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ORIGINAL PAPER

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THE OPTIMIZATION OF PEPTIDES – PRODUCING CONDITIONS FOR ANTIOXIDANT PEPTIDES IN GOAT MILK BY *LACTOBACILLUS CASEI* L61

He Chen[1]✉, Yaolong Mao[1], Guanli Du[2], Xiangyun Li[2], Zemin Liu[2], Jianbo Kou[1]

[1]School of Food and Biological Engineering, Shaanxi University of Science and Technology
Xuefu Middle 6, 710021 Xi'an, Weiyang district, **China**
[2]Department of Research and Development, Xi'an Xiyangyang Biological Technology Co.
Xi'an, **China**

ABSTRACT

Background. The chemical synthesis of antioxidants has exposed more and more of their disadvantages. Therefore, the development of safe, healthy and efficient natural antioxidants has become a research hotspot. At present, antioxidant peptides are gradually becoming more popular due to their strong antioxidant activity and high safety.

Materials and methods. The aim of this experiment was to obtain the optimum fermentation conditions for producing antioxidant peptides from *Lactobacillus casei* L61. The effects of various factors (fermentation temperature, fermentation time, concentration of regenerated milk and inoculum) on the production of antioxidant peptides by *Lactobacillus casei* L61 were studied using a single factor test. Four methods (DPPH radical scavenging activity, the ability to chelate iron, hydroxyl radical scavenging rate, total reduction force) were used to determine antioxidant activity *in vitro*. The optimum fermentation conditions were determined by orthogonal experiment.

Results. The results showed that the optimal fermentation conditions for the production of antioxidant peptides by L61 were 11% reconstituted milk, 5% inoculated milk, fermentation at 41°C and 16 hours of fermentation time. Under these conditions, the results of the orthogonal test showed that the DPPH radical scavenging rate of the whey sample was 59.97 ±0.87%, which was higher than those of the other 9 groups, and significantly higher than that of the control group (41.97 ±1.37%).

Conclusion. The concentration of reconstituted milk and the amount of inoculated milk had a significant effect on the production of antioxidant peptides by *Lactobacillus casei* L61. It provides a reference for the optimization of the fermentation nutritional composition of antioxidant peptides produced by *Lactobacillus casei* L61.

Keywords: goat milk, antioxidant peptide, fermentation, *Lactobacillus casei*

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✉2739854893@qq.com, https://orcid.org/0000-0001-6046-7583, phone 0086 15619239983, fax 0086 86168293

INTRODUCTION

Harman theorized about free radicals in the middle of the last century, and since then it has been found that free radicals accumulate in the body during many diseases, including heart disease, diabetes, cancer, cataracts, and aging (Harman, 2006). They have a strong oxidation capacity in the body, and when the body produces too many free radicals, it will cause a serious impact on the organs and tissues of the body (Das, 2016). Studies have found that free radicals produced by oxidation can attack proteins and nucleic acids, causing cell damage and breaking the balance of the body's antioxidant defense system, leading to diseases in the body. Therefore, it is necessary to take in exogenous antioxidants to remove free radicals in the body (Clancy and Birdsall, 2013; Park et al., 2001; Persichetti et al., 2014; Sila and Bougatef, 2016).

In recent years, with the continuous improvement of human research and knowledge, more and more disadvantages of the chemical synthesis of antioxidants have been exposed (Goodman et al., 1990). Therefore, the development of safe, healthy and efficient natural antioxidants has become a research hotspot. Nowadays, antioxidant peptides are gradually becoming more popular on account of their strong oxidation resistance and high safety, and have shown excellent prospects for application in food, medicine and other fields. The antioxidant peptide is an important biological peptide belonging to a special protein fragment with a low molecular weight and strong antioxidant capacity, which can effectively remove free radicals accumulated in the body (Abeyrathne et al., 2018; Kitts and Weiler, 2003). In general, antioxidant peptides are unable to express their activity normally, but they can be released by enzymatic hydrolysis or fermentation, showing specific functions (Lorenzo et al., 2018). Compared with enzymatic hydrolysis, fermentation has the advantages of a lower cost, higher yield and more convenient operation. At present, the preparation of antioxidant peptides from milk is mainly by enzymolysis at home and abroad, but research on the preparation of antioxidant peptides by probiotic fermentation is lacking. At the moment, some scholars have screened *Lactobacillus fermentum* M4, *Lactobacillus acidophilus* TS2004, *Lactobacillus suis* ASCC983, *Lactobacillus bulgaricus* l34.5 and *Streptococcus thermophilus* SP1.1, and determined the best conditions for producing peptides (Bao et al., 2009; Elfahri et al., 2016; Guo, 2003; Panchal et al., 2020; Ramesh et al., 2012; Virtanen et al., 2007). In this study, a single factor test was used to investigate the effects of fermentation time, fermentation temperature, inoculum and concentration of reconstituted milk on the production of antioxidant peptides in fermented goat milk, and the single factor optimum conditions were obtained. A four-factor and three-level orthogonal test was designed to determine the optimum production process conditions, which laid a foundation for subsequent experiments.

MATERIALS AND METHOD

Microorganism and growth medium

Microorganism. *Lactobacillus casei* L61 was obtained from the College of Food and Biological Engineering, Shaanxi University of Science and Technology (Xi'an, China).

Strain activation. The strain was inoculated three successive times with the MRS medium at 37°C for 24 h until the viability of the bacteria remained stable. The third-generation bacteria solution obtained above was inoculated in a medium of reconstituted goat milk according to 5% (v/v). After fully mixing, it was fermented in a constant temperature incubator at 37°C to complete curdling, and then stored in a refrigerator at 4°C for standby.

Preparation of the fermented goat milk and whey samples. The reconstituted milk with the required concentration of skimmed goat milk powder was pasteurized at 95°C for 15 minutes. After it was cooled, it was inoculated with the activated bacteria according to a specific amount of inoculation and fermented in a constant temperature water bath at a specific temperature. After the fermentation, the fermented milk was taken out for testing.

The obtained fermented milk was shaken and poured into a 100 mL clean beaker to measure the pH value of the fermented milk. First, the fermented milk was adjusted to 3.4–3.6 with HCl (1 mol/L). After centrifugation at 5000×g for 15 minutes, the supernatant

was collected and adjusted to pH 8.3 with NaOH (1 mol/L) and centrifuged at 5000×g for 15 minutes. The supernatant was collected as the whey sample for the determination of antioxidant activity (Shu et al., 2019).

In vitro antioxidant assay

There are many methods for evaluating the antioxidation *in vitro*, but the principles are different and the results may not be the same. Therefore, the combination of various methods can evaluate the antioxidant capacity comprehensively and accurately. Therefore, this study adopted several commonly used methods to detect the antioxidant activity, and evaluate the antioxidant peptide activity.

DPPH radical scavenging activity. 2.0 mL of the sample was taken to be tested and placed in a test tube. DPPH ethanol solution (0.1 mmol/L) of equal volume was added, and mixed thoroughly in a whirlpool mixer. It was then incubated at 25°C in the dark for 30 min. Finally, the absorbance of the reaction solution was measured at 517 nm. The calculation formula is as follows (Zeng et al., 2012):

$$\text{DPPH radical scavenging activity} = \left(1 - \frac{A_1 - A_2}{A_3}\right) \times 100$$

where:

A_1 – the absorbance of the sample group,
A_2 – the absorbance of the blank group containing 2 mL 95% ethanol and 2 mL sample mixed solution,
A_3 – the absorbance of the control group containing 2 mL 95% ethanol and 2 mL DPPH solution.

The ability to chelate iron (Fe^{2+}). 0.5 mL of the sample solution to be tested was placed in a test tube, 1 mL $FeCl_2$ (0.02 mmol/L) and 1 mL ferrozine (0.5 mmol/L) were added in turn and thoroughly mixed in a vortex blender. After being placed at room temperature for 10 min, the absorbance value A_1 was measured at 562 nm. Distilled water was used as a control instead of the sample. The chelating capacity was calculated as follow (Kong and Xiong, 2006):

$$\text{Chelating rate} = \frac{A_2 - A_1}{A_2} \times 100\%$$

where:

A_1 – the absorbance at 562 nm of sample,
A_2 – the absorbance at 562 nm of control.

Hydroxyl radical scavenging rate. 1 mL of the sample solution was taken to be tested and put into a test tube, equal volumes of $FeSO_4$ (9 mmol/L) and H_2O_2 (10 mmol/L) were added in turn and mixed well in a vortex mixer. They were then incubated at 37°C for 10 min, then 1 mL of salicylic acid solution (9 mmol/L) was added and they were incubated for 30 min. The absorbance was measured at 510 nm as A_1. Distilled water was used as a control instead of the sample (Li et al., 2015).

$$\text{Hydroxyl radical scavenging rate} = \frac{A_2 - A_1}{A_2} \times 100\%$$

where:

A_1 – the absorbance at 510 nm of sample,
A_2 – the absorbance at 510 nm of control.

Total reduction force. 2.5 mL of the sample solution was taken to be tested and put into a test tube. 0.2 mmol/L phosphoric acid buffer solution (pH = 6.6), 1% potassium ferricyanide solution and 10% trichloroacetic acid solution of equal volume were added in turn and mixed well in a vortex mixer. They were mixed well and their absorbance at 700 nm was measured. There was a positive correlation between absorbance and reduction ability (Dorman, et al., 2004).

RESULTS AND DISCUSSIONS

The effects of reconstituted milk concentration on the antioxidative activity of goat milk fermented by *Lactobacillus casei* L61

50 mL of reconstituted goat milk with 5, 7, 9, 11, 13% (w/v) concentration was prepared and sub packed in a 100 mL anaerobic flask. It was sterilized in a 90°C constant temperature water bath for 15 minutes and taken out. After this, it was cooled to room temperature, the activated *Lactobacillus casei* L61 was added to it according to 6% (v/v) inoculation amount, and then it was fully mixed and fermented in a 41°C constant temperature water bath for 14 h. It was taken out after fermentation and centrifugated to prepare the whey supernatant sample. The DPPH radical scavenging activity, chelate iron ion activity, hydroxyl radical

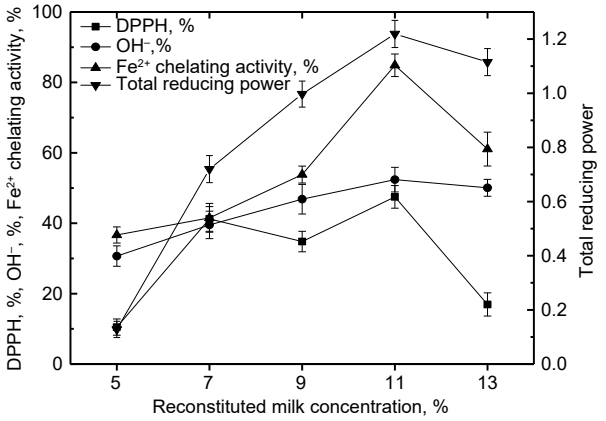

Fig. 1. Effects of goat milk density on antioxidant ability

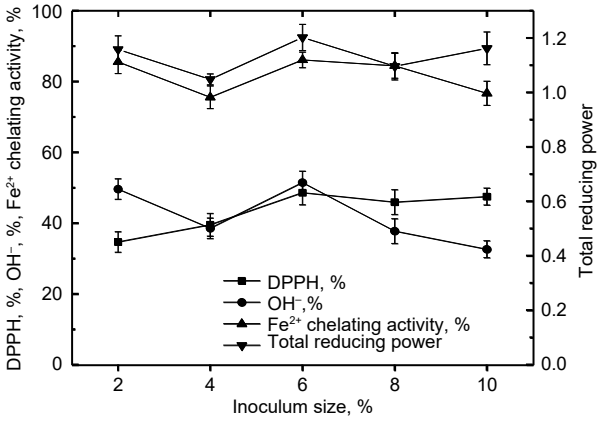

Fig. 2. Effects of inoculum on antioxidant ability

scavenging activity and total reducing power were determined. The results are shown in Figure 1.

It can be seen from Figure 1 that the concentration of reconstituted milk has a significant impact on the ability of goat milk to produce antioxidant peptides. With an increase in the concentration of reconstituted milk, chelating iron ion capacity, and hydroxyl radical scavenging rate, total reducing power first increased and then decreased. When the concentration of reconstituted milk reached 11%, the DPPH scavenging rate hydroxyl radical scavenging rate and total reducing power reached the maximum, which were 84.87%, 52.38% and 1.22, respectively. With an increase in reconstituted milk concentration, DPPH radical scavenging activity first increased and then decreased and then increased, and when the concentration was 11%, it reached the maximum value of 47.49%. It can be seen that an 11% concentration of reconstituted milk is the best result of a single factor. This is because, under the same conditions, increasing the concentration of the reaction substrate is conducive to promoting the reaction. When the concentration of reconstituted milk is too high, it will inhibit the protease production of *Lactobacillus casei* L61, which will reduce the content of antioxidant peptides and reduce the activity.

The effects of the inoculation amount of *Lactobacillus casei* L61 on the antioxidation of fermented milk

The activated *Lactobacillus casei* L61 was inoculated into 11% reconstituted goat milk according to 2%, 4%, 6%, 8%, 10% respectively. The goat milk was well blended and fermented at 41°C for 14 hours to prepare the supernatant sample of whey. The DPPH free radical scavenging rate, chelating iron ion ability, hydroxyl free radical scavenging rate and total reducing power were measured. The results are shown in Figure 2.

It can be seen from Figure 2 that with an increase in inoculation amount, the chelating iron ion capacity and hydroxyl radical scavenging activity show a trend of decreasing first, then increasing and then decreasing. When the inoculation amount reaches 6%, the scavenging rate of free radicals reaches the maximum, which is 86.11% and 51.46% respectively; when the concentration of recovered milk increases, the scavenging rate of DPPH radical first increases and then decreases, which also reaches the maximum value of 48.13% when the inoculation amount is 6%. It can be seen from the change trend of the total reducing power that when the inoculation amount reaches 10%, the total reducing power reaches the maximum. Therefore, 6% inoculation amount was selected as the best condition for single factor. This is because when the reaction substrate is fixed, *Lactobacillus casei* L61 in a certain range can make full use of the substrate to produce antioxidant peptides; when the number of bacteria continues to increase, it is difficult for the substrate to meet the needs of more microbial growth, and it is possible to decompose and utilize the generated antioxidant peptides so as to reduce the antioxidant activity.

The effects of fermentation time on the antioxidation of fermented milk by *L. casei* L61

L61 activation solution was added to an 11% reconstituted milk medium according to a 6% inoculation amount. The mixture was well mixed and fermented at 41°C for 8, 10, 12, 14 and 16 hours to prepare the supernatant sample of whey, and a fermentation for 0 h was designed as the control group. The DPPH radical scavenging rate, chelating iron ion ability, hydroxyl radical scavenging rate and total reducing power were measured. The results are shown in Figure 3.

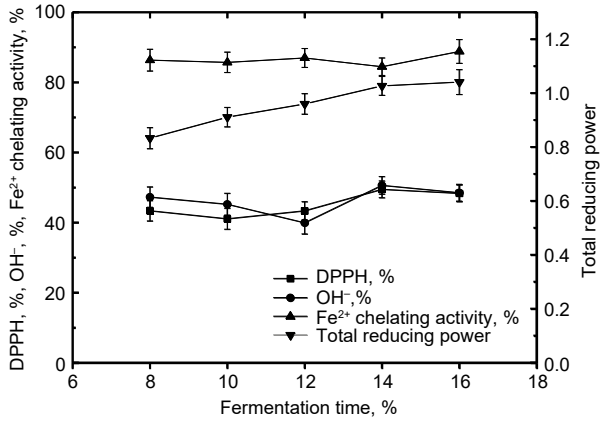

Fig. 3. Effects of fermenting time on antioxidant ability

From Figure 3, it appears that DPPH scavenging rate and hydroxyl scavenging rate decreased first, then increased and then decreased with an increase in fermentation time. When the fermentation time reached 14 h, DPPH scavenging rate and hydroxyl scavenging rate reached the maximum, 49.47% and 50.60% respectively, while the scavenging rate of DPPH free radical in the control group was 32.23%. The antioxidant activity of the experimental group was significantly higher than that of the control group. The ability to chelate iron ion showed a trend of decreasing first and then increasing; when the fermentation time was 16 h, the ability to chelate iron ion reached the maximum of 84.67%; when the fermentation time reached 14 h, the total reducing power reached the maximum of 1.06. Based on the above results, the optimal fermentation time of 14 h was determined.

The effects of temperature on the antioxidative activity of fermented milk by *L. casei* L61

L61 activation solution was added to an 11% reconstituted milk medium according to a 6% inoculation amount. It was mixed evenly, put in a constant temperature water bath at 35°C, 37°C, 39°C, 41°C and 43°C for 14 h, then taken out and prepared for the whey sample. The DPPH radical scavenging rate, chelating iron ion ability, hydroxyl radical scavenging rate and total reducing power were measured. The results are shown in Figure 4.

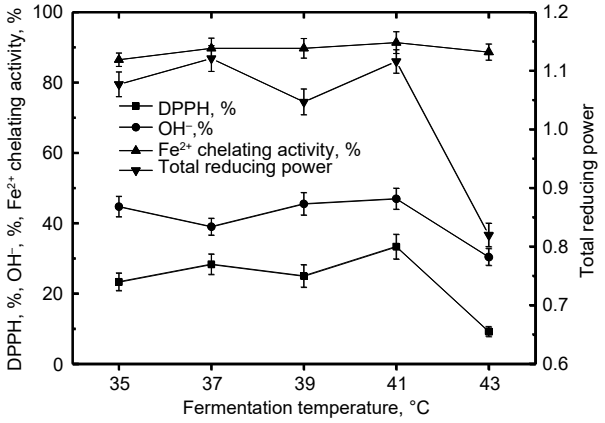

Fig. 4. Effects of temperature on antioxidant ability

As can be seen from Figure 4, the antioxidant indexes of the four groups showed a trend of increasing first and then decreasing. When the fermentation temperature was 41°C, the antioxidant activity was stronger, the scavenging rate of DPPH radical, chelating iron ion ability, scavenging rate of hydroxyl radical and total reducing power were 35.87%, 90.34%, 46.69% and 1.14, respectively.

The optimization of fermentation conditions for antioxidant peptide production by *Lactobacillus casei* L61 by orthogonal test

The effects of inoculation amount, concentration of reconstituted milk, fermentation temperature and fermentation time on the antioxidation of fermented milk by *L. casei* L61 were investigated. The optimum single factor conditions were determined as follows:

inoculation amount 6%, concentration of reconstituted milk 11%, fermentation temperature 41°C, and fermentation time 14 h. According to the results of the single factor experiment, taking the reconstituted milk concentration (*A*), inoculation amount (*B*), fermentation time (*C*) and fermentation temperature (*D*) as variables, DPPH scavenging rate (*Y*) as indicators, four factors and three levels of orthogonal experiment were designed to determine the best combination of fermentation conditions for the production of antioxidant peptides, and the orthogonal results were analyzed. The results are shown in Table 1 and Table 2. The control group had inoculation 5%, reconstituted milk concentration 10%, fermentation temperature 41°C, fermentation time 12.3 hours.

K_1, K_2 and K_3 are the sum of index of factor level 1, 2 and 3 respectively. The mean value is expressed by k, and k_1, and k_2 and k_3 are the average of experimental results at each level respectively. The range, R, reflects the effects of various factors in the experiment. The larger the R value, the higher the significance of the corresponding factor and the greater the influence on the experimental index. The highest value of k in each factor constitutes the optimal horizontal combination. It can be seen from the analysis of the range of orthogonal test results in Table 1 that each factor has a certain impact on the production of antioxidant peptides in fermented goat milk, among which the inoculation amount of bacteria has the most significant impact on the DPPH scavenging rate of fermented goat milk, and the significant effect of each factor on DPPH clearance rate was $B > A > C > D$, that is, the inoculation amount > reconstituted milk concentration > fermentation time > fermentation temperature, and the best combination was $A_1B_2C_3D_3$, that is, the concentration of reconstituted milk was 11%, the inoculation amount was 5%, the fermentation time was 16 h, and the fermentation temperature was 41°C.

From the results of ANOVA in Table 2, it can be seen that the F values of the reconstituted milk concentration (*A*) and inoculation amount (*B*) were 7.062 and 34.783, respectively, which had significant effects compared with the $F_{0.05}$ values. Among them, the inoculation amount had the most significant effect on the results, while fermentation time and temperature had no significant effect. The results of ANOVA were consistent with those of the range analysis. So, the optimal combination of orthogonal test was $B_2A_1C_3D_3$, that is, the concentration of reconstituted milk was 11%, the inoculation amount was 5%, the fermentation time was 16 h, and the fermentation temperature was 41°C.

Table 1. Results of orthogonal experiment on fermenting conditions of *Lactobacillus casei* L61

Trial no.	Factor number				*Y*, %
	A, %	*B*, %	*C*, h	*D*, °C	
1	1(11)	1(4)	1(14)	1(37)	46.31
2	1	2(5)	2(15)	2(39)	56.62
3	1	3(6)	3(16)	3(41)	56.91
4	2(12)	1	2	3	42.50
5	2	2	3	1	53.96
6	2	3	1	2	49.13
7	3(13)	1	3	2	46.17
8	3	2	1	3	54.33
9	3	3	2	1	53.26
K_1	159.840	134.979	149.769	153.531	
K_2	145.590	164.910	152.379	151.920	
K_3	153.769	159.300	157.041	153.741	
k_1	53.280	44.993	49.923	51.177	
k_2	48.530	54.970	50.793	50.640	
k_3	51.253	53.100	52.347	51.247	
R	4.750	9.977	2.424	0.607	
Best	$A_1B_2C_3D_3$				

Table 2. The ANOVA of DPPH scavenging rate

Factors	SS	DF	*F*-value	$F_{0.05}$
A	34.086	2	7.026*	6.940
B	168.749	2	34.783*	6.940
C	9.042	2	1.864	6.940
D	0.661	2	0.136	6.940
Residual	9.70	4		

SS – sum of squares, DF – degree of freedom.

Jia, Bo and Li (2002) investigated the preparation of antimicrobial peptides from 15 strains of *Lactobacillus plantarum* fermented starch, screened one strain of *Lactobacillus plantarum*, and studied the fermentation time of its peptide production (Jia et al., 2002). The peptide activity was measured every 5 hours. The experimental results showed that the peptide activity reached the maximum at 15 hours of fermentation, and began to decline after 15 hours, which was basically consistent with the influence of fermentation time on the production of antioxidant peptides in this study. Therefore, as the fermentation time continues to extend, the peptide production capacity will decline. Zhang studied the clotting effects of 123 kinds of lactobacilli, selected *three* Lactobacillus strains CH9--34.0, SH34.3 and SB5 from them, and optimized their fermentation conditions for protease production, and determined that the optimal reaction temperature of protease was 42°C (Zhang, 2014). It can be seen that temperatures which are too high will lead to the inactivation of protease and affect its ability to produce antioxidant peptides. At the same time, high temperatures can inhibit or even kill the growth of bacteria, so it shows the decline of antioxidant activity.

The results showed that the DPPH radical scavenging rate of the whey sample was 59.97 ±0.87%, which was higher than that of the above 9 groups, and significantly higher than that of the control group (41.97 ±1.37%). Therefore, it is reliable to optimize the fermentation conditions of *Lactobacillus casei* L61 by orthogonal test. The importance and benefits of antioxidants are well known. This study provides more methods and theoretical basis for the preparation of safe antioxidants and also paves the way for the preparation of antioxidant foods such as antioxidant milk powder and dairy products.

CONCLUSION

The optimal fermentation conditions for the production of antioxidant peptides by *Lactobacillus casei* L61 were a reconstituted milk concentration of 11%, an inoculum amount of 5%, a fermentation temperature of 41°C, and a fermentation time of 16 hours. The DPPH scavenging rate of the fermented milk was 59.97 ±0.87%, 18% higher than that of the control group (41.97 ±1.37%).

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