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Full Research Paper

Using several fruit and vegetable juices as substrates for producing non-dairy probiotic beverages

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Abstract

Probiotics are living microorganisms that provide beneficial effects when they are eaten with food. The probiotic dairy products raise the risks associated with increased cholesterol and lactose intolerance. In this research, fruit and vegetable juices of apple, banana, carrot and tomato were used as substrates for producing probiotic beverages and the viability of two LABs of *L. acidophilus* and *L. plantarum* in these products was investigated. Fruit and vegetable juices were inoculated with bacterial suspensions to obtain a concentration of 10^5 CFU/ml for each LAB. Samples were incubated at 37°C for 72 hours and at 24-hour intervals, pH levels and viable cell count in products were determined based on CFU/ml. Fermented products were transferred to the refrigerator and the viability of LABs was determined at 4°C for 4 weeks. The results show that, in all products, the pH decreased over time, so that there was a significant difference between the two examined bacteria during the experiment ($P<0.05$). Both LABs were able to grow well in products and to ferment the fruit and vegetable juices properly implying that all the beverages were able to provide suitable conditions for the growth of two strains of LABs. *L. plantarum* showed a higher viability in cold storage at 4°C. In general, considering the high growth rate of these bacteria in the products and pH reduction and their viability during cold storage at 4°C, fruit and vegetable juices of apple, banana, carrot and tomato can be a good substrate for producing non-dairy probiotic beverages.

Keywords: Viability, Cold storage, Fermented Fruit and Vegetable Juices, *L. acidophilus, L. plantarum*

Introduction

Fermentation of food is a desirable process in the food industry, and microorganisms and their enzymes play an important role in this field. Fermentation improves the flavor and taste of foods; increases the shelf-life of foods and enhances the nutritional value of products (Karovicova and Kohajdova, 2003).

Food enrichment with probiotics has been considered as one of the methods of producing fermentation products. Probiotics are living microorganisms that provide beneficial effects for the host if they are eaten with food in adequate amount. Some of the most important health effects of adding probiotics to foods include improving digestive system function (Vasudha and Mishra, 2013), preventing diarrhea (Fuller *et al*., 2008), reducing constipation (Ouwehand *et al*., 2002), improving the lactose digestion (Li *et al*., 2012), reducing serum cholesterol levels (Pereira *et al*., 2002), diminishing the inflammatory bowel disease (Fooks *et al*., 2002), decreasing the risk of colon cancer (Rafter, 2004), enhancing both innate and acquired immunity in the immune system (Fuller *et al*., 2008), decreasing the risk of recurrent urogenital tract infection (Dani *et al*., 2002), inhibiting *Helicobacter pylori* infection (Hamilton-Miller *et al*., 2003), and preventing allergies (Delcenserie *et al.*, 2010).

To provide these health effects, probiotic bacteria should be presented alive in foods, and their concentrations be high enough in the food. The viability of probiotic organisms under difficult conditions, such as low pH of foods and against gastrointestinal enzymes, is one of the most important concerns in the processes and production of probiotic foods. In this regard, the final number of probiotic bacteria must be at least 10^6 - 10^7 CFU/ml or g at the time of food consumption, in order to be

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suitable for providing health (Vasudha and Mishra, 2013; Shaikh Uzma *et al.*, 2018).

The use of different species of probiotic bacteria for producing dairy products has become popular since the late 1970s, and nowadays, dairy products are the most popular probiotic products. Milk products have high cholesterol content, and the increasing population of people with high cholesterol level is regarded as one of the most important food concerns in the modern era. So, consumers prefer more vegetarian diets with lower cholesterol levels. Besides, lactose intolerance in some people is another problem associated with consuming the dairy products (Vasudha and Mishra, 2013).

Therefore, among the foods suitable for adding probiotics, there have been a great demand for non-dairy probiotic products due to a number of reasons; such as milk lactose intolerance in some individuals and high levels of cholesterol in dairy products. Since fruits and vegetables contain beneficial substances such as minerals, antioxidants, dietary fiber and vitamins and are free of sensitizing ingredients present in milk, they can be good substrates for producing non-dairy probiotic beverages (Carlos *et al*., 2007; Nematollahi *et al*., 2013). More than 90% of probiotic foods contain Lactobacillus and Bifidobacterium species (Perricone *et al.*, 2015). Meanwhile, several strains of *L. plantarum* and *L. acidophilus* were used as probiotics in fruit substrates due to their tolerance to acidic conditions (Peres *et al.*, 2012).

High intake of carotenoid-rich fruits and vegetables are associated with a reduced risk of various cancers, including colon cancer. A study conducted on individuals consuming carrot and tomato juices indicated that a diet rich in carotenoids, especially high-dose b-carotene and lycopene, can modify luminal processes relevant to colon carcinogenesis (Schnabele *et al.*, 2008).

The present study aimed at using fruit and vegetable juices of apple, banana, carrot and tomato as substrates for producing non-dairy probiotic beverages by two lactic acid bacteria (LAB) of *L. acidophilus* and *L. plantarum*.

Materials and methods

Bacterial Strains

The strains of the tested LABs were *Lactobacillus acidophilus* (PTCC[1] 1643) and *Lactobacillus plantarum* (PTCC 1745). They were purchased from the Iranian Research Organization for Science and Technology (IROST) in a lyophilized form. Then, they were recovered in MRS[2] broth medium (Merck, Germany) for 24 h at 37°C in an anaerobic jar in the microbiology laboratory of the Azadshahr branch, Islamic Azad University. MRS broth medium supplemented with 20% glycerol was used to store standard strains at -20°C (Pakbin et al., 2014; Yoon et al., 2005).

Fermentation of fruit and vegetable juices by LABs

Fruits and vegetables, including banana, apple, carrot, and tomato were bought from the local market and then were juiced with a juicer (Hitachi, Japan). Fruit and vegetable juices were pasteurized at 80°C for 5 minutes. Then they were transferred to sterile tubes (25×200 mm) and were inoculated with bacterial suspensions of *L. acidophilus* and *L. plantarum*, so that suspensions containing 10^5 CFU/ml of each of the bacteria in the fruit and vegetable juices were obtained. For this purpose, first turbidity equal to 0.5 McFarland $=1.5\times10^8$ CFU/ml was prepared from each acid lactic bacteria. Then, by adding this bacterial suspension to the samples, fruit and vegetable juices containing 10^5 CFU/ml of each of the bacteria were obtained.

Fermentation of fruit and vegetable juices was performed at incubator of 37°C for 72 hours and the number of LABs per ml of fruit and vegetable juice based on colony forming unit (CFU/ml) was determined by serial dilution and pour plate culture method in MRS agar at intervals of 24 hours and they were incubated at 37°C for 72 hours in an anaerobic jar (Pakbin *et al*., 2014; Yoon *et al*., 2005; Sivudu *et al*., 2014). The number of bacteria per ml of fruit and vegetable juices was

1 Persian Type Culture Collection
2 Man, Rogosa and Sharpe

determined based on colony forming unit (CFU/ml) (Pakbin *et al.*, 2014; Yoon *et al.*, 2005; Sivudu *et al.*, 2014).

The viability of LABs in fermented fruit and vegetable juices at 4°C

After 72 hours of fermentation, the fermented fruit and vegetable juices were stored at 4°C for 4 weeks and samples were taken at weekly intervals, and the viability of *L. acidophilus* and *L. plantarum* in fermented fruit and vegetable juices was determined and expressed as colony forming units (CFU/ml) (Pakbin *et al.*, 2014; Sivudu *et al.*, 2014; Yoon *et al.*, 2005).

Measuring pH

During fermentation and at 24-hour intervals, pH of each sample of fermented fruit and vegetable juices was measured with a pH meter (WTW, Inolab 720, Germany). Calibration was carried out using KCL solutions at pH 7, 10 and 4.

Statistical Analysis

All experiments were performed in triplicate and the mean values were reported. The Significant differences ($P< 0.05$) between means were determined by Duncan`s multiple range test.

Results and discussion

pH Changes During Fermentation in Fruit and Vegetable Juices

The results of pH changes during the fermentation process in fruit and vegetable juices, inoculated with *L. acidophilus* and *L. plantarum* are presented in Figures 1 and 2, respectively.

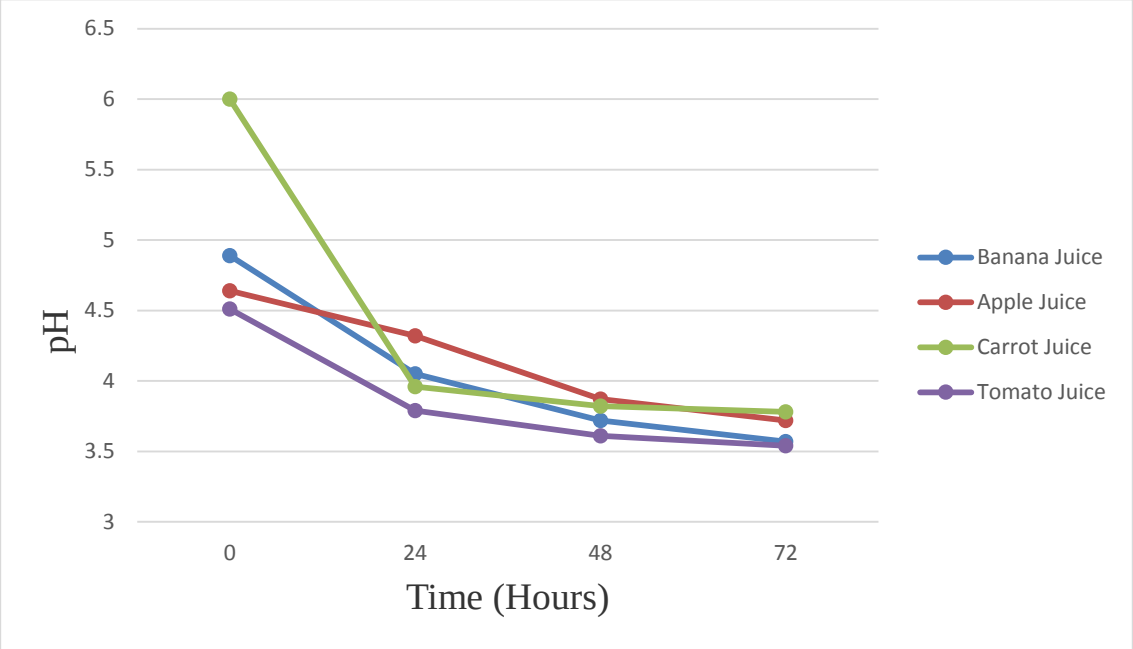

Fig. 1. Changes in pH During Fermentation by *L. acidophilus* in Fruit and Vegetable juices

In all products inoculated with these two bacteria, the pH decreased over time. However, there was a significant difference between the two bacteria during all days of the experiment ($P<0.05$). The trend of pH change in the fermented fruit and vegetable juices has been also observed in other studies (Yoon *et al.*, 2004, 2005, 2006; Kaur *et al.*, 2016; Kohajdova *et al.*, 2006) which is justifiable due to the production of lactic acid by *L. acidophilus* and *L. plantarum*. Among the fermented products, the process of reducing the pH of carrot juice from 6 to 3.78 and 3.74 for *L. acidophilus* and *L. plantarum*, respectively, was significant in the present research (Figure. 1 and 2).

In the study of Ayaseh et al. (2017) in order to produce probiotic carrot juice with *Lactococcus lactis*, the pH of fermented carrot juice decreased from 6.63 at the time of inoculation to 3.62 after 24 h.

Yoon et al. (2005) conducted a study on the fermentation of beet juice by different lactic acid bacteria. They found that *L. acidophilus* and *L. plantarum* produced more lactic acid than other species, and pH of fermented beet juice decreased from 6 to less than 4.5 after 48 hours incubation at 30°C.

Kohajdova et al. (2006) also tested different varieties of vegetables, including cabbage, tomatoes, pumpkin and courgette for preparing probiotic vegetable juices during lactic acid fermentation. From the point of view of lactic acid production and pH reduction, during the fermentation process, in all vegetable juices, the pH reduction was reported to be between 6.15- 6.5 to 3.35- 3.8 and all products were considered as suitable substrates for lactic acid fermentation by *L. plantarum* were introduced. Studies have shown that during lactic acid fermentation, the pH levels of vegetable and fruit juices decreased from about 6-6.5 to 3.8-4.5. Certainly, the rapid reduction of pH in the initial stages of fermentation to obtain a high quality product is of great importance and it may be considered as an advantage. Because in an environment with low acidity, lactic acid fermentation is inhibited by bacteria producing butyric acid (Viander *et al*., 2003; Holzapfel, 2002, Kohajdova *et al*., 2006).

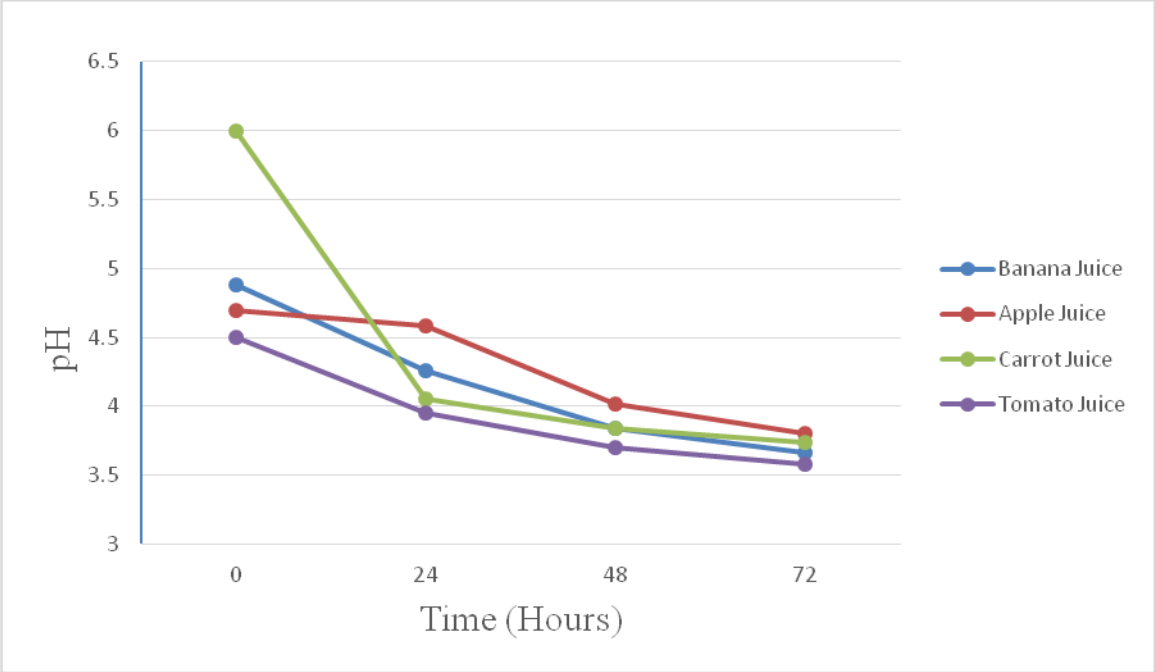

Fig. 2. Changes in pH During Fermentation by *L. plantarum* in Fruit and Vegetable juices

Growth kinetics of *L. acidophilus* and *L. plantarum* during fermentation of fruit and vegetable juices

The growth kinetics of *L. acidophilus* and *L. plantarum* during the fermentation of fruit and vegetable juices are shown in Figures 3 and 4, respectively.

The results showed that both of these LABs were able to grow well in the fruit and vegetable juices without any additives and to ferment the fruit and vegetable juices appropriately. So that the log of *L. acidophilus* count in products, which were prepared from 4.07– 4.23 CFU/ml at the beginning of fermentation (Time= 0), increased to 11.6–12.68 CFU/ml on the third day (Figure 3). This increase was also observed for *L. plantarum* in the prepared products from 3.56– 3.77 CFU/ml

at the beginning of fermentation to 10.46–11.63 CFU/ml on the third day (Figure 4). This indicated that all the prepared products (fruit and vegetable juices) were able to provide the proper conditions (acidic pH, and nutrients) for the growth of these two lactic acid bacteria. Tuorila and Cardello also reported that fruit and vegetable juices could be a good environment for probiotic growth (Tuorila and Cardello, 2002)..

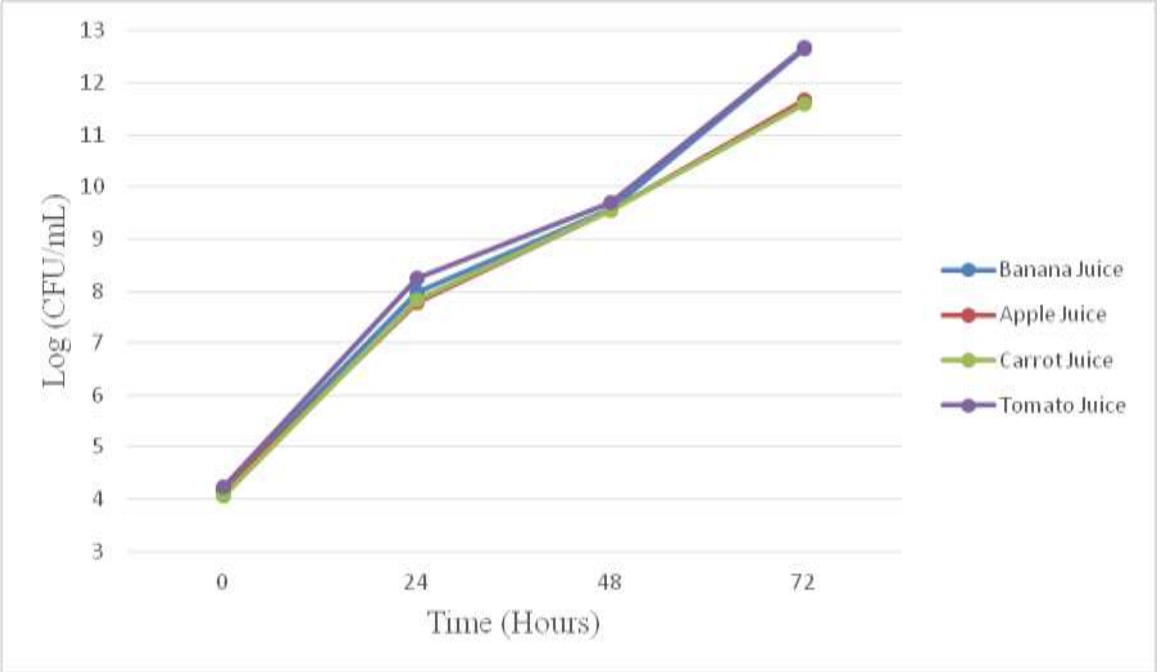

Fig. 3. Growth Kinetics of *L. acidophilus* During Fermentation of Fruit and Vegetable juices

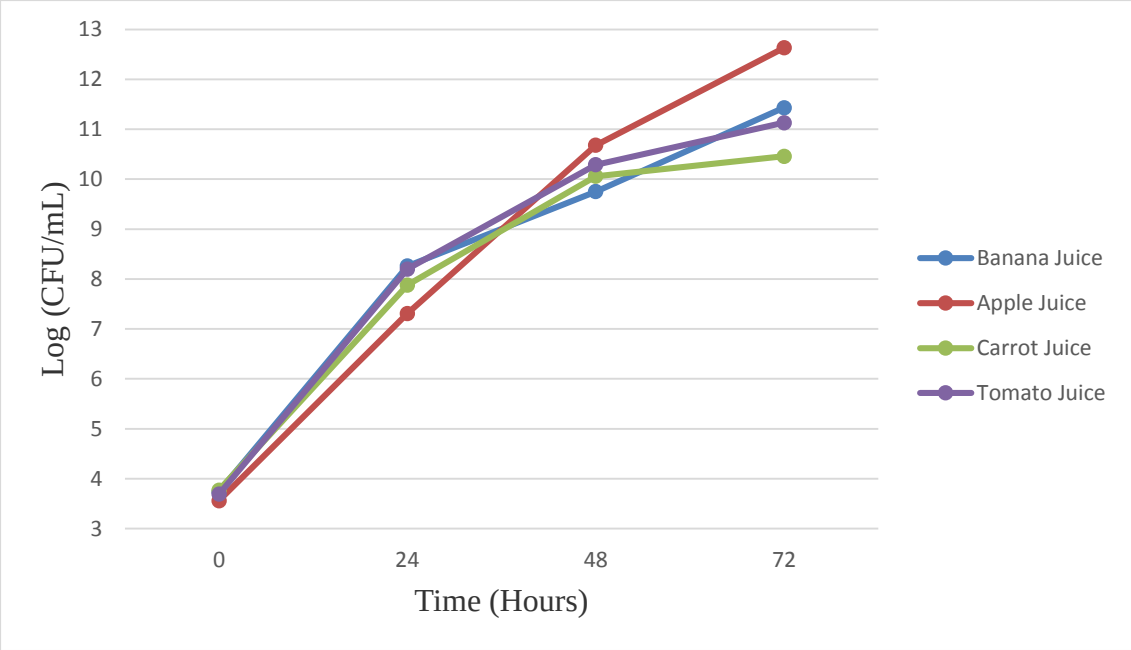

Fig. 4. Growth Kinetics of *L. plantarum* During Fermentation of Fruit and Vegetable Juices

Yoon et al. (2005) also investigated the fermentation of beet juice by lactic acid bacteria and proposed *L. acidophilus* and *L. plantarum* as probiotic candidates. Wheat and barley extract have also been reported as suitable environments for the growth of *L. acidophilus* and *L. plantarum* (Charalampopoulos *et al.*, 2003).

As it can be seen, among the tested products, tomato juice had the lowest initial pH level (Figure 1) and the results of the growth kinetics of *L. acidophilus* in this product also showed that the highest bacterial growth rate (Viable counts) was related to the tomato juice among all tested products (Figure 3). It could be justified due to the acidophilic nature of *L. acidophilus* (Peres *et al.*, 2012). These cases, along with the proper viability of this bacterium in tomato juice in cold storage conditions (Figure 5.) suggested that tomato juice was the best substrate among the products tested for the growth of *L. acidophilus* and the preparation of a non-dairy probiotic beverage. The probiotic suitability of this bacterium in tomato juice has also been reported in other studies (Kaur *et al.*, 2016; Yoon *et al.*, 2004)

Effect of Cold Storage on the Viability of Lactic Cultures in Fermented Fruits and Vegetables Juice at 4°C

In order to obtain the health benefits of the product, the presence of a large number of live probiotic bacteria in the final product is of great importance. In this regard, the number of probiotic bacteria surviving in the food at the time of consumption, must be at least 10^6 to 10^7 CFU/ml or g to be useful in providing health benefits (Vasudha and Mishra, 2013; Shaikh Uzma *et al.*, 2018).

The comparison of the viability of the two tested LABs in the prepared products and the process of reducing the number of *L. acidophilus* and *L. plantarum* during 4 weeks of cold storage at 4°C are shown in Figures 5 and 6, respectively. As it can be seen, *L. plantarum* presented higher viability during cold storage at 4°C than *L. acidophilus*.

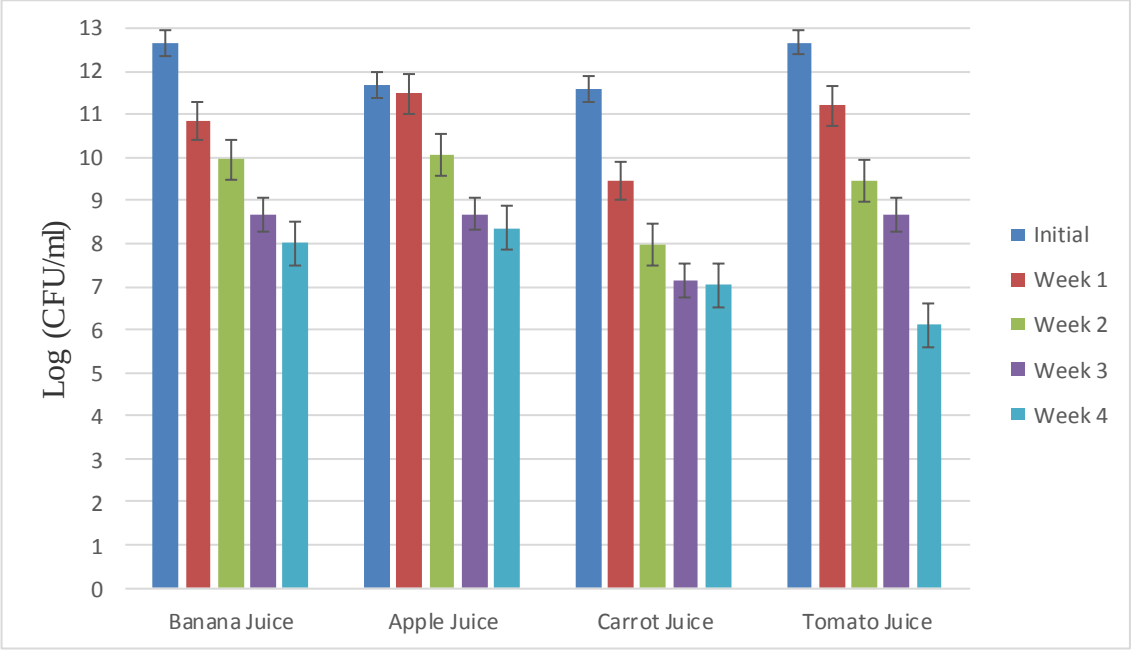

Fig. 5. Viability of *L. acidophilus* in Fermented Fruit and Vegetable Juices in 4°C

Accordingly, the log of *L. plantarum* count in products prepared at the initial of the cold storage was within the range of 10.46 - 12.63 CFU/ml and this number decreased to 8.11 - 9.23 CFU/ml at the end of the fourth week of storage at 4°C (Figure 6.). While, the log of

the number of *L. acidophilus* at the beginning of the cold storage in fruit and vegetable juices was within the range of 11.6– 12.68 and this number decreased to 6.1– 8.37 at the end of the fourth week (Figure 5).

The lower viability of *L. acidophilus* compared to *L. plantarum* in cold storage conditions has also been reported in other studies (Yoon *et al*., 2004 and 2005; Mousavi *et al*., 2011; Claude and Gardner, 2008).

Moreover, Yoon et al. (2004) evaluated the fermentation of beet juice and tomato juice by 4 lactic acid bacteria, which showed the less viability of *L. acidophilus* during storage at 4°C compared to *L. plantarum*, *L. casei* and *L. delbrueckii*. *L. plantarum* that showed the highest viability at cold storage among the four lactic acid bacteria tested. Mantzourani et al. (2019) also considered the possibility of using *L. plantarum* as a probiotic in pomegranate juice, and they evaluated the viability of this bacterium at cold storage at 4°C. Mousavi et al. (2011) conducted a study on the fermentation of pomegranate juice by *L. plantarum*, *L. paracasei*, *L. delbrueckii* and *L. acidophilus* and they showed that *L. plantarum* and *L. delbrueckii* created better conditions compared to the other two strains in terms of growth in pomegranate juice and the reduced pH and viability at 4°C. Claude and Gardner also reported sensitivity of *L. acidophilus* among 10 species of lactobacillus in 10 varieties of fruit juices mixed with milk components during 80 days of storage at 4°C (Claude and Gardner, 2008).

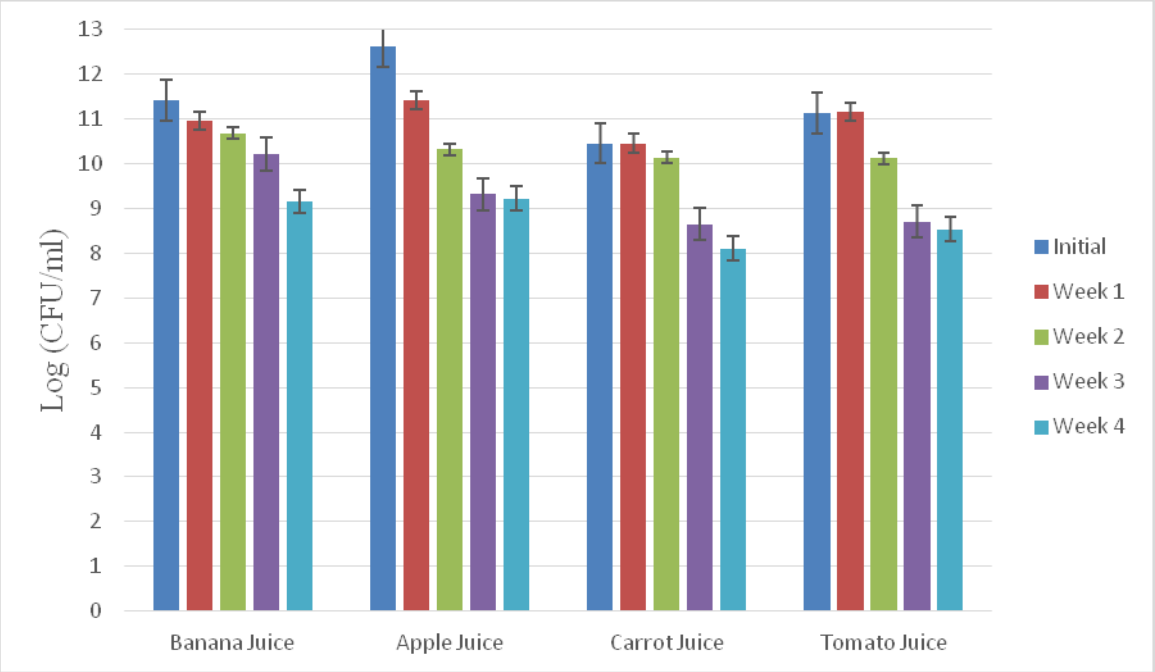

Fig. 6. Viability of *L. plantarum* in Fermented Fruit and Vegetable Juices in 4°C

The reason for the sensitivity to cold storage conditions may be due to the inability of the bacteria to survive in low pH stress conditions and high acidity of the products at low temperature (4°C) (Mousavi *et al*., 2011). Sheehan et al. (2007) reported that low pH of fruit juices within the range of 2.5 to 3.7 led to the sensitivity of bacteria to stress conditions such as cold. Claude and Gardner also showed that the viability of probiotics increased in cold storage at 4°C by increasing the pH of fruit juices from 3.8 to 4.2 (Claude and Gardner, 2008). Several factors could affect probiotic viability and survival in fruit and vegetable juices. The most important of these factors include intrinsic food parameters such as acidity, pH, oxygen, water activity, the presence of salt, sugar and chemical or

microbial preservatives such as hydrogen peroxide and bacteriocins. Also processing parameters such as incubation temperature, cooling rate and storage methods and finally microbiological factors, the most important of which are type of probiotic strains, compatibility of strains and inoculation rate (Tripathi and Giri, 2014).

Among all these factors, pH is the most important factor in the viability of probiotics in fruit juice. Fruit juices are naturally high in organic acids with low pH. It is assumed that acidic environment and the intrinsic antimicrobial activity of accumulated organic acids together affect probiotic bacteria. Among probiotics, lactobacilli generally found to be more resistant and survive in fruit juices with pH ranging from 4.3 to 3.7, while bifidobacteria are less acid tolerant; even pH 4.6 is unfavorable for their survival (Tripathi and Giri, 2014). In general, pH exerts a detrimental effect, but protein and dietary fiber could protect cells from acidic stress (Perricone *et al.*, 2015).

Although the pH is a drawback for probiotic survival in fruit and vegetable juices, Ranadheera et al. (2015) assumed that the incorporation of lactic acid bacteria into fruit juices with low pH may enhance the resistance of bacteria to subsequent stressful acidic conditions in the gastrointestinal tract.

Conclusion

Overall, the results of this study showed that banana, apple, carrot and tomato juice can provide the raw materials needed for growth of *L. acidophilus* and *L. plantarum* and due to the proper growth of these lactic acid bacteria in these products and decreased pH and the viability of these two bacteria in these products of plant origin in cold storage at 4°C, banana, apple, carrot and tomato juices can be suitable substrates for producing the non-dairy probiotic products.

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استفاده از چند آب‌میوه و سبزی به‌عنوان محیط پایه برای تولید نوشیدنی‌های پروبیوتیک غیرلبنی

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چکیده

فراورده‌های پروبیوتیک لبنی خطر افزایش کلسترول و عدم تحمل لاکتوز را مطرح می‌سازند. در این تحقیق از آب‌میوه‌ها و سبزی‌های سیب، موز، هویج و گوجه به‌عنوان محیط پایه برای تولید نوشیدنی‌های پروبیوتیک استفاده شد و قابلیت زنده‌مانی دو باکتری اسید لاکتیک *لاکتوباسیلوس اسیدوفیلوس* و *لاکتوباسیلوس پلانتاروم* در این محصولات مورد بررسی قرار گرفت. آب‌میوه‌ها و سبزی‌ها با سوسپانسیون‌های باکتریایی تلقیح شدند به‌طوری که سوسپانسیون حاوی 10^5 CFU/ml از هریک از باکتری‌ها در آب‌میوه‌ها و سبزی‌ها حاصل شد. نمونه‌ها به مدت ۷۲ ساعت در ۳۷ درجه سانتی‌گراد گرمخانه‌گذاری شدند و در فاصله‌های ۲۴ ساعته، pH و تعداد باکتری در هر میلی‌لیتر از آب میوه و سبزی براساس واحد تشکیل‌دهنده کلنی (CFU/ml) تعیین شد. محصولات تخمیر شده به یخچال منتقل شده و زنده‌مانی باکتری‌های اسید لاکتیک طی چهار هفته در ۴ درجه سانتی‌گراد مورد بررسی قرار گرفت. نتایج نشان داد که در همه محصولات تلقیح شده با این دو باکتری، میزان pH با گذشت زمان کاهش یافت به‌طوریکه تفاوت ایجاد شده در همه روزها بین دو باکتری مورد مطالعه، معنی‌دار بود ($P<0/05$). هر دو باکتری اسید لاکتیک توانستند در محصولات تولید شده بدون هر گونه افزودنی به‌خوبی رشد نمایند و به‌طور مناسبی آب‌میوه‌ها و سبزی‌های مورد آزمون را تخمیر نمایند. *لاکتوباسیلوس پلانتاروم* زنده‌مانی بیشتر را در ۴ درجه سانتی‌گراد نشان داد. به‌طور کلی با توجه به رشد مناسب این باکتری‌های اسید لاکتیک در محصولات مذکور و کاهش pH و زنده‌مانی آنها در شرایط نگهداری در ۴ درجه سانتی‌گراد، آب‌میوه‌های موز و سیب و آب سبزی‌های هویج و گوجه‌فرنگی می‌توانند سوبسترای مناسبی برای تولید محصولات پروبیوتیک غیرلبنی باشند.

واژه‌های کلیدی: زنده‌مانی، نگهداری در سرما، آب‌میوه و سبزی تخمیری، *لاکتوباسیلوس اسیدوفیلوس*، *لاکتوباسیلوس پلانتاروم*

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