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EFFECTS OF NACL, PEPTONE AND GLUCOSE ON THE PRODUCTION OF ANTIOXIDANT PEPTIDES FROM GOAT MILK FERMENTED BY *LACTIPLANTIBACILLUS PLANTARUM* L60

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Abstract
Dairy products are rich in nutrients. Among them, fermented goat milk (FGM) has gradually become a hot topic because it contains antioxidant peptides to remove free radicals and inhibit the occurrence of cardiovascular diseases. In this paper, this study uses goat milk as a raw material, the DPPH free radical scavenging rate (DPPH-FRSR) and viable counts as response values, and uses the response surface method to study the effects of NaCl, peptone, and glucose on the production of antioxidant peptides from goat milk fermented by *Lactiplantibacillus plantarum* L60. The results show that NaCl and peptone have a significant interaction on the response value ($p<0.05$), and peptone and glucose have a very significant interaction with DPPH-FRSR ($p<0.01$). The optimal additive amounts of NaCl, peptone and glucose were 1.02%, 0.82% and 0.72%, respectively. The DPPH-FRSR and viable counts in the prepared FGM were 80.23% and 1.09×10^9CFU/mL respectively. The findings can guide the progression of novel probiotic fermentation goat milk.

Keywords: antioxidant peptides, goat milk, *Lactiplantibacillus plantarum* L60

Introduction

When covalent bonds break in a compound molecule, atomic or ionic groups with unpaired electrons are created, known as free radicals. Because of their high chemical reactivity, free radicals often combine with other molecules in the body to produce new oxides or free radicals (Kehrer & Klotz, 2015). Reactive oxygen species are oxygen radicals converted from oxygen in the body, such as superoxide, monoclinic oxygen, hydrogen dioxide, hydrogen and hydroxyl radicals (Martemucci *et al*., 2023). When the equilibrium of the body of free radicals is disturbed, there is excessive accumulation of free radicals. At this time, free radicals, as a kind of

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cytotoxic oxidant, will harm the body's lipids, proteins, and nucleic acids, thus destroying cell structure. This oxidative stress caused by excess free oxygen may lead to human cardiovascular disease, Alzheimer's disease, and cancer (Song *et al*., 2020; Wu *et al*., 2021; Escamilla Rosales *et al*., 2023).

Antioxidants not only preserve oxidative equilibrium and physical health by scavenging free radicals, but also decrease the incidence of hypertension and other cardiovascular diseases (Lv *et al*., 2022). The antioxidant peptide is a kind of natural antioxidant that has become one of the most talked-about topics due to its low molecular weight, straightforward composition, remarkable antioxidant capacity, and security (Sarmadi and Ismail, 2010).

Food-derived antioxidant peptides have been extensively studied in five main areas: cereals and potatoes (Aderinola and Duodu, 2022; Liu *et al*., 2023), animal foods (Xing *et al*., 2021), legumes and nuts (Acevedo-Juárez *et al*., 2022; de Fátima Garcia *et al*., 2021), fruits and vegetables (Sosalagere *et al*., 2022) and energy-only foods (Pan *et al*., 2022). Yu *et al*. (2023) identified a novel peptide, Arg-Tyr-Leu-Leu (RYLL), with a strong antioxidant capacity from corn gluten meal hydrolysis products. Chen *et al*. (2023) extracted walnut antioxidant peptides from defatted walnut powder, and its DPPH free radical scavenging rate (DPPH-FRSR) was 89.59%. Wang *et al*. (2024) isolated M-2 (<3 KDa) with the strongest antioxidant activity from the hydrolysate of chicken blood hemoglobin by ultrafiltration, and its DPPH-FRSR was 82.91%. Huang et al. (2023) screened six novel antioxidant peptides from the hydrolysis products of pearl shellfish meat. Zare-Zardini *et al*. (2013) found that Snakin-Z, a fresh peptide originating from Ziziphus jujuba fruits, has inhibitory activity on acetylcholinesterase and butyrylcholinesterase, which could offer guidance for treating Alzheimer's disease. Chang *et al*. (2015) isolated three novel antioxidant peptides from protein hydrolysate extracted from oil palm kernel.

Although antioxidant peptides have been studied extensively, the preparation methods are relatively homogeneous, and enzymatic hydrolysis is currently the most commonly used method for the preparation of antioxidant peptides, which is used primarily to prepare antioxidant peptides by degradation of food-derived proteins (Ashaolu, 2020). Fermentation of probiotics to prepare antioxidant peptides is a method that has emerged gradually in recent years. It can reduce manufacturing costs in addition to streamlining operational procedures. Aguilar-Toalá *et al*. (2017) found that peptides <1 kDa had higher antioxidant activity when studying various *L. plantarum* fermented milk whey. A new strain of wild *L. plantarum* with significant antioxidant activity was isolated by Chen *et al*. (2021) from Kefir, which fermented goat milk with strong DPPH free radical scavenging activity.

In previous papers, the effects of the nitrogen source, inorganic salt and source of carbon on the antioxidant capacity of FGM were studied, and peptone, NaCl, and glucose were found to be the main influencing factors (Shu *et al*., 2017; Hu *et al*., 2023). In this paper, the Box-Behnken design is utilized to investigate the impacts of NaCl, peptone, and glucose on DPPH-FRSR and viable counts of FGM, which will guide the progression of probiotic fermentation goat milk.

Materials and methods

Microorganism

L. plantarum L60 from the Food Science and Technology School, SUST, was injected into the MRS Broth medium that had been sterilized at 121°C for 15 minutes and cooled, incubated at 37°C for 24 hours, activated for two generations, and incubated at 37°C for 18 hours to obtain the third-generation activated suspension of microorganisms. For later use, store it refrigerated at 4°C.

Preparation of FGM

First, whole goat milk powder (Shaanxi Yatai Dairy Co., Ltd., Shaanxi, China) and distilled water were combined in a 1:8 (w/v) ratio to obtain reconstituted goat milk with a concentration (w/w) of 14%. Second, the reconstituted milk was pasteurized for 15 minutes at 90°C and then allowed to cool to about 30°C. Third, the activated third-generation strains were added to the reconstituted goat milk at a rate of 5%. Finally, the combination was then thoroughly shaken and allowed to ferment at 42°C in a water bath until all goat milk curdled.

Preparation of the whey sample to be tested

First, the FGM is shaken well and its pH is determined with a pH meter (PHS-3C). Second, using a 1 mol/L HCl solution, FGM's pH was brought to 3.5, and centrifugation was used to extract the supernatant for 15 minutes at 8000 rpm. Third, the pH of FGM was brought to 8.3 using a 1 mol/L NaOH solution. Finally, the milk was centrifuged for 15 minutes at 8000 rpm and the supernatant was collected as the sample to be measured.

DPPH-FRSR assay

Regarding the method of Shu *et al*. (2019a), the mixed liquid of each experiment was thoroughly shaken and mixed, then placed in a dark place for 30 minutes for reaction and its absorbance was determined at 517 nm. Meanwhile, the DPPH-FRSR of Trolox solution at 0.25 mg/mL served as the control. The following formula was used to get the DPPH-FRSR:

$$DPPH - FRSR\ (\%) = \left[1 - \frac{X1-X2}{X}\right] \times 100\% \qquad (1)$$

where X1, as the test group, represents the absorbance value of 8 mL of DPPH free radical solution (0.1 mmol with ethanol as solvent, Sigma, US) +2 mL sample for examination. X2 is the blank group, which represents the absorbance value of 8 mL of 95% anhydrous ethanol+2 mL solution to be tested. X, as the control group, indicates the absorbance value of 8 mL of DPPH free radical solution +2 mL ethanol solvent.

Determination of viable counts

Referring to Shu et al. (2019b). After cooling and solidification of the MRS agar medium, 0.1 mL diluent of bacteria was taken for coating. Three dilution gradients were selected for each sample, and three parallel gradients were made for each gradient to obtain the average value.

Results and discussion

Response surface optimization design

Three factors of NaCl (A), peptone (B) and glucose (C) served as separate variables (Table 1), and DPPH-FRSR (R1) and viable counts (R2) served as the values of response to perform response surface regression analysis. A total of 15 trials were conducted in this response surface analysis. Among them, 3 groups were center point trials, and another 12 groups were analyzed for factorization tests.

Table 1. The experimental factors and levels of Box-Behnken.

level	factors		
	A: NaCl (%)	B: Peptone (%)	C: Glucose (%)
-1	1.00	0.80	0.70
0	1.02	0.82	0.72
1	1.04	0.84	0.74

Response surface modeling and result analysis

Design Expert 10 software was utilized to fit the data acquired from Table 2 by multiple regression. The regression equations for R1 and R2 of FGM as functions of the three separate variables (A, B, and C) and their linear and quadratic interactions are as follows:

$R1 = 81.95-1.48A-1.07B-0.80C-3.90AB+0.32AC+4.66BC-5.70A^2-5.27B^2-2.93C^2$,

$R2 = 11.63-0.15A+0.22B+0.18C-0.75AB-0.10AC+0.20BC-0.69A^2-1.14B^2-0.99C^2$,

and the model can be used for response surface analysis.

Table 2. The experimental design and results of Box-Behnken.

Test number	NaCl %	Peptone %	Glucose %	DPPH-FRSR %	Viable counts $*10^8$ CFU/mL
1	-1	-1	0	71.21	9.2
2	1	-1	0	73.87	10.2
3	-1	1	0	75.89	10.9
4	1	1	0	62.93	8.9
5	-1	0	-1	75.92	10.1
6	1	0	-1	74.49	10.2
7	-1	0	1	71.50	9.9
8	1	0	1	71.36	9.6
9	0	-1	-1	78.69	8.8
10	0	1	-1	68.22	9.1
11	0	-1	1	69.95	9.5
12	0	1	1	78.13	10.6
13	0	0	0	81.37	11.8
14	0	0	0	81.63	11.6
15	0	0	0	82.84	11.5

The model underwent an analysis of variance (ANOVA) and significance test to determine the model viability and the impact of each factor's involvement on the model equation. The results are displayed in Table 3.

From the table of ANOVA of DPPH-FRSR and viable counts, it is evident that the significance level of the model corresponding to DPPH-FRSR and viable counts as values of response both are $p<0.05$, and the two models were significantly different. The item of Lack of Fit: p (DPPH-FRSR) = 0.0819>0.05, and p (viable counts) =0.0504>0.05 respectively, which is not a noteworthy distinction, demonstrating that the two models fit well. The closer the correlation coefficient is to 1, the better it fits the model. R_1^2=0.9479, R_2^2=0.8987, indicating that both models can reach a relatively ideal level and have a good fit. The decline along with an increase in NaCl concentration and model 2 coefficient of variation are 2.83 and 5.18 respectively, which are less than 10, indicating that the test repeatability is better. Precision are 10.123 and 6.125 respectively, which are both greater than 4, showing that the test values and the model suit each other well. In conclusion, this model can be used for predictive analysis of antioxidant peptides.

Table 3. ANOVA of the response variables for DPPH-FRSR and viable counts of *L. plantarum* L60.

Source	Df	DPPH-FRSR				Viable counts			
		S-S	MS	F	p	S-S	MS	F	p
Model	9	404.11	44.90	10.10	0.0101*	12.21	1.36	4.93	0.0469*
A-NaCl	1	17.61	17.61	3.96	0.1032	0.18	0.18	0.65	0.4555
B-peptone	1	9.14	9.14	2.06	0.2111	0.40	0.4	1.47	0.2794
C-glucose	1	5.09	5.09	1.14	0.3336	0.25	0.25	0.89	0.3888
AB	1	61.00	61.00	13.72	0.0139*	2.25	2.25	8.17	0.0355*
AC	1	0.42	0.42	0.094	0.7720	0.04	0.040	0.15	0.7187
BC	1	86.95	86.96	19.56	0.0069**	0.16	0.16	0.58	0.4803
A^2	1	120.00	120.00	26.99	0.0035**	1.77	1.77	6.42	0.0523
B^2	1	102.58	102.58	23.07	0.0049**	4.81	4.81	17.48	0.0086**
C^2	1	31.66	31.66	7.12	0.0444*	3.63	3.63	13.19	0.0150*
Residual	5	22.23	4.45			1.38	0.28		
Lack of Fit	3	21.00	7.00	11.37	0.0819	1.33	0.44	19.00	0.0504
Pure Error	2	1.23	0.62			0.05	0.023		
Cor Total	14	426.34				13.59			

According to Table 3, the DPPH-FRSR model can be used to determine the magnitude of the effect of each factor of the primary term on the DPPH-FRSR during *L. plantarum* L60's fermentation process in the order of A (NaCl) > B (peptone) > C (glucose), in which NaCl has the greatest effect on DPPH-FRSR. The interaction term AB is significant, and BC is very significant. The quadratic terms A^2 and B^2 were very significant, and C^2 was significant. The viable counts model determined the effect of the primary term factors on the viable counts in the fermentation process of *L. plantarum* L60 in the order of B (peptone) > C (glucose) > A (NaCl), in which peptone has the greatest effect on viable counts. B^2 was very significant, and C^2 was

significant. It shows that the variation of the response values of the two models is quite complex, and the effect of each specific experimental factor on the response surface is not a simple linear relationship.

Factor interaction response surface analysis

To better understand the effect of NaCl, peptone, and glucose on viable counts and DPPH-FRSR after the interaction of any two of these factors, Design Expert 10 analysis was used, and 3D and 2D graphs were acquired for the interaction of the response values with any two of these factors. The contour plot examines the effect caused by each of the two factors on the dependent variable, and the contour lines are formed from the fitted equations, which are 2D planar graphs. The contour plot is the projection of the 3D response surface plot on the bottom surface, from which better ranges can be found. If the graph is oval, it denotes that the response value is more significantly influenced by the interaction of the two components, and the more elliptical the graph and the faster the color change of the graph, the better the interaction. If the graph is circular, the interaction between the two factors is not good. The 3D response surface diagram can more intuitively see the influence of the variables corresponding to the two factors and can intuitively find the optimal range. Response surface steepness and flatness show how the change of a factor value affects the response value. The relatively gentle slope of the response surface indicates that changes in factor values have less impact on the response values, while the steeper slope indicates that changes in factor values have more impact on the response values.

Figure 1 shows 3D and 2D graphs of the impact of every element on the response value R1. As illustrated in the figure, the 2D graphs of AB and BC were elliptical, demonstrating a strong interaction with each other, while the 2D graph of AC was close to circular, suggesting a meek interaction between the two factors, and the results were consistent with the results in Table 3. The 3D graphs were arch-shaped, *i.e*., the DPPH-FRSR displayed a gradual increase and then a slow decrease with the increase of each substance addition, with the maximum value appearing near the center point.

Figure 2 shows 3D and 2D graphs of the effects of each factor on the value of response R2. As demonstrated in the figure, the 2D graph of AB was elliptical, demonstrating a strong interaction with each other, while the 2D graphs of AC and BC were close to circles, suggesting a meek interaction between the two factors. The 3D graphs were arch-shaped, *i.e*., viable counts showed a gradual increase and then a slow decrease with increasing each substance addition, with the maximum value appearing near the center point.

This study found that supplementing the medium with appropriate amounts of nutrients was beneficial in increasing the viable bacteria count and antioxidant activity of FGM. The reason for this is that when *L. plantarum* L60 fermented goat milk, it created proteases. These proteases enhanced the antioxidant activity of the milk by stimulating the breakdown of milk protein and producing more antioxidant peptides. This is in line with the claim made by Ding *et al*. (2023) and Hu et al. (2023) that the culture conditions and medium affect the biomass of probiotics and the synthesis of antioxidant peptides.

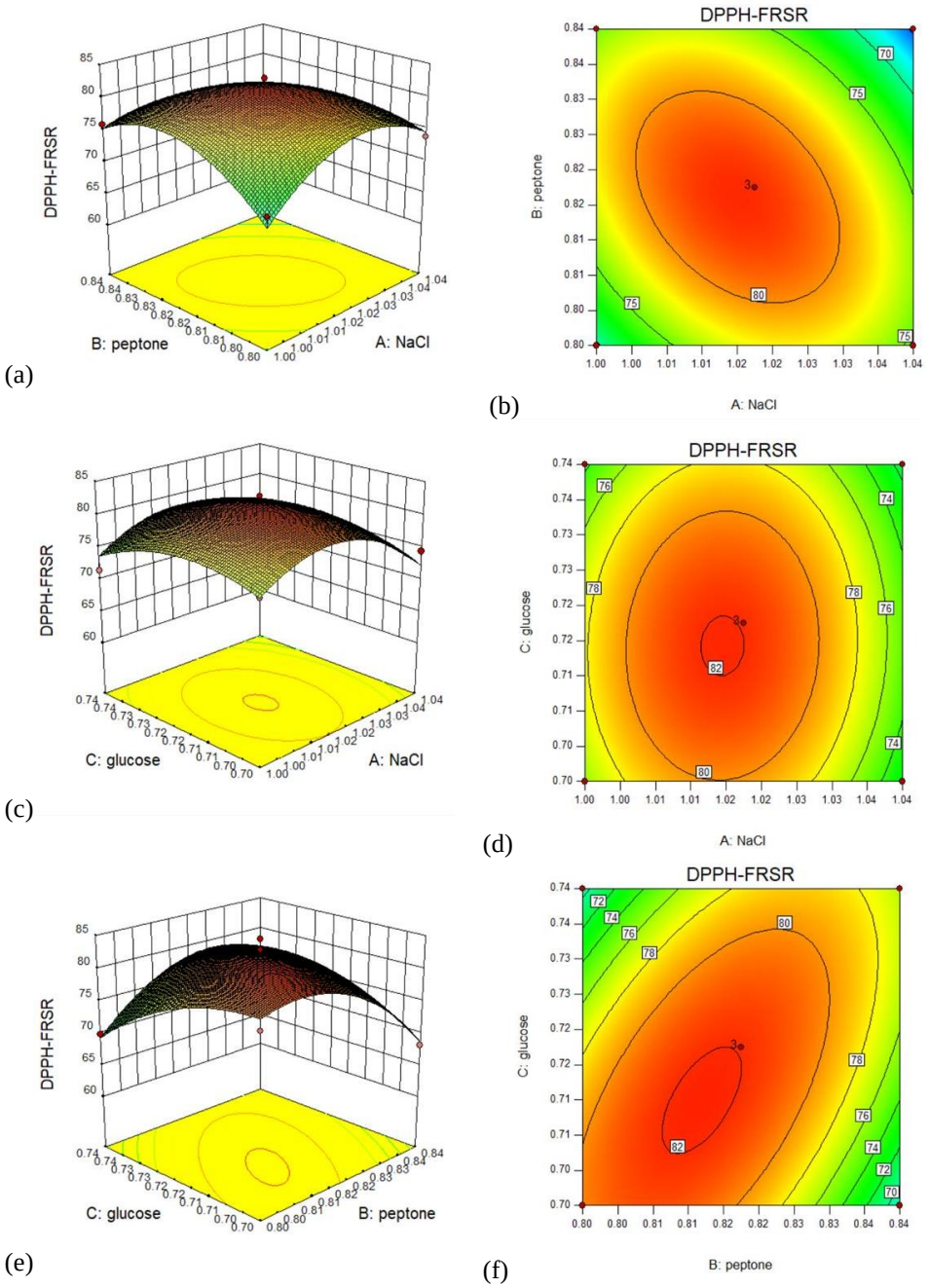

Figure 1. Response surface (right) and contour (left) plots of A (NaCl), B (peptone), C (glucose) and its mutual interaction to DPPH-FRSR.

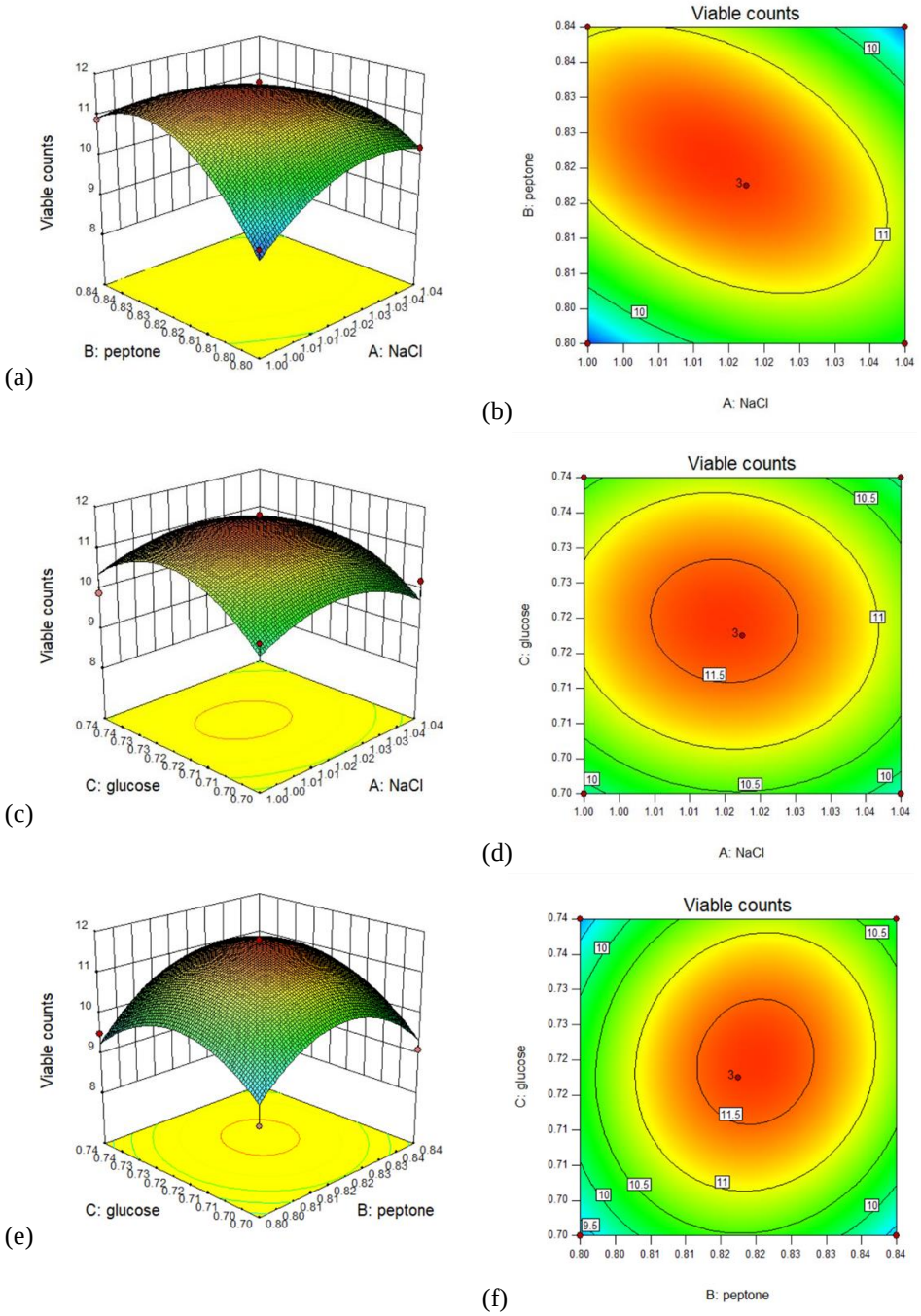

Figure 2. Response surface (right) and contour (left) plots of A (NaCl), B (peptone), C (glucose) and its mutual interaction to viable counts.

Salt concentration is one of the determinants of bacterial growth, and ions in salt solution are involved in the active transport of nutrients, which promotes bacterial growth and development. In addition, the addition of NaCl in the fermentation environment provides a selective environment for the growth of desired microorganisms. Lower salt concentrations provide an ideal environment for fermentation by promoting a rise in lactic acid, a reduction in pH, and the growth of lactic acid bacteria. Excessive salt concentrations can induce osmotic stress, which alters the structure of bacterial cell walls, prevents germs from growing, and ultimately leads to a decrease in antioxidant activity (Dalla Rosa et al., 2016). Zhang *et al*. (2016) simulated the effect of gastrointestinal digestion on the antioxidant activity of peptide components of hydrolysate of *Pseudosciaena crocea* and found that the peptides of *Pseudosciaena crocea* demonstrated good stability when combined with sugar and trace levels of NaCl. Tkaczewska *et al*. (2019) found that the antioxidant properties of fish hydrolysates declined along with an increase in NaCl concentration because antioxidant peptides emitted after hydrolysis may consist of polar and non-polar amino acids and peptides may self-aggregate under the effect of salt addition. López De La Paz *et al*. (2002) discovered that antioxidant peptides form amorphous aggregates at greater concentrations of NaCl, which may cause a loss of antioxidant activity.

Peptones give microorganisms carbon, minerals, and several vitamins in addition to organic nitrogen, which can encourage the growth of L. plantarum. (Tuysuz *et al*., 2021). However, too much peptone can lead to excessive nitrogen sources in the medium, which in turn leads to high osmotic pressure in the medium, inhibiting the growth of the strain and reducing the number of proteases and antioxidant peptides.

Glucose can promote microbial growth. Antioxidant peptide production increased with an increase in glucose content because glucose content can provide sufficient energy for microbial growth, but when the glucose addition level exceeded a certain amount, antioxidant peptide production decreased, possibly due to inhibition of fermentation caused by excessive substrate (Rai *et al*., 2010; Jiang *et al*.,2020). To sum up, it is of great significance for *L. plantarum* L60 to produce antioxidant peptides in fermented goat milk by selecting appropriate nutrients and adding amount.

Response surface validation test

Design Expert 10 software was used to solve DPPH-FRSR and viable counts in response surface analysis, and optimal nutrients of goat milk fermented by *L. plantarum* L60 were obtained as follows: The additive amounts of NaCl, peptone, and glucose were 1.02%, 0.82% and 0.72%, respectively. Under optimal conditions, DPPH-FRSR and viable counts were 80.23% and 1.09×10^9CFU/mL respectively, and both were nearly in line with the predicted values. In contrast to the control group without the inclusion of NaCl, peptone, and glucose, DPPH-FRSR and viable counts were increased by 4.25% and 0.36×10^9CFU/mL respectively. In summary, the feasibility of the model is demonstrated.

Conclusions

In this paper, the effect of NaCl, peptone, and glucose on the antioxidant activity of fermented goat milk was examined. The results showed that under optimal conditions of 1.02% NaCl, 0.82% peptone, and 0.72% glucose in goat milk, the DPPH-FRSR and viable counts were 80.23 % and 1.09 $\times 10^9$CFU/mL respectively, which were close to the predicted values, suggesting that the model was real and effective. This study can offer direction for the advancement of functional probiotic fermentation of goat milk.

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