, Shandong, China and determined as *Bacillus subtilis* and *Bacillus licheniformis* by 16s rDNA identification.

Preparation of bacterial douchi

Soybean was rinsed and mixed with tap water (1:3, w/v), then soaked at 12℃ for 11h followed by being autoclaved at 121℃ for 40min after decanting the water. The suspensions of bacteria (10^7cfu/mL) were inoculated to the autoclaved soybean (AS) in sterile petri dishes according to the ratio of 1% (v/w). Then AS containing inoculums were incubated at 37℃ for 48h, subsequently at 50℃ for 7 d in incubator. BD1 to BD8 bacterial douchi were obtained by fermentation of AS with BBDC1, BBDC2, BBDC3, BBDC4, BBDC5, BBDC6, BBDC3+BBDC4 and BBDC1+BBDC3, respectively. AS and resulting douchi were dried at 40℃ under vacuum and milled to pass through 60 mesh screen, then was kept in tightly sealed containers at -20℃ until used.

Analysis of protein, amino acid nitrogen (AAN) and trichloroacetic acid soluble Protein (TCASP)

Protein content was determined by the microKjeldahl method and the factor 5.71 was used to convert the nitrogen value to protein. AAN was determined by formol titration. Five grams sample was added to 100mL water and stirred in 75℃ water bath for half an hour, then made up to scale by water in 200mL volumetric flask. Forty milliliter water extract was mixed with 60mL water and then was titrated to pH 8.2 with 0.05M sodium hydroxide. After adding 10 mL formalin solution (20%, v/v), titration was continued to obtain pH 9.2 with 0.05M sodium hydroxide. TCASP was based on a fraction prepared by adding 40mL of aqueous solution of 30% (w/v) TCA to 40mL water extract followed by keeping the mixture at room temperature for half an hour. This was then centrifuged at 3000rpm for 20min. Aliquots from the fraction was then analyzed by the micro-Kjeldahl method. The factor 5.71 was used to convert the nitrogen value to protein.

Analysis of amino acid and peptide

Sample preparation for analysis of amino acid

About 30 mg of each sample placed in 5mL ampoule was hydrolysed with 5mL 6 M hydrochloric acid under vacuum at 150℃ for 1.5 h. After hydrolysis, 50μL hydrolyzed sample solution was placed into 0.5mL eppendorf tube and the solvent was completely removed at 40℃ by vacuum drying. Then 50μL ethanol: water: triethylamine (2:2:1, v/v) was added and the sample was dried again. The residue was dissolved in 100μL phenylisothiocyanate : ethanol : water : triethylamine (1:7:1:1, v/v) by ultrasound and stood at room temperature for 30 min for derivatization . Excess reagent was removed at 40℃ by vacuum drying. Derivatized sample was dissolved in 500μL 0.005M disodium hydrogen phosphate (pH7.4, adjusted by phosphoric acid: acetonitrile, 95: 5, v/v) and centrifuged at 10000rpm for 20min. The supernatant was filtered with 0.45μm membrane.

Sample preparation for analysis of peptide

About 1g sample was added into 25mL volumetric flask and well shaken with mobile phase, then made up to scale by mobile phase and filtered through 0.45 μm membrane.

Apparatus and condition for HPLC

Amino acid and peptides were analyzed using a Waters 2695 separations module HPLC system equipped with two pumps, an in-line degasser, an auto-sampler and a Waters 2996 photodiode array detector (Waters Corp., Milford, MA, USA). Equipment controls, peak identification and integration were performed by Empower Pro software. Separation of amino acid and peptide were carried out on a Kromasil C18 column (5μm 4.6mm×250mm) (Elite Analytical Instruments Co., Ltd., Dalian, China) and TSK-GEL G2500PW×L (7.8×300mm) (Tosoh Corp., Tokyo Japan), respectively.

The injection volume for amino acid and peptide analysis both were 10μL, while the solvent flow rate, column temperature and detector wavelength were 1.0 and 0.5mL/min, 38℃ and room temperature, 254 and 214nm, respectively. The mobile phase for amino acid analysis consisted of (A) 0.1M sodium acetate: acetonitrile (97:3, v/v) and (B) acetonitrile: water (4:1, v/v). The solvent linear gradient was as follows: 0 min, 100% A; 13min, 93%A-7%B; 23min, 77%A-23%B; 29min, 65%A-35%B; 35min, 60%A-40%B; from 40min to 45min, 100%B; from 47min to 50min, 100%A. The mobile phase for peptide analysis was acetonitrile: water: Triflouroacetic acid (45:55:0.1, v/v/v).

Amino acids were identified by comparing the retention times with standard mixture of amino acids running by the same method. The percent composition of amino acid was expressed as the ratio of individual peak area to total definable peak area. Calibration curve of molecular weight (MW) of peptide was gained by running five peptide standards (MW189, 451, 1450, 6500, 12400Da) at the same condition. The area under the curve was determined in four segments (5000–2000Da, 2000–1000Da, 1000–500Da and 500-100Da) and was expressed as the percentage of the total area of four segments.

Analysis of lipid and fatty acid

Sample preparation

Lipid was extracted from about 1g sample with 10mL chloroform:methanol (C:M, 2:1, v/v) containing 10mg/L butylhydroxytoluene at room temperature for 24h, then the mixture was filtered into a separatory funnel. About 5 mL 0.9% sodium chloride was used to rinse the funnel. Subsequently, the sample was shaken well and left to stand until demixing. The lower phase was then evaporated and made up to scale by chloroform in a 10mL volumetric flask. Then for lipid content determination, 1mL sample was added to a tube with constant weight

Table 1. Protein content, amino acid nitrogen and trichloroacetic acid soluble protein of bacterial douchi and autoclaved soybean.

	Protein content (%DM)	Amino acid nitrogen (%DM)	TCA soluble protein (%DM)
BD1	41.0±0.1 ^{f}	1.6±0.3 ^{b}	3.0±0.3 ^{c}
BD2	39.6±0.0 ^{h}	1.3±0.0 ^{d}	2.2±0.3 ^{d}
BD3	42.5±0.1 ^{b}	1.4±0.0 ^{cd}	3.0±0.2 ^{c}
BD4	40.3±0.1 ^{g}	1.4±0.2 ^{cd}	3.5±0.3 ^{b}
BD5	42.0±0.2 ^{d}	1.7±0.1 ^{ab}	3.0±0.1 ^{c}
BD6	41.3±0.1 ^{e}	0.5±0.1 ^{e}	0.7±0.1 ^{f}
BD7	42.7±0.1 ^{a}	1.6±0.1 ^{bc}	4.0±0.1 ^{a}
BD8	42.2±0.1 ^{c}	1.8±0.0 ^{a}	3.4±0.1 ^{b}
AS	41.4±0.2 ^{e}	0.2±0.1 ^{f}	1.3±0.2 ^{e}

BD1 to BD8 represent bacterial douchi fermented by BBDC1, BBDC2, BBDC3, BBDC4, BBDC5, BBDC6, BBDC3+BBDC4 and BBDC1+BBDC3, respectively. AS=autoclaved soybean, DM=dry matter; Values are expressed as mean ± SD, n=3. Significant difference between line means of each sample is indicated by different superscript letters (one way ANOVA, p<0.05).

Table 2. Amino acid composition of bacterial douchi and autoclaved soybean

AA	BD1	BD2	BD3	BD4	BD5	BD6	BD7	BD8	AS
Asp	10.0±0.2	10.0±0.4	9.8±0.2	9.8±1.0	9.1±0.4	9.4±0.1	10.1±0.5	8.7±1.2	10.5±0.7
Glu	15.2±0.5	13.8±0.5	13.9±0.2	13.2±1.6	12.8±0.3	11.3±0.8	12.9±0.5	11.9±0.7	12.7±0.7
Ser	3.5±0.4 ^{c}	3.5±0.2 ^{c}	3.3±0.4 ^{c}	2.6±0.2 ^{d}	3.7±0.2 ^{c}	4.7±0.2 ^{b}	3.7±0.6 ^{c}	3.6±0.1 ^{c}	5.6±0.2 ^{a}
Gly	6.6±0.6	6.9±0.4	7.3±0.6	6.8±0.6	7.6±0.2	7.8±0.3	7.3±0.5	7.3±0.5	7.2±0.4
His	2.0±0.2 ^{ab}	1.8±0.0 ^{bcd}	1.8±0.1 ^{cd}	2.0±0.1 ^{abc}	1.8±0.2 ^{d}	1.8±0.1 ^{bcd}	1.7±0.1 ^{d}	1.7±0.2 ^{d}	2.1±0.1 ^{a}
Arg	4.0±0.1 ^{b}	3.9±0.2 ^{bc}	3.9±0.2 ^{bc}	3.3±0.3 ^{d}	3.6±0.2 ^{cd}	4.2±0.2 ^{b}	4.1±0.1 ^{b}	3.4±0.3 ^{d}	4.6±0.0 ^{a}
Thr	2.9±1.0	2.3±0.1	2.2±0.1	2.3±0.0	2.3±0.1	2.5±0.0	2.1±0.1	2.4±0.1	2.8±0.1
Ala	8.2±0.1 ^{abc}	8.0±0.8 ^{bc}	7.7±0.6 ^{bc}	8.5±0.7 ^{ab}	8.6±0.4 ^{ab}	7.4±0.4 ^{c}	8.9±0.3 ^{a}	8.3±0.2 ^{ab}	7.7±0.4 ^{bc}
Pro	11.8±0.6	12.6±2.0	12.1±1.9	12.9±1.4	12.3±2.1	13.6±2.9	11.2±2.3	14.5±4.0	11.1±1.9
Tyr	3.7±0.5	3.9±0.2	3.8±0.10	4.0±0.1	4.1±0.3	3.8±0.3	3.8±0.4	4.0±0.2	3.8±0.8
Val	5.8±0.3	6.0±0.5	6.1±0.3	6.2±0.6	5.7±0.5	6.0±0.2	6.2±0.2	5.7±0.2	5.7±0.3
Met	1.9±0.1	1.9±0.1	1.9±0.1	1.9±0.1	2.1±0.1	2.0±0.1	1.9±0.2	2.0±0.2	1.8±0.1
Cys	0.6±0.1 ^{c}	0.7±0.1 ^{bc}	0.7±0.1 ^{bc}	0.5±0.1 ^{c}	0.7±0.2 ^{bc}	0.9±0.1 ^{ab}	0.7±0.2 ^{bc}	0.7±0.1 ^{bc}	1.0±0.1 ^{a}
Ile	5.1±0.5	5.3±0.4	5.4±0.1	5.4±0.2	5.2±0.4	5.3±0.3	5.6±0.0	5.1±0.2	5.3±0.3
Leu	9.2±0.8	9.3±0.3	9.7±0.5	10.0±0.1	9.5±0.3	9.4±0.2	9.7±0.0	9.6±0.4	9.4±0.5
Phe	4.8±0.2	5.0±0.3	5.3±0.4	5.1±0.7	5.2±0.4	4.9±0.4	5.0±0.5	5.4±0.3	4.7±0.2
Lys	4.8±1.0	5.2±0.4	5.2±0.4	5.5±1.5	5.8±0.2	5.2±0.5	5.1±1.2	5.7±0.3	4.1±0.8
EAA_7	34.4	35.0	35.8	36.4	35.8	35.3	35.7	36.0	33.7
EAA_9	40.5	40.8	41.5	41.7	41.2	41.4	41.5	41.0	40.3

AA=amino acid, EAA_7=Thr, Val, Ile, Leu, Phe, Lys and Met, EAA_9=EAA_7, His and Arg. Values are % of total amino acid expressed as mean ± SD, n=3. Significant difference between row means of each sample is indicated by different superscript letters (one way ANOVA, p<0.05).

and evaporated by N-EVAP model 111 (Organomation Associates Inc., BERLIN, MA, USA), then was keep in the desiccator for 24h.

For analysis of fatty acid, extracted lipid was hydrolyzed and derivatized to methyl ester as follows: 1mL lipid sample, 3mL 0.9 M Sulfuric acid in methanol and 1mL Methyl benzene were added in the teflon tube and shaken strongly, then submerged in a water bath at 70℃ for 2 h. Then 2mL n-hexane and 1mL 0.9% sodium chloride were added to the teflon tube and centrifuged at 1200rpm for 15min. For removing water-soluble components and dehydration, the supernatant was dropped into two consecutive tubes containing 2mL water and a little sodium sulphate anhydrous, respectively and then was filtered by Sep-pak silica column (Alltech, Associates Inc., Illinois, USA) for removing sterols. The fatty acid methyl esters (FAMEs) in eluent were evaporated by N-EVAP model 111 and resolved in 100μL n-hexane, then were sealed and kept at -20℃ until use.

Apparatus and condition for GC

Analysis of FAMEs was carried out on a Shimadzu GC-14C system equipped with a flame ionization detector (Shimadzu corp., Kyoto, Japan), a fused silica capillary column (Agilent Technologies, Inc., Palo Alto, CA , USA; DB-23, 60m×0.248mm×0.25μm) and the N2010 Chromatography Data System (Zhida Information Technologies, Inc., Hangzhou, China.). The condition for GC was set up according to that of Yang *et al*..[19]

Fatty acids were identified by comparing the retention times with standard mixture of FAMEs running by the same method. The percent composition of fatty acid was expressed as the ratio of individual peak area to total definable peak area.

Table 3. The essential amino acid score of bacterial douchi and autoclaved soybean.[a]

Amino acid	BD1	BD2	BD3	BD4	BD5	BD6	BD7	BD8	AS
Thr	85	69	65	67	69	74	61	70	81
Val	165	171	174	176	163	171	177	163	162
Met+Cys	100	102	102	96	111	113	104	108	115
Ile	180	191	194	191	186	188	201	183	189
Leu	140	141	147	152	143	143	147	146	142
Phe+Tyr	136	140	144	145	148	137	139	150	135
Lys	83	89	90	95	99	89	88	99	71
His	105	96	93	104	92	97	89	88	111

[a]Essential amino acid (EAA) score was calculated as follows: EAA score = test amino acid (% of protein) × 100 / reference amino acid pattern for 2-5years old children (FAO/WHO, 1990) (% of protein)

Table 4. Molecular weight distribution of peptides in bacterial douchi and autoclaved soybean.

	Molecular weight (Da)			
	2000-5000	1000-2000	500-1000	100-500
BD1	0.8±0.4 ^{f}	9.1±0.3 ^{h}	13.7±0.6 ^{d}	76.5±1.1 ^{a}
BD2	11.7±1.2 ^{e}	17.8±0.5 ^{f}	23.1±0.5 ^{b}	47.4±2.0 ^{c}
BD3	16.0±0.5 ^{b}	20.4±0.6 ^{b}	22.8±0.1 ^{b}	40.9±0.9 ^{f}
BD4	12.6±0.6 ^{de}	18.9±0.4 ^{de}	23.3±0.1 ^{ab}	45.2±1.1 ^{d}
BD5	14.1±0.5 ^{c}	19.4±0.9 ^{cd}	23.2±0.7 ^{ab}	43.2±1.0 ^{e}
BD6	24.4±0.8 ^{a}	16.6±0.2 ^{g}	17.2±0.3 ^{c}	41.8±1.0 ^{ef}
BD7	15.8±1.0 ^{b}	21.4±0.2 ^{a}	23.9±0.3 ^{a}	38.9±0.7 ^{g}
BD8	13.2±0.1 ^{cd}	20.2±0.5 ^{bc}	23.3±0.1 ^{ab}	43.3±0.5 ^{e}
AS	0.5±0.6 ^{f}	18.3±0.3 ^{ef}	8.2±0.5 ^{e}	73.1±0.4 ^{b}

Values are % of total peptide expressed as mean ± SD, n=3. Significant difference between line means of each sample is indicated by different superscript letters (one way ANOVA, $p<0.05$).

Apparatus and condition for IATROSCAN

Analysis of lipid composition was performed on extracted lipid using an Iatroscan MK-6s TLC-FID analyzer (Iatron Laboratories, Tokyo, Japan). The ChromStar 6.1 software was used to monitor the instrument and analyze chromatogram.

One microliter of the lipid extract was spotted onto a silica gel chromarod (Chromarod SⅢ, Iatroscan Laboratories) and allowed to dry. Then it was developed in the following solutions in turn at room temperature: first, petroleum ether: diethyl ether: acetic acid (65:15:0.1, v/v/v); second, petroleum ether: diethyl ether (56:4, v/v). After developing, chromarods were dried and then scanned at a rate of 30s/rod. The flow rate of hydrogen and air were set at 160mL/min and 2L/min respectively. Lipid fractions were expressed as percent of the sum of all lipid classes.

Statistical analysis

Data from all assays were obtained from at least three replicates on a dry-weight basis and analyzed using the one way ANOVA procedure followed by a post hoc test in SPSS statistical software (version 11.0) (SPSS Inc. Chicago, USA). The level of significance was set at $p < 0.05$.

Results

Protein content, AAN and TCASP of BD and AS are given in table 1. Protein content of BD3, BD5, BD7 and BD8 (42.5%, 42.0%, 42.7% and 42.2%, respectively) was significantly higher than that of AS (41.4%). AAN of BD ranged from 0.5% of BD6 to 1.8% of BD8, and was significantly higher than that of AS (0.2%). TCASP of BD except BD6, ranged from 2.2% of BD2 to 4.0% of BD7, was also significantly higher than that of AS (1.3%).

The amino acid compositions of BD and AS are listed in table 2. Predominant amino acids of BD were glutamic acid (11.3-15.2%), proline (11.2-14.5%), aspartic acid (8.7-10.1%), leucine (9.2-10.0%) and alanine (7.4-8.9%), same as those of AS (12.7%, 11.1%, 10.5%, 9.4% and 7.7%, respectively). Each percentage of histidine, arginine, cysteine and serine of BD was significantly lower than that of AS. BD had EAA_7 and EAA_9 values of 34.4-36.4% and 40.5-41.7% respectively, slightly higher than AS (33.7% and 40.3%, respectively).

The EAA score of proteins in BD and AS was given in table 3. The EAA score in all BD except BD1, threonine (61-74) was the lowest. Nevertheless, lysine (71) in AS took the place of threonine (81) as the lowest one. In general, each EAA score of threonine, histidine, methionine + cysteine in BD was lower than that in AS, whereas each EAA score of valine, lysine, phenylalanine + tyrosine was higher than that in AS.

Molecular weight distribution of peptide (100-5000Da) in BD and AS was summarized in table 4. The percentage of peptide (100-500Da) was significantly higher in AS (73.1%) than in BD (38.9-47.4%) except BD1. On the contrary, the percentage of peptide (2000-5000Da) was significantly lower in AS (0.5%) than in BD (11.7-24.4%). The percentage of peptide (500-1000Da) was also significantly lower in AS (8.2%) than in BD (13.7-23.9%).

The lipid content and composition of BD and AS were shown in table 5. Lipid content of BD1, BD5 and BD6 (21.2%, 23.5% and 21.6%, respectively) was significantly higher than that of AS (19.7%). Phospholipid (PL) of BD2, BD4 and BD5 (13.8%, 15.5% and 16.4%, respect-

Table 5. Lipid content and composition of bacterial douchi and autoclaved soybean.

	Lipid content (%DM)	TAG (% of total lipid)	PL (% of total lipid)	PS (% of total lipid)
BD1	21.2±0.3 ^{b}	88.2±2.6 ^{a}	9.6±2.5 ^{e}	2.2±0.4 ^{abc}
BD2	20.7±0.7 ^{bc}	85.0±1.5 ^{b}	13.8±1.1 ^{bc}	1.2±0.6 ^{bc}
BD3	22.0±1.2 ^{abc}	87.4±0.4 ^{a}	10.9±0.2 ^{de}	1.6±0.2 ^{c}
BD4	22.6±2.0 ^{abc}	82.8±1.5 ^{c}	15.5±1.5 ^{ab}	1.7±0.5 ^{bc}
BD5	23.5±0.5 ^{a}	82.4±1.0 ^{c}	16.4±0.8 ^{a}	1.2±0.7 ^{abc}
BD6	21.6±0.4 ^{b}	88.0±2.0 ^{a}	10.1±1.7 ^{e}	1.9±0.7 ^{abc}
BD7	21.6±1.9 ^{abc}	84.9±1.8 ^{b}	12.2±1.7 ^{cd}	2.9±0.4 ^{a}
BD8	21.4±2.1 ^{abc}	87.5±1.5 ^{a}	11.3±1.7 ^{de}	1.2±0.2 ^{c}
AS	19.7±0.4 ^{c}	87.2±1.5 ^{a}	10.3±1.4 ^{de}	2.5±0.2 ^{ab}

TAG=triacylglycerol, PL=phospholipid, PS=phytosterol. Values are expressed as mean ± SD, n=5-10. Significant difference between line means of each sample is indicated by different superscript letters (one way ANOVA, p<0.05).

Table 6. Fatty acid composition of bacterial douchi and autoclaved soybean.

Fatty acid	BD1	BD2	BD3	BD4	BD5	BD6	BD7	BD8	AS
C12:0	0.5±0.1 ^{a}	0.3±0.2 ^{abc}	0.2±0.0 ^{bcd}	0.2±0.1 ^{bcd}	0.3±0.0 ^{ab}	0.1±0.0 ^{cd}	0.2±0.2 ^{bcd}	0.2±0.2 ^{bcd}	0.0±0.0 ^{d}
C14:0	0.1±0.0	0.1±0.1	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0
C15:0	0.2±0.0 ^{bc}	0.2±0.1 ^{d}	0.2±0.0 ^{cd}	0.2±0.0 ^{bc}	0.3±0.0 ^{a}	0.1±0.0 ^{e}	0.2±0.0 ^{cd}	0.3±0.0 ^{ab}	0.1±0.0 ^{e}
C16:0	11.3±0.1	11.0±0.1	11.0±0.3	10.9±0.4	11.1±0.2	10.8±0.4	11.3±0.2	10.6±1.4	11.1±0.1
C17:0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.1	0.1±0.0	0.1±0.1	0.1±0.1	0.1±0.0	0.1±0.0
C18:0	2.7±0.0	2.8±0.1	2.8±0.1	2.6±0.2	2.7±0.1	2.6±0.1	2.8±0.0	2.5±0.3	2.7±0.0
C20:0	0.3±0.0	0.3±0.2	0.2±0.0	0.3±0.1	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0
C22:0	0.3±0.1	0.4±0.2	0.2±0.1	0.4±0.1	0.3±0.1	0.2±0.1	0.3±0.1	0.3±0.0	0.3±0.1
∑SFA	15.4	15.2	14.7	14.8	15.2	14.1	15.2	14.3	14.6
C14:1	0.1±0.0 ^{a}	0.1±0.0 ^{ab}	0.1±0.0 ^{bc}	0.1±0.0 ^{ab}	0.1±0.0 ^{ab}	0.0±0.0 ^{c}	0.0±0.0 ^{bc}	0.1±0.0 ^{ab}	0.0±0.0 ^{bc}
C15:1	0.1±0.0	0.3±0.2	0.3±0.0	0.3±0.2	0.1±0.0	0.3±0.2	0.1±0.0	0.2±0.1	0.3±0.1
C16:1	0.1±0.0	0.1±0.1	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0
C18:1 n-9	21.6±0.1	21.3±0.3	21.9±0.2	21.3±0.3	21.9±0.2	20.5±0.4	21.6±0.2	20.6±0.8	21.4±0.1
C20:1	0.2±0.0	0.3±0.2	0.3±0.0	0.4±0.4	0.2±0.0	0.3±0.0	0.2±0.0	0.3±0.0	0.3±0.0
C18:2 n-6	54.3±0.1	54.2±1.1	54.3±0.1	54.6±0.3	54.3±0.2	55.6±0.4	54.3±0.1	55.4±1.9	54.7±0.3
C18:3 n-3	8.3±0.0	8.6±0.2	8.4±0.1	8.5±0.1	8.2±0.1	9.1±0.3	8.4±0.1	9.1±1.0	8.5±0.1
∑EFA	62.6	62.8	62.7	63.1	62.5	64.7	62.7	64.5	63.3
n-6/n-3	6.5	6.3	6.4	6.4	6.7	6.1	6.5	6.1	6.4
P/S	4.1	4.1	4.3	4.3	4.1	4.6	4.1	4.5	4.3

SFA=saturated fatty acid, EFA=essential fatty acid, PUFA=polyunsaturated fatty acid, P/S= ∑PUFA/∑SFA. Values are % of total fatty acid expressed as mean ± SD, n=3. Significant difference between row means of each sample is indicated by different superscript letters (one way ANOVA, p<0.05).

tively) was significantly higher than that of AS (10.3%), meanwhile phytosterol (PS) of BD3 (1.6%) and BD8 (1.2%) was significantly lower than that of AS (2.5%).

The fatty acid composition of BD and AS is summarized in Table 6. The fatty acid pattern of BD was same as that of AS. Palmitic acid (10.6-11.3%) was the most abundant SFA, followed by stearic acid (2.5-2.8%). Oleic acid (20.5-21.9%) and linoleic acid (54.2-55.6%) were the most abundant monounsaturated fatty acid (MUFA) and PUFA respectively. α-linolenic acid (8.2-9.1%) was the unique n-3 PUFA in BD. There was no significant difference in all fatty acids except lauric, myristoleic and pentadecanoic acid between BD and AS.

Discussion

In this study, soybean was fermented by pure cultures of five strains of *Bacillus subtilis* and one strain of *Bacillus licheniformis* to make eight kinds of BD. Furthermore, this is the first report on nutritional evaluation on protein and lipid profiles of BD.

According to Chinese Trade Standard SB82-80[20], protein content and AAN should be no less than 20% and 0.6%, respectively. Protein content and AAN of BD except BD6 were much higher than the standard. The colour of BD except BD6 was brunneus or pitchy and also consistent with the standard. So all BBDC except BBDC6 can be used to make douchi meeting the basic standards and may be applied into industrial production.

Because of the much higher cost of animal protein, plant protein as another abundant source of protein has been increasingly popular. In our study, BD was rich in protein, amounted to about 40% of total dry matter, similar to raw soybean[21], also comparable to other fermented soybean products such as soy-dawadawa[22], kinema[23], sufu[24], and much higher than doenjang.[25] Some studies have reported an increase in protein content after soybean fermentation.[22, 26, 27] It was consistent with the results of some BD in our study. This phenomenon may be attrib-

uted to the incorporation of the bacterial protein into the product[26] and more expenditure of the carbohydrate.[28]

The quality of protein is as important as its quantity for human health, and the profile of EAA was an index of protein quality. BD was rich in EAA, comprising more than one third of total amino acid, and the ratio of EAA to total amino acid in BD was comparable to that in aspergillus and mucor douchi [29], natto[30] and sufu[31], although a little lower than tempeh[30] and kinema.[23] The EAA score of BD was evaluated according to the EAA profile of reference pattern for children 2–5 years old established by FAO/WHO (1990).[31] Only threonine was lower, while others were higher or almost equivalent. Therefore, threonine was the limited amino acid (LAA) in BD. Meanwhile, threonine was lower in BD than in AS. The formation of 2,5-dimethylpyrazine during fermentation by *Bacillus subtilis* at the expense of threonine may account for the decline of threonine. [32] Consumption of BD can fulfil the EAA requirements with the exception of threonine. Although in raw soybean, methionine was the exclusive LAA, there was a variation of the LAA among the fermented soybean products. For example, methionine was the LAA in aspergillus douchi[29], natto[30] and sufu.[31] Both methionine and lysine were the LAA in mucor douchi.[29] Both valine and lysine were the LAA in tempeh.[30] There was no LAA in kinema. [23] The reason for this may be partly due to the different species of microorganism playing roles in fermentation and different ways of pretreatment.

During fermentation, protein in the soybean was hydrolysed into smaller molecular compounds such as many kinds of peptides, amino acids by the proteases produced by *Bacillus spp.*.[14] Therefore there should be a great increase in peptide and free amino acid content after fermentation. In the present study, there was significant difference in AAN and TCASP between BD and AS. AAN reflecting the total free α-amino nitrogen content[28] and TCASP reflecting the small molecular weight peptide and free amino acid content[33], both were indications of the degree of hydrolysis of protein. Fermenting soybean with *bacillus subtilis* led to an approximate 9–fold increase in AAN at the highest level which accounted for approximately 25% of the total nitrogen and an approximate 2–fold increase in TCASP at the highest level which accounted for approximately 9% of the total protein. Similar trends were seen in other studies.[14,27-30]

Bacillus fermentation of soybean was related to the improvements in digestibility and absorbability.[14,27,34] As we know, protein should be hydrolyzed into amino acids and small molecular weight peptides first, before it can be absorbed and utilized by human body. Compared with the unfermented soybean, although there was even higher percentage of peptide (100-500Da) in AS than in BD, BD contained more quantity of low molecular weight peptide according to the area under the curve in chromatogram (data not shown here) and TCASP. Moreover, a large number of amino acids were released in BD during fermentation. Therefore we can conclude that BD may be digested and absorbed more easily than unfermented soybean.

Because proteolysis was a prominent character of fermentation, a great deal of interest has been placed on bioactive peptides, usually containing 3-20 amino acid residues and their activity being based on their amino acid composition and sequence.[35] Our study indicated that BD possessed a large number of small molecular peptides and their molecular weight distribution was mainly below 2000Da. It is possible that some bioactive peptides were present in BD.

Apart from the protein profile, the lipid profile was another important factor in the nutritional value of BD. Lipid content in some BD were significantly increased compared with AS, it was consistent with reported other fermented soybean products.[24,36] This phenomenon may be explained as the result of lipid synthesis as well as better assimilation of carbohydrate.[28]

There was no change in lipid and fatty acid variety owing to fermentation. Like AS, TAG was the dominant lipid in BD, followed by PL and PS. Proportion of palmitic, oleic, linoleic and α-linolenic acid in BD were as same as in soybean oil[37], AS and other fermented soybean products.[25] The production of extracellular lipase by Bacillus has been reported[38] and the increase of free fatty acids has been found during soybean fermentation.[27] However, no free fatty acid has been detected in BD in the present study. This may be due to the very low activity[39] of lipase produced by *Bacillus spp.* used in our study, and those free fatty acids depleted by esterification formed TAG again.[40] Thus, the rancidity may not be the problem related to BD. At the same time, the moderate PL content may improve the nutritional value of BD.

Conclusions

Bacterial fermentation of soybean increased amino acid nitrogen and trichloroacetic acid soluble protein except BBDC6, decreased molecular weight of 100-500 and increased 500-1000 and 2000-5000 peptides. The composition of lipids, fatty acids, and amino acids were not significantly changed after soybean bacterial fermentation. These findings provide opportunities to enhance the functional properties of soybean.

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