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Preparation and Impact of Fermented Sheep Bone Powder on Sausage Quality

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Abstract: Sheep bones are a rich resource in China, but their deep processing is limited by outdated technology. Sausages are popular among various consumer groups due to their unique flavor. The purpose of this study was to optimize the preparation process of fermented-enzymatic sheep bone powder and develop calcium-fortified functional sausages with an excellent flavor, aroma, and taste. In this experiment, the fermented-enzymatic sheep bone powder was prepared by optimizing the two-bacterial fermentation process of Lactobacillus Plantarum *BNCC336421* and Pediococcus Pentosaceus *BNCC193259*. The nutritional indexes and micro-structure were analyzed. Additionally, different ratios of fermented sheep bone powder were added into the sausages to investigate their effects on the nutritional indexes, physicochemical properties, and organoleptic quality of the sausages. The results showed that the optimal process conditions for the fermented sheep bone sludge were as follows: a strain inoculation of 3%; a compounding ratio of 1:1; a bone sludge concentration of 1:20; and fermentation time of 24 h. Under these conditions, the Ca^{2+} content and protein hydrolysis degree of the sheep bone were 2441.31 mg/100 mL and 23.78%, respectively. The fermented sheep bone powder analyzed using SEM, and the particle size analysis showed it was loose and porous with a small particle size. The addition of fermented sheep bone powder to the sausage increased its amino acid and calcium ion contents, improved the texture indexes such as cohesion, and enhanced both the L* value and sensory scores. The best result was observed in the 1% group ($p < 0.05$). It serves as a data source for developing fermented sheep bone powder and its application in sausage, offering a fresh idea and approach to achieving the high-value utilization of sheep bone.

Keywords: sheep bone powder; fermented-enzymatic; characteristic; sausage; quality

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1. Introduction

Calcium, as a macronutrient, is one of the most important components that make up the human body. It exists in ionic form, and plays a crucial role in blood clotting, bone formation, muscle contraction, and neurotransmitter transmission. The organism can obtain calcium ions through its diet [1]. Sheep bones are a rich resource of this, accounting for about 20–30% of the total body weight of live sheep [2]. They have a high nutritional value, being enriched with functional collagen and a variety of the essential amino acids required by the human body, as well as minerals such as calcium, phosphorus, magnesium, and iron. The ratio of calcium to phosphorus is approximately 2:1, which corresponds to the optimal ratio of calcium and phosphorus in the normal human body [3]. Additionally, animal bone cells are a natural source of calcium supplementation due to their affinity for cells in the same tissue. However, sheep bones are often underutilized and discarded, resulting in wasted resources and environmental pollution. In recent years, the development of bone-based food has shown promise due to the improved comprehensive utilization rate of meat by-products. Currently, biochemical technology can extract various edible bio-products from livestock and poultry bones, such as gelatin, fish bone meal, horse bone puree, pig bone broth, and other food products [4–7]. However, there have been few studies on sheep bone products. Bone powder is a meal product made using animal bones as raw materials. It is one of the main products in China's bone food processing and utilization industry and can be used for nutritional food research and development. At present, collagen peptide extracted from bone has been circulating in the market as a health product. Bone meal is also widely used in western sausages, bone noodles, chutneys, drinks, biscuits, and other food [8]. The only nutrient that can be utilized for bone strength is calcium, as other nutrients are lost during high-temperature cooking [9]. Therefore, biochemical methods can be used to produce bone calcium to improve the utilization rate of nutrients and enhance the value of bones. However, the calcium in bone powder exists in the form of hydroxyapatite crystals ($Ca_{10}(PO_4)_6(OH)_2$) and amorphous calcium phosphate ($CaHPO_4$), which are difficult for organisms to absorb and utilize.

Enzymatic digestion is a common form of protein hydrolysis that can release calcium ions from bound calcium, allowing for its direct absorption and use by an organism's intestines [10]. However, due to the specificity of bone structure, enzymatic hydrolysis can only release one sixth of the bone's calcium content, with the rest remaining poorly soluble in water. Microbial fermentation is the process of utilizing microorganisms to convert raw materials into products needed by humans through specific metabolic pathways under suitable conditions. On one hand, microorganisms can decompose collagen fibers using fermentation enzymes, and on the other hand, they can produce soluble calcium lactate through the action of fermentation acid production on calcium phosphate. Microbial fermentation can not only convert bound calcium in bones into free calcium ions, but also produce bacterial-containing calcium supplements from livestock bones that provide amino acids and phosphorus to promote calcium absorption and regulate the intestinal micro-ecological environment [11]. The combination of enzymatic and microbial fermentation will enhance the benefits of both processes, further breaking down the nutrients in the bone powder to release more calcium ions and catalyze protein hydrolysis for added flavor [12].

Sausage is a traditional meat product that offers a wide diversity, rich nutrition, strong aroma, unique flavor, low cost, and easy portability to meet the preferences of different consumer groups. Though sausage contains high levels of protein and fat, its calcium content is poor and cannot fulfill the recommended daily dietary calcium intake for the population. At present, domestic research on sausage is focused on improving its quality and extending its storage period, with little emphasis on the development of functional sausages such as high-calcium varieties. Therefore, in this paper, an enzymatic method combined with microbial fermentation was used to prepare and optimize fermented sheep bone powder. The sausages were treated as research objects, and the optimal amount of fermented sheep bone powder was derived by adding different levels of it to study its effects on the calcium ion content, texture, color, and organoleptic qualities. It provided technical parameters for the development and utilization of fermented sheep bone powder, a theoretical basis for the development of calcium-enriched sausages, and new ideas and approaches for the comprehensive utilization of meat by-products.

2. Materials and Methods

2.1. Materials

Fresh lamb shoulder blade was purchased from Aofili Food Co., Ltd. (Inner Mongolia, Bayan Nur, China). Alcalase (≥200,000 U/g) was acquired from Coolaber Co., Ltd. (Beijing, China). Lactobacillus Plantarum *BNCC336421* and Pediococcus Pentosaceus *BNCC193259* were provided by BeNa Culture Collection Co., Ltd. (Beijing, China). All the essential ingredients for sausage production, such as pork, beef, 28 mm collagen casing, starch, salt, sodium nitrite, cooking wine, sesame oil, and monascus pigment, etc., were procured from local suppliers. The remaining chemicals were of analytical grade.

2.2. Sample Preparation

2.2.1. Preparation of Enzymatic Bone Powder Solution

The process of producing the sheep bone powder was slightly modified based on Hu's method [13], including steaming in boiling water for 15 min to remove meat and tendons, autoclaving at 121 °C for 30 min to soften the bones, washing off the surface fat, drying at 110 °C for 6 h, and finally crushing into a powder (80 mesh). The sheep bone powder was mixed with anhydrous ethanol at a ratio of 1:10, and stirred for 24 h at room temperature (anhydrous ethanol was renewed each 8 h). The precipitate was obtained via centrifugation (4 °C, 3000 r/min, 15 min) and defatted sheep bone powder was produced after rinsing with water and drying at 60 °C.

The defatted bone powder and distilled water were weighed in a certain proportion (bone sludge concentration: 1:10, 1:15, 1:20, 1:25, and 1:30), and alkaline protease was added for stirring with a magnetic stirrer (HJ-6, Jiangsu Dongpeng Instrument Manufacturing Co., Ltd., Changzhou, China). The pH was adjusted using HCl or NaOH with a pH meter (Matthaus Company, Erlangen, Germany). Enzyme digestion was conducted in a rotary bath shaker (SHZ-82A, Changzhou Zhongbei Instrument Co., Ltd., Changzhou, China) for 4 h while maintaining a constant pH. After four hours, the enzyme was inactivated in a water bath at 100 °C for 15 min and the enzymatic bone powder solution was obtained upon cooling to room temperature.

2.2.2. Activation and Domestication of Strains

Lactobacillus Plantarum *BNCC336421* and Pediococcus Pentosaceus *BNCC193259* were inoculated into a liquid MRS medium at an inoculum level of 4%, followed by three generations of activation, resulting in a final concentration range of 10^8–10^9 CFU/mL. During the domestication process of the strains, the proportion of enzymatic bone sludge in the liquid MRS medium was gradually increased and the ratios were adjusted as follows: 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, and finally reaching a ratio of 0:10. The inoculum concentration was set at 4%, while the incubation temperature and duration were maintained at 37 °C and 30 h, respectively.

2.2.3. Preparation of Fermented Sheep Bone Powder

The domesticated strains were inoculated into a 5% glucose-added enzymatic bone powder solution. The strain inoculation concentrations (1, 2, 3, 4, and 5%), strain ratios (3:1, 2:1, 1:1, 1:2, and 1:3), and fermentation durations (6, 12, 18, 24, 30, and 36 h) of the Lactobacillus Plantarum *BNCC336421* with Pediococcus Pentosaceus *BNCC193259* were controlled to investigate the variations in pH level, hydrolysis degree, and Ca^{2+} content, as well as to determine the optimal fermentation protocol. After fermentation, the samples were sterilized in a water bath at 100 °C for 15 min, cooled to room temperature, and freeze-dried to obtain the fermented sheep bone powder.

2.2.4. Preparation of Sausage

The minced raw meat was mixed with various seasonings to make the meat filling (fermented sheep bone meal addition: 0, 0.2, 0.6, and 1%, recorded as C, L1, L2, and L3 groups), marinated at 4 °C for 24 h before enema, and finally boiled to make sausage. (Formulation: 25.6 kg of lean pork, 6.4 kg of fat pork, 8 kg of beef, 1 kg of starch, 23.16 g of monosodium glutamate (MSG), 1.5 kg of salt, 38.61 g of pepper, 7.5 g of sodium nitrite, 0.5 kg of cooking wine, 150 g of sesame oil, and 38 g of monascus pigment).

2.3. Degree of Hydrolysis

The degree of hydrolysis was determined with reference to Venuste's method [14]. Specifically, the sample enzyme solution was mixed with distilled water in a 1:5 (v/v) ratio in a triangular flask and titrated with 0.1 M NaOH (standard solution) to pH 8.2. Then, 10 mL of formaldehyde solution was added and titrated to pH 9.2, while noting the volume of the NaOH solution consumed from pH 8.2 to pH 9.2 as V1; using distilled water as a control, the volume was noted as V0.

$$\text{Degree of Hydrolysis (\%)} = \frac{(V1 - V0) \times C \times V \times 0.014}{(5 \times TPN)} \times 100$$

$V0/V1$—the NaOH consumption (mL); C—the actual concentration of NaOH (M); V—the total volume of mixture (mL); 5—the sample volume (mL); and TPN—the protein content of the sample.

2.4. Calcium Content

The calcium content was measured following flame atomic absorption spectrometry (FAAS). The sample, ranging from 0.2 to 3 g with a precision of 0.001 g, was meticulously weighed and digested in a digestion tube before being cooled and diluted to 25 mL. A lanthanum solution of 20 g/L was added to achieve a final dilution concentration of 1 g/L, while a blank sample was also prepared. The absorbance was detected at 422.7 nm using an atomic absorption spectrophotometer (AA-3300, Thermo Fisher Scientific Inc., Waltham, MA, USA) and quantified by a comparison with the standard series.

2.5. Scanning Electron Microscope (SEM) and Particle Size Analysis

The microstructure of the sheep bone powder was characterized with a scanning electron microscope (Hitachi S-3400N, Hitachi, Ltd., Tokyo, Japan). The appropriate amount of sample was applied onto the conductive adhesive, secured in place, coated with gold, and imaged using an electron beam at a probe voltage of 30 kV, current of 50 pA, and electron beam accelerating voltage of 5 kV for a micro-morphological analysis. The particle size of the bone powder was measured using a particle analyzer (Nano-ZS ZEN3600, Malvern Panalytical Ltd., Malvern, UK), and the mean particle size was expressed as median diameter (D50).

2.6. Basic Nutrients

Hu's method was employed to determine the basic nutrient contents [15]. The moisture content was quantified by desiccating the samples at 105 °C for 12 h, while the protein content was obtained through a Kjeldahl determination using a fully automated Kjeldahl nitrogen analyzer (Kjeltec 8400, FOSS, Hillerød, Denmark). The ash content, on the other hand, was determined by combusting the samples in a muffle furnace (AFD-4-10, Henan Aofeida Instrument Co., Ltd., Zhengzhou, China) at 550 °C.

2.7. Amino Acid Assay

The amino acid content was assayed using the acid hydrolysis method [16]. An adequate amount of samples (10–20 mg protein) was weighed and subjected to hydrolysis with 6 M HCl at 110 °C for a duration of 22 h. After filtration through a 0.22 μm aqueous membrane, the amino acid content was analyzed using an ultra-high-speed fully automated amino acid analyzer (LA8080, Hitachi, Ltd., Tokyo, Japan).

2.8. Color Evaluation

The sausage color was tested with a TCP2 colorimeter (Shanghai Biological and Biochemical Experimental Instrument Co., Ltd., Shanghai, China). The interior brightness (L*), redness (a*), and yellowness (b*) of the samples were measured using an instrument calibrated with a white ceramic disk at an observer angle of 2 °C, aperture size of 50 mm, and illuminant D65.

2.9. Texture Determination (TLP)

The texture was determined using a CT3-type texture analyzer (US), referring to Rigdon's method [17]. The samples were peeled and cut into squares of $1 \times 1 \times 1$ cm^3, which were then measured using a P/36R probe with the following parameter settings: a pre-test speed of 2 mm/s, a test speed of 2 mm/s, a post-test speed of 5 mm/s, and a compression ratio of 50%.

2.10. Sensory Evaluation

The sensory evaluation of the fermented sheep bone powder sausage was consistent with the sensory evaluation standard established by Meng et al., and altered depending on the product's characteristics [18]. The middle section of the sausage was carefully selected and sliced into 4–5 mm pieces for the sensory evaluation. A panel of ten food professionals conducted the evaluation, scoring on a scale of 1 to 10 based on appearance (20%), texture (30%), and flavor (50%) (Table 1).

Table 1. Fermented sheep bone powder sausage sensory scoring criteria.

Item	Total Score	Score Criteria
Flavor	5	strong flavor in meat; smooth in taste; no impurities (4–5); slightly strong flavor in meat; slightly rough taste; a few impurities (2–3); light flavor in meat; rough in taste; lots of impurities (1–2).
Texture	3	Firm and elastic (3); slightly firm and elastic (2); loose and non-elastic (1).
Color	2	Bright, rose-red in color (2); slightly dull, dark red in color (1); very grayish color (0).

2.11. Statistical Analysis

The results were expressed as the mean ± standard deviation (SD) of three independent experiments and subjected to an analysis of variance (ANOVA) using SPSS Statistics 23 software (IBM, New York, NY, USA). Statistical significance was set at $p < 0.05$, followed by post hoc testing with Duncan's multiple range test.

3. Results and Discussion

3.1. Optimization of Fermented Sheep Bone Sludge

By optimizing the strain inoculation amount, bone sludge concentration, compound ratio, and fermentation time, we developed an optimal process for fermenting sheep bone sludge (Figure 1). During the screening process for the optimal conditions, a pattern of initial increase followed by decrease was observed in both the hydrolysis degree of the sheep bone protein and the calcium ion content. With the exception of the fermentation time, there was a trend of decreasing pH values followed by an increase in all the other process conditions. The hydrolysis degree of the sheep bone protein reached maximum values of 20.75%, 13.87%, 18.78%, and 21.86% at an inoculum amount of 3%, bone sludge concentration of 1:20, strain compounding ratio of 1:1, and fermentation time of 24 h, respectively. Additionally, the Ca^{2+} level also reached its peak value under these conditions with measurements of 1787, 1898, 2274, and 2368.33 mg/100 mL for each respective sample group; the pH achieved a minimum value ranging from 5.02 to 5.20 under these conditions. The pH value decreased from 5.10 at 24 h of fermentation time to its lowest point of 4.33 at 36 h. Considering the pH value, degree of hydrolysis, and free calcium content, optimal results were achieved with a composite strain inoculum of 3%, bone sludge concentration of 1:20, strain compounding ratio of 1:1, and fermentation time of 24 h.

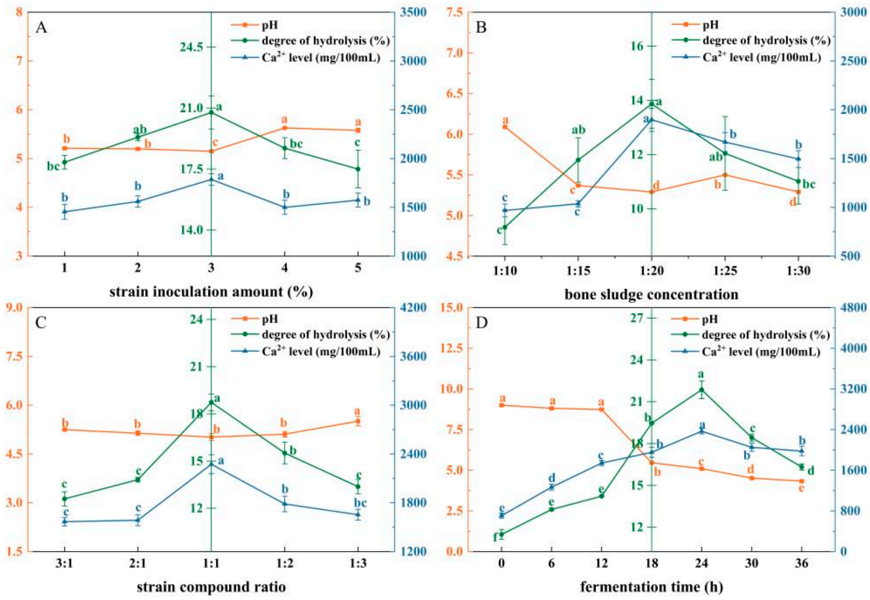

Figure 1. The optimization of fermented sheep bone sludge. (**A**–**D**) The optimization of the strain inoculation amount, bone sludge concentration, strain compound ratio, and fermentation time for fermented sheep bone sludge. Different letters indicate significant differences ($p < 0.05$), and same letters indicate insignificant differences ($p > 0.05$).

3.2. Basic Ingredients of Fermented Sheep Bone Powder

The fundamental ingredients of the fermented sheep bone powder are displayed in Figure 2. The fermented sheep bone powder exhibited significantly lower levels of protein, moisture, and ash compared to the sheep bone powder ($p < 0.05$). Conversely, the free Ca^{2+} and amino acid contents were significantly higher in the fermented sheep bone powder than in the sheep bone powder ($p < 0.05$). Moreover, the fermented sheep bone powder exhibited a calcium ion content that was approximately 39 times higher than that of the unfermented bone powder. This proved that fermentation could facilitate protein hydrolysis and calcium ion release.

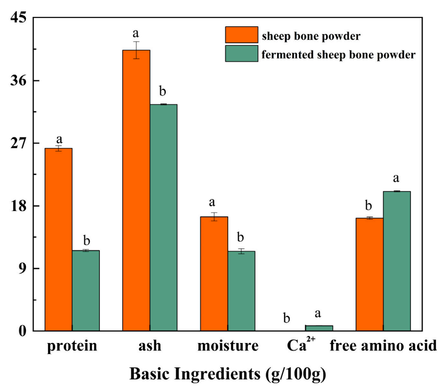

Figure 2. The basic ingredients of fermented sheep bone powder. Different letters indicate significant differences ($p < 0.05$), and the same letters indicate insignificant differences ($p > 0.05$).

3.3. Microstructural Analysis of Fermented Sheep Bone Powder

We observed the microstructure of the fermented sheep bone powder using SEM and a nano-particle size analysis (Figures 3 and 4). Based on Figure 3(A1,B1), it is evident that the fermented sheep bone powder had a more porous and loosely structured surface in comparison to the original bone powder, which had a less porous and denser surface. Within the 100 μm field of view (Figure 3(A2,B2)), it is evident that the fermented bone powder particles were smaller compared to the original bone powder particles. This suggested that some of the substances in the fermented bone powder were free, which was conducive to the release of nutrients.

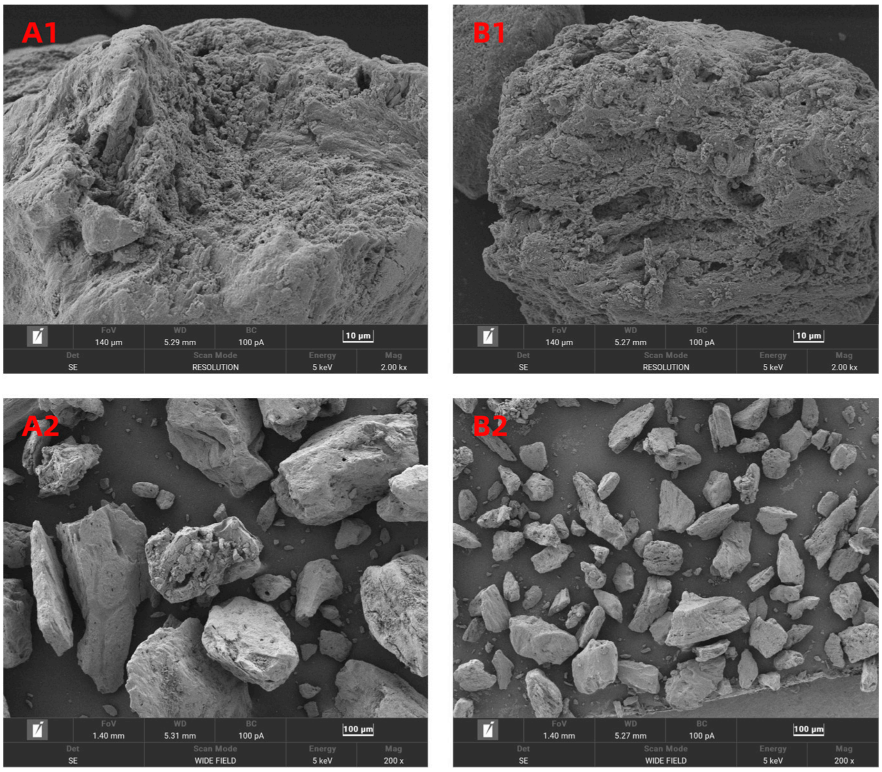

Figure 3. The SEM image of sheep bone powder. (**A1**,**A2**) The SEM image of non-fermented sheep bone power. (**B1**,**B2**) The SEM image of fermented sheep bone power. Bar: (**A1**,**B1**)—10 µm; (**A2**,**B2**)—100 µm.

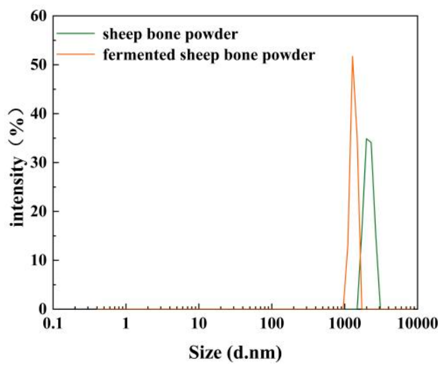

Figure 4. The particle size of fermented and unfermented sheep bone powder.

As seen in Figure 4, the crude sheep bone powder treated by simple crushing and drying had an average particle size of 3.08 µm with a wide range of particle size distribution, consistent with the SEM observations. In contrast, the sheep bone powder treated under optimal fermentation conditions exhibited an average particle size of 2.35 µm, indicating a significant reduction in particle size due to fermentation.

3.4. Effect of Fermented Sheep Bone Powder on the Basic Composition of Sausages

It was observed that the addition of fermented sheep bone powder had varying effects on the base composition of the sausage in Figure 5. The addition of fermented sheep bone powder significantly improved the calcium content, with the sausage group containing 1%

fermented sheep bone powder having a content as high as 176.32 mg/100 g, which was 7.8 times higher than that of the control group ($p < 0.05$, Figure 5B). The ash and protein ratio in the sausages supplemented with fermented sheep bone powder was significantly higher than that of the non-supplemented groups ($p < 0.05$, Figure 5C,D). There was a significant increase in the moisture content in groups L1 and L2 in comparison to groups C and L3 ($p < 0.05$, Figure 5A). These results suggested that the addition of fermented bone powder to sausage could enhance its calcium content while maintaining its original nutrient composition.

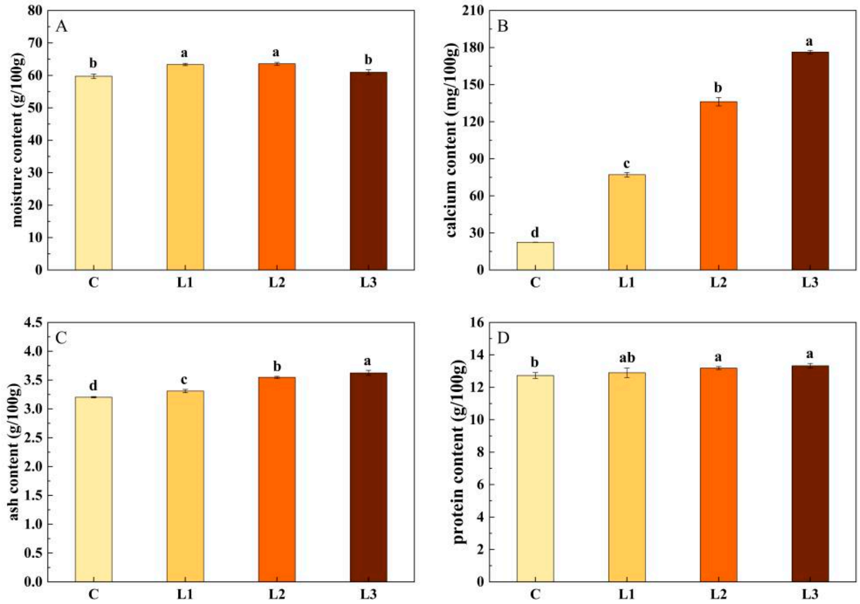

Figure 5. The basic composition of sausage. (**A**–**D**) The content of moisture, calcium, ash, and protein for sausage. Different letters indicate significant differences ($p < 0.05$), and the same indicate insignificant differences ($p > 0.05$).

3.5. Effect of Fermented Sheep Bone Powder on the Amino Acid Content of Sausages

A comprehensive list of the 16 amino acids detected in the sausages is presented in Table 2, comprising eight essential amino acids (Thr, Val, Met, Ile, Leu, Phe, Lys, and His) and eight non-essential amino acids (Asp, Ser, Glu, Gly, Ala, Tyr, Arg, and Pro). The amino acid contents in the sausages were correlated with the fermented sheep bone powder. The L3 group had the highest total amino acid and hydrophobic amino acid contents in the sausages among all groups, with values of 10.62 g/100 g and 4.01 g/100 g, respectively. Aspartic acid, proline, glycine, alanine, phenylalanine, methionine, and arginine contents were statistically significant higher in the 1% group relative to the control group ($p < 0.05$). These findings revealed that incorporating fermented bone powder could enhance the nutritional value and flavor profile of sausages.

Table 2. Amino acid content of sausages.

Item	C	L1	L2	L3
Asp	0.95 ± 0.033^{b}	1.05 ± 0.011^{a}	1.08 ± 0.037^{a}	1.05 ± 0.011^{a}
Thr * #	0.51 ± 0.024^{a}	0.52 ± 0.013^{a}	0.51 ± 0.011^{a}	0.52 ± 0.010^{a}
Ser	0.48 ± 0.019^{a}	0.50 ± 0.08^{a}	0.49 ± 0.009^{a}	0.50 ± 0.008^{a}
Glu	1.92 ± 0.074^{a}	2.02 ± 0.068^{a}	1.91 ± 0.057^{a}	1.97 ± 0.048^{a}
Pro #	0.48 ± 0.015^{bc}	0.47 ± 0.016^{c}	0.51 ± 0.009^{ab}	0.52 ± 0.011^{a}
Gly	0.56 ± 0.018^{b}	0.54 ± 0.014^{b}	0.67 ± 0.009^{a}	0.69 ± 0.025^{a}
Ala #	0.67 ± 0.019^{b}	0.69 ± 0.008^{ab}	0.71 ± 0.008^{a}	0.72 ± 0.005^{a}

Table 2. *Cont.*

Item	C	L1	L2	L3
Val * #	0.44 ± 0.014 [a]	0.42 ± 0.014 [a]	0.42 ± 0.010 [a]	0.42 ± 0.010 [a]
Met * #	0.18 ± 0.004 [b]	0.24 ± 0.002 [a]	0.24 ± 0.004 [a]	0.24 ± 0.008 [a]
Ile * #	0.85 ± 0.022 [a]	0.87 ± 0.018 [a]	0.84 ± 0.015 [a]	0.86 ± 0.023 [a]
Leu * #	0.39 ± 0.014 [a]	0.38 ± 0.018 [a]	0.37 ± 0.007 [a]	0.38 ± 0.009 [a]
Tyr	0.37 ± 0.013 [a]	0.38 ± 0.014 [a]	0.37 ± 0.014 [a]	0.36 ± 0.0152 [a]
Phe * #	0.30 ± 0.014 [b]	0.31 ± 0.007 [b]	0.31 ± 0.008 [b]	0.35 ± 0.001 [a]
His *	0.41 ± 0.009 [a]	0.41 ± 0.011 [a]	0.41 ± 0.009 [a]	0.41 ± 0.006 [a]
Lys *	0.94 ± 0.022 [a]	0.93 ± 0.009 [a]	0.92 ± 0.011 [a]	0.92 ± 0.008 [a]
Arg	0.67 ± 0.023 [b]	0.68 ± 0.009 [ab]	0.69 ± 0.014 [ab]	0.70 ± 0.009 [a]
Total Amino Acid	10.11 ± 0.338 [b]	10.44 ± 0.234 [ab]	10.48 ± 0.232 [ab]	10.62 ± 0203 [a]
Essential Amino Acid (EAA)	4.02 ± 0.122 [a]	4.10 ± 0.093 [a]	4.03 ± 0.074 [a]	4.10 ± 0.075 [a]
Hydrophobic Amino Acid	3.82 ± 0.126 [b]	3.91 ± 0.096 [ab]	3.93 ± 0.071 [ab]	4.01 ± 0.077 [a]

Note: *—Essential amino acids, #—Hydrophobic amino acids. Different letters in peer data indicate significant difference ($p < 0.05$), same letter indicates non-significant difference ($p > 0.05$).

3.6. Effect of Fermented Sheep Bone Powder on the Texture of Sausages

In a comparison between the sausage group with the fermented sheep bone powder added and those without, it was discovered that the former exhibited increases in hardness, springiness, cohesiveness, and chewiness (Figure 6). The L3 group showed a significantly greater hardness than the other test groups ($p < 0.05$) and it also demonstrated a notably higher level of hardness compared to the control group ($p < 0.05$), as illustrated in Figure 6A. The additive of fermented sheep bone powder resulted in significant improvements in springiness, cohesiveness, and chewiness and a statistically significant relationship was observed at 1% added compared to the control group ($p < 0.05$, Figure 6B–D).

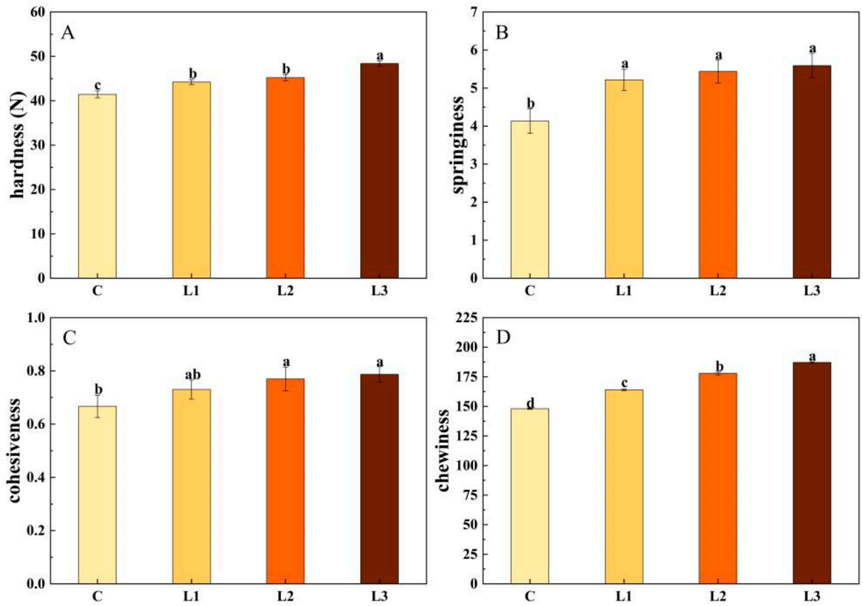

Figure 6. The texture of sausage. (**A**–**D**) Hardness, springiness, cohesiveness, and chewiness of sausage. Different letters indicate significant differences ($p < 0.05$), and the same indicate insignificant differences ($p > 0.05$).

3.7. Effect of Fermented Sheep Bone Powder on the Color of Sausages

The impact of different levels of fermented sheep bone meal on the color of the sausages is depicted in Figure 7. Additions of fermented sheep bone powder gave an increment in the lightness value (L*) of the sausages, while the redness value (a*) and yellowness value (b*) had little variation. As seen in Figure 7A, L* was significantly raised in groups L1 and L3 in comparison to group C ($p < 0.05$), whereas L2 was not statistically significant ($p > 0.05$).

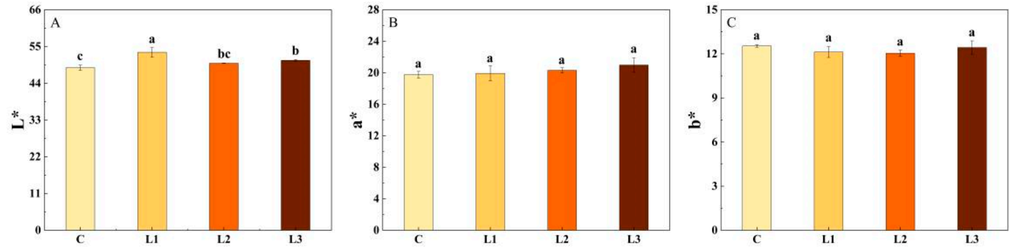

Figure 7. The color of sausage. (**A**–**C**) L*, a*, and b* of sausage. Different letters indicate significant differences ($p < 0.05$), and the same indicate insignificant differences ($p > 0.05$).

3.8. Effect of Fermented Sheep Bone Powder on the Sensory Evaluation of Sausages

Based on the scoring of the sausage sensation by a professional panel, the results obtained are shown in Figure 8. There was no significant difference ($p > 0.05$) between the sensory attributes of groups C and L1, nor between those of groups L2 and L3 ($p > 0.05$), while the latter differed significantly from the former ($p < 0.05$). Sausages with a 1% added amount appeared to have a slight sheep odor.

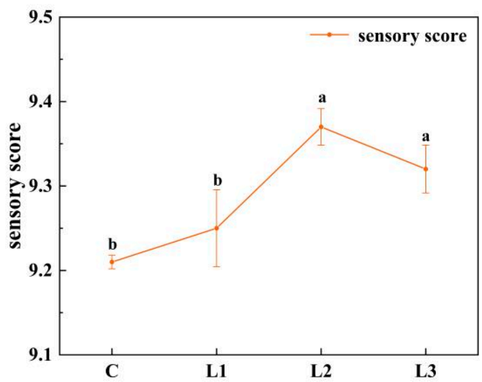

Figure 8. The sensory score of sausage. Different letters indicate significant differences ($p < 0.05$), and the same indicate insignificant differences ($p > 0.05$).

4. Discussion

Skin, blood, and internal organs are all by-products generated during the slaughter and processing of livestock and poultry. According to statistics from EU countries, nearly 330 million livestock are slaughtered every year, resulting in over 18 million tons of meat by-products [19]. Skeleton is also one of the main sources of livestock and poultry by-products. Presently, it has been used in a variety of applications in the international market, such as bone meal, bone sludge, bone mineral, bone oil, active polypeptide, and so on. However, less than 10% of these are deeply processed in China, with most being used for animal feed, low-end product processing, or simply being abandoned, which imposes a heavy burden on the environment [20]. Enzymatic fermentation co-processing technology is a novel method for extracting the free calcium from bones, which effectively combines the advantages of two major technologies, including enzymatic hydrolysis and bio-fermentation, achieving simultaneous improvements in product content, purity, and safety. In this paper, we used enzymatic hydrolysis and fermentation technology to prepare fermented sheep bone powder with high levels of hydrolysis products and calcium content. Furthermore, we optimized the process and added it to sausage, resulting in a functional high-calcium sausage with both color and aroma.

The quality of the fermented sheep bone sludge was closely correlated with its conditions during fermentation (strain inoculation amount, bone sludge concentration, compounding ratio, and fermentation time). The degree of hydrolysis is regarded as an indicator of the extent of protein breakdown, which affects both the length and sequence of peptide chains. Bio-fermentation was found to promote the release of free iron calcium from bone powder [21]. The pH value serves as an indicator of the bacterial growth status, with a rapid decline indicating a strain's strong adaptability to a medium environment and fast reproduction. At this stage, microorganisms exhibit their strongest ability to decompose substrates, resulting in increased calcium ion release from acidic systems. With increases in inoculation amounts and fermentation time, the number of viable bacteria in the system increased. This caused a downward trend in the overall pH value and a higher degree of hydrolysis due to peptide bond destruction under sufficient nutrient conditions. However, when the ratio of bone sludge was 1:10, an excessive use of bone sludge raw material lowered the dissolved oxygen levels, which affected the bacterial growth and resulted in a sub-optimal degree of hydrolysis and other indexes [22]. Thus, the hydrolysis degree, pH, and free calcium content of the sheep bone powder reached their peak at a certain level. During late fermentation, the substrate consumption accelerated and the proportion of bone sludge declined, leading to insufficient nutrients for normal bacterial growth [23]. In addition, lactobacillus accumulated a large number of metabolites during growth and reproduction, in which negative feedback regulated their growth. Hence, the hydrolysis degree and Ca^{2+} content of the system decreased, while the pH value increased. The reduction in free Ca^{2+} may also be attributed to its binding with excess strain metabolites, which converted the free calcium into a bound state and resulted in a lower conversion rate. The fastest drop in pH was observed in the medium when the strains were combined at a ratio of 1:1, indicating that Lactobacillus Plantarum *BNCC336421* and Pediococcus Pentosaceus *BNCC193259* had the best growth status and highest bacterial count among the two strains at a 1:1 ratio. Depending on the optimal fermentation conditions derived, three replications were conducted to confirm that the free Ca^{2+} content in the sheep bone was measured to be 2441.31 mg/100 mL, and the degree of hydrolysis was 23.78%.

The calcium ion and amino acid composition are the key indicators for evaluating fermented sheep bone powder. We found that the enzymatic fermentation technology could increase both the calcium ion and amino acid contents in the sheep bone powder (Figure 2). This was due to an increase in protein hydrolysis, which released more calcium ions, causing a higher nutritional value in the bone powder. As a result, the high-value development of sheep bone could be achieved. Meanwhile, the increased amino acid content further enhanced the flavor of the bone powder [24]. Furthermore, the characterization and microstructural analysis revealed that the particles of the fermented sheep bone powder observed under SEM were small, loose, and porous, making them easier to be digested and absorbed by the human body [25]. Particle size is a pivotal factor that reflects the characteristics of fermented bone powder. The smaller the particle size, the higher the solubility and bio-availability of the fermented sheep bone powder [26]. Enzymatic hydrolysis and fermentation techniques effectively disintegrate the macro-molecular conjugates in sheep bone, transforming them into a free state with a uniform particle size distribution and narrowing this range.

Sausage is a category of meat product that is dehydrated, salted, cured, enrobed, and cooked at high temperatures, and its quality evaluation involves a basic composition, color, and texture [27]. In this study, the base constituents of sausage, such as its calcium ions and ash content, were positively correlated with the amount of bone powder added, indicating that fermented bone powder could incorporate its own nutrients into the sausage and increase the inorganic content of the sausage to enhance its functionality. In contrast, the moisture content in the L3 group decreased significantly, probably due to the increase in positive valent ions (Figure 5A). Amino acids are the basic building blocks of proteins, providing energy for the normal metabolism of the human body and serving as precursor substances for sausage flavor. It has been found that aspartic acid, arginine, and proline, as well as alanine, are precursors to meat flavor [28]. There was an upsurge in content of Asp, which is also the main source of the freshness flavor of sausage [29]. Meanwhile, Gly and Ala conferred sweetness to the food. Overall, all amino acids with a good flavor were elevated in the sausages, which may have been related to their corresponding amino acid contents in the fermented sheep bone powder. Taste was affected by the texture of the sausages. Lee et al. demonstrated that adding calcium compounds improved the gelling properties of Pacific cod surimi [30], while Yin et al. found that incorporating 0.1% fish bone meal increased both the breaking force and distance in surimi, which is consistent with our results [31]. Sausages' color is correlated with consumer satisfaction. A higher L* value was observed in the L3 group with a high mineral content (Figure 7A) [32]. Interestingly, sausages made from broilers fed with Calcium Anacardate exhibited a good color stability [33]. It was also investigated that calcium had no effect on the color of the fermented sausages [34]. The sensory properties of sausages determine the consumer acceptance and future market of the product [35]. Currently artificial food additives or other substances are added to alter the meat quality of sausages, especially the flavor of the sheep. This was also true for our high-calcium sausages, the flavor of which could be enhanced in this way. Apart from the advantages mentioned above, calcium salts can be added to sausages as a substitute for traditional sodium chloride in order to produce low-salt, high-calcium sausages [36]. Furthermore, the addition of calcium also reduces the nitrite residues in sausages, significantly enhancing the food safety [37].

5. Conclusions

In summary, the best fermented sheep bone budge with a Ca^{2+} content of 2441.31 mg/100 mL and degree of protein hydrolysis of 23.78% was obtained when the compound ratio of Lactobacillus plantarum *BNCC336421*, Pediococcus Pentosaceus *BNCC193259* was 1:1, with an inoculum amount of 3%, a bone sludge concentration of 1:20, and a fermentation time of 24 h. The particle size of the sheep bone powder became smaller, the structure became looser and more porous, and amino acids and calcium ions were released after enzymatic fermentation. Moreover, adding fermented sheep bone powder could help prepare high-calcium sausage while effectively improving its quality in terms of its basic nutrition, flavor, and texture. It provides a novel method for the high-value utilization of sheep bone powder and innovative functional sausage preparation.

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