

# Survival of Probiotics in Synbiotic Apple Juice During Refrigeration and Subsequent Exposure to Simulated Gastro-Intestinal Conditions

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**ABSTRACT:** *The aim of this work was to produce synbiotic apple juice and investigate the survival of *Lactobacillus acidophilus* and *Lactobacillus plantarum* in apple juice during the refrigerated storage (4 °C) for 42 days and then the ability of the mentioned probiotic bacteria in gastrointestinal tolerance under gastrointestinal tract conditions, with simulated gastric and bile juices. Eight-treatment combination Plackett-Burman design was used to evaluate the influence of seven variables such as probiotic strain, inoculum size, fructooligosaccharide content, inulin concentration, patulin content, ascorbic and citric acids concentration on the viability of mentioned probiotic strains. The results showed that the survivability of probiotics in apple juice depends significantly ( $P \leq 0.05$ ) on the inoculum size, inulin concentration, kind of probiotic strain, and ascorbic and citric acids' concentration, respectively. The highest viability was achieved by inoculation of  $10^8$  CFU/mL of *Lactobacillus acidophilus* ATCC 4356 to the apple juice contaminated with 110 µg/L patulin content, containing 2.5% (w/v) inulin, 4 g/L citric acid, and 200 mg/L ascorbic acid. No significant difference was observed in the organoleptic properties of the synbiotic apple juice and the control sample. After sequential incubation in the simulated gastric (2 h) and intestinal juices (pH 7.4, 2 h), the highest number of surviving cells was around 3.5 log (CFU/mL).*

**KEYWORDS:** *Viability; Probiotic; Functional food; Prebiotic; Simulated gastro-intestinal juices.*

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1021-9986/2019/2/159-170

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## INTRODUCTION

Functional food is defined as food containing health-promoting components. These foods have been enriched with active components such as prebiotics and probiotics. Probiotics are known as live microorganisms, which in adequate amounts confer a health benefit on the host including control of gastrointestinal infections, irritable bowel syndrome, inflammatory bowel disease, help to the metabolism of lactose, stimulation of the immune system, suppression of cancer, improvement of digestibility of food, and reduction of serum cholesterol as well as many other health beneficial impacts [1]. In order to exert their health benefits, the minimum concentration of live probiotic bacteria at the expiry date of the product should be around  $10^7$  CFU/mL [2]. This population has been suggested because, during the passage through the gastrointestinal tract, a significant number of bacterial cells die [3]. Most of the probiotic foods are produced from milk. Other non-dairy beverages that can be ideal substrates for the culture of probiotics are fruit juices as they contain beneficial nutrients such as antioxidants for lactose intolerant consumers. Also, *Tuorila* and *Cardello* suggested that fruit juice is a suitable carrier for delivery of probiotics due to its sugar, vitamin, antioxidant, and mineral content [4].

Lactobacilli, Bifidobacteria, and nonpathogenic yeasts are the most commonly used probiotics in functional foods [5]. Survival of probiotic in fruit juices may be affected by several environmental conditions e.g. pH, strain, the method of culture preparation, additives and the temperature of storage [6]. *Perricone et al.* suggested that fruit juices are a good carrier for *Lactobacillus (L.) reuteri* DSM 20016 but their viability is strongly affected by the kind of juices [7]. In this study, *L. reuteri* showed high viability in pineapple, orange and apple juices, while the viability was reduced in red-fruit juice. *Espirito-Santo et al.* stated that apple juice is suitable for *Lactobacillus* growth and viability [8].

A prebiotic is known as a non-digestible food ingredient that has beneficial effects on the host by stimulating the activity or growth of a limited number of beneficial bacteria in the colon in a selective manner [9]. The beneficial effects of prebiotics are fermentation of dietary oligosaccharides and generation of short-chain fatty acids and miscellaneous gases with different biological roles. Inulin and fructooligosaccharide (FOS)

are two of the most used prebiotics in juices, which are tolerant to digestion by gastric and pancreatic enzymes both *in vitro* and *in vivo*. Inulin is one of the best documented prebiotic oligosaccharides and has demonstrated specific and different functional attributes, such as modulation of the gut microbiota, prevention of adhesion and colonization by pathogens, stimulation of anti-inflammatory effects, reduction of food intake, modulation of bowel movements, and regulation of alterations in lipid and glucose metabolism. Also inulin is one of the most known prebiotics that influences the growth and survival of *L. acidophilus* and *L. plantarum* [10]. *Pimentel et al.* stated that inserting oligofructose to probiotic apple juice did not change the physicochemical characteristics, acceptability and storage stability of the product, but it enhanced the probiotic survival during 28 days of cold storage [11]. Dose ranges of 2.5 to 5 g/day have demonstrated a prebiotic effect [12]. *Roble et al.* used FOS as a prebiotic for the production of synbiotic fresh-cut apple slices [13].

To achieve beneficial effects, probiotics must be capable of resisting stressful conditions during production processes and GIT [2]. Resisting the acidic conditions in the stomach and the bile acids at the beginning of the small intestine are properties that indicate the ability of a probiotic microorganism to survive the passage through the GIT [14].

Although significantly less amount of information is available regarding the factors influencing probiotic survival in fruit juices compared to dairy products, potential factors could be the probiotic strain [15], inoculum size [3], prebiotic content [12], ascorbic acid and citric acid concentration [1].

*Marhamatizadeh et al.* stated that the survival of *L. acidophilus* bacteria was better than *Bifidobacterium bifidum* in probiotic apple juice production [15]. Also investigation on the sensorial properties of probiotic apple juice with *L. acidophilus* and *L. plantarum* showed no significant influence on color and flavor in the product [16]. It is suggested that ascorbic acid is an effective additive for cell survival during cold storage [1]. On the other hand, this organic acid is a beneficial vitamin for human health. It is reported that high levels of citric acid play an important role in the survival of *L. plantarum* and *L. acidophilus* in fruit juices during the refrigerated storage [1]. Additionally, inserting ascorbic acid and citric acid to apple juice leads to a reduction of pH

to about 3.5, and certain lactic acid bacteria strains to provide more surface (S) layer proteins at low pH values [17].

Patulin (PAT) is a toxic metabolite produced by various species of *Penicillium* and *Aspergillus* in the pre-processing stages of apple fruit (pre-harvest and post-harvest). Various acute and chronic effects have been attributed to PAT including nervousness, convulsions, lung congestion, gastrointestinal tract distension, intestinal hemorrhage, epithelial cell degeneration, and DNA and RNA synthesis inhibition. Due to its toxicity, the European Commission has accepted 50 µg/L as the maximum concentration of PAT in fruit juices and nectar [18]. Fuchs *et al.* [19] and also Zoghi *et al.* [20] showed that *L. acidophilus* and *L. plantarum* can remove PAT from aqueous solution successfully. Also, they showed that the adsorption of PAT by probiotics depends strongly on the concentration of mycotoxin. Probiotics adsorb PAT by surface binding due to great adhesive properties of S-layer proteins in their cell wall and high efficiency of PAT removal depends on thicker S-layer proteins. Certain probiotic bacteria that show good adhesion to intestinal cells lose this property when binding to PAT and will rapidly pass through the gastrointestinal tract [21].

So, the overall aim of this study was to investigate the effect of seven variables on the survival of *L. acidophilus* ATCC 4356 and *L. plantarum* ATCC 8014 in apple juice supplemented with prebiotic substances and kept in cold-storage facilities, using a Plackett-Burman Design (PBD). Moreover, viable counts of the mentioned probiotic strains under simulated GIT conditions were evaluated after 42 days of refrigerated storage.

## EXPERIMENTAL SECTION

### *Chemicals and media*

All chemicals used in the experiments were obtained from Carlo Erba (Chaussée du Vexin, Val-de-Reuil, France). De Man-Rogosa-Sharpe (MRS) broth was obtained from Liofilchem (Abruzzi, Italy). Bile salts (Oxgall), Pepsin (from porcine stomach mucus, P-7000), Pancreatin (from the pancreas, P-1500) and PAT were all supplied by Sigma Aldrich (Vienna, Austria). Inulin and FOS were purchased from the Sensus Company (Roosendaal, Netherlands).

### *Probiotic strains and growth conditions*

*L. acidophilus* ATCC 4356 and *L. plantarum* ATCC 8014 were obtained from Tak Gene Company (Tehran,

Iran). Both the Probiotic strains were inoculated separately into 10 mL of MRS broth (pH 6.2) and incubated at 37 °C for 24 h and 48 h, respectively. The number of live bacteria was determined by total plate count on plate count agar. Viable cell counts were calculated as colony-forming units per milliliter (CFU/mL), and data were expressed as log values.

### *Preparation of synbiotic apple juice*

Commercial apple concentrate was obtained from Takdaneh group company (Marand, Iran) and kept at 4 °C before using. Apple concentrate and vessels were autoclaved before inoculation. Apple juice was produced by 14 g concentrate and 85 mL distilled water (the mixture included 0.49 g titratable acidity, 11.36 g total sugar and 7.54 g reducing sugars in 100 g apple juice, brix 11, pH 3.7). According to PBD (Table 1), defined amounts of PAT (150 or 110 µg/L), prebiotic (FOS and inulin), citric acid (0 or 4 g/L) and ascorbic acid (0 or 200 mg/L) were added to the apple juices; then the samples were transferred into sterile glass flasks and pasteurized for five minutes at 90° C. All samples were then inoculated with determining the amount of probiotic strain (1 mL of MRS broth containing 10<sup>8</sup> or 10<sup>10</sup> CFU/mL of *L. acidophilus* or *L. plantarum* according to Table 1). It is to be mentioned that each flask of the samples contained 100 mL synbiotic apple juice. The juices were stored in a refrigerator at 4° C for 42 days. Samples were collected weekly and analyzed for viable cell counts by pour plate counting. The colonies were counted after 72 h of incubation at 37° C [22]. The survival experiments were conducted in duplicate for each treatment combination (basis in Table 1).

Krasaekoopt and Chea reported that the fermentation provided a high number of cells in the fruit juices, but the organoleptic properties of the products were not acceptable to the consumers [23]. Therefore, to prevent the possibility of fermentation, the samples (without fermentation time) were kept in the refrigerator at 4 °C for 6 weeks. Moreover, it is reported that *L. acidophilus* and *L. plantarum* showed acceptable viability with less than one log (CFU/mL) reduction at 4° C for a long shelf life [24].

The viability of probiotics was assessed after 1 day in the refrigerator (week 0) and then after 1, 2, 3, 4, 5 and 6 weeks. At last, PAT concentration for the best treatment

**Table 1: Eight-treatment combination PBD<sup>a</sup> to study the effect of seven factors (A-G) on probiotics survival in apple juice.**

Treatment combination number	A: Probiotic strain	B: Inoculum size (CFU/mL)	C: FOS <sup>b</sup> (% w/v)	D: Inulin (% w/v)	E: PAT <sup>c</sup> (µg/L)	F: Ascorbic acid (mg/L)	G: Citric acid (g/L)
1	L. <sup>d</sup> plantarum	10 <sup>8</sup>	2.5	0	150	200	4
2	L. acidophilus	10 <sup>8</sup>	0	2.5	110	200	4
3	L. acidophilus	10 <sup>10</sup>	0	0	150	0	4
4	L. acidophilus	10 <sup>10</sup>	2.5	0	110	200	0
5	L. plantarum	10 <sup>10</sup>	2.5	2.5	110	0	4
6	L. acidophilus	10 <sup>8</sup>	2.5	2.5	150	0	0
7	L. plantarum	10 <sup>10</sup>	0	2.5	150	200	0
8	L. plantarum	10 <sup>8</sup>	0	0	110	0	0

a) Plackett-Burman Design, b) Fructooligosaccharide, c) Patulin, d) Lactobacillus

the combination was determined by High Performance Liquid Chromatography (HPLC) [25]. The extraction of PAT was performed according to AOAC [25]. The Merck Hitachi (Tokyo, Japan) chromatographic system with a high pressure pump (L-6200A) and autosampler (AS-2000A) was used.

The column (SC-02-100 Prontosil C18, 100 × 2.1 mm, 3 µm particles; Bischoff Chromatography, Leonberg, Germany) was equilibrated with acetonitrile/water (5:95 v/v) as the mobile phase. 20 µl of the samples were injected and eluted and detected by a UV-visible spectrophotometer (L-4250; Merck Hitachi, Tokyo, Japan) at 276 nm. Also, organoleptic properties of the best trials were assessed because a challenge for the probiotic fortification of juices is product acceptance by consumers.

Evaluation of organoleptic properties was carried out by the scoring of the synbiotic apple juice (without adding PAT) and control sample by ten trained assessors daily for 4 days after the first day of production. These assessors were trained in the general sensory analysis of sensory attributes in juices. The samples were served in plastic cups with no color or odor at 20 °C. The arrangement of the presentation of cups to each assessor was in random order. The assessors were asked to evaluate each sample for flavor and color. An evaluation sheet with a 1–6 scales was utilized to indicate the score of the samples as extremely dislike equaled 1, and extremely like equaled 6 [26].

#### Preparation of simulated gastric and intestinal juices

Simulated gastric and small intestinal juices were prepared according to Charteris et al. [27], with some modifications.

Simulated gastric juice was prepared by suspending pepsin (3 g/L) in a sterile sodium chloride solution (0.5%) and adjusting the pH to 2.0 with concentrated HCl using a pH meter. Simulated small intestinal juice was prepared by suspending pancreatin (1 g/L) and bile salts (1.5 g/L) in sterile sodium chloride solution (0.5%) and adjusting the pH to 8.0 with 0.1 mol/L NaOH. Both gastric and intestinal juices were sterile filtered through a membrane (0.45 µm, Nalge Co., Rochester, NY, USA). Simulated gastric and intestinal juices were prepared daily. Anyway, *in vitro* studies about the simulation of gastric juice are only an overestimation of the viability of probiotic microorganisms, while many food components may lead to temporarily change *in vivo* [28]. 1 g of the synbiotic apple juice samples (after 6 weeks of refrigerated storage) was added to 4 mL of tempered (37 °C) simulated gastric juice, mixed well by vortexing for 10 s, and incubated for 2 h at 37 °C. 5 mL of the simulated intestinal juice tempered at 37 °C was then added and incubated for 2 h at 37 °C with periodical shaking. Surviving bacteria after set times of sequential incubation were enumerated by pour, plate counts in plate count agar at 37 °C for 48 h for both the probiotic bacteria [22].

#### Experimental design

The experiments were designed using a PBD [29]. This design allows for the study of  $k = (N-1) / (L-1)$  factors with L levels and N experimental treatment combinations. The usefulness of PBD is in determining the effects of several variables (7, 11 and 15) on the response, independently. The effective factors and their levels were selected based on the literature review [1,3,12,15,19].

The numerical range of each variable should be wide enough but practically reasonable [30]. The important criteria to choose each factor settings for any two-level screening design have been mentioned elsewhere. Table 1 shows the selected experimental variables and their levels, as well as a PBD for conducting 8 treatment combinations. The PBD was constructed using a generating vector.

### Statistical analysis

Statistical analysis of the results was performed using Design Expert software (version 9.0). All data are presented as mean value  $\pm$  standard deviation ( $\pm$  SD) of two independent experiments on different days. The data from the sensory analysis were exposed to Kruskal-Wallis H non-parametric test. A Mann-Whitney U test was used to specify the statistical significance of the means. A  $p$ -value below 0.05 (presented as  $P \leq 0.05$ ) was considered statistically significant.

## RESULTS AND DISCUSSION

When considering the therapeutic properties of probiotic food products, it is essential to remember that they must contain a satisfactory number of live and active cells at the moment of consumption. The evolution of the experimental viability of *L. plantarum* and *L. acidophilus* during the refrigerated storage in apple juice and the log difference between the initial viable count and week 6 [ $\log N_{\text{initial}} - \log N_{\text{week6}}$ ] are presented in Table 2. In all trials, the cell concentrations were reduced significantly during the storage ( $P \leq 0.05$ ). As it is clear, in most cases except treatment combinations numbers 1 and 8, the numbers of cells were higher than 7 log (CFU/mL) during 4 weeks cold storage. According to the results shown in Table 2, in all treatment combinations, the number of cells started to decrease slowly in the first week of storage and significantly reduced ( $P \leq 0.05$ ) by weeks 5 and 6. The highest cell survival was observed for the treatment combination numbers 2 and 6. Decreasing the viability of probiotics in different fruit juices during the refrigerated storage was reported earlier by many researchers [1, 31-34].

There are some strong factors that could limit probiotic survival in fruit juices; Tripathi and Giri [35] grouped them as follows: (i) food parameters such as pH, titratable acidity, molecular oxygen, water activity, presence of salt, sugar, and chemicals, artificial flavoring

and coloring agents; (ii) processing parameters including pasteurization, cooling rate, packaging materials, storage methods, oxygen levels, and volume, and (iii) microbiological parameters such as strains of probiotics and proportion of inoculation.

In a report, the viable cell counts of *L. casei* and *L. acidophilus* were decreased in a mixture of barberry and black cherry juice after fourth weeks of storage at 4 °C. The reason was addressed to the lack of cultures' ability to survive in the stressful conditions of low pH and high acidity of the mentioned juices [34]. Moreover, Sheehan *et al.* [36] reported that probiotic viability in fruit juice was reduced 1 log cycle during each week of refrigerated storage and a similar result was obtained in the present study (Table 2).

These results differ from the findings of Periera *et al.* [24]. They found that *L. casei* grew during the cold storage and the viable cell counts from an initial value of 7.48 log (CFU/mL) reached to more than 8 log (CFU/mL) during 42 days of storage at 4 °C. This could be due to the difference between bacteria strains and other components of the juices. Anyway, results obtained in this study are in agreement with the results of Alegre *et al.* [31], who reported that *L. rhamnosus* remained viable in orange and apple juices over 12 and 4 weeks of cold storage at 4 °C, respectively. In the present investigation, the viable cell counts of *L. acidophilus* ATCC 4356 and *L. plantarum* ATCC 8014 strains in all runs were around  $10^7$  CFU/mL at the fourth week of cold storage at 4°C (except run 1). So, all runs, except Sample 1, had probiotic property until 28 days of cold storage. The lowest survivability of *L. plant-arum* in treatment combination number 1 can explain as low tolerance of this probiotic strain in the highly acidic environment, in contrast to *L. acidophilus* in treatment combination number 2. This is similar to another report [33], which showed the viability of probiotics in pomegranate juice during the cold storage. 2 log (CFU/mL) decrease in the viable cell count of *L. plantarum* after one week of cold storage.

This decrease was due to the low pH and existence of some metabolites such as organic acids. Also, a significant decrease ( $p \leq 0.05$ ) in the viability of *L. plantarum* was observed in grape juice during 28 days of cold storage [32]. Statistical calculations for the PBD of viable count of probiotics for 6 weeks are summarized in Table 3. Each variable, which related  $p$ -value is lower than 0.05,

**Table 2: Viability of probiotic strains of eight-treatment combination PBD<sup>a</sup> in apple juice during 6 weeks of refrigerated storage<sup>b</sup>.**

Run number	Initial <sup>f</sup> Log (CFU/mL)	Week 0 <sup>d</sup> Log (CFU/mL)	Week 1 Log (CFU/mL)	Week 2 Log (CFU/mL)	Week 3 Log (CFU/mL)	Week 4 Log (CFU/mL)	Week 5 Log (CFU/mL)	Week 6 Log (CFU/mL)	$[\log N_{\text{initial}} - \log N_{\text{week6}}]$
1	8.00 ± 0.12	8.40 ± 0.01	7.81 ± 0.12	5.80 ± 0.30	5.08 ± 0.30	4.23 ± 0.20	4.05 ± 0.12	3.82 ± 0.13	4.18 ± 0.12
2	8.30 ± 0.20	8.53 ± 0.06	7.99 ± 0.08	7.74 ± 0.12	7.24 ± 0.13	7.00 ± 0.05	6.81 ± 0.12	6.51 ± 0.21	1.79 ± 0.20
3	10.04 ± 0.10	10.44 ± 0.06	9.57 ± 0.27	9.09 ± 0.22	8.40 ± 0.10	7.38 ± 0.38	6.86 ± 0.05	6.49 ± 0.15	3.55 ± 0.12
4	10.00 ± 0.04	10.43 ± 0.04	9.35 ± 0.36	8.80 ± 0.20	8.27 ± 0.44	7.60 ± 0.30	6.90 ± 0.05	6.60 ± 0.30	3.40 ± 0.17
5	10.17 ± 0.29	10.52 ± 0.11	9.86 ± 0.10	9.31 ± 0.40	8.63 ± 0.22	7.84 ± 0.05	7.01 ± 0.29	6.02 ± 0.09	4.15 ± 0.19
6	8.11 ± 0.08	8.46 ± 0.03	8.06 ± 0.01	7.88 ± 0.03	7.63 ± 0.08	7.14 ± 0.18	6.76 ± 0.10	6.09 ± 0.12	2.02 ± 0.10
7	10.07 ± 0.15	10.36 ± 0.00	9.85 ± 0.10	8.78 ± 0.18	8.00 ± 0.30	7.90 ± 0.06	7.20 ± 0.29	6.43 ± 0.11	3.64 ± 0.13
8	8.11 ± 0.10	8.47 ± 0.03	7.98 ± 0.08	7.40 ± 0.46	6.80 ± 0.05	6.34 ± 0.04	5.85 ± 0.10	5.44 ± 0.20	2.67 ± 0.15

a) Plackett-Burman design, b) Mean ± standard Deviation, c) Just after inoculation, before cold storage, d) One day after refrigerated storage

**Table 3: Statistical data for analysis of variance of probiotics viability in synbiotic apple juice\*.**

variables	Week 0 <sup>a</sup>		Week 1 <sup>b</sup>		Week 2 <sup>c</sup>		Week 3 <sup>d</sup>		Week 4 <sup>e</sup>		Week 5 <sup>f</sup>		Week 6 <sup>g</sup>	
	Coef <sup>h</sup>	P value <sup>g</sup>	Coef <sup>h</sup>	P-value	Coef <sup>h</sup>	P value	Coef <sup>h</sup>	P-value	Coef <sup>h</sup>	P-value	Coef <sup>h</sup>	P-value	Coef <sup>h</sup>	P-value
Probiotic strain	0.01	0.633	-0.06	0.149	0.28	0.067	0.38	0.017	0.35	0.004	0.400	0.006	0.50	0.002
Seed size, CFU/mL	0.99	<0.0001	0.85	<0.0001	0.89	0.001	0.82	0.001	0.75	0.0002	0.56	0.001	0.46	0.003
FOS <sup>i</sup> , % w/v	0.00	1.000	-0.03	0.361	-0.15	0.245	-0.10	0.340	-0.22	0.020	-0.25	0.029	-0.29	0.016
Inulin, % w/v	0.01	0.547	0.13	0.027	0.33	0.042	0.37	0.019	0.54	0.0008	0.51	0.002	0.34	0.010
PAT <sup>j</sup> , µg/L	-0.03	0.245	0.01	0.726	-0.21	0.130	-0.23	0.078	-0.27	0.011	-0.21	0.047	-0.22	0.044
Ascorbic acid, mg/L	-0.02	0.494	-0.05	0.211	-0.32	0.045	-0.36	0.021	-0.24	0.014	-0.19	0.063	-0.08	0.314
Citric acid, g/L	0.02	0.469	0.0006	0.987	-0.11	0.359	-0.17	0.157	-0.32	0.006	-0.25	0.029	-0.21	0.045

a) Week 0 means one day after cold storage,  $A_0 = 9.45$  (mean of the experimental yield), standard error,  $S_b = 0.027$ ; b)  $A_0 = 8.80$ ,  $S_b = 0.038$ ; c)  $A_0 = 8.10$ ,  $S_b = 0.110$ ; d)  $A_0 = 7.50$ ,  $S_b = 0.097$ ; e)  $A_0 = 6.92$ ,  $S_b = 0.060$ ; f)  $A_0 = 6.43$ ,  $S_b = 0.075$ ; g)  $A_0 = 5.92$ ,  $S_b = 0.079$ ;

h) Coefficient; i) Fructooligosaccharide; j) patulin

\*Values of "P" less than 0.0500 indicate that the model terms are significant. Values greater than 0.1000 indicate that the model terms are not significant.

is significant. Coefficient values in this table show the relative effect of each variable on the viability of probiotics. Table 3 shows that the best results were achieved at the negative level of FOS, PAT, ascorbic acid, and citric acid contents during most of the cold storage time. At week 1 *L. plantarum* survival was better than *L. acidophilus*.

Regarding Table 3, the most significant variables affecting the viable count of probiotics in apple juice during our storage condition were inoculum size and inulin concentration, respectively ( $p \leq 0.05$ ). Kind of probiotic strain showed an impact on the viability of probiotics in apple juice. Also, ascorbic acid content played an important role during weeks 2, 3 & 4.

Other significant variables in weeks 4, 5 and 6 included FOS, PAT and citric acid content ( $p \leq 0.05$ ).

So, according to data analysis, best conditions for the viability of probiotics in apple juice during 6 weeks of cold storage should involve  $10^{10}$  CFU/mL of *L. acidophilus* and 2.5% (w/v) inulin. In PBD, when the coefficient of a variable is not high enough, two reasons may exist: the variable is not significant, or the selected range finding is not wide enough to ensure a measurable response. Also when a variable leads to a significant difference in response, this may mask the effect of other variables. So like other screening designs, in PBD, the researcher can claim which variables are more important than others in the selected range.

*Nualkaekul* and *Charalampopoulos* found that pH and citric acid were the main factors influencing the survival of lactic acid bacteria strains' during refrigerated storage [1]. They reported that high levels of citric acid supported the survival of *L. plantarum* in model solutions. But it is in contrast with our results because this factor was significant only in weeks 4, 5 and 6. However, our results demonstrated that inserting 4 g/L citric acid to the apple juice samples containing  $10^8$  CFU/mL of *L. acidophilus*, 2.5% (w/v) inulin, and 200 mg/L ascorbic acid improved the probiotic survival (according to Table 3). There is limited information regarding the role of citric acid in lactic acid bacteria survival during storage.

Ascorbic acid has been associated with the good survival of *L. acidophilus* in yogurt as it acts as an oxygen scavenger [37]. The same results were obtained in the present study in treatment combination number 2 (Table 2). This is in contrast to the results of another

study by *Nualkaekul* and *Charalampopoulos* [1] which showed that ascorbic acid did not have an effect on the cell survival of *L. plantarum*. It might be because of the kind of bacteria strains and the presence of inulin (that helps the survival of probiotic strains). The results obtained in our experiments indicated that adding prebiotic to apple juice could increase the survival of probiotic strains during storage time. As shown in Table 2, inserting 2.5 % (w/v) inulin to the samples, in comparison to FOS, had a significant effect on the viability of *L. acidophilus* in treatment combination number 2. It can be explained that inulin beneficially affects the growth, activity, and survival of probiotic strains. This has been confirmed by some other studies [1, 2, 13].

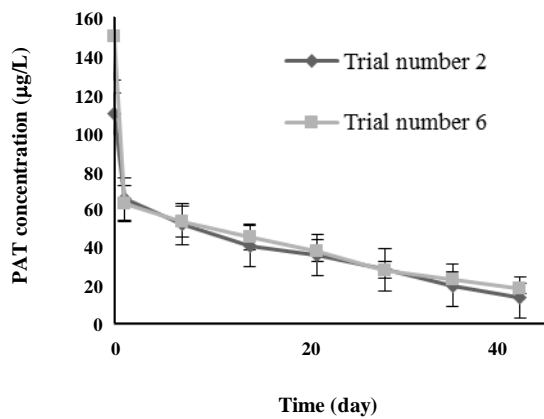
It is reported that the number of live *Bifidobacterium bifidum* DSM 20215 cells was maintained at the level of  $10^6$  CFU/mL in carrot juice supplemented with raftiline, raftilose, and inulin during the first 28 days of cold-storage when  $10^8$  CFU/mL of bacterial cells were initially added to the juice [9].

The survival rates of *L. plantarum* and *L. acidophilus* in the apple juice samples (after 6-week storage at refrigerator) during 2-hour incubation in the presence of simulated gastric juice and 2 hours exposure to simulated intestinal juice are shown in Fig. 1. As shown, *L. acidophilus* in treatment combinations number 2 and 4 and also *L. plantarum* in treatment combination number 7 had the highest viability (around 3.5 log CFU/mL), respectively after 4 h. of exposure to the simulated GI juices, and around 3-4 log cycles decline was observed for all treatment combinations.

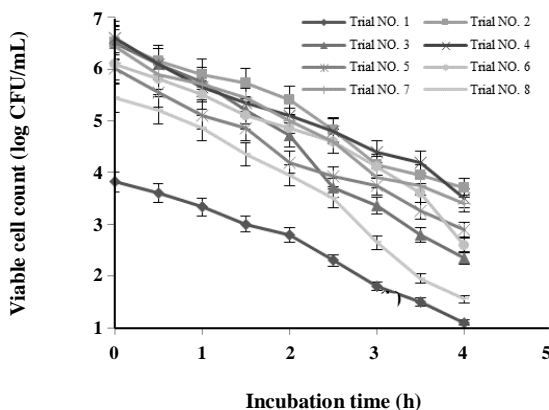
It can be concluded that both of the mentioned bacteria strains in a different environment of storage before consumption could tolerate the simulated GI conditions for 4 h. In our experiments, both *L. acidophilus* ATCC 4356 and *L. plantarum* ATCC 8014 were capable of tolerating the treatments with simulated gastric pancreatic juices (for 4 h); however, it strongly depended on the initial storage conditions.

As demonstrated in Fig. 1, samples of treatment combinations number 3 and 8 (that contained no prebiotic sources and ascorbic acid) had the most reduction in probiotic viability after 4 h of exposure to simulated GI juices.

On the contrary, higher viability was observed for the other samples with at least one prebiotic source.



**Fig. 1:** The viability of *Lactobacillus (L.) plantarum* ATCC 8014 and *L. acidophilus* ATCC 4356 in synbiotic apple juice samples in 8 treatment combinations according to Plackett-Burman design (after 42 days of storage at refrigerator) during 2 h incubation (at 37 °C) in simulated gastric juice and 2 h exposure to simulated intestinal juice (at 37 °C). Surviving bacteria after set times of sequential incubation were enumerated by pour plate counts in plate count agar at 37 °C for 48 h.



**Fig. 2:** Reduction of patulin (PAT) concentration by *Lactobacillus (L.) acidophilus* ATCC 4356 in synbiotic apple juice sample in the best condition (number 2 and 6) of Plackett-Burman design, during 42 days of refrigerated storage. Run number 2 contained  $10^8$  CFU/mL *L. acidophilus* ATCC 4356, 110 µg/L PAT, 2.5% (w/v) inulin, 4 g/L citric acid, and 200 mg/L ascorbic acid. Treatment combination number 6 contained  $10^8$  CFU/mL *L. acidophilus* ATCC 4356, 150 µg/L PAT, 2.5% (w/v) inulin, 2.5% (w/v) fructooligosaccharide. PAT concentration was determined by high-performance liquid chromatography (HPLC) method.

Nazzaro *et al.* investigated the viability of the probiotic strain *L. acidophilus* DSM 20079, after its passage through the simulated gastric and pancreatic juices, as a function of its pre-growth in a medium containing the known prebiotics pectin or inulin, and was compared to glucose as control [2]. They showed that pectin and inulin, in contrast to glucose, induced cell stress resistance against GI juices ( $\Delta_{\log}$  1 and 2 CFU/mL, respectively), which is in agreement with the results of our study. Annan *et al.* evaluated the probiotic *Bifidobacterium adolescentis* 15703T with the objective of enhancing survival during exposure to the adverse conditions of GIT [28]. They found that the populations of bifidobacteria declined over the 2 h incubation period with the final decrease of 3.45 log (CFU/mL). In general, the tolerance of probiotics was improved by the presence of one source of prebiotic and ascorbic acid. In contrast, the citric acid did not show a remarkable effect on the probiotic survival in simulated GI juices (Fig. 1 and Table 1).

As demonstrated in Table 2, treatment combinations number 2 and 6 were the best runs because of the least reduction in log cycle during 6 weeks of refrigerated storage. Therefore, reduction of PAT concentration by probiotics for mentioned treatment combinations was investigated during 42 days of storage in the refrigerator, and the results are shown in Fig. 2.

Results in this figure showed a sharp decrease in PAT concentration after the first day of juice production and PAT removal continues slowly if the shelf life lasts 42 days.

Based on the data given in Fig. 2, by producing synbiotic apple juice the PAT content of contaminated juice was reduced by *L. acidophilus* during 6 weeks of refrigerated storage. In addition, the PAT concentration of the best treatment combinations (numbers 2 and 6) showed 87.26% and 87.67% reduction after the cold storage time, respectively. It should be noted that about 50% of PAT removal caused during the first day after inoculation of probiotics then removal continued slowly during 6 weeks which may be due to gradual binding of free sites at the probiotic surface to PAT. So, it can be concluded that after the first day of juice production (which is usual for transfer of products to shelf), it is safe for customer use. Role of S-layer proteins in the binding of probiotics to PAT was detected as the mechanisms



**Table 4: Average sensory flavor and color scores for control and synbiotic apple juice in treatment combinations number 2 and 6 of PBD<sup>a</sup>.**

	Color			Flavor		
	Run No. 6	Run No. 2	Control	Run No. 6	Run No. 2	Control
Av. score <sup>b</sup>	5.3	5.5	5.7	5	4.5	5
Total score	53	54	56	52	47	50

a) Plackett-Burman design, b) The average score in the range of 1 to 6.

**Table 5: Ethanol concentration<sup>a</sup> of eight-treatment combination PBD<sup>b</sup> in synbiotic apple juice samples after 6 weeks of refrigerated storage<sup>c</sup>**

Trial number	Ethanol concentration g/100 mL
1	1.46 ± 0.07
2	0.28 ± 0.08
3	0.33 ± 0.11
4	0.44 ± 0.06
5	0.15 ± 0.05
6	0.68 ± 0.02
7	0.13 ± 0.02
8	0.08 ± 0.06

of PAT removal by probiotics [20]. Also, Bacterial cell wall peptidoglycans and polysaccharides are responsible for the PAT binding by bacteria [38]. In addition, the findings of earlier reports confirmed the ability of bacteria for PAT elimination [19, 20, 38].

The organoleptic properties of the treatment combinations number 2 and 6 were assessed (Table 4). As it is clearly shown in Table 4, sample numbers 2, 6, and the control sample were not different significantly ( $P > 0.05$ ) in the organoleptic properties; this is similar to the findings of some researchers who showed that probiotics did not affect the overall acceptance of functional foods [39-43] and also juices; e.g., *Perricone et al.* [7] for pineapple juice containing *L. reuteri*, and *Ellendersen et al.* [44] for apple beverage fermented with *L. casei*. So, further studies are required about sensory properties, with the trained panel and consumer acceptance. Also, due to alcohol free diet, the amount of ethanol in the 6<sup>th</sup> week samples was assessed and the results are demonstrated in Table 5. In all trials, probiotics provided the negligible amount of ethanol after 42 days of cold storage because fermentation time was eliminated for all samples.

## CONCLUSIONS

The most significant variables on the survival of *L. acidophilus* ATCC 4356 and *L. plantarum* ATCC 8014 in synbiotic apple juice were inoculum size, inulin concentration, probiotic strain, and concentration of ascorbic acid and citric acid, respectively. The basis of data analysis for achieving better results, mentioned variables except for ascorbic acid and citric acid, should be at a positive level. It can be concluded that *L. acidophilus* ATCC 4356 as a probiotic strain and inulin as a prebiotic are more suitable for the production of synbiotic apple juice, in comparison to *L. plantarum* ATCC 8014 and FOS, respectively. In addition, in the organoleptic study, no significant difference was observed between the produced synbiotic apple juice and the control sample. Also, this product is safe for humans with non-alcoholic diet. Both of the probiotic strains applied in this research, after 6 weeks of cold storage could resist the simulated GI conditions for 4 hours. The results showed that *L. acidophilus* has the capacity of PAT removal from apple juice. It is to be noted that for a commercial application, instead of fresh cells, which were used in this study, the use of either frozen or freeze-dried cells is recommended. This aspect needs to be investigated further, as it is likely the preparation of these cells will affect their survival during storage in the juices. Since PBD is typically used as a preliminary optimization technique, more accurate quantitative analysis of the effect of these variables for PAT reduction from apple juice is required.

## Acknowledgements

We would like to thank the National Nutrition and Food Technology Research Institute, and Shahid Beheshti University of Medical Sciences of Iran for financial and technical support of this research project.

Received : Nov. 4, 2017 ; Accepted : Mar. 7, 2018

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