Songklanakarin J. Sci. Technol.
31 (5), 481-490, Sep. - Oct. 2009

Songklanakarin Journal of Science and Technology
http://www.sjst.psu.ac.th

Original Article

Optimization of prebiotics in soybean milk using mixture experiments

Phakkhateema Sugkhaphan and Kongkarn Kijroongrojana*

Department of Food Technology, Faculty of Agro-Industry,
Prince of Songkla University, Hat Yai, Songkhla, 90112 Thailand.

Received 25 November 2008; Accepted 17 August 2009

Abstract

A mixture experiment was used to optimize prebiotic mixtures in soybean milk formulation. Inulin (I), galacto-oligosaccharides (GOS), and isomalto-oligosaccharides (IMO) were the prebiotic ingredients added (4% w/v) to soybean milk. Thirteen formulations of soybean milk were compared using the general descriptive analysis and the growth of probiotics (*Bifidobacterium bifidum* DSM 20456, *Lactobacillus plantarum* TISTR 875, and *Lactobacillus acidophilus* TISTR 1034). There were no significant differences ($p>0.05$) in all sensory attributes (color, thickness, beany flavor, sweetness, viscosity, sweetness aftertaste) among the samples. Various mixtures of the prebiotics had only a slight effect on the soybean milk color and viscosity ($p<0.05$). The best formulation was determined by the highest growth of the probiotic bacteria. GOS was the prebiotic that stimulated the highest growth in all probiotic strains as shown by its highest growth coefficient. The optimized formulation (0.11I + 0.62GOS + 0.27IMO), after fermentation with the probiotics, stimulated the growth of *B. bifidum* DSM 20456 from 6.58 to 7.54 $\log_{10}$ CFU/ml (after 48 hrs), *L. plantarum* TISTR 875 from 6.59 to 8.65 $\log_{10}$ CFU/ml (after 24 hrs), and *L. acidophilus* TISTR 1034 from 6.83 to 8.44 $\log_{10}$ CFU/ml (after 24 hrs) and was not different when compared with glucose ($p>0.05$). The soybean milk supplemented with the optimized prebiotic mixture had higher ($p<0.05$) carbohydrates, total soluble solid, total solid content, and viscosity than the control (without prebiotic). However, it had a lower L^* value (lightness) and a higher a^* value (redness) than the control ($p<0.05$).

Keywords: prebiotic, soybean milk, inulin, galacto-oligosaccharides, isomalto-oligosaccharides

1. Introduction

In recent years, the market for probiotics and prebiotics as functional foods and dietary supplements is positioned for growth due to increased consumer awareness of the relationship between diet and health. Prebiotics and/or probiotics beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria (healthy bacteria), while limiting the ability of harmful bacteria to multiply and thrive in the colon, thus improving the host's health (Tuohy *et al.*, 2003). 'Optimal' gut microflora can improve the intestinal microbial balance, increase the resistance to pathogenic bacteria, stimulate the immune response, reduce the risk of cancer, improve calcium absorption, and alleviate lactose intolerance (Holzapfel and Schillinger, 2002; Manning and Gibson, 2004). However, as we age, and when we either encounter stress or ingest antibiotics, the populations of these good bacteria tend to diminish inside the body, leaving us more vulnerable to gastrointestinal disorders and diseases (Rice, 2002). Above the age of 55, faecal bifidobacterial counts have been shown to remarkably decrease compared to those in younger people, resulting in a diminished ability to resist colonization by invading pathogens. Prebiotics may be utilized as a dietary intervention to restore the gut microflora balance in elderly population, thus indirectly providing them with antipathogenic protection (Manning and Gibson, 2004). Manning and Gibson (2004) indicated that at least 4 g/days, but preferably 8 g/days, of fructo-oligosaccharides (FOS) would be needed to significantly elevate bifidobacteria (ca. one $\log_{10}$) in

*Corresponding author.
Email address: kongkarn.k@psu.ac.th

human gut.

The range of foods, which prebiotics can be added to, is much wider than probiotics, where culture viability needs to be maintained. This has the advantage that heat stability, or exposure to oxygen is not an issue (Manning and Gibson, 2004). One of the interesting foods for this purpose is soybean milk, because soybeans and soy foods are particularly abundant sources of isoflavones, which have anti-carcinogenic effects on hormone-related diseases such as cancer. Moreover, they can also alleviate menopausal symptoms and osteoporosis, and suppress the onset of arteriosclerosis by improving the metabolism of lipids such as cholesterol (Prabhakaran *et al.*, 2006), thus reducing the risk of coronary heart disease (Food and Drug Administration, 1999). Due to the purported beneficial effects of both prebiotic and isoflavone, it may be desirable to develop a product supplemented with prebiotics for the benefit of human health. Thus, the objective of this study was to develop soybean milk supplemented with optimum combination of three types of prebiotics, i.e. inulin, galacto-oligosaccharides, and isomalto-oligosaccharides. Sensory properties and growth of probiotics were used as criteria in optimizing the prebiotic mixture.

2. Materials and Methods

2.1 Ingredients and chemicals

Soybeans (Chiang Mai 60) obtained from Chiang Mai Field Crops Research Center, Department of Agriculture, Chiang Mai, Thailand, and were used for all the experiments. The basic ingredients of soybean milk (sugar and salt) were purchased from a local supermarket. Food grade chemicals, e.g. sodium bicarbonate, tri-calcium phosphate, potassium citrate, and κ-carrageenan were obtained from Union Chemical 1986 Co., Ltd, Thailand. The commercial prebiotics used were Inulin (90% purity, Frutafit® HD, Sensus, Roosendaal, The Netherlands), Galacto-oligosaccharides (55% purity, Oligomate55, Yakult, Tokyo, Japan), and Isomalto-oligosaccharides (50% purity, Biotose50, Nihon Shokuhin Kako Co., Ltd, Tokyo, Japan).

2.2 Experimental design

The augmented simplex-centroid design for mixtures of three components with three point replications (Table 1) was employed, with concentration of inulin (I), galacto-oligosaccharides (GOS) and isomalto-oligosaccharides (IMO) as variables. The maximum level (component proportion = 1) of each variable was 4 % (w/w based on total formulation). The response variables were the sensory attributes selected by trained panel to describe the soybean milk characteristics and the growth of probiotics (comparing the *in vitro* fermentation of prebiotic oligosaccharides by probiotics bacteria). The models fitted for the growth of probiotics cultures were used to optimize the prebiotics proportion using software Design Expert version 7.0.3 (Stat-Ease, Inc., Minneapolis,

Table 1. Augmented simplex-centroid design for prebiotic mixture and physical properties of various prebiotics supplemented soybean milks.

Treatment	Proportion of prebiotics**			Total soluble solid (°Brix)	Mean				
	I	GOS	IMO		Total solid content (%)	Viscosity (cPs)	Color CIE		
							L* value	a* value	b* value
P1	1	0	0	17	16.04[a]	20.70[a]	83.83[c]	1.02[e]	17.28[a]
P2	0	1	0	17	15.22[c]	19.87[c]	84.09[b]	3.15[a]	15.37[d]
*P3	1	0	0	17	16.01[a]	20.63[a]	83.89[c]	1.06[e]	17.26[a]
P4	0.17	0.67	0.17	17	15.22[c]	18.40[d]	84.54[a]	3.15[a]	15.31[d]
P5	0.17	0.17	0.67	15	13.59[e]	18.27[d]	83.94[c]	3.02[b]	15.58[c]
*P6	0	1	0	17	15.27[c]	19.97[c]	83.62[d]	3.16[a]	15.39[d]
P7	0.5	0.5	0	16	15.21[c]	19.83[c]	83.68[d]	2.98[bc]	17.19[a]
P8	0.5	0	0.5	15	14.45[d]	18.43[d]	83.81[c]	3.00[bc]	16.32[b]
P9	0.33	0.33	0.33	15	14.46[d]	18.39[d]	83.93[c]	2.89[d]	15.58[c]
P10	0	0	1	15	13.55[e]	17.27[e]	84.17[b]	2.92[cd]	16.28[b]
*P11	0	0	1	15	13.54[e]	17.10[e]	84.12[b]	2.98[bc]	16.27[b]
P12	0.67	0.17	0.17	17	15.89[b]	20.17[b]	84.55[a]	2.88[d]	15.31[d]
P13	0	0.5	0.5	16	13.59[e]	17.17[e]	83.92[c]	1.05[e]	16.29[b]

I = inulin, GOS = galacto-oligosaccharides, IMO = isomalto-oligosaccharides.

* The replicated design point.

** Code values I + GOS + IMO = 1.

[a-e] The same letters under the same column indicate no significant differences ($p>0.05$).

MN, USA).

2.3 Preparation of soybean milk

Soybean seeds were washed and soaked in 0.5% (w/v) sodium bicarbonate solution with soybean to solution ratio of 1:3 at 4°C for 16-18 hrs. The hydrated beans were drained, rinsed, and blended with warm water (50-60°C), using a blender (Moulinex, Model AY46, La Defense, France). The ratio of dry soybeans to water was 7:1 (w/v). The soybean slurry was filtered through 4 layers of cheesecloth, 60 mesh and 100 mesh sieve, respectively, to obtain raw soybean milk. The raw soybean milk was boiled and stirred for 20 min. After the soybean milk was boiled for 10 min, 0.3% potassium citrate was added. After 13 min boiling, 0.1% tri-calcium phosphate and 0.05% salt were added, and after 15 min boiling, 5% sugar and 0.015% κ-carrageenan were added. The boiled soybean milk was placed in a container and cooled down rapidly to 5°C or below in an ice-water bath and the pasteurized soybean milk was then stored in a refrigerator (4°C). For the prebiotics supplemented soybean milk formulations, 4% (w/v) of prebiotic mixture (based on purity) were added after the addition of sugar and κ-carrageenan.

2.4 Sensory evaluation

The selection and training of sensory panel members were performed according to International Standard ISO 8586-1 (1993). Panelist members between 23-30 years old were recruited from graduate students at the Faculty of Agro-Industry, Prince of Songkla University. The panelists underwent 6 sessions of training, 2 hrs in each session. The 18-member trained sensory panel was then used to evaluate 13 samples using general descriptive analysis. The intensity scale of each attribute was agreed on a 15 cm line, with 1.25 cm point as "weak" and 13.75 cm point as "strong". The panelists tested the control (soybean milk without prebiotics) and soybean milk supplemented with 4% inulin in order to select the attributes that describe the soybean milk characteristics. The samples were presented to the panelists according to the "balance order and carry-over effects design" (Macfie et al., 1989), over three sessions of 4 or 5 products each. The sensory testing was performed in replicate.

2.5 Bacterial strains and batch culture fermentations

Pure cultures of probiotic bacteria *Lactobacillus acidophilus* TISTR 1034 and *Lactobacillus plantarum* TISTR 875 were obtained from Thailand Institute of Scientific and Technological Research (TISTR), Bangkok Microbiological Resources Centre; Bangkok MIRCEN, Thailand. *Bifidobacterium bifidum* DSM 20456 were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Germany. The bacteria were grown in 10 ml of De Man Rogosa Sharpe (MRS) broth (Himedia, Mumbai, India) under anaerobic conditions at 37°C for 24 hrs. The 1 ml of each culture (1×10^6 CFU per ml) was transferred to a minimal medium (2 g/l peptone water, 2 g/l yeast extract, 0.1 g/l NaCl, 0.04 g/l K_2HPO_4, 0.04 g/l KH_2PO_4, 0.01 g/l $CaCl_2$. $6H_2O$, 0.01 g/l $MgSO_4.7H_20$, 2 g/l $NaHCO_3$, 2 ml/l Tween 80, 0.5 g/l cysteine–HCl, 0.5 g/l bile salt, 0.02 g/l hemin, 10 ml vitamin K_1) to which was added 1% (w/v) of prebiotic mixture (I, GOS, IMO) as the carbon source according to the experimental design (Table 1). As control, glucose was used for growth instead of the prebiotics. All cultures were incubated at 37°C for 72 hrs under anaerobic conditions in screw cap tubes (sparged with N_2) containing 0.05 % (w/v) cysteine-HCl (substrate reducing) and rezasurin (indicator). The growth of each strain was monitored by measuring the optical density (OD) of the cultures at 660 nm using microplate reader after 0, 6, 12, 24, 36, 48 and 72 hrs of fermentation. The prebiotics combination that stimulated the highest growth of probiotics was selected.

2.6 Growth of probiotics cultured in the optimized prebiotics

To validate the predicted growth values, three optimized prebiotics proportions based on the maximum growth of probiotics (Table 6) were used to culture the probiotics. The pure cultures of probiotic bacteria were grown under the same conditions as described before. The viable colonies of each strain after 0, 6, 12, 24, 36, 48, and 72 hrs fermentation, using MRS agar (Himedia, Mumbai, India), were enumerated. *Lactobacillus acidophilus* TISTR 1034 and *Lactobacillus plantarum* TISTR 875 were spread plated on MRS agar and *Bifidobacterium bifidum* DSM 20456 was pour plated on MRS agar. The plates were incubated at 37°C for 48 hrs under anaerobic conditions.

2.7 Chemical and physical analysis

The control soybean milk (5% sugar) and soybean milk (2.6% sugar) supplemented with optimized prebiotic formulation were analyzed for proximate composition including crude protein, crude fat, ash, carbohydrate, and moisture according to the method of A.O.A.C. (2000). Briefly, protein content was determined by the Kjeldahl method using a conversion factor of 6.25. Lipid was analyzed using the soxhlet extraction; the soybean milk samples were freeze dried before fat analysis. Ash content was determined using a furnace. Moisture content was measured using a hot air oven. Carbohydrates content was determined by subtracting the moisture, protein, fat and ash percentages from 100%. Total soluble solid was determined by using an Atago Automatic Temperature Compensation refractometer to measure degree Brix (Model ATC-1, Tokyo, Japan). Total solid content was determined according to procedures described by Priepke *et al.* (1980). Viscosity of soybean milk was measured using a Brookfield Viscometer (Model RVDII$^+$; Brookfield Engineering Laboratories, Middleborg, MA, USA) with spindle No.1, speed 100 rpm and temperature of

45°C. The color of soybean milk, the CIE L^*, a^*, b^* values, was determined by Hunter Lab (ColourFlex, Hunter Associates Laboratory Inc., Reston, VA, USA).

2.8 Statistical analysis

The SPSS package (SPSS 11.0 for windows, SPSS Inc, Chicago, IL, USA) was used for analysis of the variance. Duncan's Multiple range was used to determine significant differences among means. Analysis of variance for regression and mathematical model was performed using software Design Expert version 7.0.3 (Stat-Ease, Inc., Minneapolis, MN, USA). Significance of differences was defined at $p< 0.05$.

3. Results and Discussion

3.1 Sensory and physical properties of soybean milks

The physical properties of all samples are shown in Table 1. The results indicated that an increase in the proportion of inulin in the formulation P1 and P3 resulted in a significant increase in soybean milk viscosity and total solid content ($p<0.05$). Different types of prebiotics used in this study contained different degrees of polymerization (DP). The higher DP of inulin (Frutafit®HD, average DP of 8-13) resulted in higher viscosity when compared to GOS (Oligomate55, average DP of 2) and IMO (Biotose50, average DP of 2-3) (according to manufacturer's information). Tomas *et al.* (2008) reported that the addition of long chain inulin (>23 monomers) produced a significant increase in complex viscosity (η^*) of whole and skimmed milk, in comparison with native inulin (9-12 monomers) and short chain inulin (7-9 monomers). The L^*, a^* and b^* values of soybean milk color prepared from various prebiotic proportions were significantly different ($p<0.05$).

The list of attributes selected by the trained panel to describe soybean milk characteristics and their definitions are shown in Table 2. These attributes were analyzed in the general descriptive analysis and the average scores obtained for the different samples are presented in Table 3. Various mixtures of prebiotics had no effect on all sensory attributes; therefore it was not possible to apply a predictive model. This was due to the fact that the relative sweetness of I, GOS and IMO was about in the same range, i.e. 30-60%, than sucrose (Belitz *et al.*, 2004). Even though the physical properties of all samples might be different, but these differences could not be detected by the trained panelists.

3.2 Selection of prebiotics formulation by *in vitro* probiotic fermentation

The effect of various prebiotic mixtures were studied using mixture experiments with the aim of modeling and optimizing the growth of *B. bifidum* DSM 20456, *L. plantarum* TISTR 875 and *L. acidophilus* TISTR 1034. The results of the growth of probiotic bacteria in the batch culture fermentation are shown in Table 4. Although the batch culture fermentation was carried out for 72 hrs, the growth reached the stationary phase after 48 hrs for *B. bifidum* DSM 20456, and after 24 hrs for *L. plantarum* TISTR 875 and *L. acidophilus* TISTR 1034 (data not shown). In theory, the cell densities of the enteric strains grown on the prebiotics should be very low relative to the growth on glucose. For a sugar having prebiotic activity, it should be meta-bolized by a test strain as well, or nearly as well, as glucose (Huebner *et al.*, 2007). The results showed that the increase in cell density of all strains cultured with most of high GOS proportion treatments (P2, P4, P6, and P13) were significantly higher ($p<0.05$) when compared with that of higher IMO or I pro-

Table 2. Descriptors developed for general descriptive analysis of soybean milk.

Descriptor term	Definition	Reference
Appearance		
Color (white-yellow)	Color aspect of soybean milk	White, cream and yellow color
Thickness (thin-thick)	How thick a drink looks when gently swirling cup	Soybean milk added with 0%, 0.015% and 0.035% carrageenan
Flavor		
Beany flavor (weak-strong)	Beany characteristic flavor sensed before swallowing	Unheated soybean milk
Sweetness (weak-strong)	Perception of sweetness, associated with the presence of sugars	Soybean milk added with 5% and 7.5% sucrose
Texture		
Viscosity (slightly-very)	Degree of thickness of the drink during drinking of soybean milk	Soybean milk added with 0%, 0.015% and 0.035% carrageenan
Aftertaste		
Sweetness (weak-strong)	Perception of sweetness remains in the mouth after swallowing the soybean milk	Soybean milk added with 5% and 7.5% sucrose

Table 3. Average scores of soybean milks supplemented with various prebiotic mixtures, using 15 cm scale (1.25 = weak, 13.75 = strong).

Treatment	Appearance		Flavor		Texture	Aftertaste
	Color	Thickness	Beany flavor	Sweetness	Viscosity	Sweetness
P1	6.03[a]	4.92[a]	4.63[a]	5.49[a]	4.92[a]	5.58[a]
P2	6.78[a]	4.35[a]	4.14[a]	6.26[a]	4.95[a]	5.92[a]
*P3	7.09[a]	5.22[a]	4.77[a]	4.94[a]	4.83[a]	4.93[a]
P4	6.72[a]	5.29[a]	4.08[a]	5.11[a]	4.75[a]	5.06[a]
P5	6.70[a]	4.76[a]	4.76[a]	5.22[a]	4.53[a]	5.49[a]
*P6	6.09[a]	4.98[a]	4.07[a]	5.34[a]	4.63[a]	5.36[a]
P7	6.63[a]	5.24[a]	4.66[a]	5.41[a]	5.11[a]	5.50[a]
P8	6.86[a]	5.05[a]	4.23[a]	5.11[a]	4.72[a]	5.06[a]
P9	6.47[a]	4.90[a]	3.96[a]	5.13[a]	4.56[a]	5.16[a]
P10	6.80[a]	5.00[a]	4.52[a]	5.58[a]	4.92[a]	5.40[a]
*P11	6.44[a]	4.57[a]	3.76[a]	5.74[a]	4.19[a]	5.27[a]
P12	6.47[a]	5.11[a]	4.22[a]	5.45[a]	4.33[a]	5.16[a]
P13	6.71[a]	4.85[a]	4.08[a]	5.60[a]	4.81[a]	5.53[a]

* P3, P6 and P11 were replicated design point of P1, P2 and P10, respectively.
[a] Same letters under the same column indicate no significant differences ($p>0.05$).

Table 4. The effect of prebiotics on the growth of probiotic bacteria in the batch culture fermentations. Values are mean optical density ± S.D. for *Bifidobacterium bifidum* DSM 20456 after 48 hrs fermentation, *Lactobacillus plantarum* TISTR 875 and *Lactobacillus acidophilus* TISTR 1034 after 24 hrs fermentation.

Treatment	*B. bifidum* DSM 20456	*L. plantarum* TISTR 875	*L. acidophilus* TISTR 1034
P1	0.06 ± 0.00^{f}	0.17 ± 0.01^{h}	0.41 ± 0.01^{e}
P2	0.27 ± 0.02^{b}	0.30 ± 0.01^{cd}	0.74 ± 0.05^{ab}
*P3	0.06 ± 0.00^{f}	0.17 ± 0.01^{h}	0.41 ± 0.01^{e}
P4	0.26 ± 0.03^{bcd}	0.31 ± 0.01^{bc}	0.78 ± 0.03^{a}
P5	0.24 ± 0.01^{de}	0.29 ± 0.01^{de}	0.71 ± 0.08^{bc}
*P6	0.27 ± 0.02^{b}	0.30 ± 0.01^{cd}	0.74 ± 0.05^{ab}
P7	0.25 ± 0.01^{bcde}	0.27 ± 0.01^{fg}	0.67 ± 0.03^{cd}
P8	0.25 ± 0.01^{cde}	0.25 ± 0.00^{g}	0.62 ± 0.03^{d}
P9	0.26 ± 0.00^{bcd}	0.28 ± 0.01^{ef}	0.73 ± 0.02^{abc}
P10	0.24 ± 0.00^{cde}	0.30 ± 0.01^{cd}	0.72 ± 0.03^{abc}
*P11	0.24 ± 0.00^{cde}	0.30 ± 0.01^{cd}	0.72 ± 0.03^{abc}
P12	0.23 ± 0.01^{e}	0.27 ± 0.01^{f}	0.64 ± 0.04^{d}
P13	0.26 ± 0.01^{bc}	0.32 ± 0.01^{b}	0.75 ± 0.01^{ab}
Glucose	0.30 ± 0.02^{a}	0.34 ± 0.00^{a}	0.71 ± 0.02^{bc}

* P3, P6 and P11 were replicated design point of P1, P2 and P10, respectively.
[a-h] The same letters under the same column indicate no significant differences ($p>0.05$).

portion treatments. In addition, the growth of the bacteria cultured with the formulations containing only inulin (P1 and P3) were significantly lower than that with GOS, IMO, and glucose ($p<0.05$).

Table 5 presents the equations, coefficients, probability of models, and lack of fit of determination of models obtained from the growth of probiotic bacteria. All of the models were quadratic equations; they were significant, which was evident from the probability values ($p<0.05$); the lack of fit was not significant ($p>0.05$). The coefficients of determination (R^2) varied between 0.95 and 0.98, explaining 95-98% of variability in response. This indicated a high precision and reliability of the models. The contour graph of *B. bifidum* DSM 20456, *L. plantarum* TISTR 875, and *L. acidophilus* TISTR 1034 are shown in Figure 1. GOS was the most important variable for *B. bifidum* DSM 20456, *L. plantarum* TISTR 875, and *L. acidophilus* TISTR 1034 growth as shown by the highest coefficient in Table 5, while I had the lowest effect (low coefficient) for all probiotic strains. In general, galactose-containing oligosaccharides, namely lactulose, GOS and SOS, were ostensibly more effective than the fructose-containing inulin and FOS in terms of increasing numbers of bifidobacteria (Rycroft *et al.*, 2001). Rycroft *et al.* (2001) found that IMO and GOS were effective at increasing numbers of bifidobacteria, but inulin gave a significantly smaller increase in the numbers of bifidobacteria. Prebiotics with different degrees of polymerization exhibit differences in the capacity to stimulate bacterial growth, and the ability to metabolize these prebiotics (Banuelos *et al.*, 2008). Most current prebiotics, except inulin, are of relatively small DP. It is thought that the oligosaccharides must be hydrolyzed by cell-associated bacterial glycosidases prior to uptake of the resultant monosaccharide. It is, therefore, reasonable to assume that the longer the oligosaccharides the slower the fermentation take place. Hence the further

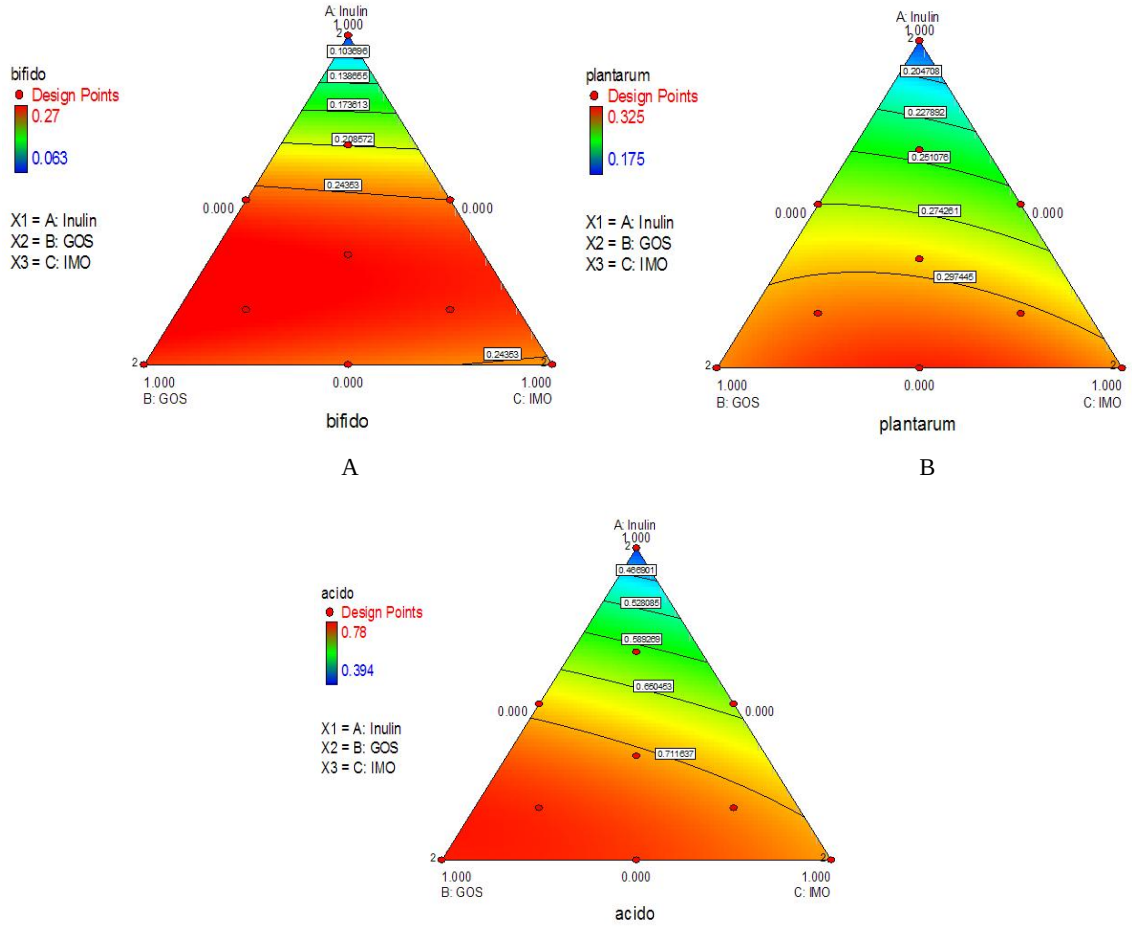

Figure 1. Contour plot for *Bifidobacterium bifidum* DSM 20456 (A), *Lactobacillus plantarum* TISTR 875 (B) and *Lactobacillus acidophilus* TISTR 1034 (C) growth in the batch culture fermentation supplemented with different mixtures of inulin (I), galacto-oligosaccharides (GOS) and isomalto-oligosaccharides (IMO).

Table 5. Predictive and regression models and goodness-of-fit obtained from the growth of probiotic bacteria.

Parameter	Regression models	R^2	p^*	Lack of fit (p)
Bb	$Y = 0.069I + 0.26GOS + 0.24IMO + 0.35I \times GOS + 0.36I \times IMO - 1.65\acute{}10^{-3}GOS \times IMO$	0.97	<0.0001	0.0593
Lp	$Y = 0.18I + 0.31GOS + 0.30IMO + 0.12I \times GOS + 0.064I \times IMO + 0.066GOS \times IMO$	0.95	<0.0002	0.0614
La	$Y = 0.41I + 0.77GOS + 0.72IMO + 0.43I \times GOS + 0.29I \times IMO + 0.056GOS \times IMO$	0.98	<0.0001	0.4430

Bb = *Bifidobacterium bifidum* DSM 20456, Lp = *Lactobacillus plantarum* TISTR 875, La = *Lactobacillus acidophilus* TISTR 1034.
I = inulin, GOS = galacto-oligosaccharides, IMO = isomalto-oligosaccharides.
p^* = Probability level.

prebiotic effect will be obtained more effectively throughout the colon (Gibson, 2004). In this study, the preference towards shorter oligosaccharides in cultures with GOS and IMO was observed in all probiotic strains. Shorter chains oligosaccharides were the first to be consumed by bifidobacteria, while predominantly long-chain structures of inulin ensure longer fermentation time in the colon (Aryana and McGrew, 2007). Roberfroid *et al.* (1998) reported that during *in vitro* fermentation of inulin by human fecal bacteria molecules with DP > 10 were fermented on the average half

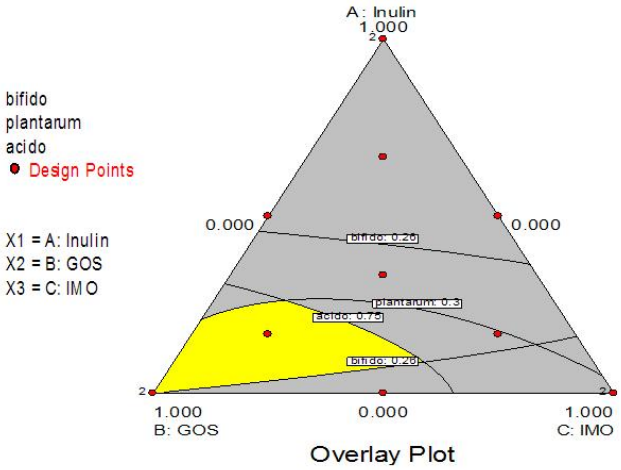

Figure 2. Over lay plot of optimum region (yellow shade) that contains 0-0.26%I, 0.36-1%GOS and 0-0.54%IMO which set the limit of optical density of *B. bifidum* DSM 20456, *L. plantarum* TISTR 875 and *L. acidophilus* TISTR 1034 at least 0.26, 0.3 and 0.75, respectively.

as quickly as molecules with DP < 10. Van der Meulen *et al.* (2004) has shown that *Bifidobacterium animalis* DN-173 010 was unable to grow on a medium with glucose or large fructan polymers (DP > 20) as the carbon source, while the fermentations with oligofructose (Raftilose P95) and a mixture of inulin and oligofructose (Raftilose Synergy1) showed changes in both growth and metabolite production due to the preferential metabolism of shorter fructan fractions over the longer chains.

The limits of *B. bifidum* DSM 20456, *L. plantarum* TISTR 875, and *L. acidophilus* TISTR 1034 growth were set at least 0.26, 0.3 and 0.75, respectively. This is based on the maximum growth of each probiotic. To obtain the optimum region, contour plots with limits of probiotic growth were superimposed. The optimum region (shade region, Figure 2.) contains 0-0.26% I, 0.36-1% GOS, and 0-0.54% IMO. Three optimized formulations predicted by the models with high desirability (0.938-0.952) were obtained from software calculations (Table 6). To confirm the predictions, the probiotic strains were cultured with three optimized formulations in the batch culture fermentation. The results shown in Table 6 demonstrated that the growth of *B. bifidum* DSM 20456, *L. plantarum* TISTR 875, and *L. acidophilus* TISTR 1034 was not significantly different ($p>0.05$). Due to higher costs of GOS, the formulation with the lowest amount of GOS was chosen (0.11I + 0.62GOS + 0.27IMO).

3.3 Growth of probiotic cultures as affected by the optimized prebiotics formulation

The viable count of *B. bifidum* DSM 20456, *L. plantarum* TISTR 875, and *L. acidophilus* TISTR 1034 in the optimized prebiotics formulation is shown in Table 7; glucose was used as control. The increase in viable count of all strains in the optimized prebiotics formulation and in the control were not significantly different ($p<0.05$) when all strains reached the stationary phase (after 48 hrs fermentation for *B. bifidum* DSM 20456, after 24 hrs fermentation for *L. plantarum* TISTR 875 and *L. acidophilus* TISTR 1034). The optimized prebiotic formulation stimulated the growth of *B. bifidum* DSM 20456 from 6.58 to 7.54 $\log_{10}$ CFU/ml (after 48 hrs fermentation), *L. plantarum* TISTR 875 from 6.59 to 8.65 $\log_{10}$ CFU/ml (after 24 hrs fermentation), and *L. acidophilus* TISTR 1034 from 6.83 to 8.44 $\log_{10}$ CFU/ml (after 24 hrs fermentation). The increases in *B. bifidum* DSM 20456, *L. plantarum* TISTR 875, and *L. acidophilus* TISTR 1034 populations were log 0.96, log 2.06, and log 1.61, respectively. The results showed that *L. plantarum* TISTR 875 grew better and faster than *L. acidophilus* TISTR 1034, followed by *B. bifidum* DSM 20456. The lowest growth of *B. bifidum* DSM 20456, suggested that the sensitivity to oxygen is one of the factors, which may cause the death of bifidobacteria (Shimamura *et al.*, 1992). Different growth of probiotics may be associated with the substrates, their enzymes and specificities. The activities of the enzymes correlated with carbohydrate catabolism. In addition, the structures that influence carbohydrate digestion (Lunn and Buttriss, 2007) and the physiochemical properties of the different fibres are fermentability. Fibre particle size and degree of related with solubility have a considerable effect on the susceptibility of fibres to bacterial fermentation since they govern the surface area exposed to bacterial degradation (Gibson, 2004).

Table 6. Optical density of probiotic bacteria cultured with three optimized prebiotics formulations and glucose. Values are mean optical density ± S.D. for *Bifidobacterium bifidum* DSM 20456 after 48 hrs fermentation and *Lactobacillus plantarum* TISTR 875 and *Lactobacillus acidophilus* TISTR 1034 after 24 hrs fermentation.

Formulation	*B. bifidum*DSM 20456	*L. plantarum*TISTR 875	*L. acidophilus* TISTR 1034
S1 (0.11I + 0.62GOS + 0.27IMO)	0.28 ± 0.00^{b}	0.31 ± 0.00^{b}	0.38 ± 0.01^{b}
S2 (0.00I + 0.64GOS + 0.36IMO)	0.28 ± 0.00^{b}	0.32 ± 0.01^{b}	0.39 ± 0.01^{b}
S3 (0.07I + 0.93GOS + 0.00IMO)	0.28 ± 0.00^{b}	0.32 ± 0.01^{b}	0.39 ± 0.00^{b}
Glucose	0.29 ± 0.00^{a}	0.34 ± 0.01^{a}	0.43 ± 0.01^{a}

[a-b] The same letters under the same column indicate no significant differences ($p>0.05$).

Table 7. Growth of probiotic bacteria in the batch culture fermentation supplemented with the optimized prebiotics formulation. Values are mean $\log_{10}$ CFU/ml ± S.D. for *Bifidobacterium bifidum* DSM 20456 at 48 hrs fermentation and *Lactobacillus plantarum* TISTR 875 and *Lactobacillus acidophilus* TISTR 1034 at 24 hrs fermentation.

Formulation	*B. bifidum* DSM 20456		*L. plantarum* TISTR 875		*L. acidophilus* TISTR 1034	
	0 h	48 h	0 h	24 h	0 h	24 h
Optimized*	6.58 ± 0.09[a]	7.54 ± 0.07[a]	6.59 ± 0.14[a]	8.65 ± 0.07[a]	6.83 ± 0.03[a]	8.44 ± 0.04[a]
Glucose	6.63 ± 0.03[a]	7.75 ± 0.10[a]	6.63 ± 0.09[a]	8.67 ± 0.07[a]	6.82 ± 0.03[a]	8.67 ± 0.06[a]

* Optimized prebiotics formulations (0.11I + 0.62GOS + 0.27IMO).
[a] The same letters under the same column indicate no significant differences ($p>0.05$).

Table 8. Chemical and physical properties of control and prebiotic supplemented soybean milk (per 100 g). Values are mean ± SD of triplicate determinations.

	Control soybean milk	Prebiotic supplemented soybean milk
Protein (N×6.25) (%)	3.18 ± 0.03[a]	2.72 ± 0.02[b]
Fat (%)	1.52 ± 0.02[a]	1.22 ± 0.07[b]
Carbohydrates (%)	7.83	12.04
Ash (%)	0.73 ± 0.03[a]	0.67 ± 0.04[a]
Total soluble solid (°Brix)	13	17
Total solid content (%)	13.26 ± 0.01[b]	16.65 ± 0.01[a]
Viscosity (cP)	15.17 ± 0.06[b]	17.37 ± 0.06[a]
Color CIE L^* value	86.88 ± 0.01[a]	85.05 ± 0.07[b]
a^* value	0.91 ± 0.05[b]	1.98 ± 0.08[a]
b^* value	16.82 ± 0.02[a]	16.90 ± 0.05[a]
Isoflavone* (mg/100ml)	13.26	16.65

* Total isoflavone = glycitin + genistin + daidzein + glycitein + genistein.
[a-b] The same letters under the same row indicate no significant differences ($p>0.05$).

3.4 Chemical and physical properties of control and optimized prebiotics supplemented soybean milk

The nutritional composition of soybean milk supplemented with the optimized prebiotics and the control is shown in Table 8. The results agree well with deMan *et al.* (1987), who reported that soymilk was composed of 94% moisture, 3.0% protein, 1.5% fat, 1.5% carbohydrates, and ash. Iwuoha and Umunnakwe (1997) demonstrated that processing method, storage temperature, and storage duration had a combined significant effect on the proximate chemical composition, physicochemical, and sensory attributes of soymilk. While Poysa and Woodrow (2002) found that genotype and year affected significantly the soymilk yield, solid levels, and pH, whereas the effects of location were much less significant. The FDA (Food and Drug Administration, 1999) recommends a total daily intake of 25 g soy protein to meet the recommended heart health claims on the reduced risk of coronary heart diseases (CHD). To meet the recommendation, at least five servings (serving size = 200 ml) per day of soybean milk beverages have to be consumed. The contents of isoflavone in the soybean milk supplemented with the optimized prebiotics and the control are 16.65 and 13.26 mg/100 ml, respectively, while in soybean seeds the content is 80.34 mg/100 g (on a dry weight basis). Kim *et al.* (2004) reported isoflavone concentration variations from 69.97 to 258.16 mg/100 g in soybean seeds. Prabhakaran *et al.* (2006) found that the amount of isoflavones in dietary supplements, soy based health products, and infant formulas ranged from 40.5 to 5,757, 4.632-133.38, and 5.95-22.68 mg/100 g of the sample, respectively. The total soluble solid, total solid content, viscosity, and color CIE L^*, a^* and b^* - values of soybean milk supplemented with the optimized prebiotics and the control are shown in Table 8. The prebiotic supplemented soybean milk had higher carbohydrate contents than the control ($p<0.05$). In addition, it also had higher values of the total soluble solid, total solid content, and viscosity ($p<0.05$). The prebiotic supplemented soybean

milk had a lower L^* value (lightness) and a higher a^* value (redness) than the control soybean milk ($p<0.05$). The decrease in lightness and increase in redness of soymilk upon heating was due to the formation of a brown pigment as a result of the Maillard reaction (Kwok *et al.,* 1999), which was caused by the supplemented prebiotics. As reported by Huebner *et al.* (2008), Raftilose P95 and NutraFlora P-95 had a visible brown color when exposed to Maillard reaction conditions. These prebiotics are composed of 95% FOS, with the remaining carbohydrates being sucrose, glucose, and fructose. The latter two are reducing sugars and were likely responsible for the formation of Maillard reaction products. In addition, Raftilose P95 contains mostly FF_n oligosaccharides, with the terminal fructose unit having reduction capacity and also able to participate in the Maillard reaction. However, the b^*-values of both soymilk formulations were not significantly different ($p>0.05$). Descriptive analysis indicated that no significant difference in all attributes were observed between the control and prebiotic supplemented soybean milk (data not shown).

4. Conclusions

The different ratios of prebiotic mixtures had no effect on sensory attributes, although the physical properties were significantly different ($p<0.05$). The addition of inulin increased the viscosity of soymilk. GOS was the most important factor contributing to the enhancement of probiotic growth. The probiotic growths (*B. bifidum* DSM 20456, *L. plantarum* TISTR 875, and *L. acidophilus* TISTR 1034) showed no significant difference ($p>0.05$) between the optimized prebiotic formulation (0.11I + 0.62GOS + 0.27IMO) and glucose. The optimized formulation increase the growth of *B. bifidum* DSM 20456 (after 48 hrs fermentation), *L. plantarum* TISTR 875, and *L. acidophilus* TISTR 1034 (after 24 hrs fermentation) by log 0.96, log 2.06, and log 1.61, respectively.

Acknowledgements

The authors express their grateful thanks to the Nutraceutical and Functional Food Research and Development Center (NFFRDC) and the Graduate School, Prince of Songkla University, for financial support. The authors are also grateful to T. Hongpattarakere for providing microbiological analysis facilities and to Helm Mahaboon Ltd., Thailand and Nihon Shokuhin Kako Co., Ltd., Japan, for ingredients.

References

AOAC. 2000. Official Method of Analysis, Association of Official Analytical Chemists, Washington, DC, USA.

Aryana, K.J. and McGrew, P. 2007. Quality attributes of yogurt with *Lactobacillus casei* and various prebiotics. Lebensmittel Wissenschast and Technologie. 40, 1808-1814.

Banuelos, O., Fernandez, L., Corral, J.M., Valdivieso-Ugarte, M., Adrio, J.L. and Velasco, J. 2008. Metabolism of prebiotic products containing β(2-1) fructan mixtures by two Lactobacillus strains. Anaerobe. 14, 184-189.

Belitz, H.D., Schieberle, P. and Grosch, W. 2004. Sugars, Sugar alcohols and Honey. In Food Chemistry, 3rd ed. Springer, Berlin, Germany, pp. 861-891.

deMan, L., deMan, J.M. and Buzzell, R.I. 1987. Composition and properties of soymilk made from Ontario light hilum soybeans. Canadian Institute of Food Science and Technology Journal. 20, 363-367.

Food and Drug Administration. 1999. Food labeling: health claims; soy protein and coronary heart disease; final rule. Federal Register. 64, 57700-57733.

Gibson, G.R. 2004. Fiber and effects on probiotics (the prebiotic concept). Clinical Nutrition Supplements. 1, 25-31.

Holzapfel, W.H. and Schillinger, U. 2002. Introduction to pre- and probiotics. Food Research International. 35, 109-116.

Huebner, J., Wehling, R.L. and Hutkins, R.W. 2007. Functional activity of commercial prebiotics. International Dairy Journal. 17, 770-775.

Huebner, J., Wehling, R.L., Parkhurst, A. and Hutkins, R.W. 2008. Effect of processing conditions on the prebiotic activity of commercial prebiotics. International Dairy Journal. 18, 287-293.

International Standard ISO 8586-1. 1993. Sensory Analysis - General guidance for the selection and monitoring of assessors. International Organization for Standardization, Geneva, Switzerland.

Iwuoha, C.I. and Umunnakwe, K.E. 1997. Chemical, physical and sensory characteristics of soymilk as affected by processing method, temperature and duration of storage. Food Chemistry. 59, 313-379.

Kim, J.J., Kim, S.H., Hahn, S.J. and Chung, I.M. 2004. Changing soybean isoflavone composition and concentrations under two different storage conditions over three years. Food Research International. 38, 435-444.

Kwok, K.C., MacDougall, D.B. and Niranjan, K. 1999. Reaction kinetics of heat-induced colour changes in soymilk. Journal of Food Engineering. 40, 15-20.

Lunn, J. and Buttriss, J.L. 2007. Carbohydrates and dietary fibre. British Nutrition Foundation. Nutrition Bulletin. 32, 21-64.

Macfie, H.J., Bratchell, N., Greenhoff, K. and Vallis, L.V. 1989. Designs to balance the effect of order of presentation and first-order carry-over effects in hall tests. Journal of Sensory Studies. 4, 129-148.

Manning, T.S. and Gibson, G.R. 2004. Prebiotics. Best Practice & Research Clinical Gastroenterology. 18, 287-298.

Poysa, V. and Woodrow, L. 2002. Stability of soybean seed composition and its effect on soymilk and tofu yield

and quality. Food Research International. 35, 337-345.

Prabhakaran, M.P., Hui, L.S. and Perera, C.O. 2006. Evaluation of the composition and concentration of isoflavones in soy based supplements, health products and infant formulas. Food Research International. 39, 730-738.

Priepke, P.E., Wei, L.S., Nelson, A.L. and Steinberg, M.P. 1980. Suspension stability of Illinois soybean beverage. Journal of Food Science. 45, 242-245.

Rice, J. 2002. Probiotics and prebiotics for healthful benefits. Available on http://www.foodproductdesign.com/archive/2002/0702AP.html, [17 May 2006].

Roberfroid, M.B., Van Loo, J.A.E. and Gibson, G.R. 1998. The bifidogenic nature of chicory inulin and its hydrolysis products. Journal of Nutrition. 128, 11-19.

Rycroft, C.E., Jones, M.R., Gibson, G.R. and Rastall, R.A. 2001. A comparative *in vitro* evaluation of the fermentation properties of prebiotic oligosaccharides. Journal of Applied Microbiology. 91, 878-887.

Shimamura, S., Abe, F., Ishibashi, N., Miyakawa, H., Yaeshima, T., Araya, T. and Tomita, M. 1992. Relationship between oxygen sensitivity and oxygen metabolism of *Bifidobacterium* species. Journal of Dairy Science. 75, 3296-3306.

Tomas, L.G., Marques, J.C. and Costell, E. 2008. Viscoelasticity of inulin-starch-based dairy systems. Influence of inulin average chain length. Food Hydrocolloids. 22, 1372-1380.

Tuohy, K.M., Probert, H.M., Smejkal, C.W. and Gibson, G.R. 2003. Using probiotics and prebiotics to improve gut health. Drug Discovery Today. 8, 692-700.

Van der Meulen, R., Avonts, L. and deVuyst, L. 2004. Short fractions of oligofructose are preferentially metabolized by *Bifidobacterium animalis* DN-173 010. Applied Environmental Microbiology. 70, 1923-1930.