Optimization of the Viability of Probiotics in a Fermented Milk Drink by the Response Surface Method

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ABSTRACT : Growth promoters were added to skim milk to retain the viability of *Lactobacillus acidophilus* and *Bifidobacterium longum* to help the product meet the "therapeutic minimum" at the time of consumption. The experiments were divided into two parts. The first part of the study used chicory inulin, isomalto-oligosaccharides and sucrose to investigate the effects of sugars on the activity of *L. acidophilus* and *B. longum*. The results indicated that the addition of isomalto-oligosaccharides stimulated growth of *L. acidophilus* and *B. longum*, resulting in a higher level of the probiotics after one month storage and yielded better β-galactosidase activity during fermentation. The second part studied the effects of three growth promoters on the viability of the probiotic cultures and the response surface method was employed to find the optimal ratio for addition of the growth promoters. The optimal ratio for added calcium gluconate, sodium gluconate and N-acetylglucosamine in fermented milk drinks were established. The response surface method proved to be a very effective way of optimizing the activity of probiotic cultures when developing a new fermented milk drink. ***(Asian-Aust. J. Anim. Sci. 2004. Vol 17, No. 5 : 705-711)***

Key Words : Probiotics, Response Surface Method, Fermented Milk

INTRODUCTION

Recently, a number of novel fermented dairy products have been developed and are being marketed under the concept of probiotic products. Due to their increasing popularity among consumers. the number of probiotic dairy products on the Taiwan, Japan. Scandinavia. Netherlands. France and United States markets have increased tremendously during the last few years. Several health benefits have been claimed to be associated with the consumption of fermented milk products. Although yogurt microflora (*Streptococcus thermophilus* and *Lactobacillus delbrueckii ssp. bulgaricus*) have been found to be beneficial for human health and nutrition, recent emphasis have focused on development of fermented milk products with added *Lactobacillus acidophilus* and bifidobacteria (known as probiotic or AB-cultures). This is due to the ability of these cultures to tolerate acid and bile. which enables them to implant in intestinal tract. The benefits derived by the consumption of AB products are well documented and have been reviewed by several workers (Kanbe. 1992; Scheinbach, 1998; Ziemer and Gibson. 1998; Lourens-Hattingh and Viljoen. 2001; Liu et al., 2002).

L. acidophilus and bifidobacteria have to retain viability and activity in the food carrier to meet the suggested 'therapeutic minimum' at the time of consumption (Playne, 1994). Several factors have been claimed to affect the viability of probiotic cultures in fermented milk products. The culture conditions. the chemical composition of the fermentation medium (e.g. carbohydrate source), the final acidity. growth promoter and inhibitors. incubation temperature. fermentation time and storage temperature have all been identified as effecting on the stability of probiotic cultures during storage (Hamman and Marth. 1983; Kneifel et al., 1993; Lankaputhra and Shah. 1994; Young and Nelson. 1978).

Bifidobacteria prefer an anaerobic environment and a low redox potential (Klaver et al., 1993). as well as exhibit a weak growth in milk. Therefore. the addition of bifidogenic factors to achieve the desired levels of growth is necessary. The purpose of this study was to study the addition of growth promoters to milk to optimize. by the response surface method (RSM). the activity and viability of the probiotics.

MATERIALS AND METHODS

Preparation of fermented milk drinks

The milk used in the experiments was reconstituted from high heat treated whole milk powder (12% total solids, Anchor. New Zealand). heat treated at 85°C for 30 min. A series of pretests were conducted and the results indicated that milk inoculated with 1% of *L. acidophilus* and 2% of *B.longum* and fermented for 10 h would produce the highest viability of probiotic cultures. Therefore. experimental samples were prepared using skim milk inoculated with 1% of *L. acidophilus* and 2% of *B. longum*. After fermentation for 10 h at 37°C (final pH about 5.75). the samples were then mixed with vegetable juice (V8 100% Vegetable Juice. Campbell Soup Company, NJ. USA) in the ratio of 1:1 to produce a new fermented milk drink (final pH about 5.4).

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Cultures and medium performance

Pure lyophilized cultures of *B. longum* (CCRC 14605) and *L. acidophilus* (CCRC 14079) were purchased from the Culture Collection and Research Center, Taiwan, ROC. *Lactobacilli* MRS (deMan, Rogosa and Sharp) was used as the selective media for *L. acidophilus* and Lithium propionate MRS agar (LP-MRS) was used as the selective media for *B. longum* (Lapierre et al., 1992).

Activity determination

For determination of the viabilities of the probiotic cultures, the populations of *B. longum* and *L. acidophilus* were measured as colony-forming units (CFU) and by the amount of β-galactosidase that was produced by them.

The suitability of the media was tested by plating decimal dilutions of the probiotic cultures. Thus, a sample of 1 g of each pure lyophilized cultures was decimally diluted into sterile peptone water (0.1%) and 0.1 ml aliquot dilutions were then plated onto the different media, in triplicate. 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, Basingstoke, Hampshire, England) to count the number of *B. longum*. The population in colony-forming units (CFU) and the characteristics of the colonies were recorded for each medium.

β-galactosidase activity was measured by determining the rate of hydrolysis of o-nitrophenol-β-galactopyranoside as described by Yu et al. (1987). 1 ml of 15 mM o-nitrophenol-β-galactopyranoside solution prepared in 0.02 M phosphate buffer (pH 7.0) was added to samples, vortexed for 1 s, and incubated for 5 to 20 min at 37°C until a faint yellow tint was observed. The reaction was terminated by the addition of 1 ml of 0.5 M Na_2CO_3. Hydrolysis of this substrate resulted in the release of o-nitrophenol, a highly chromogenic compound that was detected spectrophotometrically at 420 nm. The quantities of o-nitrophenol in samples were determined by use of a standard curve. One unit of enzyme activity released 1 μM of o-nitrophenol/min.

Experiment design

Six growth promoters were used to investe the effects on the activity of *L. acidophilus* and *B. longum*. The sugars are the sweeteners that could affect the taste of the product, so the experiments were divided into two parts. In first part selected chicory inulin, isomalto-oligosaccharides and sucrose were used. The second part studied the effects of three growth promoters on the viability of the probiotics and the response surface method (RSM) was employ to find the optimal ratio for addition of the growth promoters.

Effects of sugars

Three different samples for experiments were created by adding 4% chicory inulin, isomalto-oligosaccharides and sucrose to milk, respectively, and then following the same procedures described in the section of "Preparation of fermented milk drinks". The viability of probiotic cultures were determined by measuring the populations of *B. longum* and *L. acidophilus* as well as β-galactosidase activities on those three samples and one control (without any sugar added). Finally, the storage tests were conducted by holding samples at 5°C for a period of 4 weeks. During the storage period, viable counts of probiotic cultures were determined.

The experiments were repeated three times and the results were analyzed using Analysis of variance (ANOVA) from the SAS software package (SAS Institute 1990), with least-significant-difference (LSD) method as a multiple-comparison technique for significance to detect differences among means (Montgomery, 1991; Kleinbaum et al., 1998).

Central composite design (CCD)

According to Mitsuoka et al. (1987) and our screening test, the viabilities of *L. acidophilus* and *B. longum* were affected by three independent factors: calcium gluconate (0.0-0.5%), sodium gluconate (0.0-1.0%) and N-acetylglucosamine (0.0-1.0%). A Central Composite Design (CCD) with 6 replicates at the centerpoint was used (Chen and Lin, 2002). The CCD is the most frequently used response surface method design and the design descriptions and analyses are done best with coded factors (Myers and Montgomery, 1995). The coding schemes set -1 as the lower level of a factor, +1 as the upper level, and 0 as the middle level. A CCD can be broken down into three groups of design points (Myers and Montgomery, 1995):

Two-level factorial or fractional factorial design : The two-level factorial part of the design consists of all possible combinations of the +1 and -1 levels of the coded factors. For a three-factor case, there are 2^3=8 design points: (-1, -1, -1), (1, -1, -1,), (-1, 1, -1), (-1, -1, 1), (1, 1, -1), (1, -1, 1), (-1, 1, 1) and (1, 1, 1).

Axial (star) points : Axial (star) points have all the factors set to 0, the midpoint, except for one factor set at a value plus or minus alpha (α). For a three-factor problem, the star points are: (-α, 0, 0), (α, 0, 0), (0, α, 0), (0, -α, 0), (0, 0, α) and (0, 0, -α). There are many practical situations in which the scientist specifies ranges on the design factors, and these ranges are strict. In these cases, theα is set to 1 and the design is called the face center cube.

Center points : Data from the center points provides estimates of pure error and estimates of curvature.

For our studies with three factors, the total number of runs is equal to 20, 2^3(two-level factorial)+2×3 (axial

Table 1. Process factors and their levels for the three factors-three levels response surface design

Independent variable	Symbol	Level	
		Coded	Nature
Calcium gluconate conc. (%)	X_1	-1	0.00
		0	0.25
		+1	0.50
Sodium gluconate conc. (%)	X_2	-1	0.00
		0	0.50
		+1	1.00
N-acetylglucosamine conc. (%)	X_3	-1	0.00
		0	0.50
		+1	1.00

points)+6(center points)=20. The coded and uncoded factors. and their respective levels are presented in Table 1.

Response surface method (RSM)

The RSM procedure of the Design-Expert® software package (Stat-Ease. Inc.. USA. 2000) was used to fit the experimental data to polynomial equations of order 1 through 3 to obtain coefficients. The following relationship achieved this.

$$Y_i=f_i(X_1, X_2, X_3)+\varepsilon_i \qquad i=1,2,3 \qquad (1)$$

where Y_1. Y_2 and Y_3 are the observed numbers of *L. acidophilus*, *B. longum* and β-galactosidase activity, respectively. f_1. f_2 and f_3 represent the modeled response surfaces. X_1, X_2 and X_3, defined as natural (uncoded) factors. are the concentrations of N-acetylglucosamine. Ca-gluconate and Na-gluconate. respectively. ε_1. ε_2. ε_3 are the errors in each model. With RSM. it is convenient to transform the natural factors to coded factors ξ_1. ξ_2 and ξ_3. which are defined as dimensionless. with mean zero and the same spread or standard deviation:

$$Y_i=f_i(\xi_1, \xi_2, \xi_3)+\varepsilon_i \qquad i=1,2,3 \qquad (2)$$

An optimization of viability of AB cultures in terms of calcium gluconate. sodium gluconate and N-acetylglucosamine concentrations was calculated using the predictive equation (eq.1).

Verification of model

Five optimal producing conditions were suggested by Design-Expert software (Stat-Ease. Inc.. USA. 2000) and were verified by additional independent experiments. The experimental samples were prepared using skim milk mixed with 4% IMO and the prescribed growth promoters. then following the same procedures described in the section of "Preparation of fermented milk drinks".

Statistical analysis

The models selection was complicated by the sequential model sum of squares and by lack of fit tests from the Design-Expert® software package. The model verification experiments were repeated three times and the results were analyzed using ANOVA from the SAS software package. with least-significant-difference (LSD) method as a multiple-comparison technique for significance to detect differences among means.

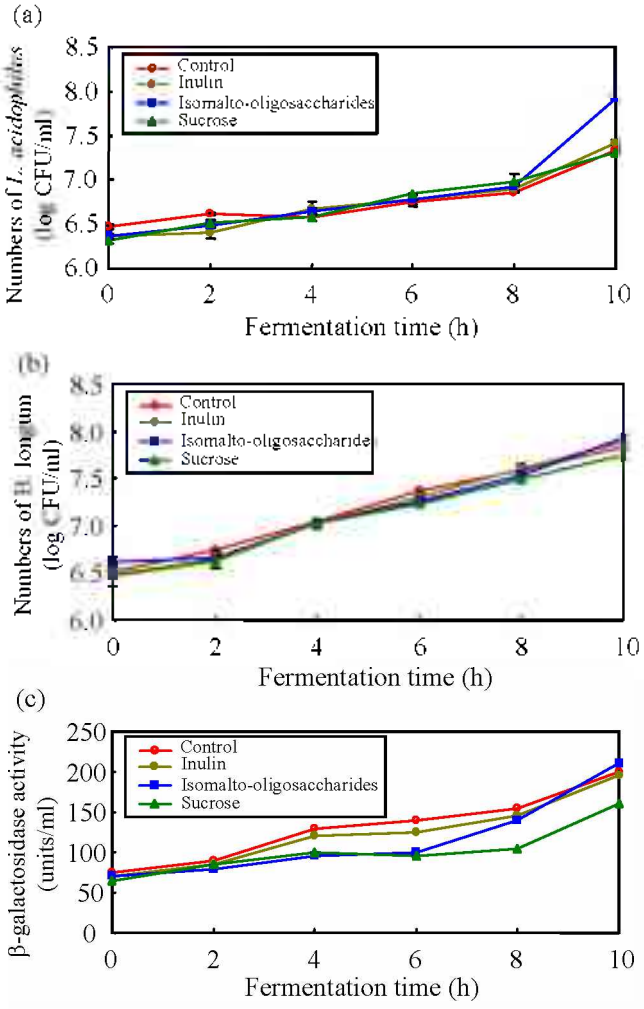

Figure 1. The effects of the addition of sugars on the activity of *L. acidophilus* and *B. longum* in fermented milk drinks during fermentation.

RESULTS AND DISCUSSION

Effect of sugars on the activities of AB cultures

Sugars are not only sweeteners but also used as growth promoters for the probiotics. Chicory inulin, isomalto-oligosaccharides (IMO) and sucrose were selected to investigate the effects of sugars on the activity of *L. acidophilus* and *B. longum*. Among the various sugars tested, supplementation of IMO yielded the highest ($p<0.05$) viable count of *L. acidophilus* (7.92 CFU/ml) after 10 h of fermentation (Figure 1(a)), whereas in case of *B.*

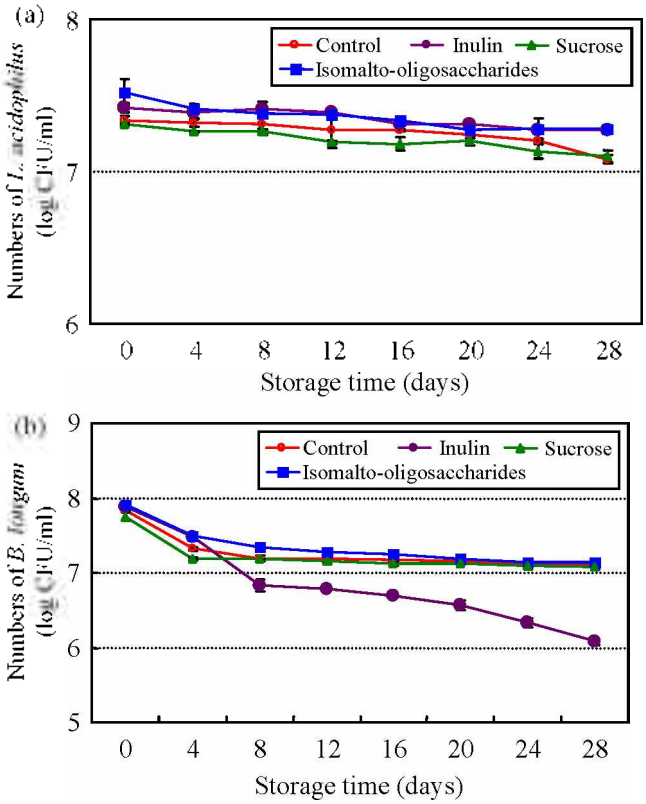

Figure 2. The effects of addition of sugars on the activity of *L. acidophilus* in fermented milk drinks during storage.

longum, a significantly higher ($p<0.05$) viable count was noted in the IMO and chicory inulin supplemented fermented milk than in the sucrose and control (milk without the added supplement). The β-galactosidase activity (Figure 1(c)) was greater ($p<0.05$) in IMO supplemented milk, while the β-galactosidase activity was lower ($p<0.05$) in sucrose supplemented milk at the end of fermentation. Among the sugars tested, IMO was the best ($p<0.05$) growth promoting substance for the AB culture and showed a higher population and β-galactosidase activity than other sugars (Figure 1). IMO has been shown to promote the growth of bifidobacteria (Kohmoto et al., 1988). Fooks et al. (1999) studied probiotics in an *in vitro* model of the human gut and showed that IMO stimulate lactic microflora as well as elevating production of butyrate. Chen et al. (2003) also confirmed that IMO improve the growth rate of probiotics. Sucrose is the most popular sweetener for dairy products. In our studies, 4% sucrose did not improve the growth of AB cultures and even inhibit the enzyme activities during fermentation. Vinderola et al. (2002) studied the influence of sugars associated with fermented dairy products and proved that some strains of bifidobacteria were inhibited by 15 or 20% of sucrose.

During storage, chicory inulin maintained the population of *L. acidophilus* at up to 7.27 log CFU/ml which was the same ($p>0.05$) as the IMO (Figure 2(a)). however inulin decreased ($p<0.05$) the viability of *B. longum* to 6.01 log CFU/ml during storage (Figure 2(b)). This could be explained by the length of polymers and enzyme activity. Chicory inulin is made up of fructose-oligosaccharides (FOS) and contains oligosaccharides with a size of 2-30 degree of polymeration. Marx et al. (2000) studied metabolization of FOS and showed that inulin polymers of molecular mass greater than ca. 4500 were not utilized by bifidobacteria. because hydrolysis of long-chain FOS is inhibited by steric hindrance at the active site of the hydrolytic enzymes. During fermentation. *B. longum* uses short chain FOS as a carbohydrate source for growth and the growth slowed or ceased once the short-chain oligosaccharides were consumed.

The results indicated that adding IMO stimulated growth of *L. acidophilus* and *B. longum*, allowed retention

Table 2. Analysis of variance for the factors as linear, quadratic and cubic terms and their interactions in a response surface model

Source	*L. acidophilus* (log CFU/ml)			*B. longum* (log CFU/ml)			β-galactosidase activity (units/ml)		
	SS[b]	DF[c]	p>F	SS	DF	p>F	SS	DF	p>F
(a) Model analysis[a]									
Mean	937.0	1		1,016.2	1	0.331	1.2×10^{-6}	1	
Linear	6.6×10^{-3}	3	0.002**	4.8×10^{-3}	3	0.007**	2,531.5	3	0.017*
Quadratic	2.2×10^{-3}	6	0.007**	1.4×10^{-3}	6	0.037*	2,168.7	6	0.001**
Cubic	7.9×10^{-3}	4	0.028*	1.5×10^{-3}	4	0.331	59.1	4	0.011*
Residual	1.1×10^{-3}	6		6.0×10^{-3}	6	0.007**	5.0	6	
Total	937.1	20		1,016.5	20	0.137	1.2×10^{-6}	20	
(b) Lack of fit tests[d]									
Linear	30×10^{-3}	11	0.015*	0.160	11	0.015*	2,227.86	11	<0.001**
Quadratic	7.9×10^{-3}	5	0.028*	1.5×10^{-3}	5	0.137	59.13	5	0.112
Cubic	0.0	1		0.0	1		0.00	1	
Total	1.1×10^{-3}	5		6.0×10^{-3}	5		5.00	5	

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

[a] Model analysis-select the highest order polynomial where the additional terms are significant. [b] SS-Sum of squares.

[c] DF-Degree of freedoms. [d] Lack of fit tests-want the selected model to have insignificant lack-of-fit.

Table 3. Analysis of variance showing the significance of the response variables

Independent factors	df	p>F		
		L. acidophilus (log CFU/ml)	*B. longum* (log CFU/ml)	β-galactosidase activity (units/ml)
Calcium gluconate (%)	4	0.0550	0.0134*	0.0048*
Sodium gluconate (%)	4	0.0116*	0.0271*	<0.0001**
N-acetylglucosamine (%)	4	0.0048**	<0.0001**	<0.0001**

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

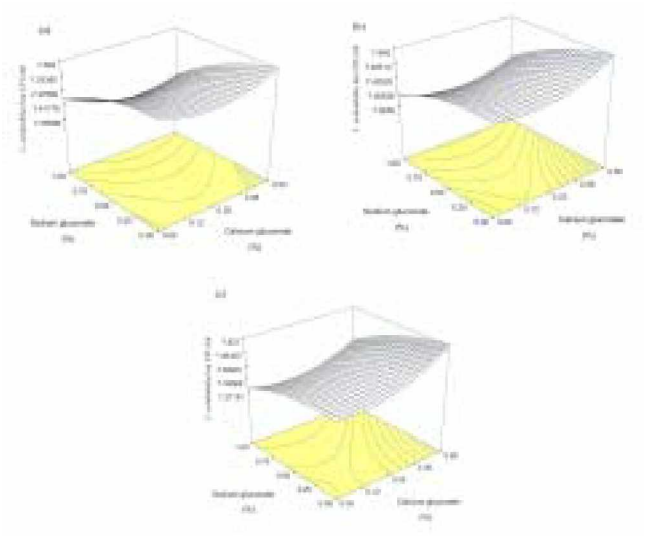

Figure 3. Response surface plots of *L. acidophilus* showing effects of calcium gluconate and sodium gluconate under the conditionsof constant N-acetylglucosamine: (a) N-acetylglucosamine=0.0%, (b) N-acetylglucosamine=0.5%, (c) N-acetylglucosamine=1.0%.

of a higher level of the probiotics during a month storage, and yielded better β-galactosidase activity during fermentation.

Effect of the prebiotics

Fitting the model : Three growth promoters (calcium gluconate, sodium gluconate and N-acetylglucosamine) were mixed with milk in order to improve the activity of *L. acidophilus* and *B. longum*. The responses as linear, quadratic and cubic functions of the factors were tested for adequacy and fitness by analysis of variance. Table 2(a) examines the probability (p>F) to see if it falls below 0.05. The highest order polynomial that is significant is selected. The "Lack of Fit Tests" (Table 2(b)) compares the residual error to the pure error from replicated design points. If there is a significant lack of fit, as indicated by a low probability value (p>F), the response predictor should be discarded. The model with insignificant lack-of-fit is selected. The model analysis results (Table 2(a)) showed that the quadratic models for all three responses that were significant (p<0.05). The lack of fit tests (Table 2(b)) indicated that the quadratic models appeared to be the most accurate for *B. longum* andβ-galactosidase activity, with no significant lack of fit (p>0.05). However, the quadratic models had a significant lack of fit for *L. acidophilus*. The

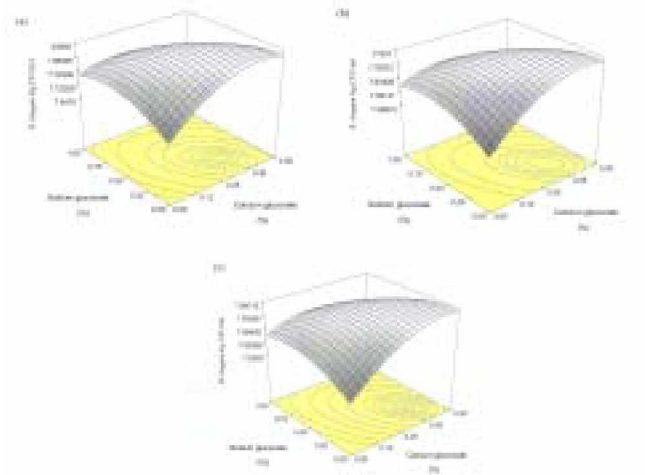

Figure 4. Response surface plots of *B. longum* showing effects of calcium gluconate and sodium gluconate under the conditions of constant N-acetylglucosamine: (a) N-acetylglucosamine=0.0%, (b) N-acetylglucosamine=0.5%, (c) N-acetylglucosamine=1.0%

second order polynomial equations (3) were fitted to the experimental data using the Design Expert procedure:

$$f_k = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{i=1}^{n-1} \sum_{j=i-1}^{n} \beta_{ij} X_i X_j \qquad k=1, 2, 3 \qquad (3)$$

where f_1, f_2 and f_3 are the three responses and β_0, β_i, β_{ii} and β_{ij} are constant coefficients and X_1, X_2 and X_3 are the uncoded independent factors. The equations of prediction for three responses were found as the following:

- *L. acidophilus* :

$f_1 = 7.56\text{-}0.38\ X_1\text{-}(2.000E\text{-}03)\ X_2\text{-}0.24X_3\text{+}0.87X_1^2\text{-}0.12\ X_2^2\text{+}0.058\ X_3^2\text{-}0.16\ X_1X_2\text{+}0.24\ X_1X_3\text{+}0.040\ X_2X_3$

- *B. longum*:

$f_2 = 7.68\text{+}1.24\ X_1\text{+}0.39\ X_2\text{-}0.61\ X_3\text{-}0.56\ X_1^2\text{-}0.30\ X_2^2\text{+}0.45\ X_3^2\text{-}0.70\ X_1X_2\text{+}0.16\ X_1X_3\text{+}0.030\ X_2X_3$

- β-galactosidase:

$f_3 = 269.32\text{+}58.25\ X_1\text{+}26.45\ X_2\text{+}8.28\ X_3\text{-}14.00\ X_1^2\text{-}56.00\ X_2^2\text{-}50.10\ X_3^2\text{-}55.00\ X_1X_2\text{-}10.00\ X_1X_3\text{+}39.10\ X_2X_3$

Table 4. The validation of the optimum producing models of *L. acidophilus* (a), *B. longum* (b) and β-galactosidase activity (c) for fermented milk drinks

No.	Calcium gluconate (%)	Sodium gluconate (%)	N-acetyl-glucosamine (%)	*L. acidophilus* (log CFU/ml)	
				Predicted value	Experimental value
(a)					
1	0.46	0.05	0.01	7.57^{x}	$7.53^{x,b}$
2	0.45	0.06	0.02	7.56^{x}	$7.50^{x,ab}$
3	0.43	0.01	0.02	7.56^{y}	$7.48^{x,a}$
4	0.49	0.12	0.00	7.58^{y}	$7.46^{x,a}$
5	0.47	0.12	0.00	7.57^{x}	$7.59^{x,c}$
(b)					
1	0.46	0.05	0.01	7.91^{x}	$7.98^{x,b}$
2	0.45	0.06	0.02	7.91^{x}	$7.91^{x,a}$
3	0.43	0.01	0.02	7.91^{x}	$7.87^{x,a}$
4	0.49	0.12	0.00	7.91^{x}	$7.88^{x,a}$
5	0.47	0.12	0.00	7.92^{x}	$7.87^{x,a}$
(c)					
1	0.46	0.05	0.01	293.3^{x}	$295.3^{x,a}$
2	0.45	0.06	0.02	292.7^{x}	$294.3^{x,a}$
3	0.43	0.01	0.02	292.0^{x}	$291.0^{x,a}$
4	0.49	0.12	0.00	293.8^{x}	$294.0^{x,a}$
5	0.47	0.12	0.00	292.7^{x}	$295.7^{x,a}$

^{x,y} Values in the same row without a common superscript are significantly different ($p<0.05$).

^{a,b,c} Values in the same column of the same partition without a common superscript are significantly different ($p<0.05$).

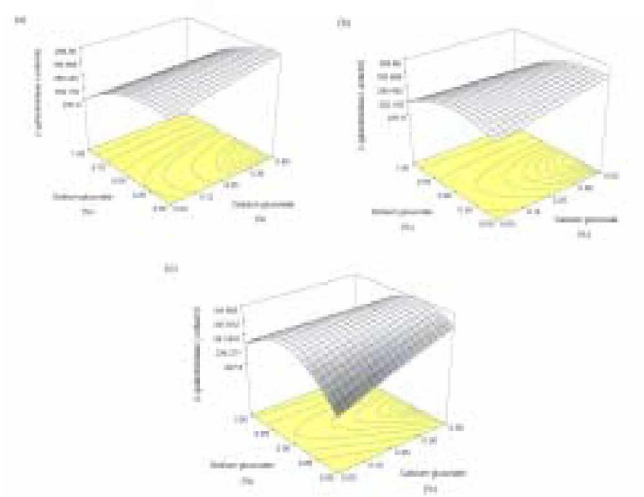

Figure 5. Response surface plots of β-galactosidase showing effects of calcium gluconate and sodium gluconate under conditions of constant N-acetylglucosamine: (a) N-acetylglucosamine=0.0%, (b) N-acetylglucosamine=0.5%, (c) N-acetylglucosamine=1.0%.

Factors affecting viability of *L. acidophilus* and *B. longum*

Estimation of the overall effects of the 3 factors affecting viability of *L. acidophilus* and *B. longum* are shown in Table 3 and indicates that all factors were significant ($p<0.05$). The relationships between the factors and the responses were also investigated by examining a series of 3-D plots generated by holding constant two of the factors of second-order polynomial equation. As Figure 3 shows, the population of *L. acidophilus* increases with increasing calcium gluconate and decreasing sodium gluconate. The maximum population could be obtained with maximum calcium gluconate and minimum sodium gluconate. The optimal values in the plots changed only slightly in relation to the variations in N-acetylglucosamine. A similar result can be observed in Figure 4. The maximum population could be obtained within the range of 0.38-0.50% calcium gluconate and 0-0.25% sodium gluconate. Rising β-galactosidase activity was obtained for increasing calcium gluconate in the range of 0-0.5% (Figure 5). The optimal values in the plots changed negatively with the addition of N-acetylglucosamine. According to the contour plots, most of the diagrams indicate that maximum activity of AB culture can be obtained with maximum calcium gluconate and minimum sodium gluconate. Mitsuoka et al. (1987) suggested the function of gluconic acid and its salts (gluconic acid, glucono-delta-lactone, calcium gluconate) as a bifidobacteria growth promoter and this was confirmed by an *in vitro* test. The current study gives similar results.

Model verification

Five optimal producing conditions were suggested by Design-Expert software and were verified by additional independent experiments (Table 4). Table 4b and c shows that the experimental values were very close ($p>0.05$) to the predicted values for *B. longum* and β-galactosidase activity. However, two out of five samples had significant differences ($p<0.05$) between predicted values and experimental values for the *L. acidophilus*. This could be due to the significant lack of fit of the predicted model for *L. acidophilus*.

CONCLUSION

In the current study, the sugar tests indicated that adding IMO stimulated growth of *L. acidophilus* and *B. longum*, allowed retention of a higher level of the probiotics during a month storage. The response surface method proved to be a very effective way of optimizing the activity of probiotic cultures when developing a new fermented milk drink.

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