International Scholars Journals

Advanced Journal of Microbiology Research ISSN 2241-9837 Vol. 13 (5), pp. 001-012, May, 2019. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Optimization of a cryoprotective medium and survival of freeze-dried *Bifidobacterium infantis* 20088

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Accepted 19 March, 2019

Supplementations of formulae with synbiotic compounds of human milk have shown to be able to decrease several gut-related diseases in formula fed infants. This study was carried out to develop a synergistic cryoprotective drying medium for infant formulae probiotic application. Response surface methodology (RSM) was employed to optimize the concentrations of skim milk and prebiotics for improvement of the cell survival of *Bifidobacterium infantis* 20088, during freeze-drying. The optimal composition was found to be 2.8% prebiotics blended with 16.1% skim milk which could protect 47.63% of cells' viability. No significant difference ($p > 0.05$) between the predicted and experimental values validated the model adequacy. Then, the protective effects of optimal composition on the survival of freeze-dried cells were evaluated through different conditions of rehydration, storage and simulated gastrointestinal tract (GIT) as compared to cells dried in phosphate buffer (control). After 120 days storage of freeze-dried stationary phase cells at 4°C, there was 2.33 log (CFU/ml) improvement in the viability of cells as compared to control. With increasing temperature to 25°C, the protective effect of optimized medium was more apparent. Direct rehydration with water led to 0.65 log (CFU/ml) increment in the mortality rate of freeze-dried cells as compared to rehydration with skim milk solution. Also, the mortality rate of cells after sequential incubation in simulated GIT conditions including gastric conditions (pH 3.0 and 4.0, 90 min) and intestinal conditions (pH 7.5, 5 h) were reduced by 1.81 and 0.35 log (CFU/ml), respectively, as compared to the control.

Key words: *Bifidobacterium longum* subsp. *infantis* 20088, freeze-drying medium, response surface methodology, storage, infantile gastrointestinal conditions.

INTRODUCTION

Intestinal microorganisms play a critical role in protecting new born infants against several diseases. Nevertheless, the type and number of micro-flora depend on the infant's diet. The unique composition of human breast milk has a great influence on the colonization of the gastrointestinal tract's micro-flora during the neonatal period (Orrhage and Nord, 1999; Harmsen et al., 2000). High incidence of diseases in formula feds, prove that their nutrition is an imperfect alternate for breast milk. The main approach in obtaining bacteriological effect is to simulate breast milk's symbiotic compounds and accordingly, its functional effects for formula feedings (Ghisolfi, 2003). Therefore, currently various strains of probiotics found in human milk (Gueimonde et al., 2007; Martin et al., 2008) and its prebiotics substances (Coppa et al., 2004) are used to

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supplement formulae. Since breast-fed infants become dominated by more bifidobacteria (Orrhage and Nord, 1999; Coppa et al., 2004), it is wise to select these microorganisms as the most appropriate food supplement to incorporate into infant formulae. According to the definition of probiotics by FAO/WHO (2001), live and active *Bifidobacterium* could be integrated in a probiotic formula to improve the health status of the host when held at a sufficient amount. The Scientific Committee on Food of the European Commission (SCFEC) has recommended that the content of viable bacteria in formulae during the shelf-life of the formulae should be 10^6 to 10^8 colony forming units (CFU) per gram of formula prepared as ready for consumption in order to effect beneficially and be known as a probiotic product. Thus, successful application of probiotic cultures for supplementation of foods depends significantly on preservation technologies employed to produce bacterial biomass (Carvalho et al., 2004). It is critical to guarantee long-term delivery of enough active viable functional cultures to the consumer (Otero et al., 2007).

To serve as a functional ingredient in infant formula powders probiotic cells should be dried. Freeze-drying is a well-documented technique used to obtain stable cultures in terms of viability and functional activity (Broadbent and Lin, 1999; Otero et al., 2007). However, decreased viability of the microorganisms during the drying process is still the most important undesirable side-effect that should be controlled (Carcoba and Rodriguez, 2000). Different factors might affect the survival of probiotics during the freeze-drying process and the following storage such as bacterial species and the cells growth phase at the moment of harvesting (Broadbent and Lin, 1999; Fonseca et al., 2000; Carvalho et al., 2004; Huang et al., 2006; Otero et al., 2007), initial concen-tration of cells (Carvalho et al., 2004; Otero et al., 2007), freeze-drying medium (Carcoba and Rodriguez, 2000; Carvalho et al., 2004; Huang et al., 2006; Otero et al., 2007) and storage conditions (Carvalho et al., 2004; Otero et al., 2007).

Moreover, reconstruction of cell powder over rehydration process has been shown to be very critical (Carvalho et al., 2004; Huang et al., 2006; Otero et al., 2007). Hence, delivery of active dried probiotics is enhanced by choosing appropriate bacterial strain as well as adjusting the production processing, storage and rehydration conditions for each consumption aim via accurate statistical techniques. Numerous compounds have been tested as medium for their ability to protect the cells viability during freeze-drying. Several studies reported that skim milk, glycerol, various sugars and some probiotic substances exhibit the highest protection among different examined cryoprotectants media (Abadias et al., 2001a; Hubalek, 2003; Huang et al., 2006; Morgan et al., 2006). On the other hand, a large amount of the medium used for freeze-drying is left over within the foods, thus, safe and nutritive compounds are preferred to utilize as the cryoprotective medium.

It is documented that in most cases the presence of prebiotics substances in drying medium can enhance the survivability of cells during freeze-drying process (Carvalho et al., 2004; Clarissa et al., 2007) in addition to their synergistic function with probiotics (Bielecka et al., 2002). Prebiotics are defined as “non-digestible food ingredients that may beneficially affect the host by selectively stimulating the growth and/or the activity of a limited number of bacteria in the colon” (Roberfroid, 2001). In terms of infant formulae applications, the nutritionally preferred compounds amongst all of cryoprotectants are skim milk and special prebiotics substances. Recently Moro et al. (2002) and Arslanoglu et al. (2007, 2008) proposed a prebiotics mixture closely mimicking the clinical effects of breastfeeding on the infantile intestinal micro-flora. It is a compound of two sugary prebiotics substances including 90% Galacto-oligosaccharides (GOS) and 10% Fructo-oligosaccharides (FOS). Currently these prebiotics substances are produced and included in some formula separately or jointly. Nevertheless, the mixture has been never used as drying medium to improve the probiotics survivability during freeze-drying process. The present study was the first attempt to develop a safe, nutritive and functional drying medium (combination of skim milk and the prebiotics mixture) for obtaining stable dried *Bifidobacterium infantis* 20088 suitable for inclusion in formulae by using RSM. Also, the protection ability of optimized drying medium was examined through the way of getting to infant’s gut. With this in mind, the stability of freeze-dried cells was assessed over 120 days storage (at 4 and 25˚C), rehydration with two feasible media (water and skim milk solution) and passage through infantile gastrointestinal tract conditions.

MATERIALS AND METHODS

Microorganism and estimation of growth kinetics

Bifidobacterium longum subsp. *infantis* DSM 20088 isolated from infant’s intestine was obtained from DSMZ Co. (Germany) and activated by growing in sterile MRS broth (Oxoid) three times successively at 37°C for 24 h. MRS broth was supplemented with filter sterilized 0.05% w/v L-cysteine-hydrochloride (Sigma Chemical Co., Castle Hill, Sydney, Australia) to create an anaerobic environment. The activated strain was stored at -80°C in 20% (v/v) glycerol (BDH Laboratory Supplies, Poole, England) as stock cultures. For growth analysis, the stock culture was reactivated over three sub-culturing and then inoculated (2%) into MRS-C broth (100 ml) and incubated anaerobically at 37°C. Samples were collected hourly for viable cells count and optical density (OD_{600}) determi-nations using spectrophotometer (Pharmacia, Sweden). The viable cells were determined as colony forming units (CFU).

Culture conditions

The stock culture was reactivated by three successive inoculations into the sterile MRS-C broth and incubated at 37°C for 24 h. The final culture for freeze-drying was achieved from the third sub-culture in a 1-L Erlenmeyer flask containing 200 ml sterile MRS

broth (Oxoid) and incubated anaerobically at 37°C for 17 h (early stationary phase according to the results of growth kinetics).

Cryoprotective freeze-drying media

The cryoprotectant agents used to protect cells during freeze-drying were skim milk (Difco) and prebiotics mixture including 90% GOS (Nissin Co., Japan) and 10% FOS (Sigma). The protective media were 13 different combinations prepared for the optimization procedure based on a central composite design (CCD) by suspending the agents in distilled water shown in Tables 1 and 2, and were sterilized at 115°C for 13 min. Control was made in which phosphate buffer instead of cryoprotectants was used.

Preparation procedure of samples for freeze-drying

Early stationary phase cells of *B. infantis* 20088 were harvested by centrifugation at 1550 × *g* at 4°C for 15 min in a centrifuge (5804 R, Eppendorf, Germany) to obtain an initial concentration of approximately 10^9 CFU/ml. The supernatants were discarded and the cell pellets were washed twice in sterilized normal saline (0.9% NaCl in distilled water) and centrifuged again prior to re-suspension in different combinations of sterile drying media. Sterilized vials were filled with 1 ml of each bacterial suspension produced as described above in triplicate and kept at -80°C for 18 h. The frozen cultures were then desiccated in a freeze-dryer (Virtis, USA) at a condenser temperature -76°C, for approximately 30 h to obtain the moisture content of 2.5 ± 0.3%. The moisture content of all test samples were measured by drying 1 g of each sample series (powdery form of treatment combinations) in a hot-air oven (Memmert, Germany) at 100 ± 1°C for 4 h and calculated, using the following equation (AOAC, 2000):

$$\text{Moisture content}(\%) = \frac{\text{loss of sample weight during drying}}{\text{intial sample weight}} \times 100 \quad (1)$$

The samples were stored in tightly sealed bijou bottles to avoid increment of moisture content during following storage study.

Determination of probiotic cells viability

Viable cells were enumerated before and immediately after freeze-drying as CFU. Decimal serial dilutions of each suspension were plated onto the surface of MRS-C ager and incubated anaerobically for 72 h at 37°C before freeze-drying. For this purpose, after freeze-drying, powdery samples were immediately rehydrated with MRS medium to the original volume (1 ml) and homogenized for 1 min with a vortex mixer and maintained at room temperature for 15 min and then plated as described above. The survival rate of cells was calculated for each drying medium as follows:

$$\text{Survival rate}(\%) = \frac{\text{Viable cells immediately after freeze} - \text{drying}\left(\frac{\text{CFU}}{\text{ml}}\right)}{\text{Initial viable cells of suspensions before freezing}\left(\frac{\text{CFU}}{\text{ml}}\right)} \times 100 \quad (2)$$

Experimental design using CCD and statistical analysis

In this study, the viability of freeze-dried probiotics is considered to be mainly affected by the type and concentrations of the freeze-drying media . For determination of the factors ranges the concentration of skim milk (5 to 20% w/v) and prebiotics mixture (0 to 6% w/v) were chosen from the results of other studies (Roberfroid, 2001) and examined through preliminary experiments. A five-level-two-variable (Table 1) CCD with 5 replicates at the center point was conducted to determine the optimum levels of variables and build the response surface model. Survival rate of *B. infantis* 20088 during freeze-drying process were used as the response for the experiments. The generalized polynomial model for linking the response to independent variables is as follows:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{12} AB \quad (3)$$

To set up a regression model, Y denoted survival rate which is calculated by the model and determined coded factor levels as follows: A: prebiotics and B: skim milk. β_0 is a constant; β_1 and β_2 are linear, β_{11} and β_{22} are squared and β_{12} is interaction coefficient. The actual factor levels corresponding to the coded factor levels are shown in Table 1. The design of experiments (DOE), analysis of data and optimization procedure were performed using the Minitab v. 14 statistical packages (Minitab Inc., State College, PA, USA). In the regression model, the response variable candidates for explanatory variables were linear, interaction and quadratic terms of coded levels of skim milk and prebiotics. The coefficient parameters were estimated using the least-squares method by multiple linear regression analysis. ANOVA was used for regression analysis of variance. Each factor in the CCD design was studied at five different levels (-1.4142, -1, 0, 1, 1.4142). The center point was repeated 5 times to calculate the repeatability of the method. ±1.4142 are the default axial points considered by software. The terms which found to be non-significant (p >0.05), dropped from the initial regression models and then the experimental data was refitted to only significant (p <0.05) independent variable effects in order to obtain the final polynomial equation (final reduced model) for optimization.

Optimization and validating procedure

The optimization procedure was conducted to obtain the optimal levels of two independent variables leading to the maximum survival of cells during freeze-drying process. For graphical optima-zation, the final reduced response model was expressed as three-dimensional (3D) surface plots to better visualize the interaction effect of main factors on the viability of cells. A numerical optima-zation technique was also carried out by the response optimizer using the Minitab software for determining the exact optimum levels of variables. For validating of predicted models, the adequacy of the regression equation was checked by graphically comparing of the experimental data with predicted values obtained from the equation.

Rehydration media

The samples of freeze-dried cells in the optimal combination of drying medium were rehydrated in equal volume (1 ml) of water, skim milk solution (20% w/v) and MRS broth (as control) and viable cells of each suspension were enumerated as described above.

Storage conditions

The samples of freeze-dried cells in the optimal combination of drying medium were kept at 4 and 25°C for 120 days. During the storage, samples were collected every 15 days and rehydrate with MRS to check for viable cells as described above. Cells freeze-dried in phosphate buffer were used as control. Survival rate (%) after each storage period was calculated as follows:

Table 1. Treatment combinations of cryoprotectants according to the CCD with experimental and predicted values of cell survival rate of *B. infantis* 20088 during freeze-drying.

Run number	Types	Variables [a]		Responses (Survival rate, %)	
		(A) Prebioticss mixture [c], %	(B) Skim milk, %	Experimental [b]	Predicted
1	fact	-1 (0.8)	-1 (7.2)	46.17±0.08	45.77
2	fact	-1 (0.8)	1 (17.8)	44.08±0.16	46.27
3	fact	1 (5.1)	-1 (7.2)	37.85±0.1	37.86
4	fact	1 (5.1)	1 (17.8)	42.74±0.06	43.08
5	axial	-1.4142 (0)	0 (12.5)	43.23±0.09	42.53
6	axial	1.4142 (6)	0 (12.5)	46.67±0.13	46.27
7	axial	0(3)	-1.4142 (5)	48.92±0.12	46.27
8	axial	0(3)	1.4142 (20)	46.38±0.03	46.27
9	center	0(3)	0 (12.5)	45.34±0.17	46.27
10	center	0(3)	0 (12.5)	36.46±0.07	34.85
11	center	0(3)	0 (12.5)	46.65±0.21	45.38
12	center	0(3)	0 (12.5	35.13±0.19	37.48
13	center	0(3)	0 (12.5)	44.79±0.02	46.03
Control	-	-	-	8.42±0.16	

[a]Code levels of concentrations; [b]the values are survival rate (mean of three replicates ± standard deviations); [c]the prebioticss mixture includes 90% GOS & 10% FOS.

Table 2. Regression coefficients, R^2, adjusted R^2, probability values and lack of fit for the final reduced models of freeze-drying of *B. infantis* 20088.

Regression coefficient	*B. infantis* 20088
β_0	46.2780
β_1	-0.1934
β_2	3.9525
β_{11}	-1.7353
β_{22}	-2.9153
R^2	88.8%
R^2 (adj)	83.2%
Regression (*p*-value)	0.001
Regression (*F*-value)	15.88
Lack of fit (*p*-value)	0.526
Lack of fit (*F*-value)	0.93
A (*p*-value)	0.764
B (*p*-value)	0.000
A^2 (*p*-value)	0.032
B^2 (*p*-value)	0.002
Linear *F*- ratio	20.16
Quadratic *F*-ratio	11.60
Pure Error	12.855
Corr Total	222.257

β_1 and β_2: the estimated regression coefficient for the main linear effects of prebiotics (A) and skim milk (B), respectively;β_{11} and β_{22}: the estimated regression coefficient for the quadratic effects of prebiotics (A) and skim milk (B), respectively.

$$\text{Survival rate}(\%) = \frac{\text{Viable cells at the time}\left(\frac{\text{CFU}}{\text{ml}}\right)}{\text{Initial viable cells after freeze} - \text{drying}\left(\frac{\text{CFU}}{\text{ml}}\right)} \times 100 \quad (4)$$

Simulated infantile gastrointestinal tract conditions

A modified method of Annan et al. (2008) was used to evaluate the tolerance of freeze-dried cells to infantile gastrointestinal conditions. To this end, the freeze-dried *B. infantis* 20088 samples (produced in the optimal combination of drying medium) were rehydrated and exposed to an *in vitro* infantile gastrointestinal tract conditions in a sequential way. Two samples of freeze-dried cells were rehydrated in triplicate and then exposed to simulated gastric juices (SGJs) at two different pH (3.0 and 4.0). Subsequently 1 ml of each sample was taken for determination of viable cells after exposing to simulated gastric conditions (SGCs). Simulated intestinal juice was then added to the residual samples (the volume adjusted to 10 ml) and pH adjusted to 7.5. Subsequently, surviving bacteria were enumerated again for determination of viable cells after exposing to whole simulated gastric and intestinal conditions (SGICs). Cells freeze-dried in phosphate buffer were used as control. Whole detailed procedure is described as follows:

Preparation of simulated gastric juice (SGJ) and simulated intestinal juice (SIJ)

Solutions of 0.5 g/l pepsin (Sigma) in saline (0.5%, v/v) were prepared and adjusted to pH levels of 3.0 and 4.0 with 12N HCl to simulate infantile gastric juices. Also Solution of 0.1% (w/v) pancreatin from porcine (Sigma) and 0.8% (w/v) porcine bile extract (Sigma) in saline (0.5%, v/v) was prepared and adjusted to pH level of 7.5 with NaOH to simulate infantile intestinal juice. They were prepared fresh (prior to use), sterilized by filtration through a membrane (0.45 µm, Minisart /CE, Germany) and heated up to 37°C prior to use in experiments.

Exposing to simulated gastric conditions (SGCs)

The freeze-dried samples were rehydrated with 1 ml of sterile MRS broth and enumerated as described above prior to exposing to SGJ. Each sample was poured into the Erlenmeyer flasks containing 4 ml SGJ and mixed thoroughly. They were incubated for 90 min at 37°C in shaker water bath (Stuart, UK) at 10 rpm. Then 1 ml of each sample was taken, washed twice with normal saline and harvested for viable cell count. The survival rate after exposure to SGCs was calculated as follows:

$$\text{Survival rate}(\%) = \frac{\text{Viable cells after exposure to SGJs}\left(\frac{\text{CFU}}{\text{ml}}\right)}{\text{Initial viable cells rehydrated in 5 ml MRS}\left(\frac{\text{CFU}}{\text{ml}}\right)} \times 100 \quad (5)$$

Exposing to simulated intestinal conditions

After incubation time in SGJs, 6 ml of simulated intestinal juice was added to the remained suspension (up to the whole volume of 10 ml), mixed thoroughly and incubated for 300 min at 37°C in shaker water bath at 10 rpm. Then 1 ml of the samples was taken, washed twice with normal saline and harvested prior to enumeration of viable cells. The survival rate after exposure to whole simulated gastrointestinal conditions (SGICs) was calculated as follows:

$$\text{Survival rate}(\%) = \frac{\text{Viable cells after exposure to SGICs}\left(\frac{\text{CFU}}{\text{ml}}\right)}{\text{Initial viable cells rehydrated in 10 ml MRS}\left(\frac{\text{CFU}}{\text{ml}}\right)} \times 100 \quad (6)$$

RESULTS AND DISCUSSION

B. longum subsp. *Infantis* was isolated from breast fed infants' intestine and digests the complex prebiotics structure of breast milk (Sela et al., 2008). It is also the predominant species of the genus in the feces of breast fed infants (Collins and Hall, 1984). Consumption of this microorganism has been shown to be beneficial for those who suffer from symptoms of irritable bowel syndrome (IBS) including bloating, gas, diarrhea, constipation, urgency and abdominal discomfort (Whorwell et al., 2006). However the research on the potential uses of this strain especially for infants is still rare.

Culture conditions

The time of cells harvesting has been reported to have a significant effect on the survival of probiotic cells over drying process and subsequent storage (Muller et al., 2009). The growth of bacteria when grown in batch culture has been divided to four phases that is, log, lag, stationary and death phases (Morgan et al., 2006). It has been demonstrated that over the stationary phase, probiotic cells exhibit their most stable feature (Corcoran et al., 2005; Muller et al., 2009). Corcoran et al. (2005) noted that the stationary phase cells appear to give the highest recovery after drying, whereas early log phase cells exhibited lower survival and in the lag phase cells showed the most sensitivity. It is due to the fact that during stationary growth phase of many bacterial species, several intense structural and physiological changes in expression levels of stress related proteins, membrane composition and cell wall structure occur, resulting in increased resistance to adverse conditions (Schwab et al., 2007). Therefore, to eliminate the effect of culture condi-tions on the viability results of cells over following experiments, we standardized initial cell concentration and age of the cultures for incorporation into all treatment combinations of freeze-drying media.

In this study for finding the exact time of incubation taken to reach the stationary phase, the growth kinetics of *B. infantis* 20088 was monitored and analyzed by direct enumeration of viable cells and checking the optical density (OD) simultaneously, over 24 h incubation. As shown in Figure 1, although the cells number decreased after 17 h, the OD did still increase with incubation. This distinction is mainly due to the fact that OD of cultures determine base on turbidity of culture media that consist of viable and dead cells together. However, another important reason for this dissimilarity between population and OD trend of *B. infantis* 20088 is

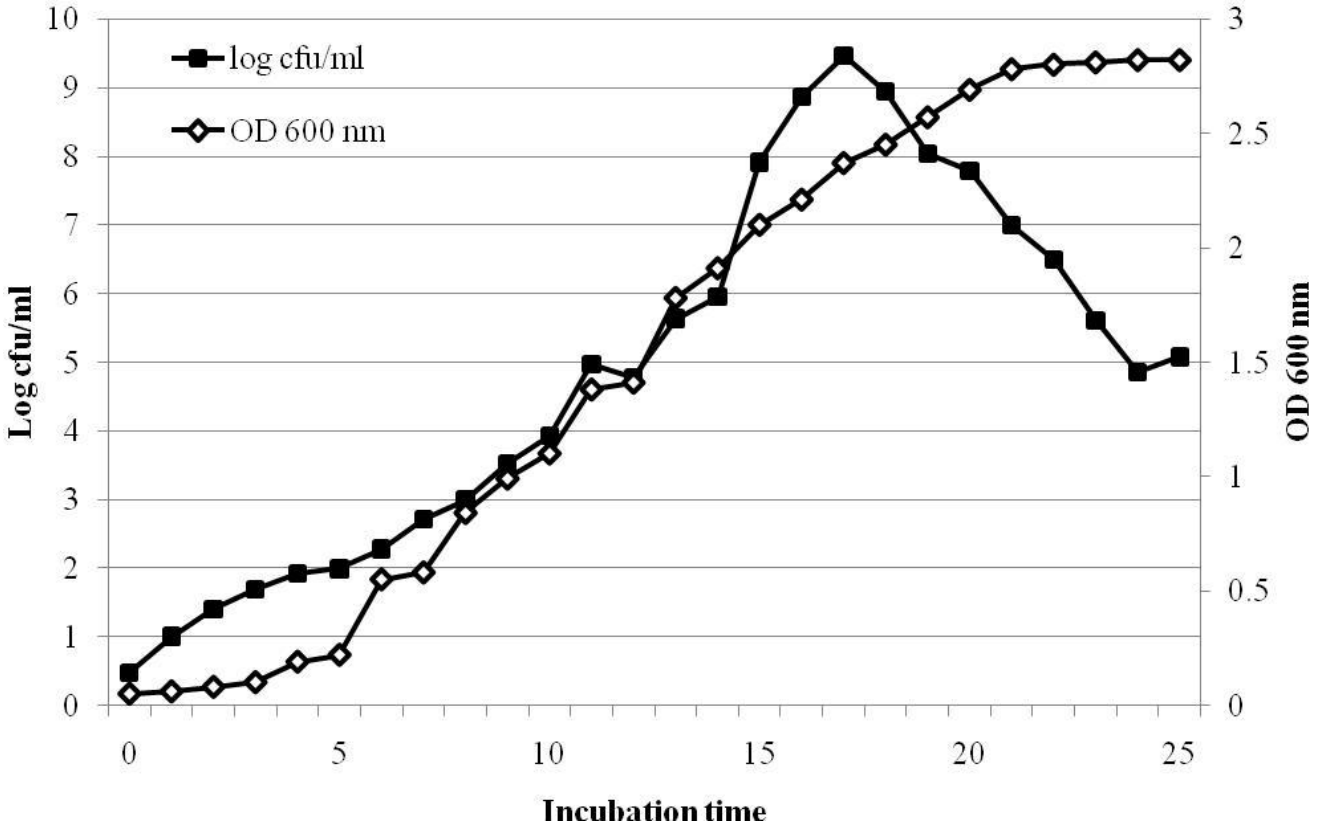

Figure 1. Growth kinetic of *B. infantis* 20088.

related to the special variable cellular morphology of bifidobacteria (Kim, 1968; Scardovi, 1986; Sun and Griffiths, 2000). The results of our study support Ellenton's idea (1998) that although OD is used often to evaluate cell numbers of microorganisms, it may not be useful for bifidobacteria, since, the change in turbidity of *Bifidobacterium* culture especially *B. infantis,* as he noted may be due to extreme branching in their morphology.

Freeze-drying; developing and checking the fitted model

Response surface methodology (RSM) was used in the present work to develop a prediction model for establishing the optimal concentrations of skim milk and infantile prebiotics mixture for maximizing the cell viability of *B. infantis* 20088 during freeze-drying. The experimental design (CCD) and the experimental and predicted responses are presented in Table 1. Fitting of data to various models (linear, quadratic, cubic and quartic) were tested by using the analysis of variance (ANOVA). The position of the axial points in the CCD is denoted by α. The value of α, along with the number of center points, determines whether a design can be orthogonally blocked and is rotatable. Rotatable designs provide the pleasant property of constant prediction variance at all points that are equidistant from the design center, consequently improving the value of the prediction. Minitab's default designs achieve rotatability when there are no blocks. Experiments were randomized to minimize the effects of inexplicable variability in the actual responses caused by extraneous factors. The items considered for the fitness of a model were the significance of the model ($p < 0.05$) and the insignificance of lack of fit ($p > 0.05$). The fitness of model to experimental data was indicated by the coefficient of determination, R^2, which measures the percent of variable that can be explained by independent variables as outlined by the previous studies (Lee et al., 2000; Weng et al., 2001; Mirhosseini et al., 2008). Joglekar and May (1987) demonstrated that for a good fit of a model, R^2 should be at least 80%.

The results from statistical analysis showed that B, A^2, B^2 were significant model terms and had direct effect on the response. The interaction term (AB) was not signi-ficant on the response and should be reduced from final model, thus the experimental data were refitted to final reduced model and the results from statistical analysis after reducing the non-significant term are shown in Table 2. Although A (prebiotics mixture) was shown to be a non-significant factor, it was remained because of its

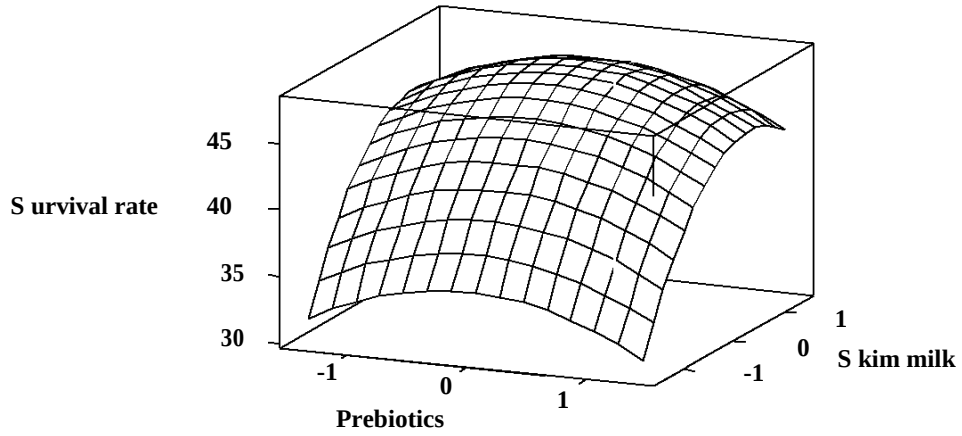

Figure 2. Response surface plot of skim milk versus prebiotics mixture on the cell survival rate of *B. infantis* 20088 over freeze-drying.

quadratic significant effect. The following quadratic polynomial equation was established to express the cell viability of freeze-dried *B. infantis* 20088:

$$Y = 46.278 - 0.1934\ A + 3.9525\ B - 1.7353\ A^2 - 2.9153\ B^2 \quad (7)$$

Where Y (%) is the cell survival rate of freeze-dried *B. infantis* 20088, and A and B are the coded values of prebiotics mixture and skim milk, respectively.

The second order model was significant with *p*-value of 0.001 and the lacks of fit was insignificant with *p*-value of 0.526. The Model *F*-value of 15.88 implies the model is significant. The coefficient of determination, R^2 of 88.8% was good. Values of *p*-value less than 0.05 indicated model terms are significant. The coefficients of estimates for each significant model terms and the individual significance *F*-ratio and *p*-value of independent variables are summarized in Table 2.

The results presented in Table 1 indicated that presence of selected cryoprotectants (skim milk and prebiotics mixture) could enhance the cell survival rate of *B. lactis* 10140 over freeze-drying process. The loss of *B. infantis* viability during freeze-drying in the presence of cryoprotectants was from 51.08% to 64.87% (log reduction maximum 0.44 CFU/ml). Whereas, in the absence of cryoprotectants (control) the results showed that there was a viability loss of 1.08 log cfu/ml. This excellent result is comparable with the results of Champagne et al. (1996) and Wang et al. (2004) for improving the cell viability of *Bifidobacterium longum* strains over freeze-drying using skim milk–sucrose–ascorbic acid mixture and soy milk as cryoprotective media, respectively.

Results from Table 2 along with Figure 2 (estimated coefficients and surface plot) show that skim milk was more effective than prebiotics for protecting the cells' viability during freeze-drying. Several studies have validated that skim milk is capable to prevent cellular injury by stabilizing the cell membrane and providing a protective coating for the cells (Hubalek, 2003; Saarela et al., 2005; Huang et al., 2006). Hubalek's study (2003) noted that skim milk is non-penetrating and protects the cells against extracellular ice formation. Although, the prebiotics mixture was not as effective as skim milk, the results show that its presence (approximately up to middle values of range) could enhance the protective effect of skim milk. The cryoprotective properties of various saccharide components have been evaluated over several studies. They have been reported to be related partly to the enhancement of membrane integrity by replacement of structural water during dehydration (Crowe and Crowe, 1986; Carvalho et al., 2004; Zhao and Zhang, 2005; Clarissa et al., 2007). Zhao and Zhang (2005) postulated that differences exhibited by various sugar components are associated with their water-binding capacity. They also noted that this ability results in prevention of intracellular and extracellular ice crystal formation.

Evaluation of the freeze-drying models visualization

Following the statistical and diagnostical analyses of this model, the graphical representations were determined by considering the response surface plot (Figure 2). The response surface plot represent the effects of varying concentration of prebiotics mixture and skim milk on the survival rate of *B. infantis* 20088 during freeze-drying, as a three dimensional shape (surface). As figure show response surface plots have four decreasing edges and one peak. This peak would be the optimum composition of the factors. Also as shown in Table 2 the quadratic terms Eigen (second derivation) values only, it can be predicted that the stationary point for the response is maximum. Therefore, the accuracy of factors ranges and

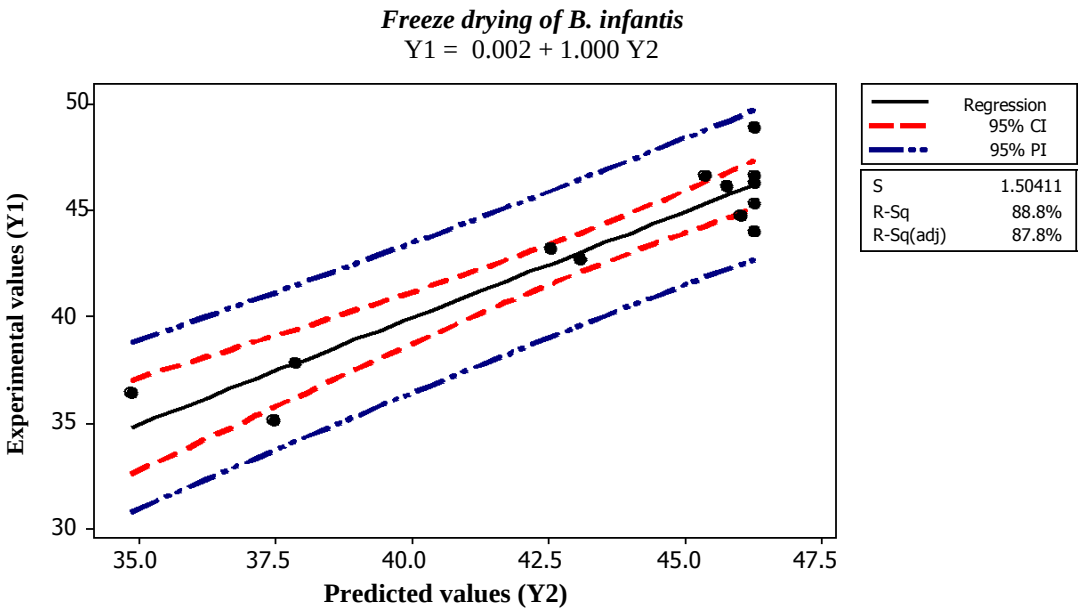

Figure 3. Fitted line plots showing the closeness between the experimental (Y1) and predicted values (Y2) obtained from the final reduced model for freeze-drying of *B. infantis* 20088.

model is confirmed.

Finding the optimum points of the factors and validating of the model

Numerical optimization was conducted by the response optimizer by means of the Minitab software for determining the exact optimum level of independent variables leading to the maximum survival of cells during freeze-drying process. The results indicated that maximum survival predicted for freeze-drying of *B. infantis* 20088 was 47.63% at the combined coded levels of -0.092% prebioticss and 0.684% skim milk. The actual concentrations would be 2.8% (w/v) for prebioticss mixture and 16.1% (w/v) for skim milk. The adequacy of the regression equations was checked graphically by drawing the fitted line plot (Figure 3). This plot is showing the closeness between the experimental (Y_1) and predicted values (Y_2) obtained from the equation.

Effect of rehydration media on recovery of freeze-dried cells

Generally, dried probiotic products need to be reconstituted before consumption. Freeze-dried bacteria powder contain cells in three statues including dead, damaged due to freezing but sub lethally and uninjured. Thus it is clear that rehydration (as reconstitution method) has a great effect on the repairing and regaining normal function of damaged bacterial cells (Font de Valdez et al., 1985a, b; Zhao and Zhang, 2005; Muller et al., 2009). Zhao and Zhang (2005) demonstrated that rehydration

Table 3. Effect of rehydration media on the survival rate of B. infantis 20088 freeze-dried in optimal combination of cryoprotectants skim milk and prebioticssa.

Rehydration media		
Water	**Skim milk**	**MRS (control)**
10.08± 0.25% *	45.17 ± 0.13% *	47.62 ± 0.09%

[a]The values are survival rate (mean ± standard deviations of tripli; * Significant at 0.05 level with control.

medium properties, such as its molarity, significantly affect the rate of cell recovery. In the present study skim milk solution and water were used as rehydration media since they can be feasible rehydration media for usual recipe of formulae consumption and MRS was used as control. The best recovery was obtained when the freeze-dried cells were rehydrated with MRS medium (Table 3), whereas direct rehydration of cells with water resulted in the highest mortality rate (extra 0.67 log CFU/ml) which is possibly due to the occurrence of osmotic pressure shock. In terms of using skim milk solution for rehydration, this favorable result as compared to MRS might be also attributed to the close osmotic properties of these solutions. Similar results were reported elsewhere about the increased viability of freeze-dried cells, when they were rehydrated in solutions used for cryopreservation. It is noted that the use of such media for rehydration provides a high osmotic pressure environment, and consequently, controls the rehydration rate, and as a result avoids from osmotic shock (Abadias et al., 2001b; Muller et al., 2009). Our results about the effects of different rehydration media on the viability of freeze-dried

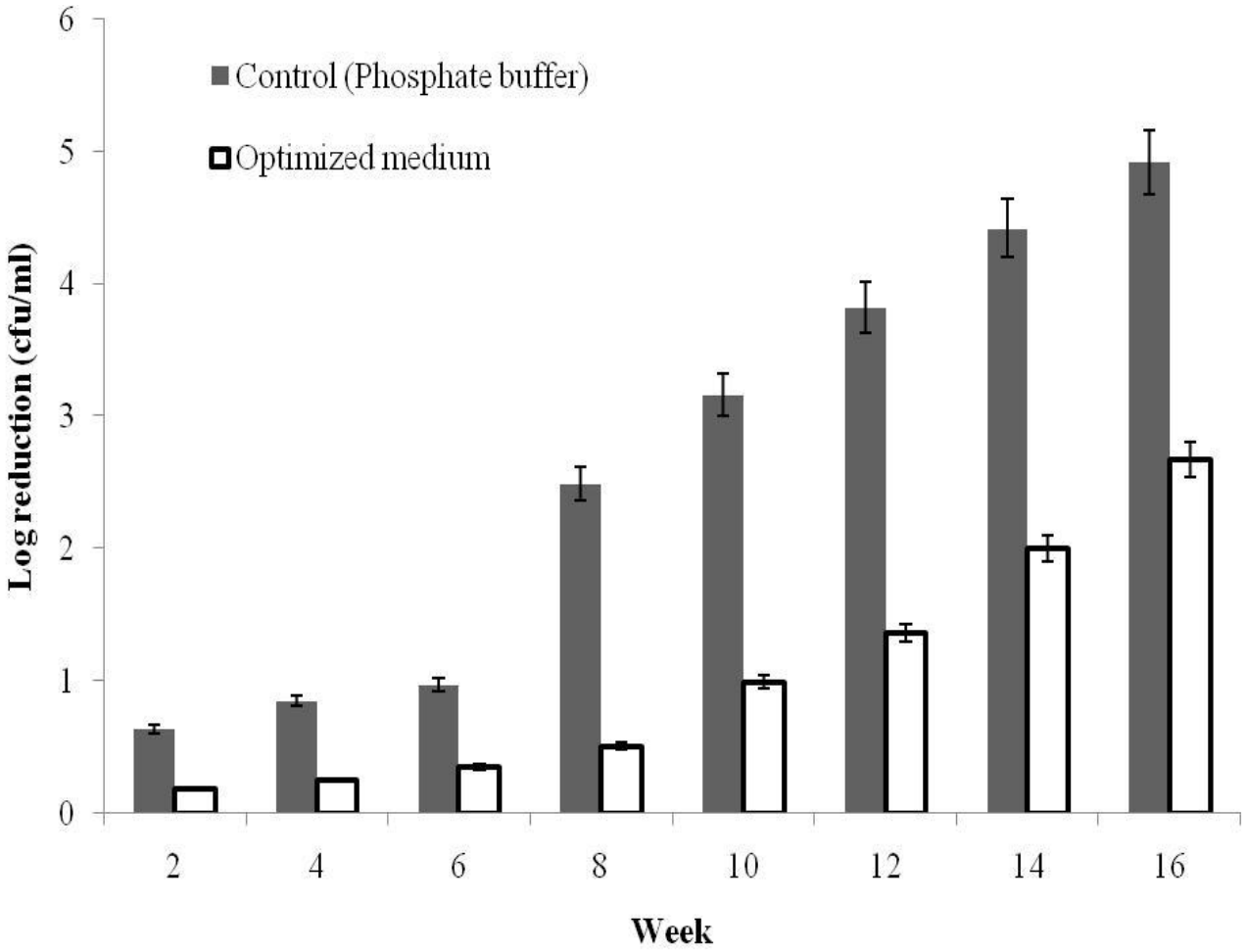

Figure 4. The effects of optimal combination of cryoprotectants of skim milk and prebiotics on the survival rate of freeze-dried *B. infantis* 20088 over 120 days storage at 4°C.

bacteria cells are similar to the results of Font de Valdez et al. (1985a, b) and Zhao and Zhang (2005). Therefore, it can be concluded that designing a powder form of probiotic cells to be wet mixed to prepared formula by consumer is more favorable than producing them as a dry mixed probiotic formula.

Stability of freeze-dried cells during storage period

Long term storage is doubtlessly one of the main factors to be considered in large scale production of any probiotic product. Apparently, proper protection of the cell membranes during freeze-drying process lead to less subsequent lethal cellular death or injuries during subsequent storage. However storage conditions particularly temperature and moisture content of dried cultures critically affect the cell viability of probiotics as reported by several studies (Wang et al., 2004; Saarela et al., 2005; Abe et al., 2009). Generally, bacteria cells survive better at a low water activity and moisture content. However, it is noted that the optimum moisture content of dried products varies with the composition of the drying medium, the species of microorganism and storage conditions (Wang et al., 2004; Abe et al., 2009). In the present study all the samples had moisture contents of below 2.8% after freeze-drying process and were kept under controlled moisture content conditions in the tightly sealed bijou bottles. The low moisture content of powdery samples is very important, since a maximum moisture content of 4% has been reported for avoiding dried milk products' deterioration (Corcoran et al., 2004; Simpson et al., 2005; Muir and Banks, 2000; Wirjantoro and Phianmongkhol, 2009).

The cells freeze-dried in optimal combination of drying medium survived satisfactory after 120 days of storage at 4°C (Figure 4), the survival rate was calculated 0.21% which means it had about 2.5 log (CFU/ml) viability loss. Whereas the viability lost of cells freeze-dried in phosphate buffer (control) after 120 days was about 5 log (CFU/ml). Nevertheless, the viability results of storage at 25°C (Figure 5) were extremely poorer than those of

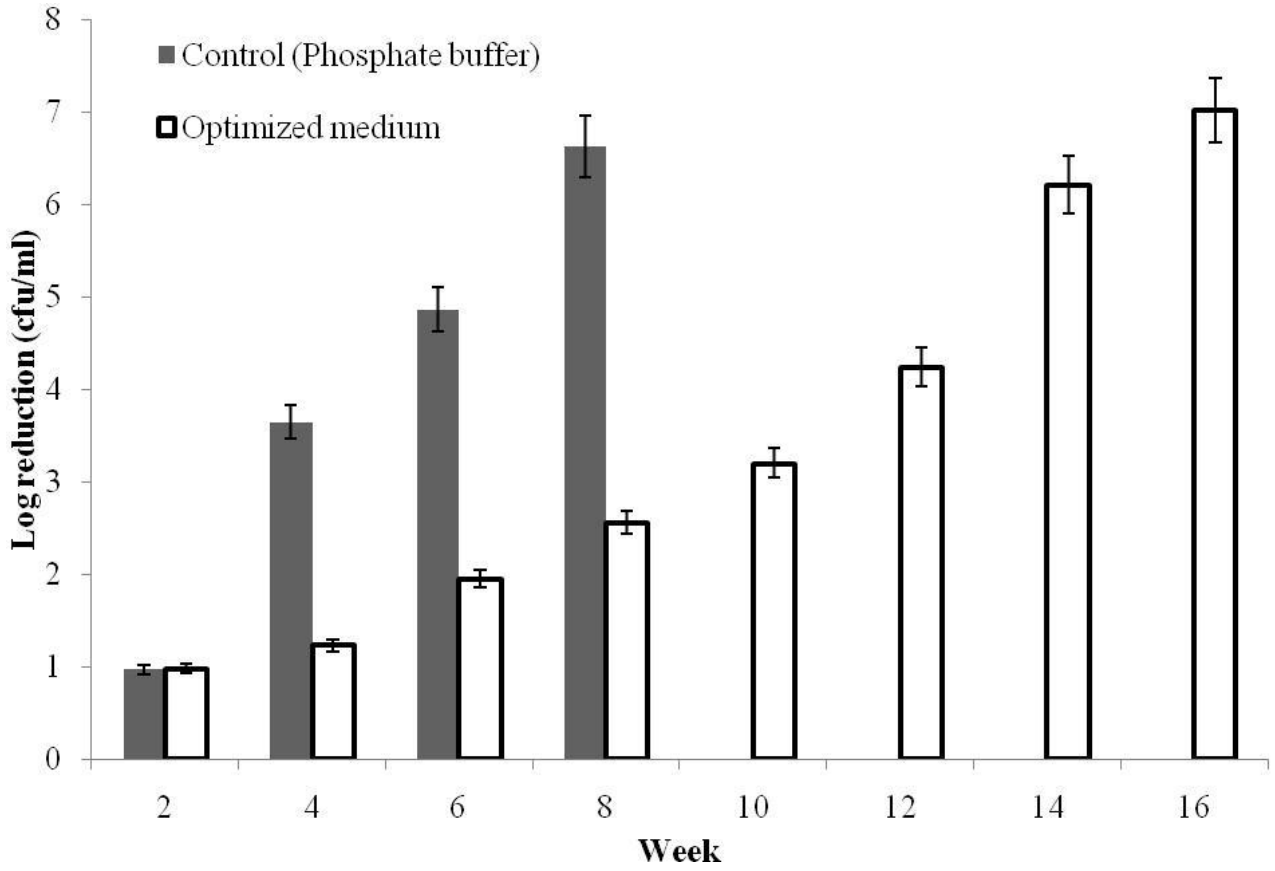

Figure 5. The effects of optimal combination of cryoprotectants of skim milk and prebiotics on the survival r ate of freeze-dried *B. infantis* 20088 over 120 days storage at 25°C.

storage at 4°C. There was 7 log (CFU/ml) viability loss after 120 days storage at 25˚C, while after 70 days storage of control samples the viable cells were determined less than 10 CFU/ml. Although the survivalability of dried cells during storage in the present study was poorer than other studies (Wang et al., 2004; Saarela et al., 2005; Abe et al., 2009), it is still significantly higher than control samples. This enhancement is probably partly due to the decreased amount of injured cells, produced during freeze-drying. According to our results, non-refrigerated storage of freeze-dried *B. infantis* 20088 can lead to a high rate of viability loss resultant efficacy lost of probiotic product.

Survival of freeze-dried cells in simulated infantile gastrointestinal tract conditions

Gastrointestinal tract (GIT) condition is the last important harsh stage before colonization of probiotics. Apparently, if the cells cannot survive during passage through it, even with a well efficiency of the products, would have no effect due to the final deterioration through host's digestion tract. Therefore produced probiotic cells should be tested for their ability to overcome the GIT conditions of the targeted consumer group. In this study, a sequential system with consideration of both gastric and intestinal stresses with all effective infantile secretions, transit time, temperature, pH and motion of peristalsis was assessed.

The results presented in Table 4 indicated that in simulated gastric conditions (SGC) at pH 3.0, initial numbers of *B. infantis* 20088 rapidly decreased by 5 log (CFU/ml), while the corresponding control samples showed about 1 log (CFU/ml) extra viability loss. The control samples' population after sequential exposure to simulated intestinal conditions (SIC), decreased to less than 10 CFU/ml (the detection limit), whereas there were still 1.81 log (CFU/ml) viable cells in the samples which had been freeze-dried in the optimal combination of drying medium.

With increasing the pH of SGC to 4.0, the survival rate of cells was also obviously increased. Generally, simulated conditions of the infantile stomach following by intestinal conditions led to a dramatic loss in the viable counts of control samples as compared to cells dried in optimal combination of cryoprotectants.

In addition to the decreased injured cell numbers which

Table 4. The effects of optimal combination of cryoprotectants skim milk and prebioticss on the survival rate of freeze-dried *B. infantis* 20088 after sequential incubation in simulated infantile gastric and intestinal conditions[a].

Freeze-drying medium	pH 3.0 [b]		pH 4.0 [b]	
	SGC	SGICs [c]	SGC	SGICs [d]
Optimized medium	$2.62\pm0.09\times10^{-3}$*	$7.65\pm0.05\times10^{-5}$*	$4.59\pm0.11\times10^{-2}$*	$2.98\pm0.07\times10^{-3}$*
Phosphate buffer (control)	$4.17\pm0.14\times10^{-4}$	CFU≤10	$9.83\pm0.12\times10^{-3}$	$6.64\pm0.06\times10^{-4}$

[a]The values are survival rate (mean of two replicates ± standard deviations); [b]The acidity of simulated gastric conditions (SGC); [c]Survival rate after sequential incubation in simulated gastric and intestinal conditions when the cells passed the SGC at pH 3.0; [d]Survival rate after sequential incubation in simulated gastric and intestinal conditions when the cells passed the SGC at pH 4.0; *Significant at 5% level with control samples in the same condition.

might be the reason for this improvement of viable cells over exposing to GIT conditions, the protection ability of drying medium through these simulated conditions was also considerable. Carrier foods can improve the stability of cells by a protective action (Zhao and Zhang, 2005) and/or by the presence of nutrients such as metabolizable sugars (Corcoran et al., 2005; Zhao and Zhang, 2005). Nowadays, there is increasing interest in developing new effective probiotics carriers such as prebiotics substances to enhance their stability. It is noted by Charteris et al. (1998) that the buffering capacity of ingested food which contains probiotics may tem-porarily increase the stomach pH and protect them from exposure to extreme pH values resulting less death rate of probiotics cells. Therefore prebiotics substances may be the most efficient components, because they are not digestible and degradable before delivery of cells to the lower part of the intestine. It is due to the fact that prebiotics are not dissolved or decomposed in the high acidity conditions of stomach, neutral pH and neither by the enzymatic activity of pancreas (Englyst et al., 1992; Sun and Griffiths, 2000; Mortazavian et al., 2007). This implies the potential application of carrier materials of probiotics to enhance the survival of cells during their passage through gastric acid and hence facilitate colonization on the intestine.

Conclusions and recommendations for future works

This is the first study that aimed to simulate synbiotic ingredients of breast milk for enriching infant formula based on clinical studies, and also to investigate the way of getting live bacteria stepwise to infant's gut. With this in mind, the clinical recommended prebiotics mixture (90% GOS: 10% FOS) for formulae fed infants' diet was incorporated into drying matrix for the first time in combination with skim milk to enhance the functional value of product and bacterial survival, simultaneously. For achieving the highest protection, the freeze-drying medium was optimized using RSM. The optimal combination was found to be 16.1% skim milk blended with 2.8% prebiotics which could protect 47.63% of cells' viability. In conclusion, this study indicated that the survival rate of *B. infantis* 20088 over freeze-drying could be enhanced by optimal combination of cryoprotectants prebiotics mixture/skim milk. The final reduced model of cryoprotective media also could improve the cells' viability over storage period and exposing to different simulated conditions of infantile gastrointestinal tract, significantly. However, more research is required about application of more efficient, synergetic, harmless and natural-like materials for supporting of probiotic cells over processing and subsequent harsh conditions.

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