Korean Journal for Food Science of Animal Resources

Korean J. Food Sci. An. 2018 December 38(6):1144~1154
DOI https://doi.org/10.5851/kosfa.2018.e32

pISSN : 1225-8563 eISSN : 2234-246X
http://www.kosfaj.org

ARTICLE

Effects of *Lactobacillus helveticus* Fermentation on the Ca^{2+} Release and Antioxidative Properties of Sheep Bone Hydrolysate

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Received February 9, 2018
Revised August 8, 2018
Accepted August 9, 2018

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Abstract Both the calcium and collagen in bone powder are hard to be absorbed by the body. Although enzymatic hydrolysis by protease increased the bio-availability of bone powder, it was a meaningful try to further increase Ca^{2+} release, oligopeptide formation and antioxidant activity of the sheep bone hydrolysate (SBH) by lactic acid bacteria (LAB) fermentation. *Lactobacillus helveticus* was selected as the starter for its highest protease-producing ability among 5 tested LAB strains. The content of liberated Ca^{2+} was measured as the responsive value in the response surface methodology (RSM) for optimizing the fermenting parameters. When SBH (adjusted to pH 6.1) supplemented with 1.0% glucose was inoculated 3.0% *L. helveticus* and incubated for 29.4 h at 36°C, Ca^{2+} content in the fermented SBH significantly increased ($p<0.01$), and so did the degree of hydrolysis and the obtaining rate of oligopeptide. The viable counts of *L. helveticus* reached to 1.1×10^{10} CFU/mL. Results of Pearson correlation analysis demonstrated that LAB viable counts, Ca^{2+} levels, obtaining rates of oligopeptide and the yield of polypeptide were positively *correlated* with each other ($p<0.01$). The abilities of SBH to scavenge the free radicals of DPPH, OH and ABTS were also markedly enhanced after fermentation. In conclusion, *L. helveticus* fermentation can further boost the release of free Ca^{2+} and oligopeptide, enhance the antioxidant ability of SBH. The *L. helveticus* fermented SBH can be developed as a novel functional dietary supplement product.

Keywords sheep bone enzymatic hydrolysis, *Lactobacillus helveticus*, ionic calcium, antioxidant activity, oligopeptide

Introduction

Calcium in bone powder exists as $Ca_{10}(PO_4)_6(OH)_2$ crystals or amorphous $CaHPO_4$. Over 90% proteins in bone are triple-helix collagen，which is hard to be utilized by our body (Pang and Huo, 2017). By hydrolyzing, ionic calcium (Ca^{2+}) get released

from bone matrix and collagen turns into peptides. Ca^{2+} is an effective form of calcium that can be directly absorbed by intestinal tracts. As a bivalent mineral nutrient, Ca^{2+} plays key roles in maintaining the overall human health and it involves in many important physiological functions, such as bone growth, nerve conduction and muscle contraction (Bass and Chan, 2006; Guo et al., 2014). Bone collagen peptide preparations have various functions far beyond their nutritional importance, such as immune-enhancing, anti-osteoporosis, antibacterial, anti-hypertensive, antioxidant effects. It's proved that most bioactive peptides are oligopeptides. Studies show that oligopeptides can enhance mineral absorption (Peng et al., 2017; Zhao et al., 2014; Zhang et al., 2017), and extensive hydrolysis is essential to render proteins immunologically unreactive (Cordle et al., 1991).

As a probiotic lactic acid bacteria (LAB), *Lactobacillus helveticus* confers a health benefit on the host (Sanders, 2008). It is suitable for dairy application and considered multifunctional. It is gaining increasing importance as a health-promoting culture in probiotic and nutraceutical food product (Giraffa, 2014). Since *L. helveticus* can proliferate in bone hydrolysate and secrete protease and peptidase, this might lead to a more extensive degrading of bone collagen and might generate much more oligopeptides, accompanied an increased liberating of free Ca^{2+}. Meanwhile, many metabolites, such as vitamins, polysaccharides, organic acids produced by *L. helveticus* will endow SBH extra nutrients (Giraffa, 2014).

Food products with antioxidant activities are currently gaining increasing significance due to the fact that aging and many pathological processes are related to the detrimental effects of free radicals (Skrzypczak et al., 2017; Wang et al., 2014). Free radicals are highly active chemicals, accumulation of which causes oxidative stress to the body. Unfortunately, synthetic antioxidants somewhat pose potential healthy risks. Therefore, antioxidant food products derived from a natural source are extremely valuable (Skrzypczak et al., 2017).

Our preliminary study worked out a recipe to prepare SBH with high degree of hydrolysis (DH) (Han et al., 2016). The present study applied the selected *L. helveticus* as the suitable LAB to ferment the prepared SBH in an effort to allow more free Ca^{2+} and oligopeptides liberated and anti-oxidant activities enhanced through fermentation, and simultaneously harvest certain amount of viable probiotics and their beneficial metabolites in the fermented SBH. It's expected that the resulted preparation would be rich in nutrition, with antioxidant effect and probiotic function.

Materials and Methods

LAB strains

L. helveticus (ATCC 15009) and *Lactobacillus paracasei* subsp. *Paracasei* (CGMCC 1.2284) are purchased from the culture collection center of China (CGMCC). *Lactobacillus sakei* was purified from the commercial starter *Lyocarni* BOM-13 composed of single bacteria strain *L. sakei*. *Lactobacillus curvatus* was isolated from the starter of *Lyocarni* VBL-97 composed of *L. curvatus* and 3 other food-grade bacterial strains. *Pediococcus acidilactici* was isolated from VBM-60 containing *P. acidilactici*, *P. pentosaceus*, *Staphylococcus carnosus* and *S. xylosus*. All above commercial starters were purchased from Danisco A/S, a Danish bio-based company. All LAB strains were activated in MRS broth.

Comparison of the protease-producing ability of different LAB strains

Temperature (30℃, 35℃, 37℃, 40℃, 42℃, and 45℃), initial pH of MRS broth (4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0), 1% carbon source (glucose, sucrose, fructose, lactose, soluble starch) were studied to determine the suitable protease-producing conditions for the tested LAB strains. The logarithm-stage culture of different LAB with equal OD_{600} was

inoculated with equal volume into MRS broth (suitable pH) supplemented with 1% determined carbon source. After 24-hour incubation at the suitable temperature, the supernatant from centrifugation (8,400×g, 15 min, 4℃) was used to check the protease activity of each strain. The mixture of 450 μL supernatant and 50 μL 1% (w/v) BSA was added to 1.5 mL sodium acetate buffer (0.1 M, pH 7.0) and incubated at 37℃. The reaction was stopped by 10% trichloroacetic acid (TCA) 5 min later. Then OD_{280} was measured. A single unit of enzyme activity (1 U) here was stipulated as the quantity of the enzyme consumed to produce 1 μM tryptophan in one minute while hydrolyzing BSA at 37℃. The protease activity was calculated out by the equation below where K stands for the dilute times of the sample, W for the amount of generated Trp (μmol), V for the volume of the reaction system (mL), and T for the reaction time (min).

$$\text{Protease activity (U/mL)} = \frac{K \times W}{V \times T}$$

SBH preparation and *L. helveticus* taming

Build a system in which 12% sheep bone powder was firstly hydrolyzed by 4.25% alcalase (activity≥200 U/mg, Solarbio) at pH 9 and 45℃ for 400 min followed by adding 3.65% flavourzyme (activity≥20 U/mg, Solarbio) to the resulted mixture (adjusted to pH 6) and incubating at 55℃ for 240 min. The final mixture was autoclaved at 121℃ for 20 min. *L. helveticus* were gradually tamed by culturing in MRS broth with increasing proportion of SBH (supplemented with 1% glucose) from 10% to 100%. The finally tamed *L. helveticus* independent of MRS broth was used as the starter in the following single-factor tests and responsive surface design tests.

Determination of proteolysis related parameters

Polypeptides are those that less than 10 kDa and can not precipitate in 10% TCA. Samples were pretreated by adding 10% TCA and centrifugated at 5,000×g for 15 minutes. Then the total soluble nitrogen (N_T, mg) and amino acid nitrogen (N_{AA}, mg) in the supernatant were determined by traditional Lowry method and neutral formaldehyde titration method, respectively.

$$\text{Yield of polypeptides (mg)} = (N_T - N_{AA}) \times 6.25$$

Obtaining rate of oligopeptide (%) was expressed as the percentage of the total soluble nitrogen in 15% TCA (Lowry method) accounting for the total nitrogen of the sample (Kjeldahl method). DH (%) can be reflected by the total number of free amino groups. In the equation below, the amount of the specific amino acid before treating and released at time t during hydrolyzing are marked as L_0 and L_t, and the amount of the specific amino acid liberated after 24-hour hydrolyzing in 6 mol/L HCl at 120℃ was labeled as L_{max}. The content of Ca^{2+} was detected by EDTA complexiметry.

$$\text{DH (\%)} = \frac{L_t - L_0}{L_{max} - L_0} \times 100$$

Determination of radical scavenging activity

The sample was fully mixed with same volume of 0.1 mM DPPH and kept in the dark for 30 min at 25℃. In the control sample volume was replaced with Milli-Q water and in the blank replaced with 95% ethanol. In the following equation, A_1,

A_2, and A_0 represent the absorbance at 517 nm of the tested sample, the blank and the control, respectively.

$$\text{DPPH scavenging rate (\%)} = \frac{1-(A_1-A_2)}{A_0} \times 100$$

Hydroxyl radical (·OH) scavenging activity was determined in the light of the reported work (You et al., 2011). The reaction system consists of 600 μL of 5.0 mM 1,10-phenanthroline, 5.0 mM $FeSO_4$, 15 mM EDTA, along with 400 μL 0.2 M sodium phosphate buffer (pH 7.4) and 800 μL 0.01% H_2O_2. The mixture was remained at 37℃ for 60 min and the absorbance at 536 nm was measured. ·OH scavenging activity (%) was interpreted as $(A_s–A_0)\times100\ /(A_c–A_0)$ where A_s, A_0, and A_c were the absorbance value of the sample, the blank and glutathione (Sigma–Aldrich, St. Louis, MO, USA) solution in the absence of H_2O_2, respectively.

The scavenging activity for the radical of ABTS was assayed using the decolorization methodology (Aazam and Fatemeh, 2016). Firstly the working solution of ABTS was prepared as below. Equal volume of ABTS (14 mM) and potassium persulphate (4.88 mM) were mixed and kept at ambient temperature for 14 hours in the dark, then the mixture were diluted with 0.1 M phosphate buffer (pH 7.4) to get an absorbance of 0.70±0.05 at 734 nm. Then 200 μL working ABTS was mixed with 10 μL sample or 10 μL phosphate buffer (blank), The absorbance was detected 5 minuntes later. In the following equation, A_{ABTS} and A_{test} represent the absorbance of the working solution of ABTS (0.70±0.05) and the sample.

$$\text{Inhibition (\%)} = \frac{A_{ABTS}-A_{test}}{A_{ABTS}} \times 100$$

Colony counting of *L. helveticus*

After fermentation, samples of each dilution were spread onto MRS agar plates in triplicates and cultured for 48 h. The viable counts of *L. helveticus* in 1 mL of crude fermented SBH were calculated.

Optimization of the fermenting parameters

Based on the range of the variables determined through preliminary single-factor tests, 29 tests in the central composite design (CCD) by Design-Expert version 7.0 were carried out in random order (Table 1). Data from 29 tests were analyzed using response surface methodology (RSM). Design Expert was applied for statistical analysis. Data were modeled by multiple regression analysis adopting backward stepwise analysis. r_{adj}^2 was used to judge the goodness-of-fit of the regressive model. Statistical significance of the terms in the model was analyzed by ANOVA for each response.

Statistical analysis

Data were presented as mean±standard deviations. Correlation coefficient between 4 parameters in Table 5 were determined by Pearson's Correlation Coefficient Test (n=29) using IBM SPSS statistics V 21.0. Statistical significance of the hydrolysis related parameters and radical-scavenging abilities before and after fermentation were examined by analysis of variance (ANOVA) using Statistix 8.1.

Results and Discussion

Table 1. Central composite design (CCD) with 4 variants at 3 levels

Tests	X_1	X_2	X_3	X_4	Tests	X_1	X_2	X_3	X_4	Tests	X_1	X_2	X_3	X_4
1	0	–1	–1	0	11	–1	0	–1	0	21	–1	0	0	1
2	0	1	0	1	12	0	–1	1	0	22	0	0	0	0
3	–1	0	0	–1	13	0	0	1	1	23	0	1	–1	0
4	0	1	1	0	14	–1	–1	0	0	24	0	0	0	0
5	–1	0	1	0	15	0	0	–1	1	25	0	–1	0	1
6	0	0	0	0	16	1	1	0	0	26	1	–1	0	0
7	0	1	0	–1	17	0	–1	0	–1	27	0	–1	–1	0
8	1	0	–1	0	18	0	0	0	0	28	0	1	0	1
9	1	0	0	–1	19	0	0	1	–1	29	–1	0	0	–1
10	–1	1	0	0	20	1	0	0	1					

SBH, sheep bone hydrolysate.
X_1, X_2, X_3, and X_4 were four variants represented fermenting temperature, initial pH of SBH, fermenting time and inoculation amount. Each variant had 3 levels of –1, 0, and 1. The 3 levels for X_1 were 35℃, 37℃, and 40℃; X_2 4, 5, and 6; X_3 10 h, 12 h, and 15 h; X_4 3%, 4%, and 5%.

Single-factor tests of *L. helveticus*

It can be seen from Fig. 1 that peak values of protease activity (U/mL) in the crude enzyme preparations increased gradually in single-factor tests. The relative suitable conditions of *L. helveticus* for protease production (listed in Table 2) and thus

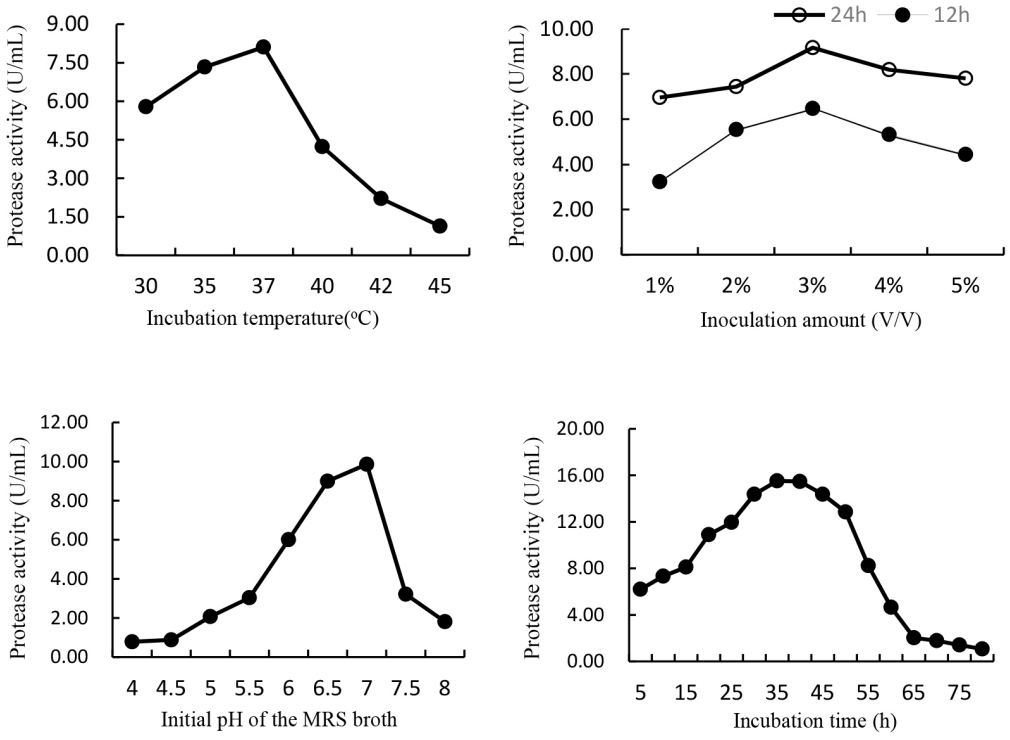

Fig. 1. Effects of incubation temperature, inoculation amount, initial pH of MRS broth and incubation time on the protease-producing ability of *Lactobacillus helveticus*. The basic conditions in single-factor tests for incubation temperature, inoculation amount, initial pH and incubation time of *Lactobacillus helveticus* were 37℃, 3%, pH 7, and 24 h, respectively. The dynamic changes of protease activity (Y-axis) are determined by changing the range of one factor (X-axis) and fixing the condition for other 3 factors in single-factor tests.

Table 2. Protease activities of LAB strains cultured under suitable conditions

LAB strains	Inoculation (%)	T (℃)	pH	Time (h)	Carbon source	Protease activity (U/mL)
Lactobacillus helveticus	3	37	7.0	40	Glucose	12.52±2.17
Lactobacillus paracasei	1	40	6.5	6	Lactose	11.36±2.04
Lactobacillus sakei	3	37	6.5	42	Sucrose	9.61±1.35
Lactobacillus curvatus	2	37	7.0	30	Glucose	8.87±1.61
Pediococcus acidilactici	3	40	7.0	30	Glucose	4.93±0.99

LAB, lactic acid bacteria.
Values of protease activity of 5 LAB strains were determined at the end of fermenting under the corresponding set of suitable conditons obtained through single-factor tests as listed in this table.

appropriate ranges for CCD were determined. The ranges for fermenting temperature, pH, inoculation amount and incubation time were 35℃–40℃, 6.5–7.0, 2%–4%, and 35–40 h, respectively. Results also suggested that glucose is the suitable carbon source for *L. helveticus* to produce protease, so we supplemented 1% glucose into SBH in later fermentation studies.

Protease-producing abilities of different LAB strains

Suitable protease-producing conditions for other 4 LAB strains and the corresponding protease activities (Table 2) were also determined using the same method as *L. helveticus.* Since *L. helveticus* displayed the biggest potentiality among the 5 tested LAB strains, it was selected as the starter for SBH fermentation. Other studies also reported the high proteolytic and peptidolytic activity of *L. helveticus* (Nielsen et al., 2009). In milk *L. helveticus* developed a proteolytic system that made it thrive (Griffiths and Tellez, 2013) and can produce extracellular protease which initiated the degradation of casein into oligopeptides (Slattery et al., 2010). Various intracellular peptidases degraded peptides into amino acids, which in turn can be converted into various cell structural constituents (Griffiths and Tellez, 2013). This proteolytic system also explains why *L. helveticus* can thrive in SBH and liberate extraneous oligopeptides during fermentation.

Optimization of *L. helveticus* fermentation parameters through RSM

Total of 29 tests listed in Table 1 were carried out to optimize the four variants in CCD, the results were showed in Table 3. By multiple regression analysis the second-order polynomial equation was obtained. Y (Ca^{2+})=2,011.6–62.58X_1–53.5X_2–37.17X_3+6.08X_4–170.38X_1^2–42.76 X_2^2 –28.51X_3^2–75.63X_4^2–12X_1X_2–74.75X_1X_3–28.5X_1X_4–47.75X_2 X_3+63.25X_2X_4–33.5X_3X_4.

Results of ANOVA analysis (Table 4) suggested that the model was significant, and the lack-of-fit showed no significance. The coefficient of the variation (CV) and r_{adj}^2 were 1.03 and 0.93, respectively. All these results implied the reliability and the precision of the experimental values, as well as high consistency between the experimental and the predicted values. Table 4 also suggested that 2 linear coefficients (X_1, X_2), quadratic term coefficient of X_2^2 and 2 cross coefficients (X_1×X_4, X_3×X_4) were significant.

Verification of the RSM model

The set of optimal conditions (pH 6.1, 3.0% inoculation volume, culture at 36.0℃ for 29.4 h) determined by RSM were also the conditions applied to verify the applicability of the model in predicting the optimal responsive values. A mean value of 19.8 mg/mL (n=3) was obtained in the verifying experiments, which was quite close to the predicated 20.3 mg/mL by the model. Therefore the model was validate and the optimal conditions obtained were reliable.

Table 3. Results of 29 tests of CCD in response surface methodology

Tests	A	B	C	D	Tests	A	B	C	D
1	99.30	88.61	1,947	9.5	16	70.35	71.21	1,652	3.0
2	90.48	87.66	1,964	8.3	17	98.12	89.66	1,984	9.0
3	94.30	86.49	1,845	6.7	18	101.87	90.41	2,014	10.8
4	89.46	84.62	1,883	7.4	19	88.81	87.13	1,893	8.9
5	95.86	84.72	1,948	9.4	20	81.35	75.65	1,674	8.1
6	107.23	91.76	2,079	12.0	21	94.57	85.22	1,884	8.6
7	88.47	85.19	1,749	8.7	22	103.28	88.73	2,007	11.8
8	79.22	79.85	1,862	6.8	23	90.35	89.17	2,017	9.4
9	80.35	81.21	1,749	6.4	24	103.28	88.73	2,011	11.8
10	94.86	77.49	1,678	5.9	25	99.64	78.74	1,946	9.7
11	99.28	89.73	1,933	8.4	26	83.44	82.78	1,863	5.9
12	100.27	84.66	2,004	8.9	27	99.30	88.61	1,947	9.5
13	84.88	81.41	1,792	8.4	28	90.48	87.66	1,964	8.3
14	90.78	86.32	1,841	7.4	29	94.30	86.49	1,845	6.7
15	90.41	86.65	1,909	10.6					

Tests of 19 combinations listed in Table 1 were performed and the results obtained were showed in Table 3 where A, B, C, and D represented total yield of polypeptide (mg/g), the obtaining rate of oligopeptide (%), ionic Ca^{2+} content (mg/100 mL) and viable counts of *Lactobacillus helveticus* ($\times 10^9$ CFU/mL).

Effect of oligopeptide and Ca^{2+} release on the proliferation of *L. helveticus*

Results of correlation analysis based on the data of CCD design (n=29) suggested that all parameters were positively correlated with each other with statistical significance ($p<0.01$) (Table 5). It can be deduced that during fermentation, more free Ca^{2+} were released along with the further extensive degrading of proteose, peptone and polypeptide into more polypeptides and oligopeptides. In turn, these released peptides and Ca^{2+} enhanced the multiplication and the metabolism of *L. helveticus*. At the end of fermentation, 1.1×10^{10} CFU/mL *L. helveticus* can be detected in the fermented SBH hydrolysates.

Level changes of Ca^{2+}, polypeptides, oligopeptides and DH caused by fermentation

It can be seen in Fig. 2, when SBH was fermented under the optimized set of conditions, all levels of tested parameters increased obviously ($p<0.05$ or $p<0.01$). It is necessary to be addressed that the acidic condition could facilitate the chelating reaction between peptidess and liberated Ca^{2+} (Han et al., 2015), and the initial pH of SBH was 6.1 in this study. It's also reported that peptides capable of chelating minerals generated during the process of bone collagen hydrolysis (Torresfuentes et al., 2011). Ca-peptide chelate formed in SBH was verified by plane scan analysis (Han et al., 2015). So more Ca^{2+} and peptides were liberated from bone than the detected levels in this study and the soluble calcium in SBH or fermented SBH was existed both in free and chelated forms, this is the same in milk or dairy product which is a well accepted good source of dietary calcium (Giraffa, 2014). Since bio-fermenting by *L. helveticus* can further boost the liberation of free ionic calcium from bone powder, the fermented SBH would be a good calcium supplementary product with high bio-availability, and the level of calcium in it was obviously higher than milk or dairy product.

DH can be considered as the percentage of the hydrolyzed bonds of peptide. After fermentation, DH increased significantly

Table 4. Variance analysis of the established regression equation

Source of variance	Sum of square of deviations	Degrees of freedom	F	Prob > F
X_1	47,000.08	1	8.18	0.012 6
X_2	34,347.00	1	5.98	0.028 3
X_3	16,576.33	1	2.88	0.111 5
X_4	444.08	1	0.08	0.785 1
X_1^2	576.00	1	0.10	0.756 2
X_2^2	22,350.25	1	3.89	0.068 7
X_3^2	3,249.00	1	0.57	0.464 5
X_4^2	9,120.25	1	1.59	0.228 3
X_1X_2	16,002.25	1	2.79	0.117 3
X_1X_3	4,489.00	1	0.78	0.391 7
X_1X_4	188,000.00	1	32.77	< 0.000 1
X_2X_3	11,859.08	1	2.06	0.172 8
X_2X_4	5,271.73	1	0.92	0.354 4
X_3X_4	37,105.30	1	6.46	0.023 5
Model	356,000.00	14	4.43	0.004 4
Error term	80,440.28	14		
Lack of fit	71,697.08	10	3.28	0.131 8
Pure error	8,743.20	4		
Cor total	437,000.00	28		
r^2=0.9157	r_{adj}^2 =0.9315	CV=1.03		

SBH, sheep bone hydrolysate.
X_1, X_2, X_3, and X_4 were 4 factors affecting SBH *Lactobacillus helveticus* fermentation, which represented fermenting temperature, initial pH of SBH, fermenting time and inoculation amount, respectively. X_1X_2 indicated the interaction between the factor X_1 and X_2, so were X_1X_4, X_2X_3, X_2X_4, and X_3X_4.

Table 5. Results of Pearson's correlation coefficient (r) (n=29) between different parameters

Parameters	ORO	Ca^{2+} content	Colony count
Yield of polypeptides	0.719**	0.749**	0.768**
Ca^{2+} content	1	0.810**	0.691**
Colony count		1	0.767**

ORO, obtaining rate of oligopeptide.
r values were obtained using IBM SPSS statistics V 21.0. ANOVA were carried out using Statistix 8.1.
** $p<0.01$.

($p<0.01$), this indicated an incomplete and limited proteolysis by protease during SBH preparation and more new peptide bonds were destroyed during later fermentation, which inevitably led to a notable lifted polypeptides ($p<0.05$) and oligopeptides content ($p<0.01$) in the final fermentation. These changes were the results of metabolic activities of *L. helveticus* and their proteolytic and peptidolytic actions on the proteose, peptones and longer peptides that alcalase and flavourzyme unnable to further hydrolyze (Elfahri et al., 2014).

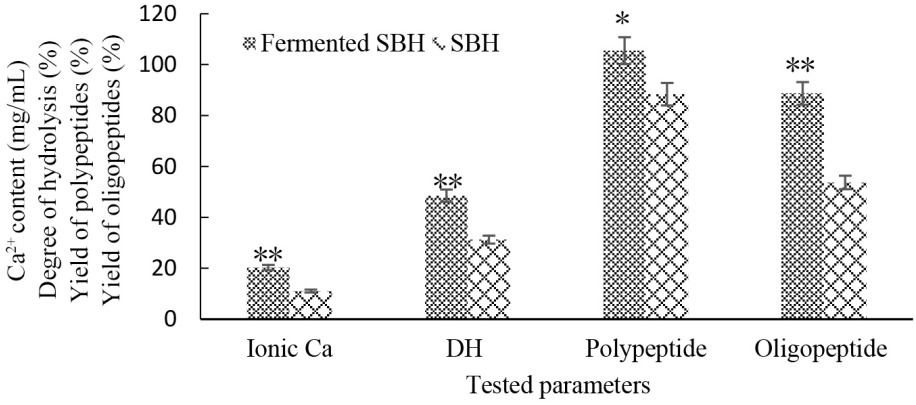

Fig. 2. Effects of *Lactobacillus helviticus* fermentation on the Ca^{2+} release, degree of hydrolysis (DH), yield of polypeptide and oligopeptide. After fermenting of SBH (pH 6.1, containing 1.0% glucose) by tamed *Lactobacillus helviticus* with 3.0% inoculation at 36℃ for 29.4 hours, supernatants from centrifugation were checked. * and ** mean that the corresponding indices were significantly higher than those of SBH at the level of 0.05 and 0.01 statistically. SBH, sheep bone hydrolysate.

Effects of fermentation on the anti-oxidant activities of SBH

DH was a widely accepted parameter in evaluating the functional activities of protein hydrolysate (Kristinsson and Rasco, 2000). The increased DH will inevitably affect the length and the amino acid sequence of peptides (Jamdar et al., 2010). Since the size and the composition of peptides were directly related to their bioactive activities (You et al., 2011), the biological activity of SBH were unavoidable altered. This was evidenced by many studies, for instance, the antioxidant activity of porcine blood plasma protein hydrolysate and peanut protein hydrolysates increased with the increase of DH (Jamdar et al., 2010; Liu et al., 2010).

In this study, as shown in Fig. 3, compared to non-fermented SBH, both DH and the antioxidant ability of fermented SBH to scavenge DPPH ($p<0.05$), hydroxyl radical ($p<0.01$), ABTS radicals ($p<0.01$) were notably increased. In addition to changes in quantity, amino acid composition and the length of the peptide, metabolites such as extra-cellular polysaccharides secreted by

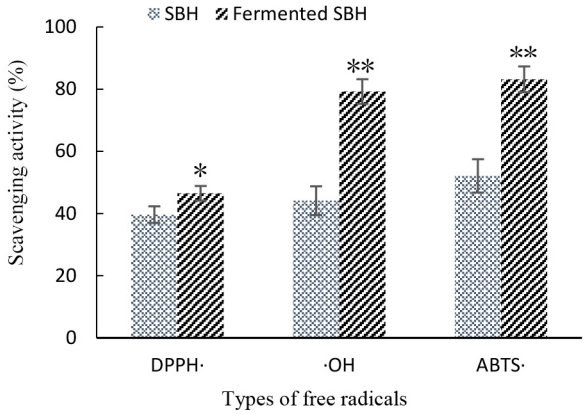

Fig. 3. Effects of *Lactobacillus helveticus* fermentation on the *in vitro* anti-oxidant activity of sheep bone hydrolysates (SBH). After fermenting of SBH (pH 6.1) supplemented 1.0% glucose by tamed *Lactobacillus helviticus* with 3.0% inoculation at 36℃ for 29.4 hours, supernatants collected after centrifugation were checked. * and ** right above the column representing fermented SBH indicated that the scavenging percentages for the corresponding free radicals were significantly higher than those of SBH at the level of 0.05 and 0.01 statistically. SBH, sheep bone hydrolysate.

L. helveticus, free amino acids liberated during fermentation might contribute to this increased extra antioxidant ability. Free radical-scavenging activity of milk and skimmed milk powder solution were also notably increased when fermented by *L. helveticus* (Rong et al., 2017). So fermentation by *L. helveticus* can further increase the anti-oxidant activity of the food matrix.

Conclusion

L. helveticus fermentation can further increase the DH, promote the further release of oligopeptide and free Ca^{2+}, hence boosted the bio-availability and the nutritive value of SBH. The increased DH of SBH by *L. helveticus* fermenting caused an elevated anti-oxidant activity, accompanied by 1.1×10^{10} CFU/mL viable counts of *L. helveticus*. Hence the fermented SBH is both probiotic, nutraceutical and functional, which can be developed as a dietary "antioxidants+Ca+probiotic" supplementary product.

Acknowledgments

This work was financially supported by grants from National Natural Science Foundation of China (No. 31201347), Scientific Research Foundation for Returned Scholars of Shanxi Province, and Shanxi Scholarship Council (No. 2014-042). Special thanks to professor MA Lizhen who kindly provided the commercial starters used in this study.

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