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FERMENTATION OPTIMIZATION OF GOAT MILK WITH *LACTOBACILLUS ACIDOPHILUS* AND *BIFIDOBACTERIUM BIFIDUM* BY BOX-BEHNKEN DESIGN*

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ABSTRACT

Background. Goat milk is only limited to the processing of goat milk powder and liquid milk, the products are mainly about milk powder and a few of them are made as milk tablet. Therefore, the study of probiotic goat milk will have great significance in the full use of goats and the development of the goat milk industry in China.
Methods. The effect of fermentation temperature (35°C, 37°C, 39°C), strain ratio (1:1:1, 2:1:1, 3:1:1) and inoculum size (4%, 5%, 6%) on viable counts of *L. acidophilus* and *B. bifidum*, total bacteria and sensory value during fermentation process of *L. acidophilus* and *B. bifidum* goat yogurt (AB-goat yogurt) was investigated.
Results. The optimum fermentation conditions for AB-goat yogurt were: fermentation temperature 38°C, the strain ratio 2:1:1, inoculum size 6%. Under the optimum conditions, the viable counts of *B. bifidum*, *L. acidophilus*, total bacteria and sensory value reached $(4.30 \pm 0.11) \times 10^7$ cfu/mL, $(1.39 \pm 0.09) \times 10^8$ cfu/mL, $(1.82 \pm 0.06) \times 10^9$ cfu/mL and 7.90 ±0.14, respectively.
Conclusion. The fermentation temperature, the strain ratio and inoculum size had a significant effect on the fermentation of AB-goat yogurt and these results are beneficial for developing AB-goat yogurt.

Key words: skimmed goat milk, *Bifidobacterium bifidum*, *Lactobacillus acidophilus*, fermentation, response surface methodology

INTRODUCTION

Goat's milk has a unique flavor, rich nutrition and special features and a kind of nutrition, and it is more easily digestible and absorbed than cow's milk for its smaller fat globule size (Park, 2006). Goats milk yogurt is considered a desirable dairy product for its nutrient-rich and easy absorption, it is known as "the king of milk" around the world (Agnihotri and Prasad, 1993; Chen et al., 2009), and recognized as the dairy product closest to human milk (Haenlein, 2004; Rafter, 2003; Saarela et al., 2002). Goat milk is the third largest dairy resource milk after cow's milk and buffalo milk, which has multiple functions of nutrition, health and diet. It is gradually becoming the preferred product of high-end consumers. In general, there is no

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significant difference in the nutritional value of goat milk and cow's milk. Because of its better nutritional and metabolic properties, goat milk has been suggested as an ideal substitute for allergic patients and infants given cow milk and other food sources (Lamblin et al., 2001; Park and Haenlein, 2006). Furthermore, goat milk is rich in protein, fat, vitamins (vitamin A, B complex and C) and minerals (Ca and P), which meets the nutritional needs of the FAO-WHO requirements for human infants, children and adults (Haenlein and Anke, 2011; Jenness, 1980; Keogh and O'Kennedy, 1998; Silanokove et al., 2010).

Probiotics are a type of beneficial microorganism that can improve the host's micro-ecological balance. Probiotics can treat diseases like atopic dermatitis, lactose intolerance, food allergy, acute gastroenteritis, Crohn's disease, help prevent cancer, and changes in microbial community structure (Kerlynn et al., 2015; Marco et al., 2006; Million and Raoult, 2012; Unno et al., 2015). Probiotic fermented milk is prepared by using probiotic lactic acid bacteria. Some bifidobacteria are used as probiotic bacteria, such as *Lactobacillus plantarum*, *L. rhamnosus*, *L. delbrueckii*, *L. lactis* subsp. *lactis*, *L. acidophilus*, *L. casei*, *B. bifidum*, *B. breve*, *B. adolescentis*, *B. longum* (Arskold et al., 2007). In recent years, more and more probiotics (such as *L. acidophilus* and *B. bifidum*) have been added to yogurt and fermented milk. Probiotics can utilize the nutrients in goat milk more efficiently than cow's milk, and their activity in probiotics goat milk can be as high as 93% (Balakrishnan and Agrawa, 2014).

Adding probiotics can not only change the taste of yogurt and the health of the intestinal tract, but also promote the diversification of yogurt products. However, most studies of the dairy products have concentrated on cow milk, and very little scientific research is available to goat milk products due to the lack of governmental, industry and academic supports. Güler-Akın and Akın (2007) used *L. bulgaricus* (LB), *S. thermophilus* (ST), *L. acidophilus* and *B. bifidum* as a yogurt starter, and the final product tasted better due to the comprehensive effects of various probiotics. Related studies showed that the fermentation capacity of composite species was better than that of single strains (Collado et al., 2007). *L. casei* and *Bifidobacterium* bifidum were used to optimize the processing of probiotic fermented milk. Consequently, the level of viable bacteria reached 4.1×10^{11} cfu/mL, and this increased by 1–2 log rank compared with previous studies. Furthermore, the viable counts of bacteria remained at 4.7×10^{10} cfu/mL after being stored at 4°C for 21 d (Liu et al., 2013). Viable counts of goat yogurt fermented by *L. acidophilus* or *L. casei* on the basis of *L. bulgaricus* and *S. thermophilus* as starter cultures reached 1.8×10^{7} cfu/mL and 1.56×10^{8} cfu/mL, respectively (Chen et al., 2013).

Goat's milk products are often produced and consumed in the European Union, for example in Greece, the Netherlands, France, Spain and Italy, and are very important to regional and social economies (Andueza et al., 2013). However, in China, whose goat milk yield is very high, production is only limited to the processing of goat milk powder and liquid milk, the products are mainly about milk powder and few of them made as milk tablet.

Therefore, the development of probiotic goat milk will have a great significance in the full use of resources of goats and the development of China's goat milk industry. In our previous study, the single factor and orthogonal experiment have been carried out to optimize the fermentation conditions of goat milk (Chen et al., 2010), the effect of inoculum size and temperature on the fermentation of goat yogurt with *L. bulgaricus* and *S. thermophilus* (Shu et al., 2014), and the effect of bacteria proportions on the fermentation of AB-goat yogurt with probiotic culture (Shu et al., 2014). The aim of the present study was to optimize the fermentation conditions of goat milk using Response Surface Methodology to improve the viable counts of bacteria in goat milk for the development of probiotic products.

MATERIAL AND METHODS

Bacterial strain and culture preparation. Starter bacteria of *L. acidophilus*, *B. bifidum*, *S. thermophilus* and *L. bulgaricus* were obtained from the School of Food and Biological Engineering, Shaanxi University of Science and Technology. The MRS (for LA, BB) and M17 (for *L. bulgaricus* and *S. thermophilus*) and tomato juice agar were purchased from a local retail market (Hopebio, Qindao, China). All the protective agents used in the experiment were of analytical grade.

Strain activation was conducted on a clean bench; the bacillus and coccus bacteria powder were

inoculated into MRS and M17 culture medium, 37°C for 24 h. The experiments were repeated several times until strain activity was stable, which is evaluated by microscopy, and then 3%–5% bacteria that had been fully activated were inoculated into an anaerobic tube containing sterile skimmed milk and mixed. *L. bulgaricus* and *S. thermophilus* were cultivated at 42°C and *L. acidophilus* and *B. bifidum* at 37°C. Following this, 3%–5% skimmed milk was inoculated in a triangular bottle sterilization of goat milk, then mixed and cultured in a constant temperature, which can be used for the production of goat yogurt after solidification.

Fermentation experiments. Adding probiotics (BB, LA) to ordinary yogurt fermentation process used *S. thermophilus* and *L. bulgaricus* as basic starter cultures. The effects of fermentation temperature, strain ratio and inoculum size on the corresponding value were optimized by using response surface methodology. The number of probiotics was determined by selective counting, and finally goat yogurt products containing probiotics were obtained. Viable bacteria counts in products and probiotics might reach more than 10^9 cfu/mL, 10^6 cfu/mL, respectively.

Determination of viable bacterial counts. There were two main ways of determining viable bacterial counts: the top agar method and the plate coating method (Shu et al., 2014). Recently, another method was used to assess the viable bacterial count in commercial milk. TEMPO (Loss et al., 2012), an automated most-probable-number method, is a cheap and rapid alternative to standard culture methods suitable for assessing viable bacterial counts in processed and raw milk samples.

MRS-LiCl (0.1%) or MRS-bile-salt (0.06%) was used to calculate viable counts of *B. bifidum* and *L. acidophilus* in fermented goat milk. MRS-gentamicin (240 u) and MRS-LiCl (0.1%) was used to count the level of probiotic bacteria in fermented goat milk. A TJA agar medium was used for counting total bacteria.

Sensory evaluation. The sensory evaluation of goat milk was assessed by five researchers professional trained in the sensory scoring of goat milk. These researchers analyzed surface appearance, taste, smell, structural state, whey precipitation and other sensory properties. The scores are shown in Table 1.

Experimental design. The most effective parameters in terms of process conditions of goat milk were fermentation temperature, strain ratio (BB : LA : (LB : ST)) and inoculum size. To optimize these factors and obtain the maximum response value, the Box-Behnken Design method was used. Response surface methodology (RSM) plays a significant role in designing, formulating, developing and analyzing

Table 1. The sense evaluation standard of goat yogurt

Project	Color (1 point)	Smell (3 points)	Taste (3 points)	Structural state (3 points)
Bad	gray or atypical color (0–0.25)	no flavor (0–0.75)	corruption/moldy (0–0.75)	adverse curd, bubbles, whey precipitation serious (0–0.75)
Common	color is uneven, pale yellow/ light gray (0.25–0.50)	flavor is slightly, slight goaty flavor (0.75–1.50)	sour and sweet taste are too strong or weak (0.75–1.50)	curd uneven, not strong, whey separation (0.75–1.50)
Good	color is uniform basically, creamy/milky (0.50–0.75)	pure yogurt flavor, slight goaty flavor (1.50–2.25)	sweet and sour moderate, little astringency (1.50–2.25)	curd is good, state is uniform and fine, little whey precipitation (1.50–2.25)
Very good	color is uniform, milky (0.75–1.00)	fragrance/pure yogurt flavor, no critical remarks (2.25–3.00)	sweet and sour moderate, no critical remarks (2.25–3.00)	no bubbles , no whey precipitation (2.25–3.00)

Table 2. The experimental range and levels of the variables in the Box-Behnken Design method

Variable parameter	Coded variable levels		
	–1	0	1
A fermentation temperature, °C	35	37	39
B strain ratio	1:1:1	2:1:1	3:1:1
C inoculum size, %	4	5	6

new scientific research, as well as in improving existing studies and products. The experimental design in the coded and actual levels is shown in Table 2. After the experiments had been performed, a second-order polynomial equation based on the data obtained was used to determine the relationships and interactions between the variables. The equation describes the effect of variables including linear, quadratic and cross-product terms:

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ii} X_i^2 + \Sigma\beta_{ij} X_i X_j$$

While Y is the predicted responses of the dependent variable, β_0 is the second-order reaction constant terms, β_i is the linear terms coefficient, β_{ii} is quadratic terms coefficient and β_{ij} is interaction terms coefficient, X_i and X_j are the independent variables (Myers, 1976).

Statistical analysis of the data. Box-Behnken Design experiments were performed using Design-Expert statistical software, version 8.0.6, which included a number of "procedures": graphical, statistical, report, process and tabulate procedures that enabled simple and rapid data to be evaluated (Katayoun et al., 2013). An Analysis of Variance (ANOVA) test was employed to describe the statistical significance of the regression coefficients. In the quadratic polynomial, non-significant terms ($p > 0.05$) were deleted and a new polynomial was recalculated to obtain a predictive model for each dependent variable (Faveri et al., 2004). Regression analysis of the experimental data was conducted to evaluate the response of independent variables. The fitting of the second-order model equations was determined by the coefficient of determination (R^2).

Table 3. Experimental design for optimizing fermentation conditions of AB-goat yogurt

Number	*A*	*B*	*C*	BB (Y_1) ×10⁶ cfu/mL	LA (Y_2) ×10⁷ cfu/mL	Total bacteria (Y_3) ×10⁹ cfu/mL	Sensory value (Y_4)
1	–1	–1	0	6.00	1.30	1.31	6.38
2	1	–1	0	8.00	4.20	1.15	6.62
3	–1	1	0	49.00	1.70	1.33	6.78
4	1	1	0	32.00	2.10	1.65	6.81
5	–1	0	–1	19.00	6.20	1.66	6.84
6	1	0	–1	28.00	7.30	1.73	6.85
7	–1	0	1	12.00	9.20	1.54	6.93
8	1	0	1	22.00	12.70	1.89	7.17
9	0	–1	–1	3.00	4.10	1.35	6.53
10	0	1	–1	51.00	3.20	1.73	6.68
11	0	–1	1	16.00	12.30	1.36	6.57
12	0	1	1	55.00	10.00	1.68	7.17
13	0	0	0	17.00	13.20	1.51	6.87
14	0	0	0	20.00	11.20	1.68	6.93
15	0	0	0	15.00	9.90	1.47	6.89

RESULTS AND DISCUSSIONS

Experimental design and results of Box-Behnken

The RSM design and the results are shown in Table 3. The viable counts of *B. bifidum* and *L. acidophilus* were represented by Y_1 ($\times 10^6$ cfu/mL), Y_2 ($\times 10^7$ cfu/mL), respectively. Total bacteria was represented by Y_3 ($\times 10^9$ cfu/mL) and the sensory value was represented by Y^4.

Establishment of regression model

Statistical software Design-Expert 8.0.6 was used to analyze the data in Table 2, and quadratic regression equation regarding three factors and total bacteria, the viable counts of *B. bifidum* and *L. acidophilus* and sensory value can be obtained as follows:

$$Y_1 = -827.32 + 46.77A + 83.54B - 51.71C - 2.38 AB + 0.13AC - 2.25BC - 0.57A^2 + 8.71B^2 + 5.21C^2 \quad (1)$$

$$Y_2 = -1291.86 + 70.46A + 33.82B - 19.93C - 0.31 AB + 0.30AC - 0.35BC - 0.96A^2 - 5.28B^2 + 1.25C^2 \quad (2)$$

$$Y_3 = 10.81 - 0.09A - 1.26B - 2.87C + 0.06AB + 0.04 AC - 0.02BC - (2.29E - 003)A^2 - 0.18B^2 + 0.16C^2 \quad (3)$$

$$Y_4 = 4.25 + 0.26A + 1.47B - 1.85C - 0.03AB + 0.03 AC + 0.11BC - (4.30E - 003)A^2 - 0.23B^2 + 0.07C^2 \quad (4)$$

where: A, B and C are the coded values of the test variables shown in Table 2. Y_1, Y_2, Y_3 and Y_4 represent the corresponding expected values including the viable counts of *B. bifidum* and *L. acidophilus*, total bacteria and sensory value, respectively.

VARIANCE ANALYSIS OF THE DATA

The results show that the linear, square and interaction terms are significant at $p \leq 0.05$, indicating that this model could be explained to a great extent using these variables. Furthermore, adjusted R-squared (R^2_{adj}) can measure the amount of variation around the mean explained by the model adjusted for the number of terms, and the predicted R-square (R^2_{pred}) can measure of the amount of variation in new data explained

Table 4. Analysis of variance (ANOVA) for response surface quadratic model

Source	df[a]	BB		LA		Total bacteria		Sensory value	
		MS[b]	*p*-value[c]	MS[b]	*p*-value[c]	MS[b]	*p*-value[c]	MS[b]	*p*-value[c]
Model	9	386.23	0.0309	26.95	0.0072	0.061	0.0173	0.074	0.0002
A	1	2	0.8666	7.8	0.1244	0.042	0.0658	0.034	0.0043
B	1	2 964.5	0.001	3	0.3044	0.19	0.0043	0.22	< 0.0001
C	1	2	0.8666	68.44	0.0028	0	1	0.11	0.0003
A×*B*	1	90.25	0.2881	1.56	0.4467	0.058	0.0405	0.011	0.037
A×*C*	1	0.25	9.53E-01	1.44	0.464	0.02	0.17	0.013	0.0271
B×*C*	1	20.25	0.5979	0.49	0.6633	9.00E-04	0.7453	0.051	0.0018
A^2	1	19.39	0.6055	54.14	0.0046	3.10E-04	0.8482	1.42E-03	0.3581
B^2	1	280.01	0.0906	44.88	0.0011	0.13	0.0098	0.19	< 0.0001
C^2	1	100.16	0.2661	102.9	0.1747	0.096	0.0166	0.018	0.0149
Lack of fit	3	102.33	0.0588	5.73	0.6273	4.43E-03	0.7943	1.683E-003	0.3761
Errors	2	6.33		1.98		0.012		9.08E-04	
R^2			0.9158		0.9549		0.9346		0.9897

[a]Degrees of freedom.
[b]Mean square.
[c]The F-test probability values.

by the model (Katayoun et al., 2013). Once the fitted regression equations were determined, the statistical software Design-Expert 8.0.6 was used to draw the response surface plots. Analysis of variance for the polynomial model developed is shown in Table 4.

The low *p*-values of all regressions indicate that most of the discrepancies in the variable response can be defined by the regression equation. The lack of fit for all the responses was significant to $p \leq 0.05$, expressing the competence of the elected model (Table 4). The model equation as expressed in Eq. (1), (2), (3), (4) was confirmed to be a suitable model to describe the response of the value of the viable counts of *L. acidophilus* and *B. bifidum*, total bacteria and sensory value, respectively.

The coefficient of determination R^2 for Y_1 reached 0.9158, indicating that 91.58% of variability could be explained by the second-order polynomial predicted equation given already. Moreover, the adjusted *R*-squared value ($R^2_{adj} = 0.7642$), which was close to the R^2 value, confirmed that the model was very significant. A negative predicted *R*-square value (–0.3016) for the Y_1 implied that the overall mean was a better predictor of the response than the current model. Furthermore, all factors examined including their quadratic and mutual interaction terms, significantly affecting the viable counts for Y_1, and except for parameter *B*, the effect of all others were not significant, as shown in Table 4. The mutual interaction between parameters *A*, *B* and *C* was weak, as shown in Figure 1. This meant that the effect of one agent concentration on viable counts of *B. bifidum* was dependent on the level of another one. Three-dimensional response surface plots show that a higher strain ratio resulted in a great increase in the viable counts of *B. bifidum*, while the effects of fermentation temperature and inoculum size were not significant (Fig. 1).

The coefficient of determination R^2 for Y_2 stood at 0.9549, indicating that 95.49% of the probability in the response was explained by the model. R^2_{adj} (0.8737) was close to the value of R^2, showing that the quadratic regression model was significant. The parameter of *C* with the probability value ($p = 0.0028 < 0.01$) showed a strong linear correlation for the viable counts of *L. acidophilus.* While the effects of parameter *A* and *B* were not significant, the mutual interaction between parameters of *A*, *B* and *C* was weak, as shown in Figure 2 and Table 4. The two-dimensional contour plots seemed to be a circle indicating that the mutual interaction of terms *A*×*B* was not significant for the responses (Fig. 2a). Furthermore, with an increase in fermentation temperature, viable counts of *L. acidophilus* first increased and then decreased. These were also suitable for the strain ratio to affect the viable counts of *L. acidophilus* (Fig. 2). The quadratic terms of A^2 and B^2 with a probability value ($p = 0.0046 < 0.01$, $p = 0.0011 < 0.01$) had significant effects on the response value (Table 4).

The coefficient of determination R^2 for Y_3 reached 0.9346, indicating that 93.46% of the probability in the response was explained by the model. R^2_{adj} (0.8169) was close to the value of R^2, showing that the quadratic regression model was significant. The parameter of *B* with the probability value ($p = 0.0043 < 0.01$) showed

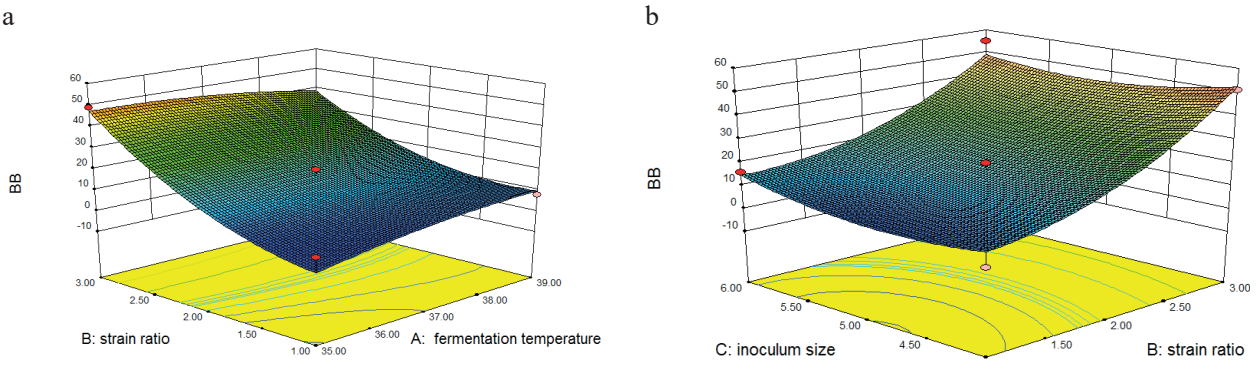

Fig. 1. Response surface graphs of viable counts of *B. bifidum*: a – fermentation temperature versus strain ratio (inoculum size, 5%), b – strain ratio versus inoculum size (37°C)

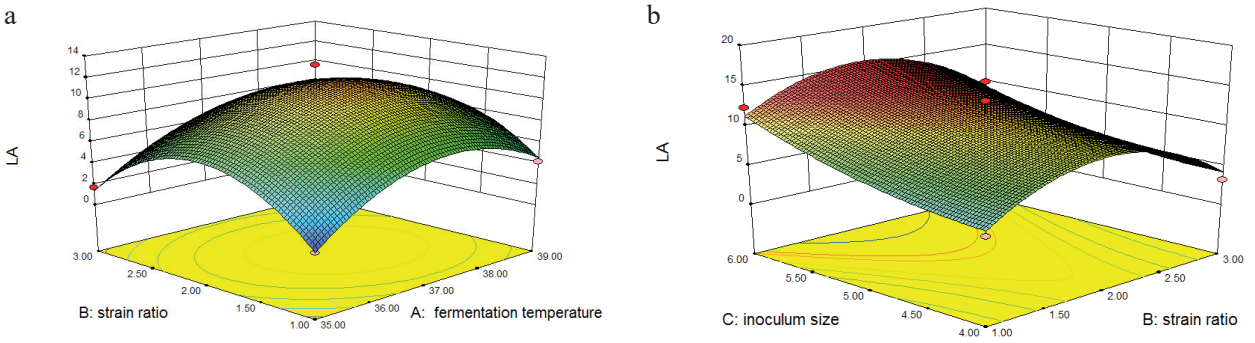

Fig. 2. Response surface graphs of viable counts of *L. acidophilus*: a – fermentation temperature versus strain ratio (inoculum size, 5%), b – strain ratio versus inoculum size (37°C)

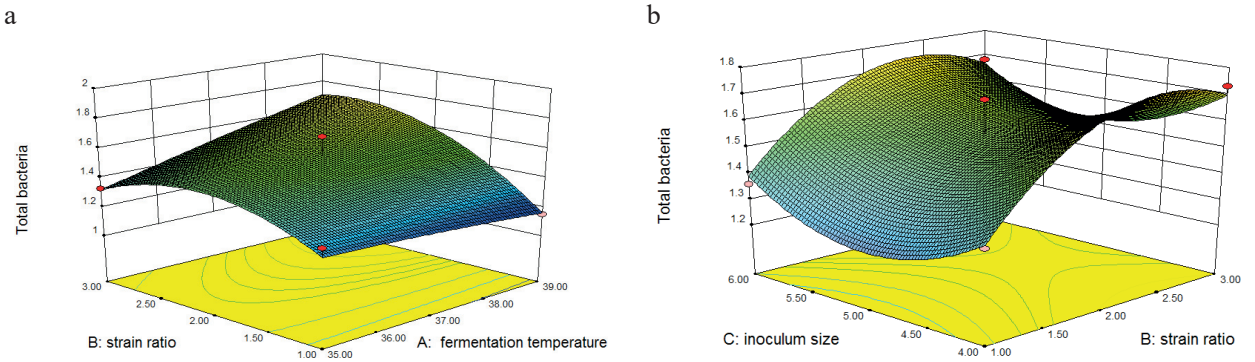

Fig. 3. Response surface graphs total bacteria: a – fermentation temperature versus strain ratio (inoculum size, 5%), b – strain ratio versus inoculum size (37°C)

a strong linear correlation for total bacteria (Table 4). However, the linear effect of parameter of A and C was not significant. The mutual interaction of $A \times B$ was significant for total bacteria, as shown in Table 4 and Figure 3, while the mutual interaction of $A \times C$, $B \times C$ was not significant. The surface effects of quadratic term A^2 and B^2 on total bacteria were extremely significant with the probability value ($p = 0.0098 < 0.01$, $p = 0.0166 < 0.05$), but the surface effects of quadratic term C^2 ($p = 0.8482 > 0.05$) were not significant. At the same time, the mean square (MS) value of each factor in the model may reflect the importance of various factors on the test indicators. The mean square (MS) value is higher, which indicates that the influence of the test index is higher. The effects of the strain ratio on viable counts of *B. bifidum* and *L. acidophilus*, total bacteria and sensory value were the same (Fig. 2-4). We can expect that the maximum response value can be obtained at the about 6% innoculum size. When the strain ratio stood at a low level, the total amount of bacteria increased with the increase in fermentation temperature, while at a high level, increasing fermentation temperature produced a decrease in the total amount of bacteria (Fig. 3a).

ANOVA showed that R^2 for Y_4 was 0.9897, which indicated that the change in the response value was 93.46% from the selected factor. Furthermore, all factors examined, including their quadratic and mutual interaction terms, significantly affected the sensory value, except interaction terms $A \times B$, $A \times C$ and quadratic term A^2, as shown in Table 4. The influence of the interaction between the strain ratio and inoculum size on the sensory was shown in Figure 4b. This shows that when the strain ratio was at a low level, the sensory value did

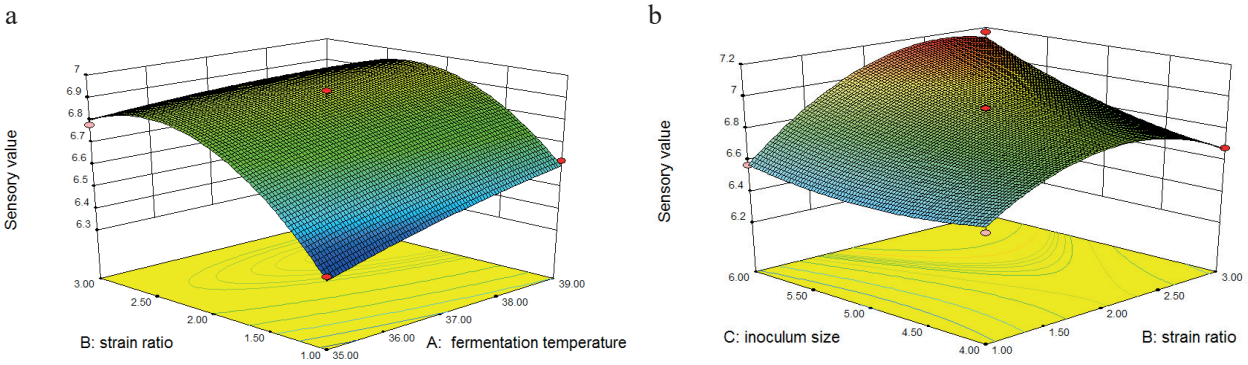

Fig. 4. Response surface graphs sensory value: a – fermentation temperature versus strain ratio (inoculum size, 5%), b – strain ratio versus inoculum size (37°C)

not change significantly with the increasing size of inoculum. However, at a high level, the sensory value increased with an increase in inoculum size.

According to the Box-Behnken experimental results and quadratic regression equation, the optimum fermentation conditions calculated by statistical software Design-Expert 8.0.6 were: fermentation temperature 38°C, strain ratio 2:1:1, inoculum size 6%. Applicability of the model equation for predicting the optimum response values was performed using these optimum conditions. Validation was conducted based on optimum conditions given by the RSM optimization approach. The viable counts of *B. bifidum*, *L. acidophilus*, total bacteria and sensory value reached $(4.30 \pm 0.11) \times 10^7$ cfu/mL, $(1.39 \pm 0.09) \times 10^8$ cfu/mL, $(1.82 \pm 0.06) \times 10^9$ cfu/mL, 7.90 ±0.14, respectively. The experimental values were found to be in high agreement with the predicted ones.

CONCLUSION

The fermentation process of skimmed goat milk with LA and BB was optimized using statistical software Design-Expert 8.0.6. The optimum fermentation temperature, strain ratio (BB : LA : (LB : ST)) and inoculum size in the goat yogurt was 38°C, 2 : 1 : 1, 6%, respectively. The viable counts of *B. bifidum*, *L. acidophilus*, total bacteria and sensory value reached 4.28×10^7 cfu/mL, 1.35×10^8 cfu/mL, 1.84×10^9 cfu/mL, 7.91, respectively. The verification result was as follows: the viable counts of *B. bifidum*, *L. acidophilus*, total bacteria and sensory value reached at $(4.30 \pm 0.11) \times 10^7$ cfu/mL, $(1.39 \pm 0.09) \times 10^8$ cfu/mL, $(1.82 \pm 0.06) \times 10^9$ cfu/mL, 7.90 ±0.14, respectively. Experiments confirmed our predictions, indicating that the optimized conditions and models used were reliable and effective, which can be proposed for fermented goat milk products.

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