British Biotechnology Journal
5(1): 37-50, 2015, Article no.BBJ.2015.004
ISSN: 2231–2927

SCIENCEDOMAIN *international*
www.sciencedomain.org

Effect of Process Variables on Survival of Bacteria in Probiotics Enriched Pomegranate Juice

Mina Khanbagi Dogahe[1*], Kianoush Khosravi-Darani[2*], Azadeh Tofighi[1], Mehrnaz Dadgar[1] and Amir Mohammad Mortazavian[3]

[1]Department of Food Science and Technology, Varamin Branch, Islamic Azad University, Varamin, Tehran, Iran.
[2]Research Department of Food Technology, National Nutrition and Food Technology Research Institute, Faculty of Nutrition Sciences and Food Technology, Shahid Beheshti University of Medical Sciences, P.O. Box: 193954741, Tehran, Iran.
[3]Department of Food Sciences and Technology, National Nutrition and Food Technology Research Institute, Faculty of Nutrition Sciences and Food Technology, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BBJ/2015/12114
Editor(s):
(1) Yan Juan, Sichuan Agricultural University, Ya'an, China.
Reviewers:
(1) Anonymous, Italy.
(2) Beatriz de Cássia Martins Salomão, Dept. of Chemical Engineering, Federal University of Rio Grande do Norte, Brazil.
(3) Anonymous, Egypt.
Complete Peer review History: http://www.sciencedomain.org/review-history.php?iid=801&id=11&aid=7083

Original Research Article

Received 18th June 2014
Accepted 3rd August 2014
Published 6th December 2014

ABSTRACT

Aims: The consumption of foods and beverages containing probiotic microorganisms is a growing, global consumer trend. In this research, production of probiotic pomegranate juice containing *Lactobacillus plantarum* and *Lactobacillus delbrueckii* was studied.
Study Design: Plackett-Burman statistical design was used to evaluate the impact of eleven process variables on the viability of both probiotics. Impact of incorporation of grape juice, tomato juice and pomegranate peel extract as well as phenolic compounds and vitamins have been investigated.
Place and Duration of Study: *Department of Food Science and Technology,* Varamin Branch, Islamic Azad University, Varamin, Tehran, Iran, between Sep 2012 and July 2013.
Methodology: Pomegranate juices were inoculated with probiotic bacteria and their survival was

**Corresponding author: Email: k.khosravi@sbmu.ac.ir, kiankh@yahoo.com;*

evaluated every week by pure plate method. The effect of 11 variables (in two levels) on survival of bacteria by the statistical design of Plackett-Burman was evaluated. For this purpose, 12 treatments in triplicate by the Minitab (version = 11.0) software at significant levels α= .01 were analyzed.

Results: The highest survival rate of *L. plantarum* (4.74 ×10^6 CFU/mL) and *L. delbrueckii* (4 ×10^6 CFU/mL) was obtained by 10% v/v inoculation of a 48 h inoculum culture in MRS broth medium to enriched pomegranate juice (10% v/v Grape juice, 5% v/v tomato juice, 0.1% v/v pomegranate peel extract and 2.0 g.L glucose) which was inoculated in anaerobic condition for 72 h at 37ºC and kept for 2 weeks at environment temperature. Sensory evaluation shows the probiotic juice was accepted by consumers with no significant difference in comparison to control in terms of taste, odour and overall acceptability (*P*>.05).

Conclusion: The results of this study suggest that grape, tomato juices and pomegranate peel extract exert a protective effect on *L. plantarum* and *L. delbrueckii* viability under acidic condition of pomegranate juice and storage time, which was associated with the chemical composition of them. This study indicates that develop of probiotic pomegranate juices with acceptable viability and stability of the probiotic is possible.

Keywords: Probiotic; pomegranate juice; survival; process variables; plackett-burman statistical design.

1. INTRODUCTION

The consumption of foods and beverages containing probiotic microorganisms is a growing, global consumer trend [1]. The term probiotic has technically defined as live microorganism which following uptake in certain numbers exerts health benefits beyond inherent general nutrition [2,3]. Functional food with probiotics, prebiotics and fibers has been available for consumers for years. Prebiotics are non digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improve host health [4]. Synbiotic food products containing probiotics with prebiotics or fibers are gaining more interest into the market [5]. Research has shown that addition of probiotics to food provides several health benefits including reduction in the level of serum cholesterol, improved gastrointestinal function, enhanced immune system and lower risk of colon cancer [6-9]. Traditionally, probiotics have been added to yogurt and other fermented dairy products, but lactose intolerance and the cholesterol content are two drawbacks related to consumption of milk products. In recent years, consumers' demand for nondairy based probiotic products has increased [10].

Fruit juice has been suggested as a novel, appropriate medium for fortification with probiotic cultures because it is already positioned as a healthy food product. It is consumed frequently by a large percentage of the consumer population [11]. Fruits and vegetables are rich in functional food components such as minerals, vitamins, dietary fibers and antioxidants. Furthermore, they do not contain any dairy allergens that might prevent usage by certain segments of the population [12]. It has been suggested that fruit juices could serve as suitable media for cultivating probiotic bacteria [13].

Pomegranate and its products, including juice, tea, wine and extracts are widely consumed and recognized for their health benefits. For instance, commercially manufactured pomegranate juice has a higher antioxidant activity than red wine and green tea [14]. The fresh juice contains 85.4% water and considerable amounts of total soluble solids (TSS), total sugars, reducing sugars, anthocyanins, phenolics, ascorbic acid and proteins and has been reported to be a rich source of antioxidants [15].

About half of the total dry matter of tomato consists of the reducing sugars, glucose and fructose, about 10% is organic acid and about 1% is skin and seeds, with the remainder being alcohol, insoluble solids (cellulose, pectins, hemicellulose and proteins), mineral (mainly potassium), pigments, vitamins and lipids. Glutamic acid is the principal amino acid found in tomato [16]. The organic acids content of tomato is responsible for a pH between 4.0 and 4.6 [17]. Tomato juice is a rich source of simple sugars, minerals and vitamins and its use has often been suggested to manufacture acceptable acidophilus milk products [18].

Grape pomace is a natural product rich in dietary fiber and polyphenols. An increasing interest on dietary phenolic compounds intake has been observed due to their well known antioxidant properties and health benefits e.g. significant reduction of cardiovascular disease risk [19]. The lactic acid bacteria (LAB) occurs naturally on grapes and their ability to grow in grape juice and wine is well documented [20].

Over centuries, fermentation has been used to improve the quality and the flavour of cereals, fruits, vegetables, legumes and meat. Nonetheless, growing body of evidence documenting the beneficial health effects of probiotics, there is a lack of research on the application of probiotics in product development [21]. Different studies have been carried out to explore the suitability of fruit juices such as tomato, beet, cabbage and juices as raw material for production of fruit drinks. *Lactobacillus plantarum, Lactobacillus acidophilus* and *Lactobacillus casei* have been employed as probiotic bacteria culture. Results have indicated that all the strains are capable to growth in the fruit juices mentioned and, as a result, the microbial population increase after 48 h of fermentation. Moreover*, L. plantarum, L. acidophilus* and *Lactobacillus delbrueckii* have shown to be resistant to high acidic and low pH conditions during storage periods at 4ºC [22-24]. The suitability of pomegranate juice as raw material for production of probiotic fruit juice has been studied. *L. plantarum*, *L. delbrueckii, Lactobacillus paracasei, L. acidophilus* were examined. *L. plantarum*, *L. delbrueckii* showed higher viability during the storage time. Pomegranate juice was proved to be a suitable media for production of a fermented probiotic drink [25]. The survival of probiotic in a model fruit juice system has been studied. The model juice containing vitamins, grape extract and green tea extract showed better survival of probiotic bacteria [26]. Table 4 summarizes kind and status of the aforementioned microorganism.

In the present work, the stability of *L. plantarum* and *L. delbrueckii* cells in pomegranate juice was studied. Pomegranate was selected as an important source of bioactive compounds with high antioxidant activity. Its peel extract has also high antioxidant activity due to high level of phenolics compounds. Fresh probiotic biomass was used instead of lyophilized bacteria. The effect of 11 variables (selected by pre-experience and literature review) on survival of bacteria was investigated by Plackett-Burman design (PBD). Grape juice, tomato juice and pomegranate peel extract were selected as antioxidant natural sources of phenolic compounds and vitamins.

2. MATERIALS AND METHODS

2.1 Starter Culture

The microorganisms used in the present study were as following: *L. plantarum* PTCC (Persian Type Culture Collection) NO: 1745 (isolated from pickled cabbage) and *L. delbrueckii*: PTCC NO: 1333, (isolated from intestine of adult) which were purchased from Iranian Research Organization for Science and Technology (IROST). The strains were maintained at 4ºC and subcultures monthly on MRS agar (Merc, Germany) plate to maintain freshness. Also strains were precultured weekly in MRS broth (Merc, Germany) at 37ºC for 24 h (stationary phase) under anaerobic conditions [27].

Number of precultured bacteria was measured by spectrophotometer and microscope to draw calibration curve of number of viable cells. First precultured bacteria was prepared by addition of 1.0 mL of the incubated tubes (with estimated viable cell of *L. plantarum* from 6 to 6.68 log CFU/mL and *L. delbrueckii* from 6 to 6.6 log CFU/mL) to 9.0 and 8.0 mL of sterilized saline solution (0.9% w/v), before use as 10^{-1} and 2×10^{-1} dilution. Then, serial dilutions of 10^{-2} to 10^{-5} were prepared as mentioned previously and the absorbance at 600 nm was measured with spectrophotometer (CT chromtech, Taiwan). The direct colony count method was used to determine cell viability too. Cell concentrations were measured by staining and microscope direct counting and calibration curve was plotted.

2.2 Preparation of Product

Pomegranates, grapes and tomato juice were prepared manually then pasteurized for 5 min at 80ºC by water bath and stored at refrigerator temperature. Pomegranates juices were weighed in 40.0 mL packages. Glucose, fructose, citric acid, pomegranate peel extract, grapes juice and tomato juice were added to the pomegranates juices at two levels according to statistical design of Plackett-Burman. Precultured bacteria (cultured in MRS broth medium for 24 and 48 h at 37ºC) were centrifuged (5000×g, 10 min, 4ºC), the pellet was washed twice with sterile saline (0.9% w/v) and the cells resuspended in sterile saline (0.9% w/v) [28,29].

The centrifuged cells were inoculated to the pasteurized pomegranates juices (10% or 20% v/v) and incubated at 37ºC for 3 and 4 days. Products were kept at 4ºC and 20ºC for 1 and 2 weeks. Fig. 1 shows a schematic diagram of fortified pomegranates juice (pomegranates juice with grape and tomato juice, pomegranate peel extract, citric acid, fructose and glucose which was inoculated with probiotic bacteria) production (Fig. 1).

2.3 Bacterial Enumeration

The pure plate method was used to determine viable cell count. Samples (products) were diluted (10^{-4} – 10^{-6}) with sterile saline (0.9% w/v) solution. The plates were incubated at 37ºC for 48 h and those showing individual colonies in the plates were counted. Viable cell counts were calculated as colony forming units per milliliter (CFU/mL).

2.4 Sensory Evaluation of Product

The taste, odour and overall acceptability of the simple pomegranates juice (not enriched) and the samples which have the highest viable cells count were judged by 30 untrained panellists. A nine points structured hedonic scale was used during a sequential presentation of samples, with one = disliked very much and nine = liked very much. The results were statistically analyzed using an ANOVA and Duncan method (.01<*P*<.05) (27).

2.5 Statistical Analysis

The statistical design of Plackett-Burman was used to figure out the optimum conditions for producing probiotic pomegranates juice with *L. plantarum* and *L. delbrueckii*. Each culture was used separately in a single product. The effect of each factor was evaluated (α = .01).

The basic equation set up for the design was as follows. The coefficients for the eleven variables were determined by:

$$A_i = (1/N)\sum_{0}^{n} X_i \cdot K_i$$

where A_i = coefficient values, X_i = experimental yield, K_i = codeed value of each variable corresponding to the respective experimental yield X_i and N = number of experiments. For Predicted yield is given by:

$$Y_i = \sum_{i=0}^{N} A_i \cdot K_i$$

for i = 0, a dummy level of +1 was used and the coefficient obtained was called A_0. The standard error was determined as the sum of the squares of the difference between the experimental and predicted yield for each run. The estimated error is given by the following equation:

$$S_b = \sqrt{S_e^2/N}$$

The student's t test was performed to estimate the significance of each variable employed (*t* value =coefficient/S_b). Since the experiments were designed to evaluate the relative effect of each variable on response, a significant level of 0.30 is acceptable [30]. However, the tabulated *t* value (degree of freedom 10) at *P*<.1 and *P*<.15 is equal to 1.2 and 0.69, respectively.

2.5.1 Selection of process variables and range finding

The first screening step is usually identification of the variables which have significant effects on response. With the aim to increase the viability of both probiotics, selection of the factors and their range (in a wide but reasonable numerical range) was conducted based on literature review and also our previous experience (unpublished data). Some changes in the response (yield) were expected for each factor over the selected range [31,32]. The variables to be evaluated are summarized in Table 1. Selected experimental variables and a Plackett-Burman design (PBD) for conducting 12 experimental trials were shown in Table 2. The elements + (high level) and - (low level) represent the two different levels of the independent variables examined.

2.6 Survival in the Simulated Gastro-Intestinal Conditions

After 30 days of storage of probiotic pomegranate juice at 4ºC, survival of probiotic bacteria of samples exposed to simulated gastro-intestinal stresses were compared to the control (a freshly prepared culture before storage) [33]. MRS broth (this type of bacterial growth medium is so-named by its inventors: De Man, Rogosa and Sharpe) was prepared and used as the base

medium (BM) to evaluate influence of pancreatic enzymes, pH and bile salts on cell viability. After sterilization by autoclave at 121ºC for 15 min, BM was adjusted at pH 2.0 by 6.0 N HCl solution for evaluation of stability at pH 2.0.

To prepare BM containing bile salt, 0.3% of bovine Oxgall was added to BM, then pH of the medium was adjusted to 6.0.

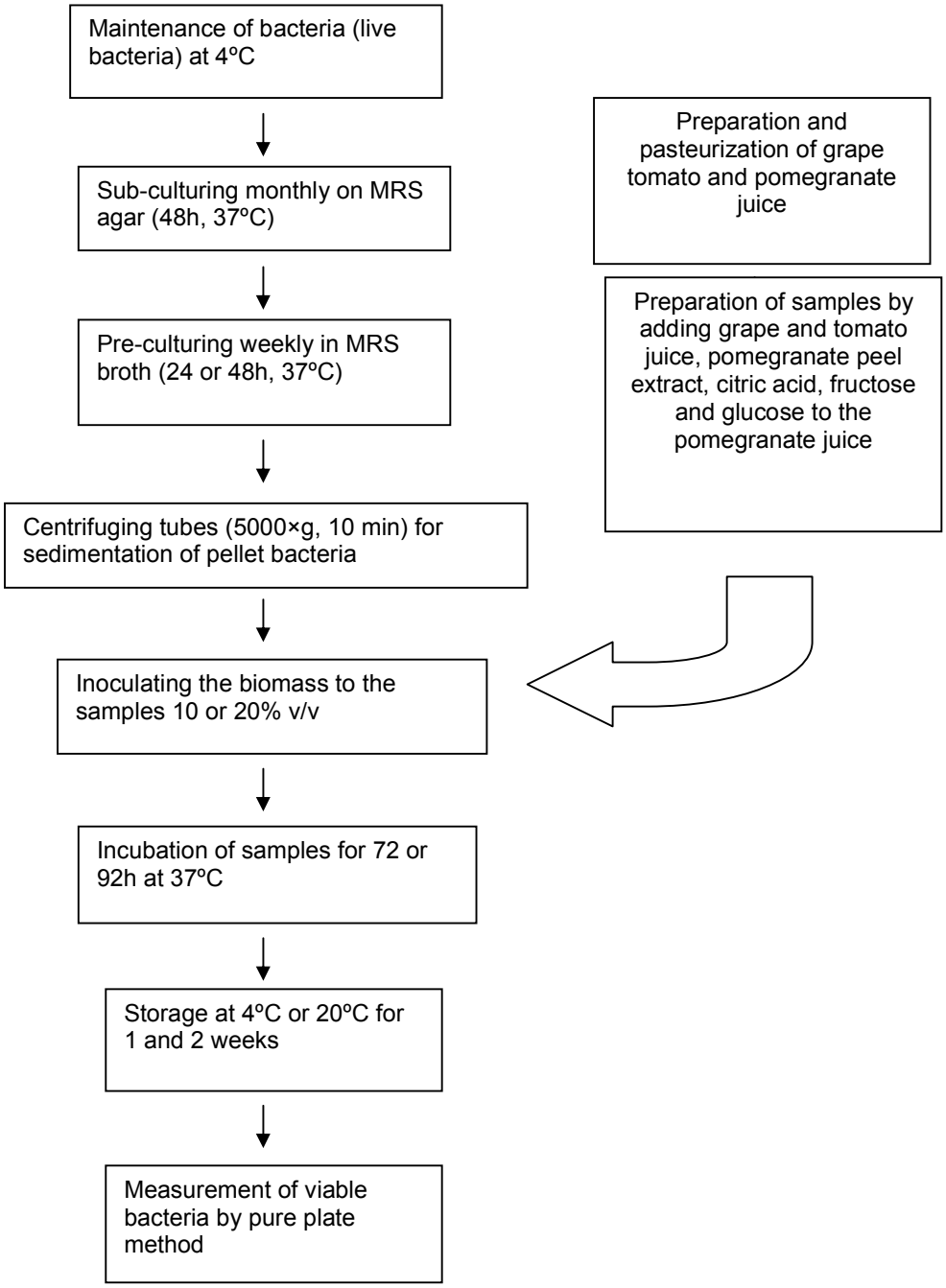


Fig. 1. Schematic diagram of enriched pomegranates juice production

Table 1. The independent variables to be evaluated and their importance in production of probiotic pomegranate juice

Independent variables	Range finding	Low level (-)	High level (+)	Suitable amount	Role	Reference
A: Grape juice content	5 and 10 (%v/v)	5	10	10 [(a,b)]	Positive effect of the total antioxidant capacity and phenolic content on bacteria viability	[35]
B: Tomato juice content	5 and 10 (%v/v)	5	10	10[(a,b)]	Positive effect of simple sugars (glucose, fructose) and minerals (Mn, Mg) on bacteria viability	[25]
C: Pomegranate peel extract content	0.01 and 0.1 (%v/v)	0.01	0.1	0.1[(a,b)]	pomegranate peel extract is a good source of the total antioxidant capacity and phenolic content	[46]
D: Fructose concentration	0 and 1 (g/L)	0	1	1[(b)], 0[(a)]	Fructose is one of the sugar that often consumed by probiotic bacteria	[25]
E: Acetic acid concentration	0 and 1 (g/L)	0	1	0[(a,b)]	As the main source of carbon used by lactic acid bacteria	[25]
F: Inoculums content	10 and 20(%v/v)	10	20	Not significent	Reaching to high cell concentration and inoculums in less percent	[25]
G: Inoculum age	24 and 48 (h)	24	48	48[(a)], 24[(b)]	Presence of glucose and fructose in the medium led to longer lag phase	[37]
H: Storage temperature	4 and 20 (ºC)	4	20	20[(a,b)]	4 as refrigerator and 20 as room temperature	[36]
I: Storage time	1 and 2 (week)	1	2	2[(a,b)]	_	_
J: Fermentation time	72 and 92 (h)	72	92	72[(a,b)]	The best fermentation time for probiotic bacteria was 72h or more of its in pomegranate juice	[25]
K: Glucose concentration	0 and 1 (g/L)	0	1	1[(a,b)]	Glucose increased the survival of lactobacilli in acidic conditions	[25]

[(a)]: Suitable range for L. delbrueckii [(b)]: Suitable range for L. plantarum

After sterilization of the solution by filtration and reaching temperature to 37ºC, medium was inoculated with 10% (v/v) of the probiotic containing pomegranate juice and incubated for 2 h. Cell counts were conducted on MRS agar and the control was base medium at pH 6.0. The "fresh culture" control was prepared by centrifuging a freshly-grown MRS culture at 3000×g for 15 min and adding the pomegranate juice to the cell pellet in order to obtain approximately 10^7 CFU/mL.

Pancreatin solution (Sigma) was centrifuged at 2000×g for 15 min at 4ºC and sterilized by microfiltration (0.22 µm pore), then 50 mg/mL of this solution was added to BM. Finally, 380 µL of the enzyme solution was aseptically added to 10.0 mL of the BM to a final pancreatin concentration of 1.9 mg/mL and pH 6.0.

3. RESULTS AND DISCUSSION

3.1 Stability in Pomegranate Juice

After the addition of ingredients to pomegranate juice and inoculation, the cell count was measured at the beginning of the storage time (time=0) as well as 1 and 2 weeks after storage at 4ºC and 20ºC.

Table 2. Studding eleven factors in probiotic juice production by adding *L. plantarum* and *L. delbrueckii* separately using the PBD statistical design

Run No	Coded setting for factors											Response[(a)] (CFU/mL)	
	A	B	C	D	E	F	G	H	I	J	K	*L. plantarum*	*L. delbrueckii*
1	+	-	+	-	-	-	+	+	+	-	+	$4.74\times10^6\pm0.11$	$4\times10^6\pm0.2$
2	+	+	-	+	-	-	-	+	+	+	-	$4.15\times10^6\pm0.13$	$3.40\times10^6\pm0.14$
3	-	+	+	-	+	-	-	-	+	+	+	$3.80\times10^6\pm0.11$	$3.10\times10^6\pm0.07$
4	+	-	+	+	-	+	-	-	-	+	+	$3.60\times10^6\pm0.03$	$2.30\times10^6\pm0.18$
5	+	+	-	+	+	-	+	-	-	-	+	$2.70\times10^6\pm0.05$	$2.50\times10^6\pm0.08$
6	+	+	+	-	+	+	-	+	-	-	-	$3.40\times10^6\pm0.17$	$2.90\times10^6\pm0.03$
7	-	+	+	+	-	+	+	-	+	-	-	$3.90\times10^6\pm0.03$	$3.60\times10^6\pm0.11$
8	-	-	+	+	+	-	+	+	-	+	-	$2.20\times10^6\pm0.06$	$1.80\times10^6\pm0.07$
9	-	-	-	+	+	+	-	+	+	-	+	$3.20\times10^6\pm0.09$	$2.40\times10^6\pm0.06$
10	+	-	-	-	+	+	+	-	+	+	-	$2.90\times10^6\pm0.10$	$2.90\times10^6\pm0.21$
11	-	+	-	-	-	+	+	+	-	+	+	$2.50\times10^6\pm0.03$	$2.70\times10^6\pm0.12$
12	-	-	-	-	-	-	-	-	-	-	-	$2.02\times10^6\pm0.10$	$1.90\times10^6\pm0.06$

[(a)]: response shows as mean±standard deviation for 3 replications

The cell viability in all samples were measured at the beginning of storage (time=0). To study effect of time on survival of bacterial cell, cell count was also conducted after 1 and 2 weeks (time=1 and 2). As Fig. 2 a shows, in some trials the number of viable cells was changed during the storage time. The maximum and minimum changes of *L. plantarum* were 0.16 and 0.02 log CFU/mL respectively and mean of changes was 0.07±0.001 log CFU/mL. Also, the maximum and minimum changes of L. delbrueckii were 0.2 and 0 log CFU/mL respectively and mean of changes was 0.09±0.002 log CFU/mL.

In the trials containing *L. plantarum*, at the beginning of storage, the highest and lowest numbers of live bacteria were respectively belonged to the test number 9 and 12. The highest numbers of viable bacteria in the seventh and fourteenth day of storage were respectively belonged to the test number 4 and 1. In other words, in sample 1 the highest numbers of viable bacteria was achieved after 2 weeks of storage. In general, survival of *L. plantarum* increased after 2 weeks so it could adapt with environment (Fig. 2a).

In the case of *L. delbrueckii* (Fig. 2b), the highest numbers of living bacteria were belonged to the test number 1 at the beginning and also after 2 weeks of storage.

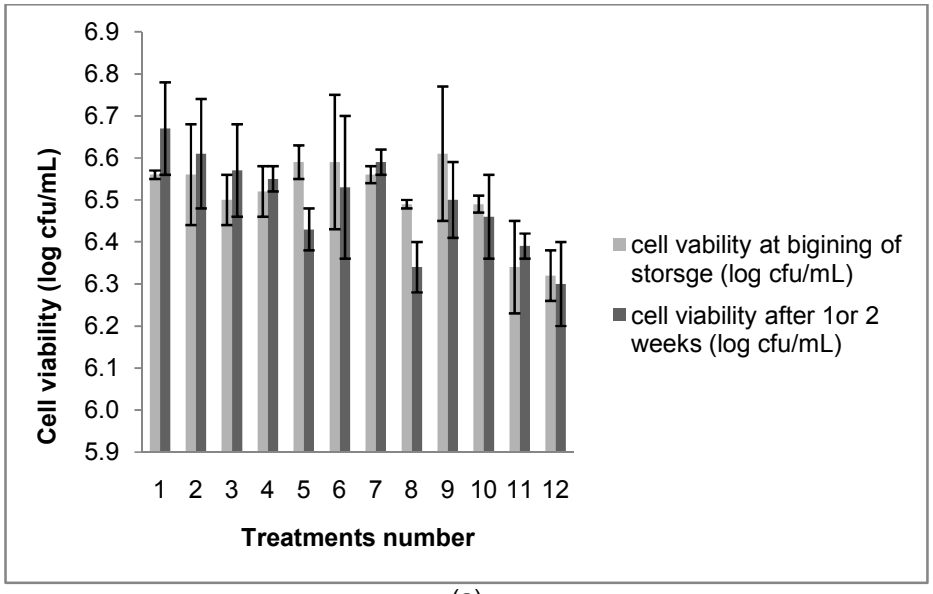
3.2 Statistical Evaluation of Cell Viability

Plackett-Burman statistical design was used to evaluate the impact of 11 process variables (in 2 levels) on the viability of probiotics. The results are shown in Figs. 3 and 4 for *L. plantarum* and *L. delbrueckii.* All variables, except for the inoculums concentration, were conceded as significant effective variables on viability of both bacteria. Storage time, pomegranate peel extract, grape juice and acetic acid concentration have been found more effective than other variables on viability of *L. plantarum* (Fig. 3a). In the trials inoculated by *L. delbrueckii* storage time, tomato juice, grape juice and acetic acid concentration have been found more effective than other variables (Fig. 3b).

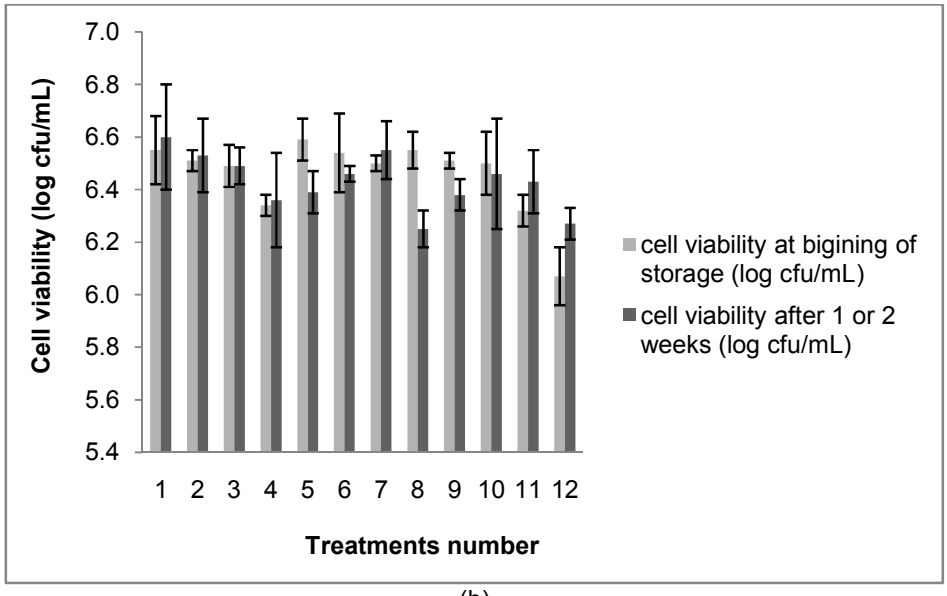
The high level of grape juice, tomato juice and pomegranate peel extract concentration, because of their total antioxidant capacity, minerals and phenolic contents leads to a positive effect on viability of both bacteria. Glucose and fructose are metabolized by *L. plantarum* and *L. delbrueckii*. As it can be concluded from Fig. 4, high concentration of glucose and fructose were found appropriate for *L. plantarum* but for viability of *L. delbrueckii* high level of glucose in the absence of fructose was more suitable. It seems that, excessive increase of sugars causes a decrease in survival of *L. delbrueckii*.

Due to the significant decrease in pH after the addition of citric acid, reducing of the survival of bacteria can be justified. Thus, absence of citric acid in the product was found suitable for both bacteria. Suitable age of inoculums for *L. plantarum* was 24 h. Studies have shown that log phase of growth has elongated in the presence of glucose and stationary phase start after 24 h [28]. So, the results indicated that inoculation with 24 h cultured cells, was more appropriate than 48 h for *L. plantarum*. In another study, it has been shown that the growth curve of *L. delbrueckii* was entered to the stationary phase after 25 h [25]. Also in the presence of glucose log phase was elongated, so, in this study,

inoculums age of 48 h seems more suitable than 24 h for *L. delbrueckii*. Microbial population of *L. plantarum* and *L. delbrueckii* decreased during fermentation time (92 h) at 37ºC but fermentation time of 72 h has been shown to be more effective. In other words suitable fermentation time was 72 h for both bacteria. Storage of product at room temperature (20ºC) for 2 weeks has shown positive effect on both bacteria but at 4ºC growth of bacteria was slower. Taste, odour and overall acceptability of probiotic pomegranate juice were examined. Samples which had the highest viable cell were compared with the control sample (pomegranate juice without any additions) by nine point structured hedonic scale (Table 3). The results were examined by ANOVA and F test. No significant difference among between samples and control in terms of taste, odour and overall acceptability were observed (.01<*P*<.05).

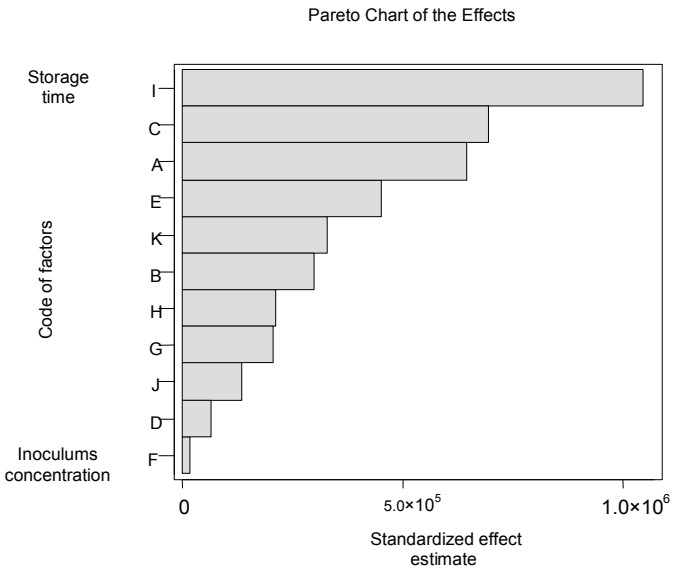


(a)

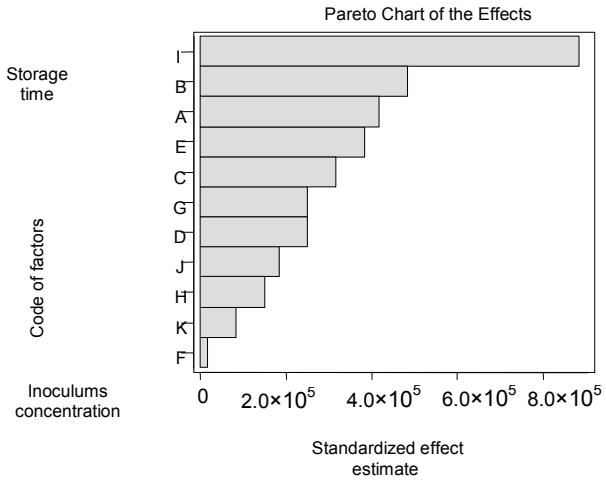


(b)

Fig. 2. Stability of fresh *L. plantarum* (a) and *L. delbrueckii* (b) in fortified pomegranate juice during storage time in 12 treatments different conditions according to the plackett-burmann design



(a)

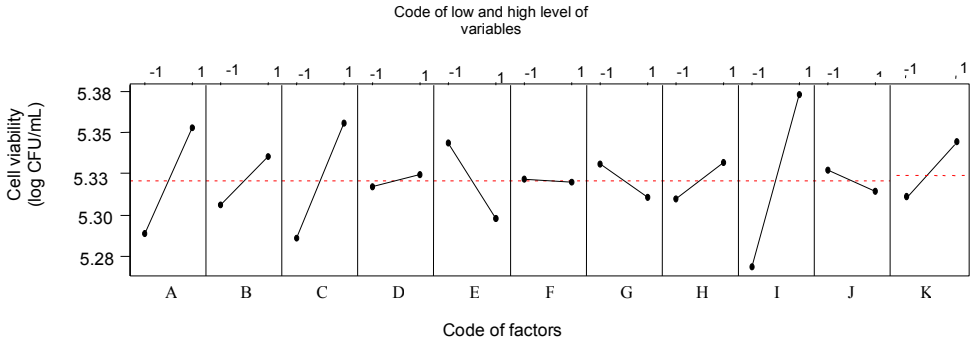


(b)

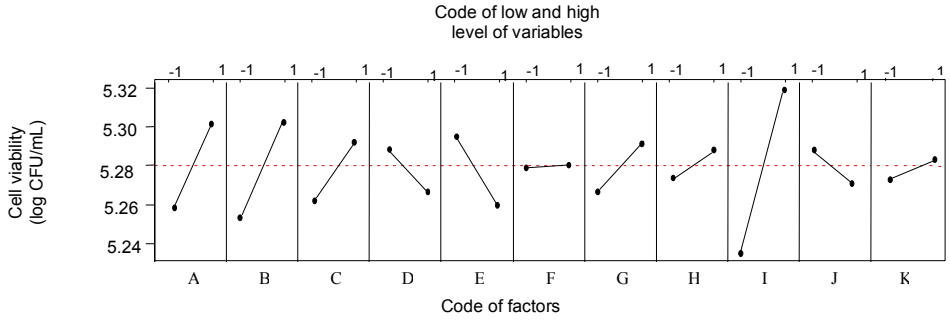
Fig. 3. Pareto diagram to evaluate the effect of eleven variables (A: grape juice content, B: tomato juice content, C: pomegranate peel extract content, D: fructose concentration, E: acetic acid concentration, F: inoculums content, G: inoculum age, H: storage temperature, I: storage time, J: fermentation time, K: glucose concentration) on survival of *L. plantarum* (a) and *L delbrueckii* (b) inoculated in pomegranate juice during storage time (significant level: α=.01)

As results show the best condition for survival of probiotic bacteria was achieved when bacteria incubated in MRS broth for 48 h and inoculated in pomegranate juice contains grape juice (10% v/v), tomato juice (5% v/v), pomegranate peel extract (0.1% v/v) and glucose (1.0 g/L) and incubated for 72 h at 37ºC in anaerobic condition then products were stored at 20ºC for 2 weeks.

The highest survival number of *L. plantarum* and *L. delbrueckii* were about 4.74×10^6 and 4×10^6 CFU/mL, respectively. Grape juice contains high rate of antioxidant, minerals and phenolic compounds which are effective on growth and viability of probiotic bacteria [34]. Vitamins, pigments and antioxidant substances in the tomato made the medium more suitable for fermentation. This result is similar with previous studies [22]. The growth and survival of probiotic bacteria also were stimulated by presence of phenolic compounds found in pomegranate peel extract. phenolic compounds not only inhibit the growth and activity of spoilage microorganisms, but also have stimulation effect on the growth of probiotic bacteria [26,37]. Using of appropriate rate of pomegranate peel in the juice leads to increase survival of bacteria without creation of undesirable flavour. Glucose was used as a carbon source by bacteria so, it had influencing role on growth and viability of bacteria. The probiotic pomegranate juice did not show any significant difference with the control and was acceptable for consumers.



(a)



(b)

Fig. 4. The main effect of different level variables (A: grape juice content, B: tomato juice content, C: pomegranate peel extract content, D: fructose concentration, E: acetic acid concentration, F: inoculums content, G: inoculum age, H: storage temperature, I: storage time, J: fermentation time, K: glucose concentration) in plackett-burmann design on survival of *L. plantarum* (a) and *L delbrueckii* (b) inoculated in pomegranate juice during storage

Table 3. Analysis result of taste, smell and acceptances for the control sample, sample number 1 for both bacteria (as best products that have the highest live bacteria)

	Sensory indicators			Total score
Sample	Taste	Smell	Acceptance	
Control	7.1	5.8	6.4	19.3
Trial no. 1 [(a)]	5.8	4.9	5.3	16
Trial no. 1 [(b)]	6.2	5.1	5.5	16.8

(a): Sample that contains the highest number of live L. plantarum;
(b): Sample that contains the highest number of live L. delbrueckii

Table 4. Probiotics used in different types of juices in liquid fermentation condition

Probiotic bacteria	Inoculum size (log CFU/mL)	Product	Survival rate (CFU/mL)	Consideration	Reference
L. plantarum *L. acidophilus* *L. casei* *L. delbrueckii*	1.64	Tomato juice	10^6 10^9 10^8 10^8	Viability in product	[22]
L. plantarum *L. acidophilus* *L. casei* *L. delbrueckii*	5.20	Beet juice	10^6 10^4 10^7 10^7	Viability in product	[23]
L. delbrueckii *L. plantarum* *L. casei*	2.9	Cabbage juice	10^7 10^5 10^5	Viability in product	[24]
L. delbrueckii *L. plantarum* *L. casei* *L. acidophilus*	0.049	Pomegranate juice	10^5	Viability in product	[25]

3.3 Viability during Simulated Gastro-intestinal Stresses

Some strains were selected to study their resistance to simulated gastro-intestinal (GI) conditions, mainly based on variable stability during storage. Indeed, *Lactobacillus rhamnosus* was the most stable cultures. Furthermore, *Lactobacillus reuteri* was of interest because it is considered to be of the few true indigenous *Lactobacillus* species in humans [38] while *L. plantarum* is rather associated with the fermentation of vegetal substrates [39]. None of the strains were significantly affected by the incubation in presence of 0.3% bile salts or the pancreatic enzymes.

Storage for 30 days in the fruit juice blend did not affect the strains sensitivity to these compounds. In comparison to control treatment (base medium at pH 6.0), viability loss of *L. plantarum* after treatment for acid (pH 2), bile (0.3%) and pancreatic enzymes was 1.8, 0.7 and 0 log CFU/mL. Also viability loss of *L. delbrueckii*. after treatment for acid (pH 2), bile (0.3%) and pancreatic enzymes was 1.6, 0.2 and 0 log CFU/mL. In probiotic selection, tolerance to the environment of the small intestine is also thought to be of importance since the acid-sensitive strains can be buffered through the stomach [40,41]. The results of this investigation show that 30 days storage in the fruit drink would not affect sensitivity of probiotics to bile or pancreatic enzymes. The previous reports indicate that a low final pH during bacterial growth induces an acid tolerance response [42,43]. The induction of pH stress may cause protection against acid, heat, osmotic or oxidative shocks [44]. The opposite observation was reported, when strong viability losses were observed by exposing at pH 2.0 for 2 h at 37ºC [45].

Existence of direct relationship between stability during storage in the juice and simulated GI condition was examined. The correlation (R^2 = 0.53) was not statistically significant (*P*=.2) due to the limited number of experimental data (only 2 strains). It has been shown that glucose enhanced the survival of lactobacilli in acidic conditions [47]. Therefore, in order to prevent viability loses during storage and in the GI stress tests, incorporation of a carbohydrate can be useful. This being stated, this classical simulated GI acid procedure at pH levels between 1.5 and 2.5 probably overestimates actual gastric viability losses due to the buffering ability of certain foods [48].

It should be mentioned that in all fractional factorial designs like PBD [49] and Taguchi Design [50], the better level of each variable can be defined to achieve the better response. In this study, PBD was applied for producing probiotic pomegranates juice with *L. plantarum* and *L. delbrueckii*. The study can not be considered as optimization process, but it is only a screening design to evaluate the effect of independent factor and finding the more suitable level (between two selected levels) of independent variable.

4. CONCLUSION

The present study indicated that some mix juice preparations have the potential in technological applications in protecting probiotic viability and

stability during processing and storage of fruit juices. The stored cultures seem to be less resistant than fresh cells to an acid challenge as would occur in the stomach following consumption of the products. This was observed with probiotic bacteria stored in a fruit juice, but raises a concern with respect to other acid foods such as pomegranate juice. The results presented here suggest that grape, tomato juices and pomegranate peel extract exert a protective effect on *L. plantarum* and *L. delbrueckii* viability under acidic condition of pomegranate juice and storage time, which was associated with the chemical composition of the aforementioned ingredients. Despite the low percentage of pomegranate peel extract (0.1% or 0.01% v/v), had a significant effect in this regard. These effects could be mainly attributed to the presence of antioxidant, minerals and phenolic compounds present in the pomegranate peel extract. These results indicate that it is possible to develop probiotic fruit juices where the juices maintain the viability and stability of the probiotic. It remains to be determined the effect of storage on the functional properties of probiotics as well as influence of the age of the stored cultures on clinical health benefits.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Verbeke W. Consumer acceptance of functional foods: Sociodemographic cognitive and attitudinal determinants. Food Qual Prefer. 2005;16(1):45-57.
2. FAO/WHO. Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. Cordoba, Argentina: Food and Agriculture Organization of the United Nations and World Health Organization Expert Consultation Report; 2001.
3. Guarner F, Schaafsma GJ. Probiotics. Int J Food Microbiol. 1998;39:237-238.
4. Gibson GR, Roberfroid MB. Dietary modulation of the human colonic microbiota: Introducing the concept of prebiotics. J Nutr. 1995;125:1401-1412.
5. Sloan AE. The top ten functional food trends. Food Technol. 2000;54:33-62.
6. Berner LO, Donnell J. Functional foods and health claims legislation: Applications to dairy foods. Int Dairy J. 1998;8:355-362.
7. Mcnaught CE, Macnie J. Probiotics in clinical practice: A critical review of the evidence. Nutr Res. 2001;21:343-353.
8. Rafter J. Probiotics and colon cancer. Best Pract Res Cl Ga. 2003;17(5):849-859.
9. Saarela M, La¨hteena¨ki L, Crittenden R, Salminen S, Mattila Sandholm T. Gut bacteria and health foods—the European perspective. Int J Food Microbiol. 2002;78:99-117.
10. Shah NP. Functional foods from probiotics and prebiotics. Food Technol. 2001;55(11):46– 53.
11. Tuorila H, Gardello AV. Consumer response to an off flavor in juice in the presence of specific health claims. Food Qual Prefer. 2002;13:561-569.
12. Luckow T, Delahunty C. Which juice is healthier? A consumer study of probiotic nondairy juice drinks. Food Qual Prefer. 2004;15:751-759.
13. Mattila Sandholm T, Myllarinen P, Crittenden R, Mogensen G, Fonden R, Saarela M. Technological challenges for future probiotic foods. Int Dairy J. 2002;12:173-182.
14. Gil MI, Tomas Barberan FA, Hess Pierce B, Holcroft DM, Kader AA. Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. J Agr Food Chem. 2000;48:4581-4589.
15. Aviram M, Dornfeld L, Kaplan M, Coleman R, Gaitini D, Nitecki S, et al. Pomegranate juice flavonoids inhibit LDL oxidation and cardiovascular diseases: Studies in atherosclerotic mice and in humans. Drugs Exp Cl Res. 2002;28:49-62.
16. Hayes WA, Smith PG, Morris AE. The production and quality of tomato concentrates. Crit Rev Food Sci Nutr. 1998;38(7):537-564.
17. Gutheil RA, Price LG, Swanson BG. pH, acidity and vitamin C content of fresh and canned homegrown Washington tomatoes. J Food Protect. 1980;43:366-369.
18. Babu V, Mital BK, Garg SK. Effect of tomato juice addition on the growth and activity of *Lactobacillus acidophilus*. Int J Food Microbiol. 1992;17:67-71.
19. Hervert Hernández D, Pintado C, Rotger R, Goñi I. Stimulatory role of grape pomace polyphenols on *Lactobacillus acidophilus* growth. Intl J Food Microbiol. 2009;136:119-122.
20. Plessis HW, Dicks LMT, Pretorius IS, Lambrechts MG, du Toit M. Identification of

lactic acid bacteria isolated from South African brandy base wines. Int J Food Microbiol. 2004;91:19-29.
21. Rivera Espinoza Y, Gallardo Navarro Y. Nondairy probiotic products. Food Microbiol. 2010;27:111.
22. Yoon KY, Woodams EE, Hang YD. Probiotication of tomato juice by lactic acid bacteria. J Microbial. 2004;42:315-318.
23. Yoon KY, Woodams EE, Hang YD. Fermentation of beet juice by beneficial lactic acid bacteria. Lebensm Wiss u Technol. 2005;38:73-75.
24. Yoon KY, Woodams EE, Hang YD. Production of cabbage juice by lactic acid bacteria. Bioresource Technol. 2006;97:1427-1430.
25. Mousavi ZE, Mousavi SM, Razavi SH, Emam Djomeh Z, Kiani H. Fermentation of pomegranate juice by probiotic lactic acid bacteria. World J Microbiol Biotechnol; 2010. DOI 10.1007/s1127401004361.
26. Shah NP, Ding WK, Fallourd MJ, Leyer G. Improving the stability of probiotic bacteria in model fruit juice using vitamins and antioxidants. J Food Sci. 2010;75(5):278-282.
27. Guergoletto K, Magnani M, San Martin J, Andrade C, Garcia S. Survival of *Lactobacillus casei* (LC1) adhered to prebiotic vegetal fibers. Innov Food Sci and Emerg Tech. 2010;11:415-421.
28. Charalampopoulos D, Pandiella SS, Webb C. Evaluation of the effect of malt, wheat and barley extracts on the viability of potentially probiotic lactic acid bacteria under acidic conditions. Int J Food Microbiol. 2003;82:133-141.
29. Michida H, Tamalampudi S, Pandiella S, Webb C, Fukuda H, Kondo A. Effect of cereal extracts and cereal fiber on viability of *Lactobacillus plantarum* under gastrointestinal tract conditions. Biochem Eng J. 2006;28:73-78.
30. Khosravi Darani K, Zoghi A. Comparison of pretreatment strategies of sugarcane baggase: Experimental design for citric acid production. Bioresource Technol. 2008;99:6986–6993.
31. Davies L. Efficiency in research, development and production: The statistical design and analysis of chemical experiments. Trends Anal Chem. 1995;4:19-50.
32. Khosravi Darani K, Vasheghani Farahani E, Shojaosadati SA. Application of the Plackett Burman design for the optimization of poly (β-hydroxybutyrate) production by *Ralstonia eutropha*. Iran J Biotechnol. 2003;1(3):155-161.
33. Charteris WP, O'Neill ET, Kelly PM. An in vitro method to determine human gastric transit tolerance among potentially probiotic lactic acid bacteria and bifidobacteria. Irish J Agr Food Res. 1994;33:203.
34. Wang H, Cao G, Prior RL. Total antioxidant capacity of fruits. J Agric Food Chem. 1996;44:701-705.
35. Kneifel W, Jaros D, Erhard F. Microflora and acidification properties of yogurt and yogurt related products fermented with commercially available starter cultures. Int J Food Microbiol. 1993;18:179-189.
36. Bialonska D, Kasimsetty SG, Schreder KK, Ferreira D. The effect of pomegranate *(punica granatum L.*) byproducts and ellagitannins on the growth of human gut bacteria. J Agric Food Chem. 2009;57:8344-8349.
37. Kneifel W, Rajal A, Kulbe KD. *In vitro* behavior of probiotic bacteria in culture with carbohydrates of prebiotic importance. Microb Ecol Health D. 2000;12:27-34.
38. Valeur N, Engel P, Carbajal N, Connolly E, Ladefoged K. Colonization and immunomodulation by *Lactobacillus reuteri* ATCC 55730 in the human gastrointestinal tract. Appl Environ Microb. 2004;70:1176–1181.
39. Alwazeer D, Cachon R, Divies C. Behavior of *Lactobacillus plantarum* and *Saccharomyces cerevisiae* in fresh and thermally processed orange juice. J Food Protect. 2002;65:1586–1589.
40. Huang Y, Adams MC. *In vitro* assessment of the upper gastrointestinal tolerance of potential probiotic dairy propionibacteria. Intl J Food Microbiol. 2004;91:253–260.
41. Leverrier P, Fremont Y, Rouault A, Boyaval P, Jan G. *In vitro* tolerance to digestive stresses of propionibacteria: Influence of food matrices. Food Microbiol. 2005;22:18–11.
42. Lorca GL, Font de Valdez G. A low-pH-inductible, stationary-phase acid tolerance response in *Lactobacillus acidophilus* CRL 639. Curr Microbiol. 2001;42:21–25
43. Hartke A, Bouch S, Giard JC, Benachour A, Boutibonnes P, Auffray Y. The lactic acid stress response of *Lactococcus lactis* subsp. lactis. Curr Microbiol. 1996;33:194–199.

44. Van de Guchte M, Serror P, Chervaux C, Smokvina T, Ehrlich S, Maguin E. Stress responses in lactic acid bacteria. A van Leeuw. 2002;82:187–216.
45. Champagne CP, Gardner NJ. Effect of storage in a fruit drink on subsequent survival of probiotic *lactobacilli* to gastro-intestinal stresses. Food Research International. 2008;41:539–543.
46. Li Y, Gue Ch, Yang J, Wei J, Xu J, Cheng Sh. Evaluation of antioxidant properties of pomegranate peel extract in comparison with pomegranate pulp extract. Food Chem. 2006;96:254-260.
47. Corcoran BM, Stanton C, Fitzgerald GF, Ross RP. Survival of probiotic lactobacilli in acidic environments is enhanced in the presence of metabolizable sugars. Appl Environ Microb. 2005;71:3060–3067.
48. Mainville I, Arcand Y, Farnworth E. A dynamic model that simulates the upper gastrointestinal tract for the study of probiotics. Int J Food Microbiol. 2005;99:287–296.
49. Khosravi-Darani K, Vasheghani-Farahani E, Shojaosadati SA. Application of the Plackett-Burman Design for the Optimization of Poly(β-hydroxybutyrate) Production by *Ralstonia eutropha*. Iran J Biotechnol. 2003;1(3):155-161.
50. Khosravi-Darani K, Vasheghani-Farahani E, Shojaosadati SA. Application of the Taguchi Design for Production of Poly(β-hydroxybutyrate) by *Ralstonia eutropha*, Iran J Chem Chem Eng. 2004;23(2):131-136.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=801&id=11&aid=7083