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Development and Verification of an Optimum Composition Model for a Synbiotic Fermented Milk Using Sequential Quadratic Programming Techniques

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ABSTRACT : The purpose of this research was to develop an optimum composition model for a new synbiotic fermented dairy product with high probiotic cell counts, and to experimentally verify this model. The optimum composition model indicated the growth promoter ratio that could provide the highest growth rate for probiotics in this fermented product. Different levels of growth promoters were first blended with milk to improve the growth rates of probiotics, and the optimum composition model was determined. The probiotic viabilities and chemical properties were analyzed for the samples made using the optimal formula. The optimal combination of the growth promoters for the synbiotic fermented milk product was 1.12% peptides, 3% fructooligosaccharides (FOS), and 1.87% isomaltooligosaccharides (IMO). A product manufactured according to the formula of the optimum model was analyzed, showing that the model was effective in improving the viability of both *Lactobacillus* spp. and *Bifidobacterium* spp. (**Key Words :** Probiotics, Synbiotics, Prebiotics)

INTRODUCTION

A number of novel fermented dairy products are being developed and marketed under the concept of probiotic products. The benefits derived by the consumption of probiotic products are well documented and have been reviewed (Kanbe, 1992; Scheinbach, 1998; Ziemer and Gibson, 1998; Lourens-Hattingh and Viljoen, 2001; Liu et al., 2002; Chen and Lin, 2005; Liu et al., 2005). However, probiotics must retain viability and activity in food carriers to meet the suggested "therapeutic minimum" at the time of consumption (Playne, 1994).

Although milk theoretically contains all the essential nutrients for the growth of probiotics, the levels of amino acids and low molecular weight peptides are insufficient to provide the ideal conditions for rapid growth or to maintain prolonged growth of these bacteria (Klaver et al., 1993; Gomes et al., 1998). A substantial number of studies have tried to improve the growth potential in milk and better results have been achieved by the addition of growth promoters and redox reduction agents (Gomes and Malcata, 1998; Chen et al., 2004). Our previous study has demonstrated that growth promoters including peptides, fructooligosaccharides (FOS) and isomaltooligosaccharides (IMO) could improve the survial of bifidobacteria in fermented products (Chen et al., 2004).

Probiotic products that contain live microbial cultures, prebiotic products that selectively stimulate the growth of probiotics, and synbiotic products that combine probiotics and prebiotics all exert a positive effect on digestive health and overall wellbeing. In order to find an optimum formula for a synbiotic drink and elucidate the effects of the different growth promoters in terms of the microbial properties of fermented milk, response surface models were developed to describe the combined effect of the factors, and the sequential quadratic programming (SQP) technique applied to attain optimal combinations for the growth promoters. A quadratic programming problem is an optimization problem involving a quadratic objective function and linear constraints. The SQP method represents the state-of-the-art in nonlinear programming methods (The Math Work Inc., 2000) and can be used to solve a series of quadratic programming problems approximating the original non-linear programming problem (Haftka and Gürdal, 1992; Chen et al., 2005a, b).

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Table 1. Independent variables and responses of the experiments

Run	X_1 peptides (%)	X_2 FOS (%)	X_3 IMO (%)	growth rate log CFU hr^{-1}	
				L[a]	B[b]
1	1.50	1.5	3.0	0.26±0.03	0.25±0.01
2	0.00	1.5	0.0	0.16±0.01	0.14±0.02
3	0.75	3.0	3.0	0.27±0.02	0.27±0.01
4	0.00	0.0	1.5	0.17±0.01	0.18±0.02
5	1.50	1.5	0.0	0.26±0.04	0.25±0.02
6	0.75	0.0	3.0	0.28±0.01	0.26±0.01
7	0.75	1.5	1.5	0.27±0.02	0.26±0.03
8	0.75	1.5	1.5	0.27±0.02	0.26±0.02
9	0.75	1.5	1.5	0.25±0.01	0.26±0.01
10	0.75	1.5	1.5	0.28±0.02	0.28±0.02
11	0.75	3.0	0.0	0.27±0.01	0.24±0.03
12	0.75	1.5	1.5	0.26±0.01	0.27±0.02
13	0.00	3.0	1.5	0.17±0.02	0.17±0.01
14	0.00	1.5	3.0	0.18±0.01	0.18±0.01
15	0.75	0.0	0.0	0.24±0.04	0.25±0.02
16	1.50	3.0	1.5	0.26±0.02	0.29±0.01
17	1.50	0.0	1.5	0.27±0.01	0.26±0.01

[a] L: *L. acidophilus+L. casei*.
[b] B: *B. Longum+B. bifidum.*

The purpose of this research was to develop an optimum composition model for a new synbiotic fermented dairy product with high probiotic cell counts, and to experimentally verify this model. The optimum composition model is the growth promoter concentration that could provide the highest growth rate for probiotics in this fermented product. Different levels of growth promoters were first blended with milk to improve the growth rates of probiotics. Then, the optimum composition model was determined by the response surface modeling and the SQP. Finally, the probiotic viabilities and chemical properties were analyzed for the samples made to verify the optimal formula.

MATERIALS AND METHODS

The experimental design

After a screening test, preliminary results were obtained revealing that the viabilities of the probiotics (*Lactobacillus spp.* and *Bifidobacterium spp.*) were affected by three independent variables: peptides (pancreatic digested casein, Cheng-Fung Co., Taipei, Taiwan), isomaltooligosaccharides (IMO, Cheng-Fung Co., Taipei, Taiwan) and fructooligosaccharides (FOS, Cheng-Fung Co., Taipei, Taiwan). Appropriate ranges, determined by the screening test, were 0-1.5% for peptides, and 0-3% for both FOS and IMO.

The experimental design was a Box Behnkin Design (BBD; Box and Behnkin, 1960), which is a three-level design based on construction of a balanced incomplete block design. This methodology allows modeling of the results using polynomial equations. Uncoded levels and experimental design are given in Table 1. A total of 17 runs, performed in duplicate, were used to search for the optimum composition model for a new synbiotic fermented milk product.

Manufacturing of the synbiotic fermented milk samples

Probiotic strains and preparation of inocula : Pure lyophilized cultures of *B. longum* (CCRC 14605), *L. casei* subsp. *rhamnosus* (CCRC 12321), *B. bifidum* (CCRC 11844) and *L. acidophilus* (CCRC 14079) were purchased from the Culture Collection and Research Center, Hsinchu, Taiwan, ROC. The preparation of inocula followed a modified method originally developed by Østlie et al. (2003). The strains were subcultured three times in Man Rogosa Sharpe (MRS) broth (Difco Labs., Detroit, MI, USA) at 37°C overnight before a final inoculation for making concentrated stock cultures. Cysteine hydrochloride (0.05%, w/v, Sigma, St. Louis, MO, USA) was added to MRS broth for culturing *B. bifidum* and *B. longum*. Early stationary phase cells were harvested by centrifugation at 14,000×g for 10 min at 4°C. The pellet was washed once in 0.05 M potassium phosphate buffer (pH 7.0) before resuspension in reconstituted skim milk (12% total solids, Anchor Foods, New Zealand) sterilized at 115°C for 1 min to 1:10 of the original volume. The fresh cultures were stored at 4°C before use.

Manufacturing of the synbiotic fermented milk : Fermented milk samples were manufactured according to the BBD (Table 1). Base on our screening test, the growth rate of *L. acidophilus*, *L. casei*, *B. bifidum* and *B. longum* were affected by three independent variables, these including: peptides (0.0-1.5%), fructooligosaccharides (FOS, 0.0-3.0%) and isomaltooligosaccharides (IMO, 0.0-3.0%). Thus, reconstituted skim milk (12% total solids) was supplemented with peptides (0.0-1.5%), FOS (0.0-3.0%) and IMO (0.0-3.0%) in order to attempt to improve the growth rate of *L. acidophilus*, *L. casei*, *B. bifidum* and *B. longum*. The mix was pasteurized at 85°C for 30 min and inoculated with 4% culture (1% *L. acidophilus*, 1% *L. casei*, 1% *B. bifidum* and 1% *B. longum)* suspensions and fermented for 6 h at 37°C.

Modeling and optimization

To carry out the response surface modeling, regression was performed on the experimental results (Table 1) to construct mathematical models. The regression model between responses (Y) and independent variables was

$$f_Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum \sum_{i \neq J=1}^{3} \beta_{ij} X_i X_j \quad (1)$$

where f_Y represents the function for the growth rate of

Table 2. (a) Model analysis (b) Lack-of-Fit tests (c) The regression coefficients of determination of the optimum composition model during 6 h fermentation

Source	L[d]		B[e]	
	Sum of squares	p>F	Sum of squares	p>F
(a) Model analysis[a]				
Mean	1.01		0.98	
Linear	0.02	<0.01**	0.02	<0.01**
Quadratic	0.01	<0.01**	0.01	<0.01**
Cubic	1.25×10^{-4}	0.76	1.14×10^{-4}	0.69
Residual	4.07×10^{-4}		2.92×10^{-4}	
Total	1.04		1.01	
(b) Lack-of-fit tests[b]				
Linear	0.01	0.06	0.01	0.07
Quadratic	1.25×10^{-4}	0.76	1.14×10^{-4}	0.69
Cubic	0		0	
Pure error	4.07×10^{-4}		2.92×10^{-4}	
(c) The regression coefficients of determination[c] (R^2)				
Linear	0.60	0.02	0.63	0.02
Quadratic	0.98	2.63×10^{-3}	0.99	2.28×10^{-3}
Cubic	0.99		0.99	

* Significant at 5% level; ** Significant at 1% level.

[a] Model analysis: select the highest order polynomial where the additional terms are significant.

[b] Lack of fit tests: want the selected model to have insignificant lack-of-fit.

[c] The regression coefficients of determination: focus on the model minimizing the "Press".

[d] L: *L. acidophilus+L. casei*.

[e] B: *B. longum+B. bifidum*.

Lactobacillus spp. or *Bifidobacterium spp.* ; β_0, β_i, β_{ii} and β_{ij} are the constant, linear, quadratic and cross product coefficients, respectively; and X_i and X_j are the levels of independent variables.

The models were then formulated as an objective function in an optimization problem, and solved using a sequential quadratic programming (SQP) approach to derive the optimal growth rate for probiotics in synbiotic fermented milk samples. Both response surface modeling and SQP were employed similarly to the work by Chen et al. (2004).

Model verification

After optimal processing conditions were found by the SQP, experiments based on the conditions determined were performed and repeated three times. The results were then analyzed using ANOVA from the SAS software package (SAS Institute Inc, 1990), with Duncan's multiple range test for significance to detect differences between predicted and observed values.

Analytical methods

Determination of probiotic viability and growth in synbiotic fermented milk samples : Lactobacilli MRS agar (Difco Labs., Detroit, MI, USA) and Lithium propionate MRS agar (LP-MRS) were used as the media for selecting *Lactobucillus spp.* and *Bifidobacteria spp.*, respectively (Lapierre et al., 1992). Viable counts were determined in fermented milk samples by using serial decimal dilutions prepared in sterile peptone water (0.1%). Plates of MRS agar were incubated aerobically for 72 h at 37°C to inhibit bifidobacteria. Plates of LP-MRS agar were incubated anaerobically (72 h at 37°C) (GasPak System; Oxoid Unipath Ltd., Basingstoke, Hampshire, England). The population, in colony-forming units (CFU), and the characteristics of the colonies were recorded for each medium.

The specific growth rate (*GR*) corresponding to each culture in synbiotic fermented milk samples was calculated using the following equation:

$$GR\left(\log CFU\ h^{-1}\right)=\frac{\left[\log\left(CFU_2\right)-\log\left(CFU_1\right)\right]}{t_2-t_1} \quad (2)$$

where CFU_1 and CFU_2 are the *CFU* at times t_1 (fermentation for 0 h) and t_2 (fermentation for 6 h).

Determination of organic acids in optimum synbiotic fermented milk sample : Determination of organic acids followed a method modified from that of Chen and Chou (1991). A 30 ml fermented milk sample was centrifuged at 10,621×g to provide a top layer, which was then removed as a supernatant and filtered through a 0.45 mm membrane filter. A filtered 10 μl sample was injected into a Gilson HPLC system (Gilson, Middleton, WI, USA). The column used for organic acid analysis was a Lichrospher RP-18 (5 μm, 250 mm×4.6 mm, Merck Inc, Germany). The flow rate for the mobile phase (2% KH2PO4, pH 2.4, w/v) was 0.5

Table 3. The coefficients of the optimum composition model during fermentation for 6 h

Coefficient\Y	L[a]	B[b]
β_0	0.14	0.15
β_1	0.21	0.19
β_2	7.71×10^{-3}	-0.01
β_3	0.02	0.03
β_{11}	-0.09	-0.08
β_{22}	-1.15×10^{-5}	1.72×10^{-3}
β_{33}	-5.26×10^{-4}	-6.21×10^{-3}
β_{12}	-3.31×10^{-3}	8.27×10^{-3}
β_{13}	-5.22×10^{-3}	-7.78×10^{-3}
β_{23}	-3.23×10^{-3}	2.22×10^{-3}

[a] L: *L. acidophilus+L. casei*.
[b] B: *B. longum+B. bifidum*.

ml min^{-1} with a Gilson Model 155 UV detector operating at 220 nm. Organic acids were identified by relative retention times and externally quantified using standards purchased from the Sigma Chemical Company (St. Louis, MO. USA).

RESULTS AND DISCUSSION

The response surface modeling

The results for probiotic growth rate are presented in Table 1. According to the experimental design (a Box and Behnken factorial plan with three factors and three levels), experimental results were modeled using linear, quadratic or cubic functions by the least square regression method. The fitted functions were tested for adequacy and fitness using analysis of variance (ANOVA). Model analysis, the Lack-of-Fit Test and the regression coefficient of determination (R^2) were used for selection of adequate models, as outlined by Lee et al. (2000) and Weng et al. (2001).

Model analysis in Table 2a compares the validities of the linear, quadratic and cubic models for the two responses according to their F-values. A model with P-values (P>F) below 0.05 was regarded as significant. The highest-order polynomial that was significant was selected. Results of model analysis showed that the second-order models were well adjusted to the experimental data for both responses. The Lack-of-Fit test measures the failure of the model to represent data in the experimental domain at points that are not included in the regression (Wang and Lu, 2005). The results of Lack-of-Fit tests (Table 2b) did not result in a significant F value, indicating that the second-order models were sufficiently accurate for predicting the growth rates for both *Bifidobacterium spp*. and *Lactobacillus spp*. for any combination of independent variable values within the ranges studied. The regression coefficients of determination (R^2) for both *Bifidobacterium spp.* and *Lactobacillus spp.*, which is a measure of how well a model can be fitted to the raw data, were 99% and 98%, respectively (Table 2c). This ensures a satisfactory adjustment of the quadratic model to the experimental data.

Hence, the second-order regression equations for both *Lactobacillus spp.* and *Bifidobacterium spp.* appeared to be the most accurate ones. The coefficients for the statistically significant models are presented in Table 3. The two responses were then added together to form a composite function (CF) whose maximum was subsequently sought by the SQP.

Optimization

Factors affecting the growth rate of probiotics : The relationships between the independent variables and the

Table 4. Randomly generated, initial searching points and optimal CF values found by SQP

Set no.	Initial searching point			Optimal point			Optimal CF value	Probability[d]
	X_1[a]	X_2[b]	X_3[c]	X_1	X_2	X_3		
1	0.7904	1.2525	2.0872	1.1224	3.0000	1.8703	0.5905	0.6667
2	1.3803	2.5066	0.6635	1.1224	3.0000	1.8703	0.5905	0.9000
3	0.5687	2.4142	1.8762	1.1224	3.0000	1.8703	0.5905	0.9714
4	0.9583	0.0275	1.5399	1.0687	0.0000	2.1623	0.5502	0.9603
5	0.2606	1.9341	1.7271	1.1224	3.0000	1.8703	0.5905	0.9870
6	1.1787	0.3371	0.8617	1.0687	0.0000	2.1623	0.5502	0.9837
7	0.5484	0.6471	2.6781	1.0687	0.0000	2.1623	0.5502	0.9814
8	1.1654	2.7279	0.2778	1.1224	3.0000	1.8703	0.5905	0.9932
9	0.5132	0.4340	0.8775	1.0687	0.0000	2.1623	0.5502	0.9923
10	1.1613	0.6224	2.4657	1.0687	0.0000	2.1623	0.5502	0.9915
11	0.2217	2.5519	2.9821	1.1224	3.0000	1.8703	0.5905	0.9968
12	0.2224	2.1393	0.2034	1.1224	3.0000	1.8703	0.5905	0.9988
13	0.9586	0.2413	2.9193	1.0687	0.0000	2.1623	0.5502	0.9986
14	0.1401	2.4561	2.7684	1.1224	3.0000	1.8703	0.5905	0.9995
15	1.3932	1.0469	2.8762	1.1224	3.0000	1.8703	0.5905	0.9998
16	0.7277	0.4876	1.6895	1.0687	0.0000	2.1623	0.5502	0.9998
17	0.2449	1.6193	2.8516	1.1224	3.0000	1.8703	0.5905	0.9999

[a] X_1: Peptides %. [b] X_2: FOS %. [c] X_3: IMO %.
[d] Probability for being the global optimum.

Table 5. Validation of the optimal composition model recommended by SQP for a synbiotic fermented milk

L[a] (log CFU h^{-1})		B[b] (log CFU h^{-1})	
Pred[c]	Exp[d]	Pred	Exp
0.30	0.29	0.29	0.29

* $p<0.05$.
[a] L: *Lb. acidophilus+Lb. casei*. [b] B: *B. longum+B. bifidum*.
[c] Pred: predicted value. [d] Exp: experimental value.

responses were investigated by examining the CF plots created by holding one of the three independent variables constant. By fixing the IMO level at 0%, 1.5% and 3%, 3-D plots of CF values as functions of peptides and FOS can be produced. It was clear that the growth rates of probiotics were sensitive to alterations of the test variables, i.e. peptides and FOS. The CF values increase in accordance with the higher levels of peptides and FOS. The FOS at the highest concentration and peptides within 1.0-1.5% lead to the highest CF value. For the effects of IMO levels, the 3.0% concentration appears to have the lowest CF values among the three different levels. From above analysis, the optimum value of CF should be at high levels of peptides and FOS.

Optimization by SQP : A global optimization program equipped with a multi-start SQP technique was coded to search for the global optimum. The program generates a series of uniformly distributed random points for initial search, and then the SQP is applied to find the optimum based on each subsequent initial point. If the probability of locating the global optimum exceeds a preset value (99.99% in this study), the global optimum is considered found. Otherwise, the next random, initial point is generated and the SQP re-executed. Table 4 lists the detailed information about the initial points and the optimal points, their respective CF value, and its probability for being the global maximum. The optimization results clearly show that determination of the optima depends on the initial search points and there are two different local optimal CF values (0.5502 and 0.5905) identified from 17 randomly generated initial points. Of these local optima, the global optimal CF is 0.5905 with 99.99% certainty. The global maximum corresponds to: 0.30 log CFU/h for growth rate of *Lactobacillus spp.* and 0.29 log CFU/h for growth rate of *Bifidobacterium spp.* The highest optimal CF value (0.5905) was attained for 10 of 17 sets and the optimal point consists of the independent variables at $X_1 = 1.12$, $X_2 = 3.00$, and $X_3 = 1.87$. In other words, the optimal combination for the growth promoters was milk blended with 1.12% peptides, 3.00% FOS, and 1.87% IMO.

Experimental verification

The optimal production formulation, derived from the SQP, was verified by independent additional experiments.

Table 6. The chemical properties and viabilities of probiotics

	Control[a]	Optimum sample[b]
pH	6.37	5.42
Acidity (%)	0.23	0.40
Lactic acid (mg mL^{-1})	0.72	2.31
Acetic acid (mg mL^{-1})	0.39	0.41
Lactobacillus spp. (log CFU mL^{-1})	6.12	8.34
Bifidobacterium spp. (log CFU mL^{-1})	5.78	7.21

[a] Control: skim milk fermented by probiotics without the addition of the peptides or prebiotics.
[b] Optimum sample: skim milk with 1.12% peptides, 3% FOS, and 1.87% IMO.

The optimal combination of the peptides and prebiotics for the fermented milk was 1.12% peptides, 3% FOS, and 1.87% IMO. Table 5 shows that the two responses (growth rates of *Lactobacillus spp.* and *Bifidobacterium spp.*) and the composite function value derived from the verification experiments are all very close to the SQP-based prediction, with no apparent significant differences ($p>0.05$) comparing the two sets.

Studies of Chen et al. (2004, 2005a) on the viability of probiotics in fermented milk and dairy tofu revealed that peptides and IMO were very efficient growth promoting agents. Fooks et al. (1999) concluded that IMO could stimulate lactic microflora while facilitating elevated production of butyrate, a desirable metabolic compound in the gut. FOS is a non-digestible oligosaccharide, which could increase the numbers of bifidobacteria and reduce the levels of clostridia, fusobacteria and bacterioides (Djouzi and Andrieux, 1998). However, in our study, medium levels of IMO and peptides (in combination of high level of FOS) produced the best result.

Chemical properties and probiotic viabilities in an optimum synbiotic fermented milk sample

A product manufactured according to the formula of the optimum model was analyzed to determine its the chemical and microbial properties (Table 6). When compared with the control group (milk fermented by probiotics without the addition of the peptides or prebiotics), additions of the peptides and prebiotics, resulted in significantly higher cell counts, lactic acid levels and acidity, and proved to be very efficient to improve the viability of both *Lactobacillus spp.* and *Bifidobacterium spp.* Comparing the results of this study with the data available on yogurt (Marsili et al., 1981), the lactic acid level was much lower than in the yogurt, whereas the acetic acid contents were similar.

CONCLUSION

A new synbiotic fermented milk product was developed.

The optimum composition model for the growth promoters was obtained by forming a surface model using the response surface method, and optimizing this model using SQP. The optimal combination of the growth promoters for the synbiotic fermented milk product was 1.12% peptides, 3% FOS, and 1.87% IMO. The optimum model was verified experimentally, showing that the model was effective in improving the viability of both *Lactobacillus spp.* and *Bifidobacterium spp.*.

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