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Production of synbiotic apple juice and evaluation of viable count of *Lactobacillus Plantarum* ATCC 8014 during refrigerated storage

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ABSTRACT

This investigation is an attempt to study the survival of *Lactobacillus plantarum* ATCC 8014 in apple juice and evaluation of independent variables (content of prebiotic, citric acid and ascorbic acid) on viability during shelf life. A central composite design was applied for considering each of the three factors at five levels to find the optimum points. The treatments were stored at 4°C for 6 weeks. The results indicated that incorporation of 2.094% (w/v) fructooligosaccharide, 207.050 mg/L ascorbic acid and 0.500 g/L citric acid to apple juice with *Lactobacillus plantarum* inoculate (4.0×10^{11} CFU/ml) improved the viability of probiotic strain during cold storage, and led to producing an optimized synbiotic apple juice. Also the probiotic showed tolerance to treatment with simulated gastric and pancreatic juice (for 4 hours). No significant different was observed between flavor (overall acceptability) of the synbiotic apple juice and the control sample until 28 days of cold storage. This product is health promoting for those who need to non-dairy probiotic products in a non-alcoholic diet.

KEYWORDS: *apple juice, functional food, prebiotic, probiotic, simulated gastro-intestinal conditions, viability*

1. INTRODUCTION

There is a major trend for consumers to purchase functional foods, which provide excellent nutrition and health benefits, especially those that can prevent disease [1]. Probiotics are described as live microorganisms that have health benefits on gastrointestinal infection, reduction in serum cholesterol, metabolism of lactose, anti-diarrheal properties, and stimulation of the immune system [2]. Probiotics are mainly used in the production of dairy products, such as yogurt and cheese, which may cause inconveniences due to their lactose and cholesterol content [3]. Other non-dairy foods that can be ideal substrates for the culture of probiotics are fruit juices as they contain beneficial nutrients such as minerals, vitamins, dietary fibers, and antioxidants for lactose intolerant consumers; also these foods support the survival of probiotics during cold storage of juices [4]. Incorporation of apple juice with prebiotics and probiotics is a health promoting product not only for those who needs to non-dairy probiotic products but also for a non-alcoholic diet.

Lactobacilli and Bifidobacteria are the most commonly used probiotics in functional foods [5, 6]. Several investigations have used *Lactobacillus* (*L.*) *plantarum* as a probiotic strain for probiotic fruit juice production [3, 4, 7, 8, 9] and they reported that *L. plantarum* showed good survival in many fruit juices. Gaudana et al. [10] demonstrated that *L. plantarum* ATCC 8014 is a good potential probiotic candidate.

To achieve beneficial effects, probiotics must be capable of resisting stressful environments during industrial processes and should survive in the product during the shelf life. In general, counts from 10^7 to 10^9 colony-forming units per milliliter (CFU/ml) are usually recommended [11]. The ability of probiotics to tolerant the normal acidic conditions of the gastric juices through the stomach allows them to enter the intestinal tract, where they can exhibit their useful effects on the intestinal microbiota [12].

Probiotic viability in fruit juices is also affected by strain, additives, pH, the temperature of storage, oxygen level, and the presence of inhibitory substances [13]. Perricone et al. [14] suggested that fruit juices are a good carrier for *L. reuteri* DSM 20016 but their viability is strongly affected by the kind of juices. Espirito-Santo et al. [8] demonstrated that apple juice is the best substrate for *Lactobacillus* growth and viability. In addition, they emphasized the importance of selection of the probiotic strain, which is the most adaptable to a given food substrate.

Prebiotics are food components mostly consisting of nonstarch polysaccharides and oligosaccharides that resist host digestion, cultivate the growth or activity of specific positive bacterial species, and enhance their survivability for the benefit of host well-being; therefore, they are fermented by the microflora present in the gastrointestinal tract (GIT). The biological effects of prebiotics depend on their impact on the gut's microbiota composition and derived metabolites [15]. Fructooligosaccharide (FOS) is glucose or fructose-terminated polymer of fructose naturally occurring in a variety of plants. Most of the commercially available FOSs are produced from sucrose and are considered as functional fiber. FOSs are one of the most used prebiotics in fruit juices. They have tolerance in digestion by gastric and pancreatic enzymes both *in vitro* and *in vivo* [16]. Pimentel et al. [17] 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.

Synbiotic products are appropriate combinations of pre- and probiotics. A synbiotic product exerts both a prebiotic and probiotic impact [15].

Although few reports are available regarding the factors influencing probiotic survival in fruit juices compared to dairy products, the most likely parameters include the prebiotic content [18] and ascorbic and citric acids concentration [2,3]. It has been reported that ascorbic acid has a positive effect on survival of *L. acidophilus* because it acts as an oxygen scavenger [2]. Besides its benefits for human health, ascorbic acid increases probiotic survival during refrigerated storage [3]. Similarly, high contents of citric acid play an important role in the survival of *L. plantarum* and *L. acidophilus* in fruit juices during the cold storage [3]. Additionally, inserting ascorbic acid and citric acid to apple juice leads to a reduction of pH and certain lactic acid bacteria (LAB) strains to provide more surface (S) layer proteins at low pH values [9]. Earlier researchers showed that *L. plantarum* can remove toxins from aqueous solution successfully. Probiotics adsorb toxins by surface binding due to great adhesive properties of S-layer proteins in their cell wall and high efficiency of toxin removal depends on thicker S-layer proteins. Certain probiotic bacteria that show good adhesion to intestinal cells lose this property when binding to toxins and will rapidly pass through the gastrointestinal tract [9]. Studies dealing with the probiotic apple juice can be found in the literature; however, to our knowledge, this is the first report on the effect of three variables (FOS, citric acid and ascorbic acid concentration) on the microbial, nutritional and sensory properties of synbiotic apple juice containing *L. plantarum* ATCC 8014 using a central composite design (CCD). So, this study investigates the survival ability, and chemical changes (content of reducing sugar and titratable acidity) of synbiotic apple juice, as well as the sensory properties of the product during 6 weeks cold storage. Furthermore, it evaluates the viable count of the mentioned probiotic strain in optimized synbiotic apple juice under simulated GIT conditions after 42 days of refrigerated storage.

2. EXPERIMENTAL SECTION

2.1. Chemicals and media

De Man-Rogosa-Sharpe (MRS) broth was obtained from Liofilchem (Italy). All chemicals used in the experiments were obtained from CarloErba (France). Bile salts (Oxgall), Pepsin (from porcine stomach mucus, P-7000) and Pancreatin (from the pancreas, P-1500) were supplied by Sigma-Aldrich (Vienna, Austria). FOS was purchased from Sensus Company (Roosendaal, Netherlands).

2.2. Preparation of probiotic culture

L. plantarum ATCC8014 as a probiotic strain [10] was obtained from Tak Gene Zist Company (Tehran, Iran). The strain was inoculated into 10 ml MRS broth (pH 6.2) and incubated at 37°C for 48 h. The number of live bacteria in the cultures was determined by total plate count and MRS agar was used for the enumeration of *L. plantarum*.

2.3. Preparation of synbiotic apple juice

Commercial apple concentrate was purchased from Takdaneh Group Company (Marand, Iran) and kept at 4°C before using. Apple juice was prepared by 14 g apple concentrate and 85 ml distilled water (brix 11, pH 3.7). For the first step, the experiments were performed with CCD, and the optimal combinations of FOS, citric acid, and ascorbic acid were pinpointed. In the second step, we used this combination for the final reconfirmation of optimum condition for production of synbiotic apple juice with the maximum viability of probiotics. The experiments were designed using a CCD [19, 20]. Three factors were studied at five different levels within the following ranges; FOS (1.5–3.5% w/v), citric acid (0.5–2.5 g/L), and ascorbic acid (100–300 mg/L). Twenty experimental trials were needed in total which six experimental trials were at the central point that is detailed in Table 1.

Table 1. The survival of *Lactobacillus plantarum* ATCC 8014 in synbiotic apple juice during refrigerated storage as a function of FOS, citric acid, and ascorbic acid in 5 levels using CCD.

Trial No.	FOS		Citric acid		Ascorbic acid	
	Real value (%w/v)	Coded value	Real value (g/L)	Coded value	Real value (mg/L)	Coded value
1	3	1	2	1	260	1
2	2	-1	1	-1	260	1
3	2.5	0	1.5	0	300	1.6
4	3	1	2	1	140	-1
5	2.5	0	0.5	-1.6	200	0
6	2	-1	2	1	260	1
7	2.5	0	1.5	0	100	-1.6
8	2.5	0	2.5	1.6	200	0
9	2.5	0	1.5	0	200	0
10	3	1	1	-1	140	-1
11	2.5	0	1.5	0	200	0
12	2	-1	2	1	140	-1
13	3	1	1	-1	260	1
14	2.5	0	1.5	0	200	0
15	2.5	0	1.5	0	200	0
16	1.5	-1.6	1.5	0	200	0
17	2	-1	1	-1	140	-1
18	2.5	0	1.5	0	200	0
19	2.5	0	1.5	0	200	0
20	3.5	1.6	1.5	0	200	0

The values for each of the factors were chosen so that they could reflect the values usually found in fruit juices. According to CCD (Table 1), defined amounts of FOS, citric acid, and ascorbic acid were inserted to the apple juice samples. After transferring the samples to sterile glass flasks and pasteurizing for five min at 90°C, all samples were inoculated with determined probiotic strain (about 13 log (CFU/ml) of *L. plantarum*), and initial viability of the samples was 11.69 ± 0.02 log (CFU/ml). It is to be mentioned that each flask of the samples contained 100 ml synbiotic apple

juice. The juices were stored at 4°C for 42 days. The samples were collected every 3 weeks and analyzed for viable cell counts. The CCD was applied to analyze the main and interaction effects of the content of FOS, citric acid, and ascorbic acid. Stepwise regressions were used to analyze the probiotic viability during cold storage. After determining the optimum amounts of FOS, citric acid and ascorbic acid (by using MINITAB statistical software, version 14), in order to insert to apple juice, optimized synbiotic apple juice was produced and its viable cell counts during the refrigerated storage were calculated; then simulated gastrointestinal juices were assessed.

2.4. Counts of viable bacteria

Viable probiotic strains were determined by pour plate counting in duplicate on MRS agar. The colonies were counted after 72 h of incubation at 37ºC; after which, they were calculated as CFU/ml and the results were expressed as log values [21]. The viability of probiotics was assessed after 1 day in the refrigerator (week 0) and then after 3 and 6 weeks.

2.5. Chemical analysis

Titratable acidity was measured by titration with titrazol 0.1N NaOH and expressed as lactic acid (g/100g) [22]. Determination of reducing sugars (g/100g) and ethanol concentration (g/100ml) was carried out by Fehling method and using digital distillation equipment, respectively (Iran National Standard No. 2685).

2.6. Sensory analysis

Evaluation of sensory attribute was carried out by scoring of the optimized synbiotic apple juice and control sample by ten experienced assessors. These assessors were trained in general sensory analysis of sensory attributes in juices. The samples were served in plastic cups with no color or odor at 20ºC as recommended for sensory evaluation of juices [23]. The arrangement of presentation of cups to each assessor was in a random order. The assessors were asked to evaluate each sample for flavor (overall acceptability). An evaluation sheet with a 1–6 scales was utilized to indicate the score of the samples as extremely dislike = 1and extremely like = 6 [24].

2.7. Preparation of simulated gastric and small intestinal juices

Simulated gastric juices were prepared by inserting pepsin to the sterile sodium chloride solution (0.5%, w/v) to a final concentration of 3 g/L and adjusting the pH to 2.0 with concentrated HCl using a pH meter. Simulated small intestinal juices were prepared by inserting pancreatin to the sterile sodium chloride solution (0.5%, w/v) to a final concentration of 1 g/L, with 1.5 g/L bile salts (Oxoid) and adjusting the pH to 8.0 with sterile 0.1 mol/L NaOH using a pH meter. Both the gastric and intestinal juices were sterile filtered through a membrane (0.45 µm, Nalge Co., Rochester, NY, USA) and were prepared fresh for use on the same day [25]. 1 g of the optimized synbiotic apple juice sample (after 6 weeks of refrigerated storage) was added to 4 ml of the 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 [26]. Surviving bacteria after setting the time of sequential incubation were enumerated by pour plate counts in plate count agar at 37°C for 72 h as described above.

2.8. Statistical analysis

Statistical analysis of the obtained results was performed using MINITAB statistical software (version 14). The data were statistically treated by analysis of variance (ANOVA). Three- and higher-order interactions were eliminated. All data are presented as the mean ± standard deviation (M ± SD) of two independent experiments at 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 among the means. A *P*-value below 0.05 (presented as $P \leq 0.05$) was considered statistically significant.

3. RESULTS SECTION

3.1. Results.

The cell concentration of *L. plantarum* ATCC 8014 during 6 weeks of cold storage in the apple juice treatments according to CCD (Table 1), and the log difference between the initial concentration and week 6 [$\log N_{initial} - \log N_{week6}$] are presented in Table 2. In all trials, the number of cells decreased during the storage; the cell concentrations were also reduced significantly ($P \leq 0.05$) by week 6 although, in most cases, they were still higher than 7 log CFU/ml. The highest cell survival was observed for trial numbers 5 (includes: 2.5% w/v FOS; 0.5 g/L citric acid; and 200 mg/L ascorbic acid) and 17 (includes: 2% w/v FOS; 1 g/L citric acid; and 140 mg/L ascorbic acid). High concentration of ascorbic acid led to low cell survival, as shown by the result of trial number 3 (includes: 2.5% w/v FOS; 1.5 g/L citric acid; and 300 mg/L ascorbic acid).

The amounts of reducing sugars and titratable acidity of all samples are shown in Table 3. Decreasing of reducing sugars and increasing of titratable acidity observed in all the 20 synbiotic apple juice samples were not significant ($P > 0.05$). In addition, since some people should have a diet without ethanol (like pregnant women or because of cancer disease); therefore, the amount of ethanol in the 6th week samples was assessed and the results are demonstrated in Table 3. In all trials, probiotics provided the negligible amount of ethanol after 42 days of cold storage because fermentation time was eliminated for all samples.

The coefficient of regression (R^2) for probiotic viability during 6 weeks cold storage was 0.93. Analysis of variance of the stepwise regression showed a first order polynomial model that fits well the data. Also, in the residual plots did not observe any trend in the distribution of the residuals around the zero line (means the goodness of fit). The value of R^2 showed good agreement between experimental and predicted values. The F ratio for lack of fit of the model for probiotic viability during 6 weeks cold storage was not significant ($P > 0.05$). The polynomial mathematic model was:

$[\log N_{initial} - \log N_{week6}] = 1.22 + 0.00440[\text{ascorbic acid}] + 0.346[\text{FOS}] + 0.655[\text{citric acid}]$

Table 2. Viability of *Lactobacillus plantarum* ATCC 8014[a] of twenty-trial CCD[b] in apple juice samples during 6 weeks of refrigerated storage[c]

Trial number	Week 0 log (CFU/ml)	Week 3 log (CFU/ml)	Week 6 log (CFU/ml)	[log $N_{initial}$ – log N_{week6}]
1	11.70 ± 0.09	9.59 ± 0.04	7.60 ± 0.04	4.09 ± 0.03
2	11.00 ± 0.02	9.27 ± 0.01	8.60 ± 0.06	3.09 ± 0.04
3	11.49 ± 0.01	9.65 ± 0.03	6.30 ± 0.01	5.39 ± 0.01
4	11.44 ± 0.01	11.00 ± 0.03	7.60 ± 0.02	4.09 ± 0.02
5	11.32 ± 0.04	11.04 ± 0.01	8.72 ± 0.00	2.97 ± 0.01
6	11.51 ± 0.01	11.00 ± 0.06	6.60 ± 0.08	5.09 ± 0.05
7	11.00 ± 0.03	10.90 ± 0.05	8.30 ± 0.10	3.39 ± 0.06
8	11.41 ± 0.00	10.95 ± 0.11	7.30 ± 0.02	4.39 ± 0.02
9	11.30 ± 0.06	9.30 ± 0.09	7.77 ± 0.00	3.92 ± 0.01
10	11.49 ± 0.10	9.47 ± 0.04	7.00 ± 0.03	4.69 ± 0.02
11	11.30 ± 0.05	9.30 ± 0.02	7.77 ± 0.04	3.92 ± 0.03
12	11.46 ± 0.01	9.84 ± 0.02	8.00 ± 0.04	3.69 ± 0.03
13	11.39 ± 0.07	10.65 ± 0.00	8.36 ± 0.12	3.33 ± 0.07
14	11.34 ± 0.09	9.30 ± 0.01	7.66 ± 0.09	4.03 ± 0.05
15	11.30 ± 0.02	9.07 ± 0.09	7.77 ± 0.07	3.92 ± 0.04
16	11.38 ± 0.08	10.47 ± 0.07	8.00 ± 0.08	3.69 ± 0.05
17	11.04 ± 0.11	10.00 ± 0.05	8.77 ± 0.01	2.92 ± 0.01
18	11.50 ± 0.01	9.11 ± 0.08	7.74 ± 0.03	3.95 ± 0.02
19	11.07 ± 0.00	9.34 ± 0.03	7.78 ± 0.01	3.91 ± 0.01
20	11.55 ± 0.03	11.00 ± 0.03	7.17 ± 0.02	4.52 ± 0.02

a. Initial viability of all trials was 11.69 ± 0.02 log (CFU/ml)
b. Central Composite Design
c. Standard deviation (± SD) calculated with 95% confidenc

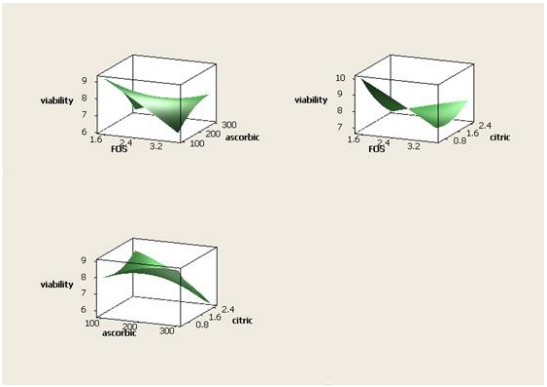


Figure 1. Response surface plot illustrating the effect of fructooligosaccharide, citric acid, and ascorbic acid concentration and their interactions on the viability of *Lactobacillus plantarum* ATCC 8014 in synbiotic apple juice after 42 days of refrigerated storage.

Fig. 1 shows the response surface plot of the viability of *L. plantarum* ATCC 8014 in apple juice as a function of FOS, ascorbic acid, and citric acid concentration. According to the plots, by increasing the citric acid concentration in apple juice, viability of the probiotic strain decreased, while FOS and ascorbic acid concentration have an optimum point in order to improve the viability of *L. plantarum* in the apple juice samples during cold storage. It means that if we use the optimum amounts of all variables in apple juice, the probiotic viability will improve significantly. On the other hand, reverse results will be achieved. According to the results obtained from data analysis by MINITAB (version 14), the optimum points for three variables include 2.094 g/100ml FOS, 207.050 mg/L ascorbic acid and 0.500 g/L citric acid. By inserting the mentioned amounts of variables to the apple juice samples and inoculation of 11.55 log (CFU/ml) *L. plantarum*, optimized synbiotic apple juice was produced.

The viability of *L. plantarum*in the optimized synbiotic apple juice (with inserting optimum amount of FOS, citric acid, and ascorbic acid) during 6 weeks of refrigerated storage is presented in Fig. 2. The cell concentration increased slightly (P>0.05) after one day of storage and then decreased approximately by 3.6 log cycles after 6 weeks of storage and reached to 8.0×10^{7}CFU/ml from an initial concentration of 3.6×10^{11} CFU/ml. It is worth mentioning that a control sample (just apple juice) was tested in order to compare with the optimized synbiotic apple juice. The control sample was inoculated by about 11 log (CFU/ml) *L. plantarum* and almost 6 log cycles decline was observed after 6 weeks of cold storage. Ethanol concentration of the optimized synbiotic apple juice after 42 days of refrigerated storage was 0.109 g/100ml.

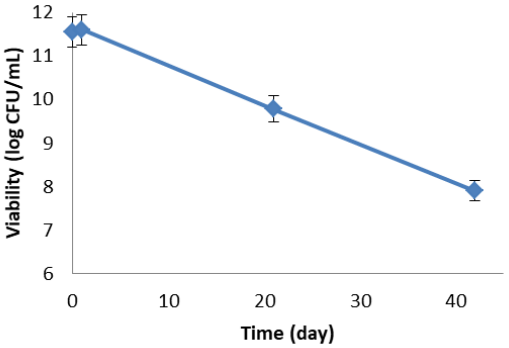


Figure 2. The viability of *Lactobacillus plantarum* ATCC 8014in optimized synbiotic apple juice (with inserting defined fructooligosaccharide, citric acid, and ascorbic acid content) during 6 weeks of refrigerated storage.

The cell concentration of *L. plantarum* (after 42 days of refrigerated storage of the optimized synbiotic apple juice) in the simulated gastric and intestinal juices is demonstrated in Fig. 3. Around 3.6 log cycles decline was observed for the mentioned probiotic strain after 4 h of exposure to simulated GI juices, and the final cell concentration reached to 4.34 log (CFU/ml). Therefore, we can say that *L. plantarum* ATCC 8014 after consumption of synbiotic apple juice can tolerance simulated GI conditions at least for 4 h.

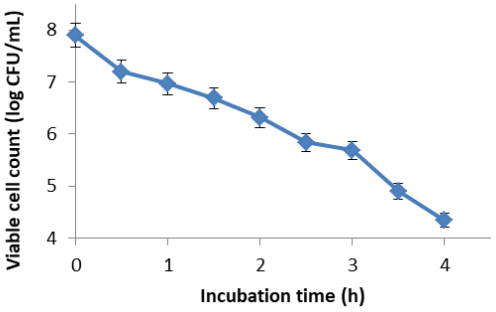
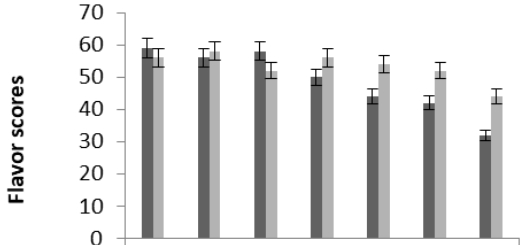


Figure 3. The viability of *Lactobacillus plantarum* ATCC 8014 in optimized synbiotic apple juice with inserting defined fructooligosaccharide, citric acid, and ascorbic acid (after 42 days of storage at refrigerator) during 2 h of incubation (at 37°C) in the presence of simulated gastric juice and 2 h of exposure to simulated intestinal juice (at 37°C). The survived bacteria after setting the time of sequential incubation were enumerated by pour plate counts in plate count agar.

At last, the results obtained from the sensory assessment of optimized synbiotic apple juice during 6 weeks of cold storage are shown in Fig. 4.



Time (week)	0	1	2	3	4	5	6
synbiotic apple juice	59	56	58	50	44	42	32
apple juice	56	58	52	56	54	52	44

Figure 4. Average sensory flavor scores for optimized synbiotic apple juice and control apple juice during 6 weeks of cold storage at 4°C (score range: 1- 6).

3.2. Discussion.

It has been reported that the fermentation provided a high number of cells in the fruit juices, but the sensory characteristics of the products were not accepted by the consumers [27]. Hence, in order to prevent the possibility of fermentation and producing ethanol by probiotics, in this study the samples (without fermentation time) were kept in the refrigerator at 4°C for 6 weeks. Moreover, Pereira et al. [28] said that *L. casei* showed an acceptable viability with less than one log (CFU/ml) reduction in cashew apple juice at 4°C for a long shelf-life. Survival of probiotics in the gastrointestinal tract in sufficient numbers needs a concentration of at least 10^7CFU/ml of probiotic strains in the product at the end of shelf-life; this helps to identify the efficiency of the product [3]. According to the results from Table2, most trials (except trials 3 (2.5% w/v FOS; 1.5 g/L citric acid; and 300 mg/L ascorbic acid) and 6 (2% w/v FOS; 2 g/L citric acid; and 260 mg/L ascorbic acid)) have more than 7 log (CFU/ml) cell concentration after 42 days shelf-life. Also, probiotic viability in the optimized synbiotic apple juice decreased from 11.55 log (CFU/ml) to 7.90 log (CFU/ml) after storage time (Fig.2). Yoon et al. [29] evaluated the production of probiotic beet juice by the four species of LAB. Although the probiotic strains in beet juice gradually lost their viability during the cold storage, the viable cell counts of these bacteria still remained at 10^6–10^8 CFU/ml after 4 weeks.

There are some important factors that could limit probiotic survival in fruit juices; *Tripathi* and *Giri* [30] 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.

Reducing the viability of probiotics in different fruit juices during cold storage has been reported by many researchers [3, 31]; this is in agreement with our findings (Table 2; Fig.2). But these results differ from the similar studies conducted by Perieraet al. [28]. They found that *L. casei* grew in cashew apple juice during 42 days of storage at 4°C, and the cell concentration from an initial value of 7.48 log (CFU/ml) reached to more than 8 log (CFU/ml). In another study, Champagne et al. [32] inoculated apple – pear – raspberry juice mixture with 4.5×10^9 CFU/250ml *L. rhamnosus* and reported the viability of *L. rhamnosus* (1.5×10^9 CFU/250ml) in the product during4 weeks of storage at 2-7°C. This could be due to the difference in probiotic strains used and other ingredients of the juices. On the other hand, Yoon et al. [33] produced a probiotic cabbage juice using LAB. After 4 weeks of cold storage, the viable cell counts of *L. plantarum* and *L. delbrueckii* decreased from an initial concentration of 1×10^9 CFU/ml to 4.1×10^7 and 4.5×10^5 CFU/ml, respectively. Espirito-Santo et al. [8] used lactobacilli strains from commercial and artisanal food origins for the production of probiotic apple juice and reported the decrease in lactobacilli counts ranging from 3.3 to 4.8 log (CFU/ml) after 28 days of refrigerated storage. They highlighted that the commercial lactobacilli strain *L. plantarum* 299 v showed higher growth and viability in the fermented apple juice than the ones isolated from artisanal products: *L. plantarum* CIRMBIA 466.

The lowest viable cell count was observed for trial number 3 (2.5% w/v FOS; 1.5 g/L citric acid; and 300 mg/L ascorbic acid) that contained the highest amount of ascorbic acid. Most probably, this decrease was because of the low pH and high acidity. This is according to Mousavi et al. [31] who investigated the viability of *L. plantarum* in pomegranate juice during the cold storage. In another study, the cell concentration in pomegranate juice decreased sharply immediately after inoculation. The main factors impacting this were most likely the low pH of the juice and the low organic acid concentration [3]. According to Sheehan et al. [34], when inserting *Lactobacillus* strains to orange, pineapple, and cranberry juices, extensive differences regarding their acid resistance were observed. All of the strains displayed a great surviving at levels above 7.0 log (CFU/ml) for at least 12 weeks in orange and pineapple juices compared to cranberry juice.

The reason for selecting the mentioned three variables and their ranges is that they have been recommended to play an important role in the survival of lactobacilli in fruit juices [3, 18]. Nualkaekul and Charalampopoulos found that pH and citric acid were the main factors influencing the LAB strains' survival during the refrigerated storage [3]. They said that high levels of citric acid supported the survival of *L. plantarum* in model solutions. But it is in contrast with our results because we found that by increasing the citric acid concentration in apple juice, the viability of the probiotic strain decreased. There are little investigations about the role of citric acid in the survival of LAB in fruit juices during cold storage. Organic acids are commonly used as preservatives because of their antimicrobial properties. The results obtained in this study (Fig. 1) regarding the effect of citric acid on cell survival are in agreement with the findings of Champagne and Gardner [13], who showed that citric acid, would have a negative effect on cell survival in the model solutions. Fermentation studies have reported that citric acid is metabolized by *Lactobacillus* species, and the main product is the acetic acid with the production of ATP [35].

Ascorbic acid has a positive effect on survival of *L. acidophilus* in yogurt because it acts as an oxygen scavenger [2]. Based on the results given in Fig. 1, ascorbic acid can improve survival of *L. plantarum* in apple juice in an optimum concentration. In contrast, it has recently been shown that high levels of citric acid supported the survival of *L. plantarum* in

different juices (orange, grapefruit, blackcurrant, pineapple, pomegranate, cranberry, and lemon) during refrigerated storage while ascorbic acid did not have an effect [3]. This can be most likely due to the role of other components of the juices, such as proteins, dietary fibers, or various antimicrobial compounds.

Roble et al. [7] used FOS as a prebiotic for the production of synbiotic fresh cut apple slices by applying probiotic bacteria (*L. rhamnosus* GG). They claimed that FOS influenced the growth and survival of probiotic strain. According to the results of our experiments (Fig. 1), FOS has a positive impact on survival of *L. plantarum* in apple juice in an optimum amount. In addition, another study reported that the number of live *Bifidobacterium bifidum* DSM 20215 cells was maintained at the level of 6 log (CFU/ml) in carrot juice supplemented with different kinds of prebiotics (raftiline, raftilose, and inulin) during the first 28 days of refrigerated storage when 8 log (CFU/ml) of probiotic strains were initially added to the juice [15].

Table 3. Changes in the titratable acidity and reducing sugars content of twenty-trial CCD[a] apple juice samples during 6 weeks of refrigerated storage and ethanol concentration after 6 weeks of refrigerated storage[b].

Trial number	Initial		Week 0		Week 3		Week 6		
	Reducing sugar g/100g	Titratable acidity[c] g/100g	Reducing sugar g/100g	Titratable acidity[c] g/100g	Reducing sugar g/100g	Titratable acidity[c] g/100g	Reducing sugar g/100g	Titratable acidity[c] g/100g	Ethanol concentration (g/100ml)
1	8.56	0.59	7.87	0.61	7.80	0.61	7.69	0.63	0.20
2	7.95	0.49	7.49	0.52	7.35	0.52	7.26	0.52	0.05
3	8.52	0.54	8.03	0.56	7.95	0.58	7.66	0.58	0.14
4	8.65	0.56	7.95	0.63	7.76	0.65	7.59	0.65	0.17
5	8.47	0.46	8.03	0.46	7.99	0.46	7.73	0.50	0.01
6	7.84	0.59	7.52	0.63	7.26	0.65	7.10	0.65	0.18
7	8.43	0.55	8.14	0.55	8.06	0.55	7.87	0.55	0.10
8	8.39	0.63	8.10	0.65	7.99	0.67	7.62	0.67	0.22
9	8.43	0.53	7.91	0.59	7.45	0.59	7.39	0.59	0.11
10	8.52	0.50	7.84	0.56	7.66	0.56	7.45	0.58	0.04
11	8.43	0.53	7.91	0.59	7.45	0.59	7.39	0.61	0.11
12	7.49	0.53	7.39	0.60	7.20	0.60	7.07	0.60	0.15
13	8.61	0.50	7.76	0.52	7.45	0.52	7.32	0.52	0.07
14	8.39	0.53	7.91	0.59	7.39	0.59	7.39	0.60	0.11
15	8.43	0.53	7.95	0.59	7.45	0.59	7.45	0.59	0.11
16	7.73	0.54	7.52	0.56	7.16	0.56	7.10	0.61	0.08
17	7.66	0.50	7.45	0.52	7.13	0.54	7.04	0.54	0.02
18	8.43	0.53	7.87	0.57	7.42	0.59	7.39	0.59	0.11
19	8.47	0.54	7.91	0.58	7.45	0.58	7.38	0.59	0.11
20	8.52	0.53	8.35	0.57	8.06	0.57	7.80	0.59	0.12

a. Central Composite Design
b. Standard deviation (± SD) calculated with 95% confidence
c. Titratable acidity is expressed as lactic acid

During 6 weeks of cold storage at 4°C, reducing sugars and titratable acidity of the samples were decreased and increased, respectively. One may attribute this phenomenon to consumption of sugars and production of organic acids by probiotics [36]. Buruleanu et al. [37] evaluated the effect of inulin as a prebiotic on the quality of produced lactic acid in carrot and beet extracts during fermentation by *Bifidobacterium* BB12. They reported that the amount of glucose reduced by the selected strain culture, and in contrast, the amounts of lactic acid and acetic acid increased; these findings are in agreement with the data of the present study. Lactic acid could have been produced from the metabolism of sugars, or the metabolism of malic acid through the action of the malo-lactic enzyme, which has been identified in *L. plantarum* [4], and acetic acid is probably produced from the metabolism of citric acid and sugars, as previously shown for *L. plantarum* [35]. Ding and Shah [4] used eight different strains of probiotic bacteria including *L. plantarum* in order to produce probiotic apple juice, and reported that all eight probiotic bacteria produced similar quantities of malic acid in the apple juice and probiotic apple juice had only a slight increase in the malic acid concentration, with an average increase of 0.15 mg/L during 6-week storage.

The results of this study are consistent with the findings of Costa et al. [38] for using sonicated pineapple juice as the substrate for the production of a probiotic beverage by *L. casei*. The results of this study showed that acidity was increased during the cold storage due to using reducing sugars by *L. casei* in order to produce organic acids. Tsen et al. [39] investigated lactic acid production in the medium based on the mashed banana by *L. acidophilus*. The selected strain metabolized low molecular weight sugars (i.e., fructose and glucose) as a carbon source for acid production.

As mentioned above, *L. plantarum* ATCC 8014 in optimized synbiotic apple juice showed tolerance to simulated GIT (Fig. 3), and 3.6 log cycles decrease was observed after 4 h. This is consistent with the findings of some other studies, including Annan et al. [26], who investigated the survival of probiotic *Bifidobacterium adolescentis* 15703T during exposure to the simulated conditions of GIT. They found that the populations of bifidobacteria declined over the 2 h incubation period with the final decrease of 3.45 log (CFU/ml). Nazzaro et al. investigated the viability of *L. acidophilus* DSM 20079, after its transmission through the simulated gastric and intestinal juices, as a function of

its pre-growth in a medium containing the prebiotics pectin or inulin in comparison to glucose as control [11]. They showed that pectin and inulin induced cell stress resistance against GI juices (Δ_{log} 1 and 2 CFU/ml, respectively), in contrast to glucose, which is in agreement with the results of our study about using FOS as prebiotic source. Anyway, *in vitro* studies about simulation of gastric juice are only an overestimation of the viability of probiotic microorganisms, while many food components may lead to a temporary change (decrease in pH) *in vivo*.

According to Fig. 4, there is no significant different (P>0.05) between the flavor (overall acceptability) of optimized synbiotic apple juice and that of control apple juice until week 4 of refrigerated storage, which is similar to the report of some researchers, who demonstrated that probiotics do not affect the overall acceptance of fruit juices [40], e.g., Perricone et al. [14], for pineapple juice containing *L. reuteri*, and Ellendersen et al. [41], for apple beverage fermented with *L. casei*. Based on obtained results, overall acceptability of control apple juice was significantly (P≤0.05) better than that of synbiotic apple juice after 4 to 6 weeks of cold storage. The study of Luckow and Delahunty [42] showed that despite better taste of conventional orange juice, consumers prefer the sensory characteristics of probiotic orange juice because of its health benefits; however, unsuitable contents of aromas and flavors have been reported.

4. CONCLUSIONS

The aim of this work was to evaluate the effect of three variables on the survival of *L. plantarum* ATCC 8014 using a CCD in apple juice containing prebiotic during 42 days of refrigerated storage. In general, the results indicated that inserting optimum amounts of FOS, ascorbic acid, and citric acid to apple juice containing *L. plantarum* ATCC 8014 improved the survival of probiotic strain during 6 weeks of cold storage, and led to the good viability of the mentioned probiotic strain in simulated gastric and bile juices for 4 h. In addition, in the organoleptic study, no significant different was observed between the optimized synbiotic apple juice and the control sample until the 4th week of storage. Also, this product is safe for humans with non-alcoholic diet.

It is to be noted that for a commercial application in juice manufacturers, maybe frozen or freeze dried cells instead of fresh cells, which were used in this study, will be used. Therefore, this aspect needs to be evaluated further, as it is likely that preparation of the cells will affect their survival during storage.

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