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Process Application of Lactic Acid Bacteria in Traditional Yak Yoghurt in Qinghai Pastoral Areas

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Abstract: *Research to investigate the effect of improving the quality of yak yogurt, composite fermentation was prepared with lactic acid bacteria strains isolated from traditional yak yoghurt in Qinghai pasture area, and the response surface methodology was used to optimize the process parameters of yak yoghurt fermentation in combination with Plackett-Burman and steepest climb tests. The results showed that the optimal process parameters of yak yoghurt with the addition of composite bacteria Y2-10 and N5-9 fermenter were: inoculum quantity of 3.5%, yak milk additive quantity of 40%, sucrose additive quantity of 4%, oligogalactose additive quantity of 3%, fermentation time of 6.5 h, fermentation temperature of 42 ℃, and sensory scores of the resultant yak yoghurt and the evaluation of the quality effect, which were higher than those of the traditional yak yoghurt, which further improved the This will further improve the acceptance of consumers and promote the development and utilisation of yak dairy products in Qinghai Plateau region.*

Keywords: *Lactic Acid Bacteria, Response Surface Method, Plackett-Burman, Traditional Yak Yoghurt*

1. Introduction

Lactic acid bacteria, as the secondary microbiota that play an important role in the post-cooking process of fermented dairy products, have a key role in the formation of taste, texture and aroma of traditional fermented dairy products[1], with unique flavour and therapeutic efficacy, and lactic acid bacteria, as one of the key factors in the fermentation of yak milk[2], have a significant impact on fermented products, and its different species of lactic acid bacteria in the fermentation process can play a Different species of lactic acid bacteria can play different roles in the fermentation process, affecting the flavour, texture and nutritional composition of fermented products[3]. At the same time, lactic acid bacteria have outstanding growth ability and can survive well in the extreme plateau environment, and the special geographic location of the Tibetan Plateau determines the diversity and complexity of lactic acid bacteria in China, among which, the lactic acid bacteria in traditional yak yoghurt from the pastoral areas of Qinghai can survive in the special climatic environment of the plateau with low temperature and pressure, and have rich natural lactic acid bacteria flora, and their microbial fermentation has a significant influence on the yak yoghurt quality[4], flavour and functional component content has a significant relationship[5], can also play a role in metabolising lactose[6],it is a probiotic with a high antioxidant effect, which can be widely applied to special populations, such as lactose intolerance, and make yoghurt become a probiotic dairy product[7]. Yak yoghurt prepared with lactic acid bacteria existing in the natural environment[8][9], it can produce rich multivitamins on the basis of retaining the original nutrients of yak milk, and its yoghurt is rich in lactic acid, amino acids, unsaturated fatty acids and other substances with higher antioxidant capacity[10], and its special flavour qualities and high nutritional value have a certain potential for development. Therefore, in this study, by analysing the acid production, tolerance and curdling properties of lactic acid bacteria, composite preparation of lactic acid bacteria fermenter, combined with Box Behnken experimental design, response surface methodology to optimize the yak yogurt fermentation process parameters, to provide a data basis for the application of the processing of lactic acid bacteria, which is of great significance to enhance the quality effect of traditional yak yoghurt.

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2. Materials and Methods

2.1 Test material

2.1.1 Sample collection

Yak milk was extracted from the fresh milk of herdsmen in Qilian County, frozen and transported back, and stored at -80 ℃; Lactobacillus strains were selected from the previous isolation and identification, and stored at -80 ℃. The lactobacillus species numbers and names are shown in Table 1 below.

Table 1 Information on Lactobacillus strains

Strain number	Strain name
G1-11	Lactobacillus plantarum
G5-8	Bacillus sp
Y2-1	Lactobacillus plantarum
Y2-9	Lacticaseibacillus rhamnosus
Y2-10	Lacticaseibacillus rhamnosus
N5-3	Lactobacillus plantarum
N5-9	Limosilactobacillus fermentum
N5-11	Limosilactobacillus fermentum
B3-9	Bacillus sp

2.1.2 Reagent equipment

The instrumental materials used during the test are shown in Table 2 below.

Table 2 Main reagent equipment information

Instrumentation	Specification	Brand name
MRS agar	250 g	Hangzhou Best Biotechnology Company
MRS Meat Soup	250 g	Hangzhou Best Biotechnology Company
Phenolphthalein	25 g	Hangzhou Best Biotechnology Company
Crystalline Violet Neutral Red Bile Salt Agar Medium	250 g	Hangzhou Best Biotechnology Company
Carbon dioxide incubator	HF240	Shanghai Lixin Scientific Instrument
Acidometer	P301	Shanghai Youke Instrumentation Company
Viscometer	3730X	Applied Biosystems
Centrifuges	LegendMicro17	Thermo
Water bath	DFD-700	Beijing Zhongxingweiye
Analytical balance	Practum	PRACTUM313-1CN
Buret	2.1 mm x 100 mm, 1.8 μm	Waters
Electrothermal constant temperature water bath	seedling	H-SWX-600BS

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2.2 Test methods

2.2.1 Strain resurrection

Referring to the methods of Yang Xing[11] and SI Mujide[12], the preserved strain sap was thawed at room temperature and then incubated in MRS broth medium at 3% inoculum 37 ℃ for 24 h. This was done for three activations so as to allow the strains to fully recover their activity.

2.2.2 Complex Lactic Acid Bacteria Preparation Fermenter

After thawing the preserved bacterial strain at room temperature, the bacterial liquid was cultured in MRS broth medium at 37 ℃ for 24 h according to the inoculum amount of 3%, and activated for 3 times to fully restore the activity; the inoculum was inoculated in the skimmed milk medium with 3% of the amount inoculated in sterilised and cooled to 42 ℃, and then left to ferment until curdling at 42 ℃, and so on, and repeated fermentation and curdling were carried out for 3 times, and the prepared fermentation agent was stored in the refrigerator at 4 ℃.

2.2.3 Optimising the yoghurt fermentation process

Yak yoghurt process, refer to Yang Xing[11], Wang Teng[13] and Liu Rongmei[14] for the production process with minor modifications.

Raw yak milk (preheated) → Sucrose+ Oligogalactose→ Homogenisation → Boiling and sterilisation → Cooling (42 ℃) → Addition of complex fermenter → Mixing → Aseptic tanking → Fermentation → Cooling to room temperature → Finished fermented milk → Inspection

2.2.4 Response surface optimisation of yak yoghurt fermentation process

Based on the results of the previous single-factor test, the central point of each main influencing factor was derived, and a Box-Behnken experimental design was selected for the inoculum amount (3%), oligogalactose addition (4%), fermentation time (6 h), fermentation temperature (44 ℃), and organoleptic evaluation (Y) as the dependent variable, respectively, using the Design-Expert 8.0.6 software, as shown in Table 3 below.

Table 3 Response surface test design factors and levels

Considerations	level		
	-1	0	1
A-Inoculum/per cent	2	3	4
E-Fermentation time/h	5	6	7
F-Fermentation temperature/℃	42	43	44

2.2.5 Sensory evaluation of yak yoghurt

The sensory evaluation was composed of 10 food students, who were required to keep their hands clean and rinse their mouths before the evaluation, and the samples were placed in room temperature environment for a period of time in advance, and then scored for colour odour, tissue state, and taste strictly according to the scoring criteria with a score of 100 out of 100, as shown in Table 4 below.

Table 4 Yak yogurt sensory scoring criteria

Scoring items	marking scheme	Score/points
Organisational status (30 points)	Good curd, uniform texture, suitable viscosity without obvious fluidity, no whey precipitation	30-21
	Homogeneous curd, soft texture, slight whey precipitation	20-11
	Poor coagulability, loose texture and fluidity, more whey precipitation	10-0
Colour Odour	Milky white colour, strong yoghurt fermentation	30-21

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(30 points)	flavour, slightly yak frankincense odour	
	Creamy white colour, average yoghurt fermentation flavour, lactic flavour not prominent	20-11
	Uneven colour, no fermented yoghurt flavour, no milky taste	10-0
how food feels in the mouth (40 points)	Delicate and smooth taste, no graininess, sweet and sour, and slightly yoghurt fermentation sour taste	40-31
	The palate is more delicate, with no obvious particles, and is more acidic or sweet.	30-21
	Coarse mouthfeel, noticeably grainy or too thin, overly acidic	20-0

2.2.6 Determination of physical and chemical indexes of yak yoghurt

(1) pH

The yoghurt samples were measured directly with a pH meter and the displayed data were recorded, three parallel sets were measured and the results were averaged.

(2) Total acidity

The acidity was determined based on Granato[15] and expressed in Thorner degree (°T), the determination of three sets of parallel, the results take the average value.

(3) Viscosity

Using NDJ-8S rotational viscometer, run with rotor No.3 at 12 r/min, read the value after 1 min, measure three parallel groups and take the average value.

(4) Water holding capacity

The water-holding capacity was determined with reference to the method of Jiang Qian[16] with the following formula:

$$\text{Water holding capacity} = \frac{\text{weight of the precipitateg}}{\text{weight of the sampleg}} \times 100\% \quad (1)$$

(5) Lactobacillus viable count determination

Reference to Bakry[17] and Lang Xiaolin et al[18] test method, the bacteria were counted by standard plate counting method. The results were obtained as the number of colony forming units per mL of yogurt (CFU/mL).

2.3 Data processing

The data were processed using Excel 2021 and SPSS 22, and the results were expressed as "± standard deviation". Design-Expert 8.0.6 was used to design the Plackett-Burman test, Box Behnken response surface test, and graphs were prepared using GraphPad Prism 2021 software for graphing.

3. Analysis of results

3.1 Analysis of the results of the Plackett-Burman experimental design

Based on the basis of the previous one-factor trial, the number of trials with N=12 was conducted, and the experimental design and response results were shown in Table 5. Through the Plackett-Burman test data ANOVA Table 6, the P-value of the test model was 0.0113< 0.05, which was reliable, indicating that the six factors selected range of the experimental design had an effect on the sensory score of yak yoghurt; the correlation coefficient $R^2 = 0.9237$, indicating that it fitted well in the whole regression region; the correction coefficient $R^2_{adj} = 0.8321$, indicating that 83.21% of the variability of the test data is available in this regression model, which can well indicate the changes of the factors; its coefficient of

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variation (CV) value is 4.27%, which indicates that the higher the test accuracy and credibility; the precision (Adeq Precisior) reaches 9.784, which indicates that the test rationality is high. Meanwhile, the regression equation was obtained by fitting multiple regression to the data: Y=75.84+2.73A+2.18B+0.46C+0.83D+3.71E-3.61F.

Table 5 Plackett-Burman experimental design and results

Test number	A	B	C	D	E	F	Sensory score/points
1	+1	+1	+1	-1	-1	-1	81.70
2	-1	+1	+1	-1	+1	+1	73.00
3	1	-1	-1	-1	1	-1	84.70
4	-1	1	1	1	-1	-1	77.60
5	1	-1	1	1	-1	1	71.50
6	1	1	-1	-1	-1	1	70.20
7	-1	1	-1	1	1	-1	83.60
8	-1	-1	1	-1	1	1	72.70
9	-1	-1	-1	-1	-1	-1	67.80
10	-1	-1	-1	1	-1	1	64.00
11	1	-1	1	1	1	-1	81.30
12	1	1	-1	1	1	1	82.00

Table 6 Plackett-Burman test factors, levels and analysis of significance

Source	Square sum	df	Mean square value	F-value	P-value
Model	477.825	6	79.6375	10.08474	0.0113*
A-Inoculum/%	89.1075	1	89.1075	11.28395	0.0201*
B-Yak milk added/%	56.7675	1	56.7675	7.188641	0.0438*
C-Sucrose addition/%	2.520833	1	2.520833	0.319221	0.5965
D-Oligogalactose addition/%	8.1675	1	8.1675	1.034275	0.3558
E-Fermentation time/h	165.0208	1	165.0208	20.89709	0.0060**
F-Fermentation temperature/°C	156.2408	1	156.2408	19.78525	0.0067**
residual	39.48417	5	7.896833		
aggregate	517.3092	11		CV%	3.71
R^2	0.9237			Pred R^2	0.5604
R^2_{adj}	0.8321			Adeq Precisi	9.784

Note: * *P*< 0.05 for significant; ** *P*< 0.01 for highly significant.

As shown in Figure 1, the standardised effect points of the influencing factors fermentation time (E), inoculum amount (A), yak milk addition (B), and fermentation temperature (F) were farther away from the fitted line, indicating that the more significant the effect was, so the influencing factors on yak yoghurt sensory scores were fermentation time, inoculum amount, yak such as the amount of yak milk added, and fermentation temperature, all of which were significant influencing factors (*P*< 0.05). The Pareto chart of standardised effects as shown in Fig. 2 further determined the effect amount value and significance of the influencing factors, whose influencing factors E, F, A, and B exceeded the t-value as the significant factors, and the order of the four significant influencing factors on yak yoghurt sensory scores from the top to the bottom was as follows: E>F>A>B. To sum up, the factors that had significant influence on yak yoghurt sensory scores should be selected Fermentation time (E), Inoculation amount (A), fermentation temperature (F), yak milk addition (B) for the follow-up test, but its yak milk addition (B) in the single-factor test of sensory scores in the middle of the square is not significant, and in this test of standardised effects of the Pareto chart is close to the value of the significant factor t value, the lowest impact on sensory scores, for the purpose of consideration of the follow-up test of the operation of the actual, the value of the B is taken with reference to the results of the single-factor test, and the no significant effect of factors C, D are the results of the single-factor test. The factors C and D, which had no significant effect, were positive effect factors, so the yak milk addition was determined to be (B) 40%, sucrose addition (C) 4% and oligogalactose addition (D) 3% according to the results of the one-factor test.

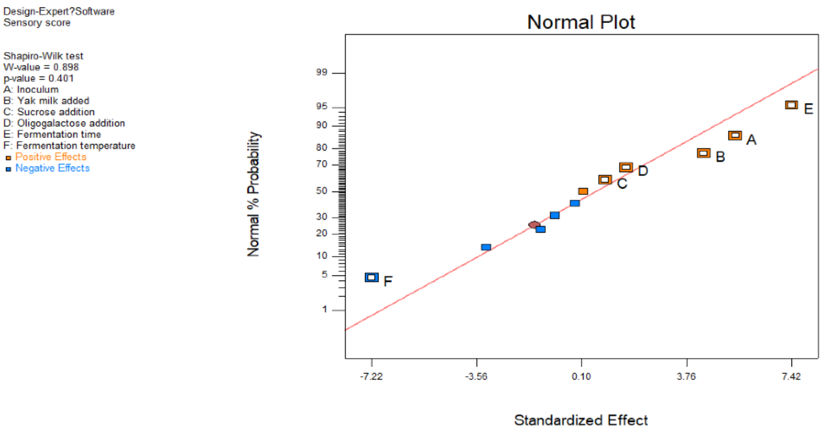

Fig.1 Normal probability effects plot for standardised effects

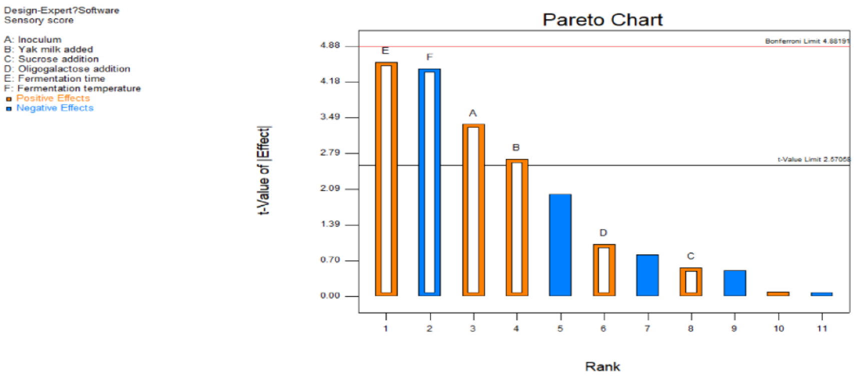

Fig.2 Pareto chart of standardisation effects

3.2 Steepest climb test design and result analysis

Combining the above multiple regression equations, selecting the most significant influencing factor E as the climbing unit and calculating the climbing direction and step size (Δ) of the other influencing factors with the coefficient of E of 3.71 can be obtained:

$$\Delta E = 1 \times \frac{7-5}{2} = 1\ \mathrm{h} \tag{2}$$

$$\Delta A = \frac{2.73}{3.71} \times \frac{4-2}{2} = 0.74\% \tag{3}$$

$$\Delta F = \frac{-3.61}{3.71} \times \frac{45-43}{2} = -0.97\ ℃ \tag{4}$$

Considering the practicality of the experimental operation process, and the inoculum amount and fermentation time are positive effect influences, and the fermentation temperature is a negative effect influence factor, therefore, the hill-climbing experimental design was chosen to gradually increase the change of the inoculum amount (ΔA) by 1%, the fermentation time (ΔE) by 1 h, and the fermentation temperature (ΔF) by 1 ℃, and the experimental design and results are shown in Table 7. From the table,

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it can be seen that the highest sensory score value of yak yoghurt was in group 2, when the inoculum amount was 3%, the fermentation time was 5 h, and the fermentation temperature was 43°C , the highest sensory score value was 81.6, so this condition was chosen as the centre point of the subsequent Box-Behnken response surface test.

Table 7 Steepest climb test design and results

Test number	A-Inoculum/%	E-Fermentation time/h	F-Fermentation temperature/°C	Sensory score/points
1	2	5	44	77.50
2	3	6	43	81.60
3	4	7	42	80.20
4	5	8	41	74.00
5	6	9	40	72.40

3.3 Analysis of response surface test results

3.3.1 Response surface test design results and analysis of variance

Based on the results of the one-way test, combined with the Plackett-Burman test and the steepest climb test, the inoculum amount (A), fermentation time (E), and fermentation temperature (F) were selected, and a three-factor, three-level Box Behnken experimental design was carried out using Design-Expert 8.0.6 software, and the results are shown in Table 8.

Table 8 Response surface analysis scheme and test results

Test number	A-Inoculum/%	E-Fermentation time/h	F-Fermentation temperature /°C	Sensory score/points
1	1	1	0	82.40
2	1	0	-1	82.70
3	-1	1	0	75.10
4	1	-1	0	77.30
5	0	0	0	83.00
6	0	1	1	75.50
7	-1	-1	0	70.20
8	1	0	1	77.20
9	0	0	0	84.60
10	-1	0	1	77.80
11	0	1	-1	82.80
12	0	-1	1	80.70
13	-1	0	-1	74.90
14	0	0	0	86.00
15	0	-1	-1	74.80
16	0	0	0	85.40
17	0	0	0	83.50

Using the software Design Expert 8.0.6 software analysis, the regression equation affecting the sensory score of yak yoghurt (R1) was obtained as $R1=84.5+ 2.7A+1.6B-0.5C+0.05AB-2.1AC-3.3BC-4.275A^2-3.975B^2-2.075C^2$, and the absolute value of coefficient in its equation magnitude responds to

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the degree of response of each factor to the sensory scores, and the model can be optimally evaluated.

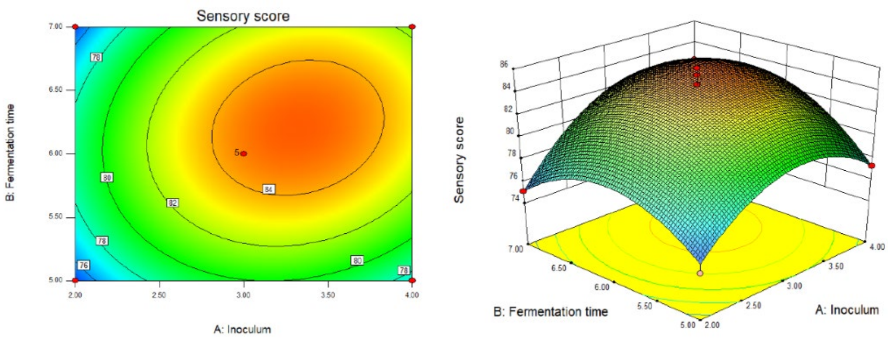
From the P-value in Table 9, the primary term B had a significant effect on the sensory score (*P*< 0.05), and the primary term A, the interaction terms AC and BC, and the secondary terms A^2, B^2, and C^2 had a highly significant effect (*P*< 0.01); and from the F-value, the order of the three factors affecting the sensory score value of the yak yoghurt in the following order from the highest to the lowest: the amount of inoculum (A)> the time of fermentation (B)> the temperature of fermentation (C).

Table 9 Response surface quadratic model ANOVA for yak yogurt sensory score values

Source	Square sum	df	Mean square	F-value	P-value	significance
Model	270.55	9.00	30.06	23.23	0.0002	**
A-Inoculum/%	38.72	1.00	38.72	29.92	0.0009	**
B-Fermentation time/h	9.68	1.00	9.68	7.48	0.0291	*
C-Fermentation temperature/℃	2.00	1.00	2.00	1.55	0.2539	
AB	4.41	1.00	4.41	3.41	0.1074	
AC	17.64	1.00	17.64	13.63	0.0077	**
BC	43.56	1.00	43.56	33.66	0.0007	**
A2	60.00	1.00	60.00	46.36	0.0003	**
B2	50.84	1.00	50.84	39.28	0.0004	**
C2	27.92	1.00	27.92	21.57	0.0024	**
residual	9.06	7.00	1.29			
mismatch test	2.74	3.00	0.91	0.58	0.6597	
pure error	6.32	4.00	1.58		0.0002	
total error	279.61	16.00			0.0009	
	R^2	0.9676		R^2_{adj}	0.92596	

Note: * *P*< 0.05 for significant; ** *P*< 0.01 for highly significant.

3.3.2 Response surface test design results and analysis of variance (ANOVA)



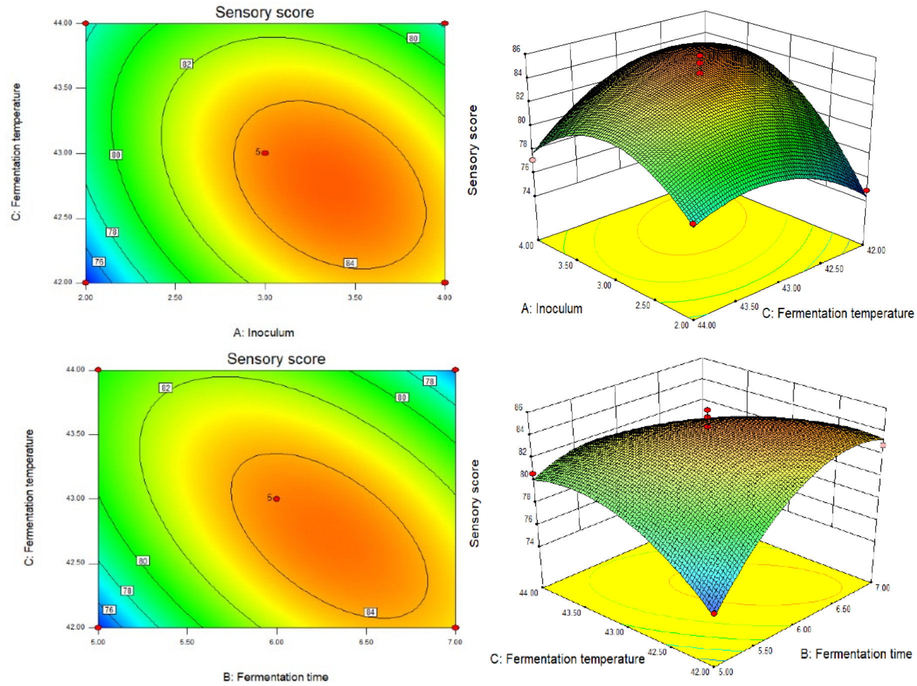


Fig.3 Contour and 3D surface map of the interaction of 3 groups of factors on sensory score

The 3D surface plot of the response surface showed that the greater the slope of the response surface, the greater the effect of this interaction factor on sensory scores. As shown in Fig.3, the response value increases from low to high and then decreases with the gradual increase of inoculum amount, increases and then decreases with the gradual increase of fermentation time, and decreases from low to high and then gradually decreases with the increase of fermentation time, in which the prominent surface of the factor interactions is the best response value. The contour lines in the graph show the intensity of the interaction between the factors, if the contour lines tend to be elliptical, it indicates that the interaction between the two factors is significant, if it tends to be round, it is not significant; the denser the contour lines are, it indicates that the factor has a higher degree of influence on the sensory scores, and vice versa, it is low; in the contour graph, the contour lines of AC and BC tend to be elliptical, it indicates that the interaction between the amount of inoculum and the fermentation temperature, and the two factors, namely, the amount of inoculation and fermentation temperature, the fermentation time and the fermentation temperature, is significant.

3.4 Optimum process parameter conditions and validation results

The process parameters of yak yoghurt were optimised using response surface methodology, and the best optimised process parameters were 3.57% inoculum according to the quadratic regression equation. The fermentation time was 6.57 h, and the fermentation temperature was 42.30℃ , and the maximum sensory score of yak yoghurt was 85.61 under this condition . In order to prove the reliability of the prediction, this optimal process parameter condition was verified and the inoculum amount was adjusted to 3.5% considering the feasibility in practice. The fermentation time was adjusted to 6.5 h, and the fermentation temperature was adjusted to 42℃ , and the sensory score value of 86 was obtained through the test, which was very close to the theoretical prediction value, and the model fit well and could predict the actual situation well.

In summary, the optimal process parameters for yak yoghurt production were: 3.5% inoculum, 40% yak milk addition, 4% sucrose addition, 3% oligogalactose addition, fermentation time of 6.5 h, and fermentation temperature of 42℃ , which resulted in the best organoleptic scores of the yak yoghurt under the conditions of this process.

4. Discussion

During yak yoghurt fermentation, different production processes, fermentation strains, fermentation time and temperature all have an impact on its quality. Traditional lactic acid bacteria, as a key factor in yak milk fermentation in pastoral areas, are abundant and diverse, and in this study, the use of composite fermenting Lactobacillus spp. is conducive to maximising the synergistic effect of lactic acid bacteria strains. Sucrose as a common food flavouring agent in yoghurt can improve the sourness produced by fermentation, and also promote the fermentation of lactic acid bacteria[19], to achieve the effect of improving the quality of yak yoghurt. The process optimisation in this study resulted in 4% sucrose addition, while Liu Xue et al[20] added 7% sucrose in yak yoghurt process optimisation, which was higher than that in the present study, probably due to the difference in fermentation strains. Ma Ruijuan[21] The study showed that the special flavour in yak yoghurt was caused by the decomposition of triglycerides present in yak milk fat globules to produce free fatty acids[22], so the addition of a certain amount of food sweeteners and so on can reduce the special flavour and the degree of over-acidity at the later stage of fermentation without changing the nutritional value of yak yoghurt, and further increase the acceptability of yak yoghurt to the general consumers. Yak yoghurt can further enhance the acceptance and popularity of yak yoghurt among consumers. In this study, the amount of oligogalactose added to yak yogurt was 3%, which was higher than that in the experiment conducted by Yang Xing[11], probably due to the different types of raw milk sources. While oligogalactose as a functional polysaccharide, can regulate intestinal flora, enhance body immunity[22], promote the absorption of minerals and other probiotic properties[24], which has been carried out in food for a wide range of applications[25], which is digested and absorbed by the stomach and intestines, supplying energy for the human body and thus regulating fat metabolism[26]. MARCELLO[27] Studies have shown that the addition of oligogalactose can promote the growth of Lactobacillus, inhibit and suppress the inhibition of harmful bacteria, such as Escherichia coli and Clostridium difficile, and thus regulate the dynamic balance of the microorganisms of the intestine; MA Chenchen[28] the study of the probiotic mechanism of oligogalactose in Lactobacillus plantarum showed that oligogalactose assisted Lactobacillus plantarum to change the intestinal microflora network and reduced the mutation of Lactobacillus plantarum genes.

5. Conclusion

Through the determination of curd performance of single strains, the combination of lactic acid bacteria with better performance was selected to prepare the fermenter, and through the one-way test, further combined with the Plackett-Burman test and the steepest-climbing test, the inoculum amount, fermentation time and fermentation temperature were selected for the three-factor, three-level Box Behnken experimental design, and the parameters of yak yoghurt fermentation were optimized by the response surface methodology. The results showed that the III (Y2-10, N5-9) curd state was better, and the post-acidification ability were all weaker combination strains, which were used to prepare the composite fermenter. Plackett-Burman and steepest climb tests were conducted with 6 single factors, inoculum quantity, yak milk addition, sucrose addition, oligogalactose addition, fermentation time and fermentation temperature as independent variables and sensory score as dependent variable to obtain the significant effect of inoculum quantity, fermentation time and fermentation temperature on the sensory score of yak yoghurt, which had a greater influence on its quality, and the Box Box Behnken test was conducted to study these three factors, and the best parameters for optimising the yak yoghurt fermentation process were: inoculum quantity of 3.5%, yak milk additive quantity of 40%, sucrose additive quantity of 4%, oligogalactose additive quantity of 3%, fermentation time of 6.5 h, fermentation temperature of 42℃ , and the best sensory score of yak yoghurt under these conditions , and the effect of yak yoghurt prepared under these conditions was higher than that of the traditional yak yoghurt in the pasture area.

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