

Improving the Growth and Stability Following of Lyophilized *Bifidobacterium breve* M4A and *Bifidobacterium longum* subsp. *longum* FA1 in Skimmed Milk Media

Mustafa Alsharafani¹, Sylvia Schnell², Stefan Ratering² and Michael Krawinkel^{1*}

¹Institute of Nutrition Science, Justus Liebig University Giessen, 35392 Giessen, Germany

²Institute of Applied Microbiology, Justus Liebig University Giessen, 35394 Giessen, Germany

Received: January 21, 2015; Accepted: May 11, 2015; Published: May 21, 2015

***Corresponding author:** M. Krawinkel, Institute of Nutrition Science, Justus-Liebig-University Giessen, Wilhelmstrasse, 20, Germany, Tel: +49-0-641/99-39048; Fax: +49-0-641/99-39039; E-mail: krawinkel@fb09.uni-giessen.de

Abstract

Among commensal bacteria, Bifidobacteria are one of the most numerous probiotics in the mammalian gut. Two strains of *Bifidobacterium* (*B*) were isolated from stool of breastfed infants and identified by their 16S rRNA genes. The strains were identical to *B. longum* subsp. *longum* FA1 and *B. breve* M4A. These isolates were conserved by lyophilisation, and the survival rates measured six months after lyophilisation, were 74.74% and 99.6% for *B. longum* subsp. *longum* FA1 and *B. breve* M4A, respectively. To improve the survival rate in skimmed milk media used for lyophilisation, the skimmed milk was supplemented with yeast extract, glucose and oligofructose. Supplementation decreased the doubling time and increased the viable cell count. Statistical analyses showed a nonlinear relationship between skimmed milk media and bacterial count number and acid production, which indicates an opposite impact of yeast extract with glucose or oligofructose supplementation in skimmed milk media. Response surface plots were applied to optimize the supplemented skimmed milk media. Both strains grew more rapidly in supplemented media than in skimmed milk-based medium alone. Acid production was higher in *B. breve* M4A than *B. longum* subsp. *longum* FA1 when inoculated into skimmed milk supplemented with yeast extract, glucose, and oligofructose. The fermentation ability in skimmed milk was dependent on nutrient availability and the carbon source, which decreased the doubling time and increased the viable cell count. The addition of yeast extract, glucose, and oligofructose to skimmed milk increased the growth rate and acid production, compared with skimmed milk alone, when incubated anaerobically at 37°C for 48 h. The purpose of this study was to evaluate the potential synergy between *Bifidobacterium* isolates with yeast extract, glucose, and oligofructose in skimmed milk after 6 months of conservation.

Keywords: Improvement; Bifidobacteria; Doubling Time; Acid Production; Oligofructose

Abbreviation

ANOVA: Analysis of Variance; *B*: *Bifidobacterium*; CFU: Colony Forming Unit; EDTA: Ethylenediaminetetraacetic acid; FOS: Fructooligosaccharids; G: Glucose; LSD: Least Significant Differences Test; MRS: de Man, Rogosa and Sharpe; Olig: oligofructose; YE: Yeast Extract

Introduction

Dairy products, especially fermented milk are considered as the best vehicle to deliver bifidobacteria to humans. Although milk is a nutritional medium for the growth of bifidobacteria, amino acids and low molecular weight peptides are only present at low concentrations. This results in the slow growth of these bacteria in milk. Many approaches have been applied to promote the development of various bifidobacteria for faster milk fermentation. The ability of organisms to grow well in milk depends on their capacity to metabolize milk protein and lactose, and this ability varies considerably among strains [1]. The yeast extract may contribute to the growth stimulation of bifidobacteria and also to the improvement of these bacteria viability in dairy products. Yeast extract is an effective growth promoter and is most often used at concentrations that vary between 0.1 and 0.3% (v/v) [2]. Regulatory authorities around the world are looking for assurance that a probiotic product can deliver viable starter organisms at sufficient number to the large intestine to provide a benefit to the consumer.

Various authors have reported that the viability of bifidobacteria is often low in fermented dairy foods [3]. Bifidobacteria have been associated with health-promoting effects and thus have been incorporated into fermented dairy foods and other dairy foods. The ultimate intent of this strategy is to provide the gastrointestinal tract of humans with elevated viable populations of bifidobacteria. Thus, viability of bifidobacteria in dairy products has received much attention [4]. The aim of the study was to improve the growth of *B. breve* M4A and *B. longum* subsp. *longum* FA1 in skimmed milk media supplemented with yeast extract, glucose and oligofructose on different levels that increase count number after conservation.

Methods

Sampling and isolation

From twelve healthy infants, samples were collected from feces of the breastfed infants. The sampling approved of children

hospital (Kinderklinik Giessen), and parent agreements were taken. None of the children suffered from gastrointestinal or any major illness or are received antibiotic therapy. Tenfold serial dilutions from the samples were prepared ranged from 10^{-5} to 10^{-7} using pour plate technique performed on de Man, Rogosa and Sharpe (MRS) selective medium (Merck, Darmstadt, Germany) supplemented with 80 mg/L mupirocin and 0.5 g/L L-Cysteine-HCl (Sigma, Japan) [5]. All plates were incubated anaerobically using anaerobic gas jar (BBL-Difco, USA) and Anaerocult A® (Merk-Darmstadt, Germany). Plates were incubated anaerobically with gas pack Anaerocult A® at 37°C for 3 days. Viability was expressed as log₁₀ CFU/mL.

Identification of bifidobacteria

After incubation, opaque white glossy colonies on MRS selective medium were counted, and the morphology of bifidobacteria growth was examined by phase contrast microscope (Axiophot 2, Carl Zeiss Microscopy-Oberkochen, Germany). Several typical colonies (2-5 mm diameter) were picked randomly from plates, and sub-cultured by streaking into MRS selective medium was made. Plates were incubated anaerobically at 37°C for three days. Two bifidobacteria isolates from two different samples were chosen for the further investigation. These two isolates were identified as *B. breve* M4A and *B. longum* subsp. *longum* FA1 using amplification and subsequently sequencing of the 16S rRNA gene using the oligonucleotide primers EUB9F (5'-GAGTTTGATCMTGGCTCAG-3') and EUB1492R (5'-ACGGYTACCTGTACGACTT-3') [6]. The PCR reactions were performed as described by Kampmann et al. [7] and the amplicons were sequenced by the company LGC Genomics (Berlin, Germany). Next relatives' sequences were obtained by homology searches using BLAST algorithm available at <http://blast.ncbi.nlm.nih.gov/Blast.cgi> and the NCBI database. The accession number was CP006716.1 and KM577186.1 for *B. breve* M4A and *B. longum* subsp. *longum* FA1, respectively. The *B. longum* subsp. *longum* FA1 was confirmed identification under subspecies with biochemical test of carbohydrate fermentation ability that was grown on basal medium.

Biochemical test of carbohydrate fermentation ability

Bifidobacteria subspecies were identified by grown on basal medium. The medium consisted of (g/L): peptone water (Oxoid Ltd. Basingstoke, UK) 2, yeast extract (Oxoid) 2; NaCl, 0.1; K₂HPO₄, 0.04; KH₂PO₄, 0.04; MgSO₄·7H₂O, 0.01; CaCl₂·6H₂O, 0.01; NaHCO₃, 2; L-Cysteine HCl (Sigma) 0.5, and (1% w/v) various media were tested with Glucose, L-Arabinose, Ribose, Oligofructose and Inulin (filter sterilized and added after autoclaving). The component media were dissolved in 10 ml bottles and then were flashed with CO₂/N₂ gas for 5 min and closed by stoppers. The bottle media were then autoclaved at 121°C for 20 minutes, once are cooled carbohydrates were filtered into add to the media to make an overall concentration of 1% (w/v). From MRS broth medium grown, one ml of *B. longum* subsp. *longum* FA1 was injected aseptically into each tube and incubated at 37°C. Each species was grown on each carbohydrate in triplicate. A blank containing medium with no added carbohydrate was also

inoculated for each Bifidobacteria species in triplicate, in order to take into account any carryover of glucose. The growth was measured at a wavelength of (650 nm) at 0, and 72 hours post inoculation [8].

Samples preparation for freeze-drying

Freeze-dried cultures of *B. breve* M4A and *B. longum* subsp. *longum* FA1 were used for the preparation of the dosage. Each of 100 mL MRS broth (Merck) culture of both strains in their stationary phase was centrifuged (Biofuge primor, Heraeus, Germany) at 2500×g for 10 min at 4°C. The supernatant was discarded, and the concentrated cells were resuspended in 20 mL of skimmed milk medium supplemented with 5% glucose (both Merck). The medium was steamed at 100°C for 30 min (Medizin & Lab. Tech. Fritz Goessner, Germany). The suspension was separated in 1 mL for each vial and the vials were frozen at -18°C overnight. Subsequently, the frozen vials were lyophilized (Lyova GT2, Steris, Germany) at -18°C, under vacuum (-2.7 mbar) for 14 hour [9]. After freeze drying, the vials were sealed under vacuum and stored in a refrigerator.

After 6 months of storage, growth activity of freeze-dried strains was measured by enumeration on MRS agar plates. Freeze-dried preparations were rehydrated with 1 mL of sterile water into each lyophilized vial for 30 min, diluted and plated. After incubation of the plates under anaerobic conditions described above, the survival rates of the cultures after lyophilization were estimated in terms of the number of surviving cell counts recorded as colony forming units per mL (CFU/mL). The results were arithmetic means of three measurements.

Optimization of bifidobacteria growth and skimmed milk media performance

Selection of enhanced growth media of Bifidobacteria strains: For growth improvement and better survival of *B. longum* subsp. *longum* FA1 and *B. breve* M4A the strains were cultured in 10% reconstituted skimmed milk media which was supplemented with different concentrations of two carbohydrates and yeast extract. For this skimmed milk (10%) powder dissolved in distilled water and were supplemented with 0.3% or 0.6% yeast extract, and oligofructose or glucose in different levels (1%, 2% and 3%). Skim powder (10%) without the addition was used as control. The skimmed milk media were heat treated at 110°C for 10 min. Skimmed milk media were inoculated with a 10% (v/v) *B. longum* subsp. *longum* FA1 and *B. breve* M4A culture after activation of lyophilized culture and were incubated anaerobically for 48 h at 37°C. Skimmed milk media samples were withdrawn for pH and turbidity measurements. For the enumeration of viable cells, culture samples in skimmed milk media were serially diluted in 0.1% (w/v) sterile pre-reduced peptone water and plated in duplicate onto MRS agar. Plates were incubated anaerobically at 37°C for 3 days. Viability was expressed as log₁₀ CFU/mL.

Growth density and mean doubling time determination: An aliquot of the culture in skimmed milk was taken at intervals (1,5,25,35,45 hours) and diluted (1:10 v/v) with 0.2% (w/v)

EDTA, (pH 12.0), and turbidity was measured (Thermo-Spectronic-Genesys 10UV, Fisher, USA) at 640 nm. The EDTA, at pH 12, chelates the calcium ions and disaggregate casein micelles, resulting in a fast loss of opacity from solubilisation of the milk solids and depleted casein micelles [10]. Un-inoculated milk media were diluted with 0.2% (w/v) EDTA and used as the blank. Specific growth rate (μ) for each culture was calculated using the following equation: $\mu = (\ln X_2 - \ln X_1) / (t_2 - t_1)$, where X_2 and X_1 are the cell density at time t_2 and t_1 . Doubling time (Td) was calculated as: $Td = \ln_2 / \mu$ [4].

Measurement count number associated supplemented media: As a matter, the desired number of bifidobacterium strains was used by supplementation with carbohydrates to enhance the growth and survival. Skimmed milk powder containing 10% solids (Merck) was reconstituted in distilled water. Skimmed milk base (containing 0.3% yeast extract) was supplemented with 3% (w/v) oligofructose for culture of *B. longum* subsp. *longum* FA1 and 3% glucose for *B. breve* M4A. The culture media were closed with stoppers and cramps. Each bottle contained 10 mL skimmed milk media and CO_2/N_2 gas was flashed the bottles, and sterilized by autoclaving at 121°C for 10min. The bottles were inoculated with 10% (v/v) *B. longum* subsp. *longum* FA1 or *B. breve* M4A cultures after the activation of each pure Lyophilized culture for 24 h. The cultures were incubated anaerobically at 37°C, and the pH was regulated: ~5 mL samples were withdrawn every 16 h for analysis.

Statistical analysis and experiment design: All experiments were repeated three (two for doubling time) times. The analyses were performed in duplicate. Statistical analyses were performed using SPSS version 22.0.0.1 (IBM software, USA). Analyses of Variance (ANOVA) was used for multiple comparisons for doubling time, and $p < 0.05$ and $p < 0.01$ were considered statistically significant and highly significant, respectively. The post hoc Least Significant Difference (LSD) test was chosen for homogenous variances. To assess the relationship between yeast extract, oligofructose, glucose and the doubling time we used three factorial ANOVA making use of the mixed procedure in SPSS to account for heterogeneity of variances in an unbalanced design.

The mediation analysis of indirect effects media supplemented with yeast extract, glucose and oligofructose affected on count number and acid production. The indirect influence of the skimmed milk media affected on pH value in such nonlinear model. The relationship were evaluated between Medium (yeast extract, oligofructose and glucose) on pH via count number as a mediator using multiple, nonlinear regression models. Multiple Regression analyses of dependent factors of pH value and count

number. The factorial design of our analysis was nonlinear relationship between independent and dependent variables.

$$y = a + b_1 * x_1 + b_2 * x_1^2 + b_3 * x_2 + e \quad (1)$$

$$y = a + b_1 * x_1 + b_2 * x_1^2 + e \quad (2)$$

with y = dependent variable (here: pH), a = constant, e = error term, $b_1 \dots b_3$ = regression coefficients, $x_1 \dots x_2$ = independent variables (glucose and oligofructose and count number), $x_1^2 = x_1$ squared (power term for nonlinear part of the model). The unbalanced present or not some ingredient and more or less concentration of glucose, and oligofructose with heterogeneity of variance, therefore, we cannot run p value and trust with it. Another ANOVA was run to its hidden and mix procedure accounting for the heterogeneity of ANOVA and correcting the p value.

Results

Lyophilization procedure sustained acceptable survival rate

The CFU of the MRS broth culture before lyophilisation was 6.77 and 6.87 log CFU/mL for *B. longum* subsp. *longum* FA1 and *B. breve* M4A, respectively (Table 1). The survival rates were 71.8% and 81.5% for *B. longum* subsp. *longum* FA1 and *B. breve* M4A, respectively. The number of viable cell counts after six months storage were 5.06 and 6.84 log CFU/mL for *B. longum* subsp. *longum* FA1 and *B. breve* M4A, respectively. The survival rates after six months storage were 74.7% and 99.6% for *B. longum* subsp. *longum* FA1 and *B. breve* M4A, respectively (Table 1).

pH reduction by utilization of yeast extract, glucose and oligofructose

Figure 1 depicts continuous reduction of pH during a 48 h incubation period for *B. breve* M4A. The pH in the control was 4.71, whereas figure 2 shows that with all treatments pH decreased to less than 4. For *B. longum* subsp. *longum* FA1, the pH in the control was 5.03, whereas the pH with all treatments decreased to 4 (Figure 1 and Figure 2).

Doubling time was improved by the use of yeast extract, glucose and oligofructose supplemented medium

Doubling time was used as a measure of the effects of yeast extract, glucose, and oligofructose concentrations on growth density of bifidobacteria. The enhancement of the growth rates of *B. longum* subsp. *longum* FA1 and *B. breve* M4A were evidenced by significantly reduced doubling times compared with the controls. As depicted in figure 3 there was a dose-dependent growth improvement of *B. longum* subsp. *longum* FA1 secondary

Table 1: Viability of *B. longum* subsp. *longum* FA1 and *B. breve* M4A before and after lyophilisation (log CFU/mL) and survival rates.

Culture	Viability before lyophilisation*	Viability after lyophilisation	Survival rate before Storage %	Viability After 6 months	Survival rate after storage %
<i>B. longum</i> subsp. <i>longum</i> FA1	6.77	4.86	71.78	5.06	74.74
<i>B. breve</i> M4A	6.87	5.60	81.51	6.84	99.6

* Lyophilisation at pH = 4

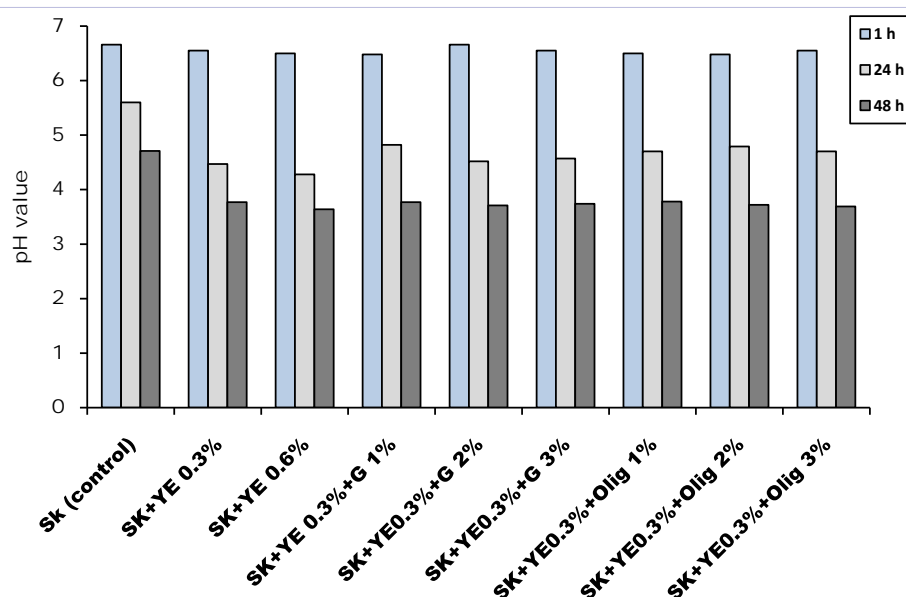


Figure 1: pH values of *B. breve* M4A after 48 h of incubation at 37°C M4A in skimmed milk media. SK = Skimmed Milk media containing variations concentrations of YE = Yeast Extract, G = Glucose, and Olig = Oligofructose. Each histogram bar represents the mean value of duplicate.

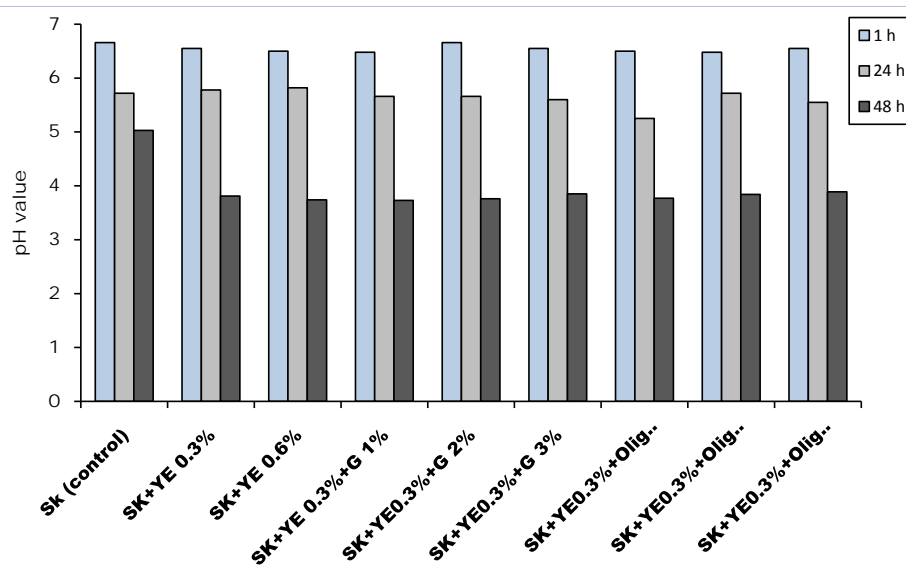


Figure 2: pH values of *B. longum subsp. longum* FA1 after 48 h of incubation at 37°C M4A in skimmed milk media. SK = Skimmed Milk media containing variations concentrations of YE = Yeast Extract, G = Glucose, and Olig = Oligofructose. Each histogram bar represents the mean value of duplicate.

to 0.6% yeast extract and 0.3% yeast extract supplemented with either glucose or oligofructose at 1%, 2%, and 3%. Among the carbohydrate sources tested, treatment with skimmed milk + 0.3% yeast extract + 3% oligofructose was the most effective, resulting in a significantly reduced doubling time. All treatments involving supplementation with oligofructose resulted in a significantly decreased doubling time compared with treatments involved supplementation with glucose and yeast extract.

Figure 4 showed the enhancement of the growth of *B. breve* M4A by various supplements. Among the carbohydrate sources tested, skimmed milk + 0.3% yeast extract + 3% glucose provided the shortest mean doubling time, and oligofructose resulted in

a significantly reduced doubling time compared with treatment involving yeast extract as the sole supplement and skimmed milk + 0.3% yeast extract + 1% glucose (Figure 3 and figure 4).

Viable cell number was improved in yeast extract, glucose and oligofructose medium

The highest viable cell number achieved by *B. longum subsp. longum* FA1 was 8.21 log CFU/ mL with the skimmed milk + 0.3% yeast extract + 1% oligofructose treatment and the lowest viable cell number was 7.70 log CFU/ mL for the skimmed milk (Control), followed by 8.11, 8.04, 8.03, 8.01, 7.96, 7.95 and 7.86 log CFU/ mL for the skimmed milk + 0.3% yeast extract + 3%

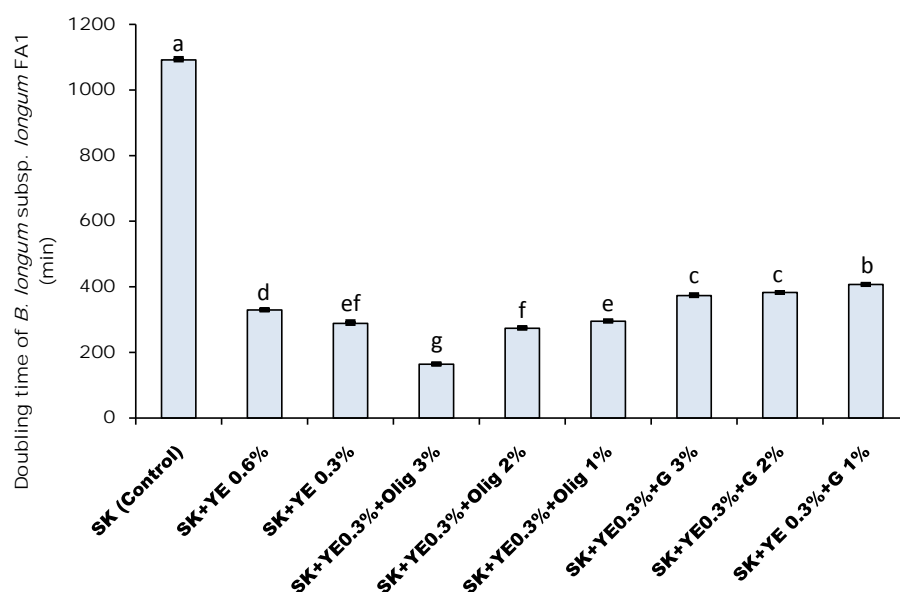


Figure 3: Doubling time of *B. longum* subsp. *longum* FA1 in SK = Skimmed Milk media containing different concentrations of YE = Yeast Extract, G = Glucose, and Olig = oligofructose. Different letters indicated highly significant ($p < 0.01$) differences among treatment means (mean \pm SD).

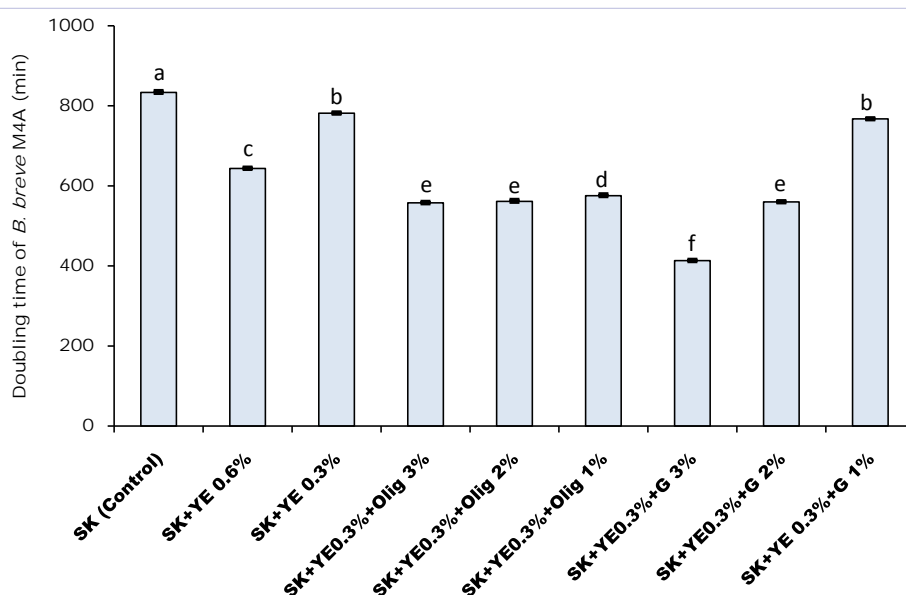


Figure 4: Doubling time of *B. breve* M4A in SK = Skimmed Milk media containing different concentrations of YE = Yeast Extract, G = Glucose, and Olig = Oligofructose. Different letters indicated highly significant ($p < 0.01$) differences among treatment means (mean \pm SD).

glucose; skimmed milk + 0.3% yeast extract + 2% oligofructose; skimmed milk + 0.3% yeast extract + 3% oligofructose; skimmed milk + 0.6% yeast extract; skimmed milk + 0.3% yeast extract + 1% glucose; skimmed milk + 0.3% yeast extract + 2% glucose and skimmed milk + 0.3% yeast extract, treatments, respectively (Figure 3). The highest viable cell number achieved with *B. breve* M4A was 8.46 log CFU/ mL in the skimmed milk + 0.3% yeast extract + 2% oligofructose treatment and the lowest viable cell number was 8.17 for the skimmed milk (Control), followed by 8.43, 8.42, 8.42, 8.41, 8.40, 8.33, 8.30, log CFU/ mL for the skimmed milk + 0.3% yeast extract + 3% oligofructose;

skimmed milk + 0.6% yeast extract; skimmed milk + 0.3% yeast extract + 2% glucose; skimmed milk + 0.3% yeast extract + 1% oligofructose; skimmed milk + 0.3% yeast extract + 3% glucose; skimmed milk + 0.3% yeast extract and skimmed milk + 0.3% yeast extract + 1% glucose treatments, respectively (Figure 5).

Optimization surface response to the supplemented media

The response surface plots in figure 6 showed the relationship between the count number (log CFU/mL) of *B. longum* subsp. *longum* FA1 and different media. The maximum count number

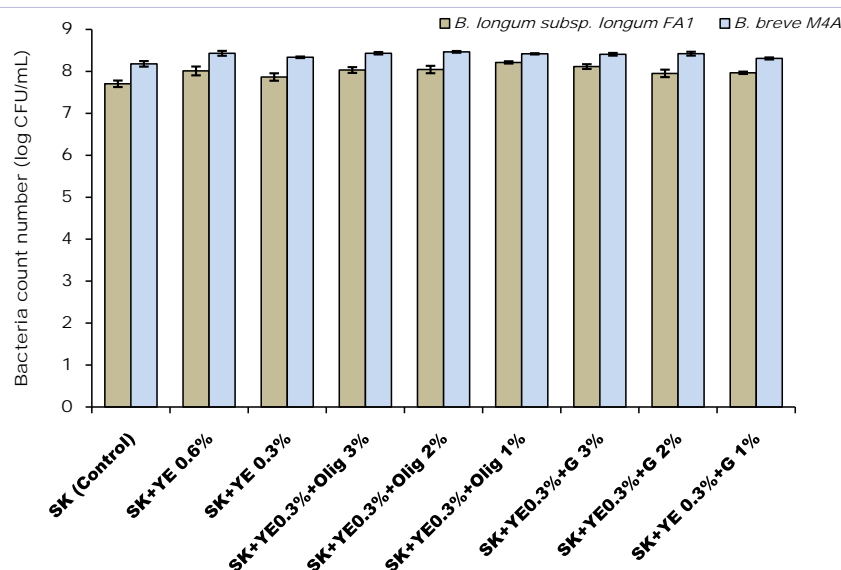


Figure 5: Treatments with supplemented skimmed milk media resulted in significant increases ($p < 0.01$) in viable cell numbers of both *B. breve* M4A and *B. longum* subsp. *longum* FA1 compared with skimmed milk medium alone (control). SK = Skimmed Milk, YE = Yeast extract, G = glucose, Olig = Oligofructose (mean \pm SD).

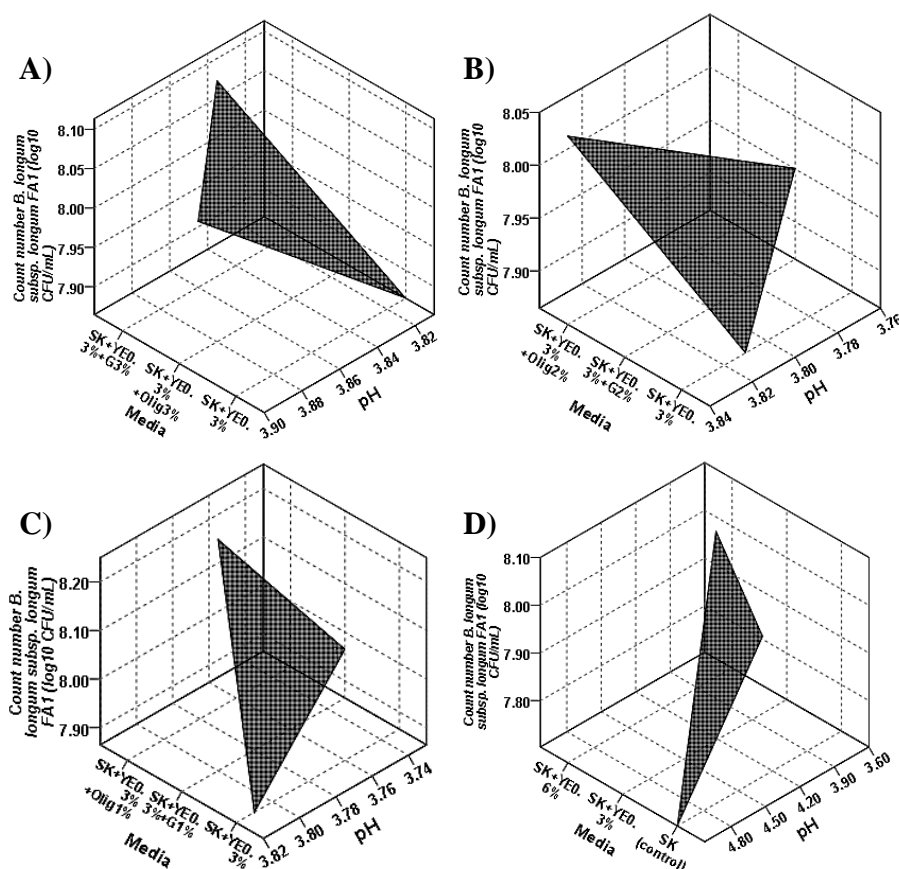


Figure 6: Response surface plots for the count number (log CFU/mL) of *B. longum* subsp. *longum* FA1 with different media. The response surface factors are; **(a):** SK + YE0.3 + Olig1%, SK + YE0.3 + G1% and SK + YE0.3% media, and the factor the pH. **(b):** SK + YE0.3 + Olig2%, SK + YE0.3 + G2% and SK + YE0.3% media, and the factor the pH. **(c):** SK + YE0.3 + Olig3%, SK + YE0.3 + G3% and SK + YE0.3% media, and the factor the pH. **(d):** SK, SK + YE0.3% and SK + YE0.6% media.

(SK; Skimmed milk, YE; yeast extract, G; glucose, Olig; oligofructose).

8.21 and 7.70 (log CFU/mL) of *B. longum* subsp. *longum* FA1 was obtained from glucose 3% followed oligofructose 1% supplemented medium, respectively (Figure 6). The highest acid production and the highest count number of the surface plot shown in figure 6a whereas the lowest levels of acid production and count number of the surface plot illustrated in figure 6b, figure 6c and figure 6d were the media with lower carbohydrate concentration and without carbohydrates.

Figure 7 showed response surface plot representing an optimized count number 8.46 and 8.43 (log CFU/mL) of *B. breve* M4A (Figure 7a) attributed to the oligofructose 3% and glucose 3% supplemented media, respectively. The supplemented skimmed milk media with 1% and 2% of carbohydrates showed in figure 7b, figure 7c and figure 7d had been less improvement of the pH values as well as count number.

Discussion

The pre-results of isolation showed that the two strains of *B. longum* subsp. *longum* FA1 and *B. breve* M4A grew faster than others and were selected for further studies.

Turbidity measurement is frequently used to determine growth rate, although turbidity measurement determines both viable and dead cells. Measuring turbidity is much faster than the direct count number such as standard plate count. For accurate determination, the viable count number is much more sensitive than turbidimetric measurement. In the present study the count number was used to measure growth in supplemented media. Anyway, the doubling time measurement method of bacterial growth is relatively easy when the measuring frequent daily achieved regarding to administer *B. longum* subsp. *longum* FA1 and *B. breve* M4A to the animal fed high fat diet (unpublished data). The culture media of *B. longum* subsp. *longum* FA1 and *B. breve* M4A were daily given to the animal trial with the anti-obesity activity reduced gain weight of bifidobacteria strains of young mice fed a high-fat diet.

Bifidobacteria are used as probiotics for the prevention and treatment of pathologies typical of newborns, such as necrotizing enterocolitis, colics, and streptococcal infections. Furthermore, allergic diseases, celiac disease, obesity, and neurologic diseases represent new potential opportunities for probiotic application of bifidobacteria [11]. Short-chain fatty acids are the major end products of carbohydrate metabolism in bifidobacteria, which could lead to lower pH and the growth of harmful organisms in the intestine as well as to an increase in intestinal wavelike movements that push the contents of the gut forward which could help to remove any potential pathogens [12]. In the present study, two strains of bacteria were used because of their origin from the human gut of breastfed infants. Several observations have been reported with *B. longum* and *B. breve*; therefore both strains were studied. These isolates were chosen for higher viability as recommended for probiotic microorganisms [13-15].

We found high differences between two bifidobacteria strains used regarding to the acid production in all tested media supplemented with different concentration of yeast extract,

glucose and oligofructose, as compared with the controls (Figure 1 and Figure 2).

Shin and colleagues [4] found significance decreased mean doubling time in three different skimmed milk media supplemented with 1% fructo-oligosaccharide, 3% galacto-oligosaccharide and 5% inulin for two commercial strains of *B. bifidum* (Bf-1 and Bf-6). Bărăscu et al. [16] monitored the growth rate of bifidobacteria, in milk supplemented with yeast extract, after 24 hour incubation time. The cell number in the presence of 0.1, 0.2, 0.3, and 0.4% yeast extract increased stepwise compared to culture grown in basal medium (Bărăscu et al. [16]). Highest CFU of bifidobacteria were obtained with basal medium plus 0.4% yeast extract (32×10^8 cfu/cm³) which was 4 times higher than with basal medium (8×10^8 cfu/cm³).

Improved culture conditions for *Bifidobacterium* strains

The viability of bifidobacteria has a limited shelf life in the conventional media. For the long-term preservation, lyophilisation has been the classical way of the preservation [17]. Viable cell counts of *B. breve* M4A and *B. longum* subsp. *longum* FA1 following lyophilisation and conservation for 6 months suggested acceptable survival rates. Skimmed milk fermentation increased the potential for acid production and decreased the doubling time in the presence of yeast extract, glucose, and oligofructose. Skimmed milk media supplemented with yeast extract and glucose or oligofructose were effective in improving the growth of *B. breve* M4A and *B. longum* subsp. *longum* FA1, depending on the carbohydrate concentration and *Bifidobacterium* strain used. Bărăscu and colleagues [16] improved the number of bifidobacteria in fermented milk with nutrients supplementation. When supplemented fermented milk with 0.3% yeast extract or 1.5% wheat germ extract within 24 h incubation. The growth was improved from 7.8×10^8 to 3.3×10^9 CFU/mL of the control and 0.3% yeast extract, respectively. The growth was improved from 1.6×10^9 to 8.5×10^9 CFU/mL of the control and 1.5% wheat germ extract, respectively.

Several studies have suggested that the existence of bifidobacteria is usually not high in most dairy products due to low pH [2,18]. In figure 1 and figure 2, the pH values at 1 h and 24 h were optimal for biomass production of different media. During the fermentation process, the pH decreased due to the accumulation of organic acids. This accumulation of organic acids inhibited the growth of bifidobacteria and consequently limited the production of more organic acids even though the media were improved with yeast extract and carbohydrate. Therefore, the acid concentration remained constant at the end of the fermentation time.

As illustrated in figure 5, the supplemented skimmed milk treatments increased the viable cell counts of both *B. breve* M4A and *B. longum* subsp. *longum* FA1 highly significantly ($p < 0.01$) in comparison with the controls. The highest viable cell number (CFU) of *B. longum* subsp. *longum* FA1 was observed with 1% oligofructose-supplemented treatment and was not influenced by higher oligofructose concentrations. In contrast, the effects

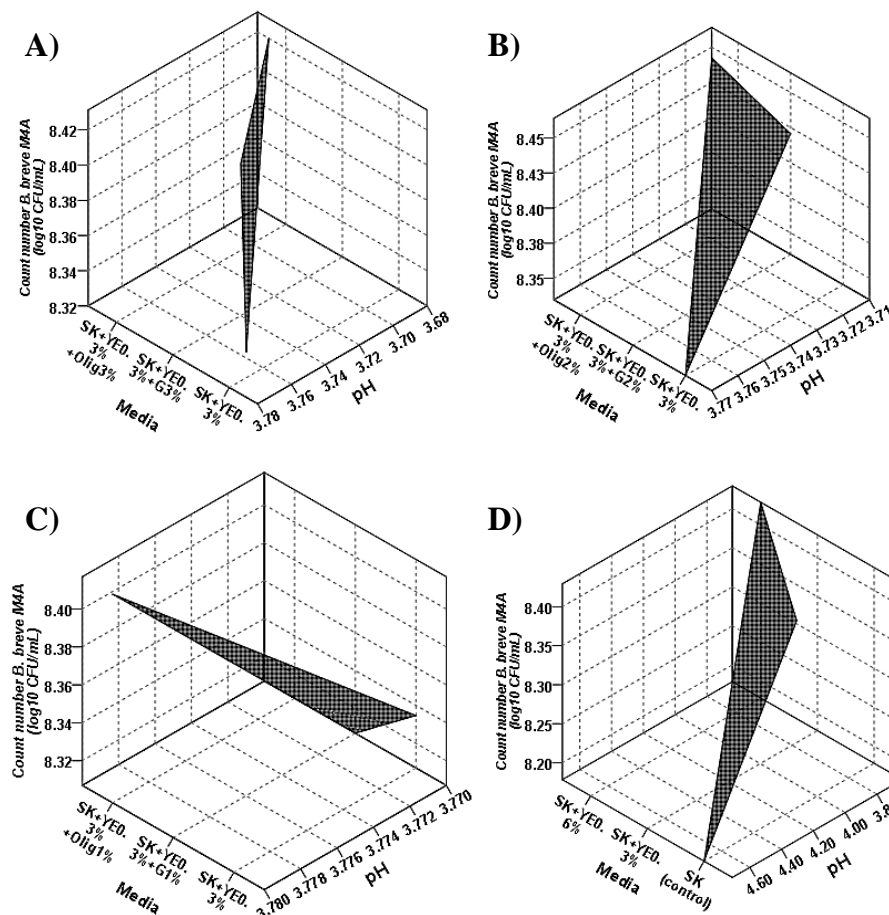


Figure 7: Response surface plots for the count number (log CFU/mL) of *B. breve* M4A with different media. The response surface factors are; **(a):** SK + YE0.3 + Olig1%, SK + YE0.3 + G1% and SK + YE0.3% media, and the factor the pH. **(b):** SK + YE0.3 + Olig2%, SK + YE0.3 + G2% and SK + YE0.3% media, and the factor the pH. **(c):** SK + YE0.3 + Olig3%, SK + YE0.3 + G3% and SK + YE0.3% media, and the factor the pH. **(d):** SK, SK + YE0.3% and SK + YE0.6% media.

(SK; Skimmed milk, YE; yeast extract, G; glucose, Olig; oligofructose).

of glucose-supplemented treatment increased with increasing concentration and were maximal in 3% glucose. For *B. breve* M4A, the highest viable cell number (CFU) was observed with treatment supplemented with 2% oligofructose. The viable cell number of *B. breve* increased with increasing oligofructose and glucose concentrations. Numbers of viable cells of both *B. longum* subsp. *longum* FA1 and *B. breve* M4A increased upon supplementation with 0.6% yeast extract compared with 0.3% yeast extract. In the other study it was suggested that each *B. breve* strain might have different carbohydrate utilization capabilities. The genetic accessibility and murine colonization capacity of this strain make it a valuable model for understanding bifidobacterial–host interactions in the gut [19]. Dubey and Mistry [20] reported that maximal counts and generation times of *B. breve*, *B. bifidum*, *B. infantis*, *B. longum* were not influenced by 0.5% fructooligosaccharides (FOS) when these organisms were grown in infant formula, but they did not investigate concentrations of FOS above 0.5%. Consistent with their findings, Shin et al. [4] observed significant differences (compared to the control) in mean doubling times when either *Bifidobacterium* Bf-1

or Bf-6 was grown in the presence of 0.5% fructooligosaccharide. The yeast extract represents a valuable source of nutrients and growth factors, being used in the majority culture medium of bacteria. Through the valuable content of amino acids, the yeast extract may contribute to the growth stimulation of Bifidobacteria and also to the improvement viability in dairy products [16].

Optimization surface response to the factors

Different levels of substrates supplemented skimmed milk media were affected on the cell growth of bifidobacteria and acid production. The mediation analysis of indirect effects skimmed milk media supplemented with yeast extract, glucose and oligofructose influenced on count number and pH value. The relationship was evaluated among supplemented media with different levels on pH via count number as a mediator using multiple, nonlinear regression models. The design not allowed to study interaction effect in such model like Plackett-Burman design [21]. The response surface plots showed that the maximum count number of *B. longum* subsp. *longum* FA1 and *B. breve* M4A was achieved at 48 h when the pH reached 4. For

every concentration levels increased of carbohydrates, the count number was increased both of *B. longum* subsp. *longum* FA1 and *breve* M4A whereas the yeast extract levels alone presented less influence toward count number. The optimal growth of *B. longum* subsp. *longum* FA1 was obtained from skimmed milk media supplemented 3% glucose, while skimmed milk media supplemented 3% oligofructose was more efficiently optimized growth of *B. breve* M4A. This result indicated that interactions between skim milk supplemented yeast extract, and glucose or oligofructose might have a stronger influence on growth rate of bifidobacteria. Consequently, the use of high carbohydrates level (3%) in skim milk media significantly improved the pH value and the count number.

In conclusion, viable cell counts of *B. breve* M4A and *B. longum* subsp. *longum* FA1 following lyophilisation and conservation for 6 months suggested acceptable survival rates. Skimmed milk fermentation increased the potential for acid production and decreased the doubling time in the presence of yeast extract, glucose, and oligofructose. Skimmed milk media supplemented with yeast extract and glucose or oligofructose were effective in improving the growth of *B. breve* M4A and *B. longum* subsp. *longum* FA1, depending on the carbohydrate concentration and *Bifidobacterium* strain used. Improved protocols for bifidobacteria fermentation and lyophilisation might be helpful for upscale and industrial production of bifidobacteria supplemented food.

Acknowledgement

Statement of authors' contributions to the manuscript

The authors listed below have certified that they meet the criteria for authorship. They have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication. All authors read and approved the final manuscript. Mustafa Alsharafani had responsibility for all parts of the manuscript.

- Mustafa Alsharafani planned and performed the experiments, statistical analyses, drafted the manuscript and had primary responsibility for the final content.
- Sylvia Schnell and Stefan Ratering supported the microbiological analysis, biochemical and molecular analysis and revised the drafted manuscript.
- Michael Krawinkel supervised the research of Mr. Alsharafani and contributed to the manuscript.

Declaration

The authors declares no conflict of interest. This article does not contain any studies with human subjects.

References

- Ostlie HM, Hell MH, Narvhus JA. Growth and metabolism of selected strains of probiotic bacteria in milk. *Int J Food Microbiol.* 2003; 87(1-2): 17-27.
- Stephenei W, Kabeir BM, Shuhaimi M, Rosfarizan, Yazid AM. Growth Optimization of a Probiotic Candidate, *Bifidobacterium pseudocatenulatum* G4, in Milk Medium Using Response Surface Methodology. *Biotechnology and Bioprocess Engineering.* 2007; 12(2): 106-13.
- Adhikari K, Mustapha A, Gruen IU, Fernando L. Viability of microencapsulated *Bifidobacteria* in set yogurt during refrigerated storage. *J Dairy Sci.* 2000; 83(9): 1946-1951.
- Shin HS, Lee JH, Pestka JJ, Ustunol Z. Growth and Viability of Commercial *Bifidobacterium* spp in Skimmed milk Containing Oligosaccharides and Inulin. *J Food Sci.* 2000; 65(5): 884-887.
- Ferraris L, Aires J, Dupriet AJW, Butel MJ. New selective medium for selection of bifidobacteria from human feces. *Anaerobe.* 2010; 16(4): 469-471. doi: 10.1016/j.anaerobe.2010.03.008.
- Lane DJ. 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M. Eds. *Nucleic acid techniques in bacteria systematics.* New York; John Wiley & Sons Inc, 1991.
- Kampmann K, Ratering S, Kramer I, Schmidt M, Zerr W, Schnell S. Unexpected stability of Bacteroidetes and Firmicutes communities in laboratory biogas reactors fed with different defined substrates. *Appl Environ Microbiol.* 2012; 78 (7): 2106-2119. doi: 10.1128/AEM.06394-11.
- Palframan RJ, Gibson GR, Rastall RA. Carbohydrate Preferences of *Bifidobacterium* Species Isolated from the Human Gut. *Curr Issues Intest Microbiol.* 2003; 4(2): 71-5.
- Milanovic NT, Kodzic A, Baras J, Brankovi SD. The influence of a cryoprotective medium containing glycerol on the lyophilisation of lactic acid bacteria. *J Serb Chem Soc.* 2001; 66: 435-41.
- Hughes DB, Hoover DG. Viability and enzymic activity of bifidobacteria in milk. *J. Dairy Sci.* 1995; 78(2):268-276.
- Di Gioia D, Aloisio I, Mazzola G, Biavati B. Bifidobacteria: their impact on gut microbiota composition and their applications as probiotics in infants. *Appl Microbiol Biotechnol.* 2014; 98(2): 563-77. doi: 10.1007/s00253-013-5405-9.
- Matsuki, T, Pe'dron T, Regnault B, Mulet C, Hara T, Sansonetti PJ. Epithelial Cell Proliferation Arrest Induced by Lactate and Acetate from *Lactobacillus casei* and *Bifidobacterium breve*. *Plos One.* 2013; 8(4): e63053-8. doi: 10.1371/journal.pone.0063053.
- Picard C, Fioramonti J, Francois A, Robinson T, Neat F, Matuchansky C. Review article: bifidobacteria as probiotic agents – physiological effects and clinical benefits. *Aliment Pharmacol Ther.* 2005; 22(6): 495-512. doi: 10.1111/j.1365-2036.2005.02615.x.
- Vitali B, Ndagijimana M, Cruciani F, Carnevali P, Candela M, Elisabetta, et al. Impact of a synbiotic food on the gut microbial ecology and metabolic profiles. *BMC Microbiology.* 2010; 10 (7): 1-13. doi: 10.1186/1471-2180-10-4.
- Yoshida Y, Seki T, Matsunaka H, Watanabe T, Shindo M, Nanako Y, et al. Clinical Effects of Probiotic *Bifidobacterium breve* supplementation in Adult Patients with Atopic Dermatitis. *Yonago Acta medica.* 2010; 53(2): 37-45.
- Bărăscu E, Iordan M, Ciumac J. Growth rate of bifidobacteria in milk supplemented with yeast extract and wheat germs. *Fascicle VIII.* 2007; 1-5.
- Heckly RJ. Principles of preserving bacteria by freeze-drying. Oakland: University of California; 1985.
- Shigwedha N, Ji L. *Bifidobacterium* in Human GI Tract: Screening, Isolation, Survival and Growth Kinetics in Simulated Gastrointestinal

Conditions. London: Intech Open Access Publisher; 2013.

19. Motherway MC, Zomer A, Leahy SC, Reunanen J, Bottacini F, Claesson MJ, et al. Functional genome analysis of *Bifidobacterium breve* UCC2003 reveals type IVb tight adherence (Tad) pili as an essential and conserved host-colonization factor. *Proc Natl Acad Sci USA*. 2011; 108(27): 11217-22. doi: 10.1073/pnas.1105380108.
20. Dubey UK, Mistry VV. Effect of bifidogenic factors on growth characteristics of bifidobacteria in infant formula. *J Dairy Sci*. 1996; 79: 1156-63.
21. Kiviharju K, Leisola M, Eerikäinen T. Optimization of a *Bifidobacterium longum* production process. *J Biotechnol*. 2005; 117(3): 299-308.