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Effect of Cold Storage on Viability of Probiotic Bacteria in Carrot Fortified Milk

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Abstract

The growing interest in new functional drinks, resulting to the development of nutritionally and healthy probiotic drinks for consumers. Since dairy based products are a good carrier for probiotics, the suitability of milk/carrot juice drink stored at 4 ± 2°C for up to 20 days for the production of non-fermented probiotic drink was studied and survival of *lactobacillus acidophilus LA5, lactobacillus plantarum, lactobacillus rhamnosus GG* and *bifidobacterium lactis BB12* was evaluated. The samples were evaluated in 5 days interval for cell number, pH and acidity. *L.acidophilus LA5* seemed more stable than three other strains in this medium with more than 98% viability during storage but other strains showed a less viability between 88-92%. The pH and acidity changes by *L.acidophilus LA5*, *L.Plantarum* and *B.lactis BB12* in storage time were low and varied according to strain from 6.43 to 6.66 and 0.13% to 0.15% (according to lactic acid) respectively and the means comparision did not show any significant differences between them. The *L.rhamnosus GG* and control (non-probiotic) samples showed more variation in pH and acidity. Changes of pH and acidity by *L.rhamnosus* varied significantly from 5.33-6.47 and 0.13-0.31% respectively. The pH level decreased from 6.48 to 5.85 and the acidity increased from 0.13% to 0.28% in the control sample. The outcome of the overall study points that milk/carrot juice drink could serve as a probiotic drink after refrigerated storage for 20 days.

Keywords: Probiotic; Viability; Functional drink; Carrot juice; Milk; Cold storage

Abbreviation: MCJ: Milk/Carrot juice; CyHcl: Cystein HCL; MRS: Man Rogosa Sharpe

Introduction

The relationship between certain foods and health benefits and development of foods that promote health and well-being is one of the key research priorities of food industry [1] .This trend has favored consumption of functional foods that enriched with physiologically active components such as prebiotics, probiotics, vitamins, minerals, dietary fiber, plant sterol and other functional ingredients [2]. Probiotics are functional ingredients that are defined as live microbial food supplements, which upon ingestion in sufficient quantities exert health benefits beyond inherent general nutrition including the improvement of the intestinal microbial balance [3,4]. The probiotic microorganisms consist mostly of strains of the genera *Lactobacillus* and *Bifidobacterium*, but not exclusively. These bacteria have been used widely in dairy and non dairy products [5].

Traditionally, probiotics have been added to yoghurt and other fermented dairy products [6,7]. On the other hand, there is a genuine interest in the development of fruit and vegetable juice/milk based functional beverages with probiotics because they have taste profiles that are appealing to all age groups and because they are perceived as healthy and refreshing foods [1,8,9]. Besides being delicious, these beverages are highly nutritious. Fruits and vegetables contain various bioactive compounds with antioxidant activities, such as vitamins A, C and E, which have a high antioxidant capacity [10,11], and phenolic compounds, which recent studies have shown to be good contributors to the total antioxidant capacity of the foods that contain them [10,12,13].

Carrot (*Daucus carota L.)* is one of the most commonly used and well-known vegetables in the everyday kitchen that is rich in functional food components such as vitamins (A, D, B, E, C, and K) and minerals (calcium, potassium, phosphorus, sodium, and iron). It has been noted that 100 g of carrot contains between 6 mg and 15 mg of carotenoids, mainly β-carotene (2-10 mg) [14,15]. Thus, an increased intake of carrot may favor the massive synthesis of vitamin A. Moreover, the carotenoids and other antioxidants present in carrot play an important role in the inhibition and/or interruption of oxidation processes, as well as in counter balancing free radical activities [14,15]. Therefore, carrot may protect humans against certain types of cancer and cardiovascular diseases. Additionally, the allergenic effect of carrot is very low or lacking [15].

An important portion of carrots wasted every year due to quality defects and lack of food industry consumptions. These carrots can be used in other products like milk or beverage. The use of carrot juice as a natural flavorings agent in the preparation of milk based drink may prove to be beneficial due to highly nutritious nature of carrot which is the richest natural source of β-carotene and contains appreciable amounts of other vitamins, anthocyanin pigments and minerals [16]. However, carrot juice is very sensitive to oxidation and the taste and appearance of it change in short time, so, it needs special treatment in processing to develop an acceptable and stable beverage.

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Studies dealing with the probiotic carrot juice can be found in literature but the present work studies the viability of probiotic bacteria in non-fermented milk/carrot juice mix beverage and also the changes of pH and acidity during cold storage. The next aim is determining of suitability of this beverage as probiotic milk based drink.

Materials and Methods

Milk

Commercial low fat UHT milk (1% fat) was prepared by Pegah Tehran Dairy Co. and used as raw material in this study.

Carrot juice

Carrots were purchased from a local store. After being washed thoroughly, they were peeled and chopped (thickness approx. 0.5 cm) and washed again. Then they were blanched in acidified water by citric acid (0.5 g/kg carrots) at 80°C for 6 min. The juice was prepared using a laboratory juice extractor (PANASONIC®, Japan). Coarse particle content was separated gravimetrically by centrifugation of carrot juice at 4000 rpm for 20 min at 20°C by SIGMA® centrifuge (model 2-16 K, Germany). The supernatant was separated with a pipette and moved to a sterile container. After pasteurization at 90°C for 1 min, it was filled in opaque glass bottles and kept in refrigerator for the next use.

Preparation of milk-carrot juice mixed drink

First, milk was heated up to 60°C and dry mix of sugar (6.5%) and high methoxy Pectin (0.2%) was added to it through a home blender with continuous stirring. Then the centrifugated carrot juice was poured to it and it was mixed thoroughly to obtain a homogenous texture. Then the product was removed from blender and heated to 70°C and homogenized immediately at 160/40 bar by a 2-stage homogenizer (APV, Denmark). The milk/carrot juice mixed drink (MCJ) then pasteurized at 80°C for 5 min and cooled down to 5 ± 1°C for next stage.

Cultures

The following probiotic strains were selected: *Lactobacillus acidophilus LA5* and *Bifidobacterium animalis subsp. lactis BB12* from CHR-HANSEN A/S, Denmark; *L.Plantarum subsp. Plantarum* (DSM No.20179); and *L.rhamnosus* (LGG ID 100271) from the Microbial Collection of Food and Science Department, Agriculture Campus, Tehran University, Iran.

Preparation of probiotic cultures

The *lactobacillus* in the frozen cultures were activated by spread plating on Man Rogosa Sharpe (MRS) agar (MERCK, Germany), after incubation anaerobically for 48 hr at 37°C (GASPAK, Darmstadt, Germany) but for *bifidobacterium lactis BB 12*, spread plating was done on MRS+0.5% cycteinHcl. Then the colonies were inoculated in MRS-broth or MRS+CYHCL (MERCK, Germany) and incubated at 37°C for 72 hr anaerobically and the cell mass were harvested by centrifugation at 5000×g for 15 min by a refrigerated centrifuge (SIGMA, model 2-16 k, Germany). After centrifugation, the cell pellets were washed in Ringers solution and the necessary inoculums to adding to product was calculated and added to pasteurized milk/carrot juice mixed drink.

Preparation of probiotic milk/carrot juice drink

After inoculations with different probiotic bacteria (each >10^6 *CFU/ml*), each probiotic MCJ was filled in some sterile glass containers (100 ml), separately and all of them were kept under refrigeration at 4 ± 2°C for 20 days. The samples were taken at 5 days intervals for microbiological and chemical analysis. The experiments were performed in duplicate.

Counts of viable bacteria

Viable probiotic concentrations were measured by pour plate counting in duplicate on MRS agar (MERCK, Germany). Samples (1.0 ml) were added to 9.0 ml of sterile Ringers solution, then, appropriate dilutions were made. Subsequently *Lactobacillus* and *Bifidobacterium* were plated onto MRS agar and MRS agar+5 ml/liter medium CyHcl (MERCK, Germany) respectively. Plates were incubated in anaerobic jar+GASPAK System. An anaerobic indicator (ANAEROTEST, MERCK, Germany) was used to control anaerobic conditions. The colonies were counted after 72 hr of incubation at 37°C.

The methods are based on International standard ISO 20128/IDF192. Colony forming units (CFU) were enumerated in plates containing 15 to 300 colonies and cell concentration was expressed as log *CFU/ml.*

Measurement of acidity and pH

Acidity of samples was determined according to titration method and based on lactic acid percentage. 10 ml of sample was titrated against N/10 NaOH in presence of phenolphthalein. The values of pH were measured by a digital pH meter (Model METTLER TOLEDO, Germany).

Determination of sedimentation

The sediment content was determined according to the standard procedure based on centrifugation (ISI 1961).

Statistical analysis

Results were expressed as mean ± SD values which were the average of triplicate experiments. Significant differences between the results were calculated by analysis of variance (ANOVA) with the help of SPSS software version 16. Differences at $p<0.05$ were considered to be significant.

Results and Discussion

Viability of probiotic bacteria

Based on existing standards and from a health point of view, it is very important that probiotic strains retain their viability and functional activity throughout the shelf life of product. Viable counts (log *CFU/ml*) of 4 probiotic strains in MCJ during storage at 4°C over 20 days are presented in Table1. All strains attained viable cell number reduction of less than 1 log cfu ml^{-1}. *L.acidophilus LA5* showed a stable level of viable cells (98.8% viability) in milk/carrot juice drink during storage. The other strains including *B.lactis BB 12, L.rhamnosus GG* and *L.plantarum* showed a viability of 91.9, 90.1 and 88.0 percent, respectively (Figure 1). *L.acidophilus* and *L.rhamnosus* remained viable

Storage days	*L.acidophilus LA5*	*L.rhamnosus GG*	*L.plantarum DSM-20179*	*B.lactis BB 12*
1	6.72 ± 0.13	6.75 ± 0.15	6.66 ± 0.13	6.29 ± 0.26
5	6.57 ± 0.27	6.61 ± 0.16	6.39 ± 0.58	6.02 ± 0.47
10	6.48 ± 0.26	6.65 ± 0.15	6.28 ± 0.53	5.96 ± 0.19
15	6.66 ± 0.24	6.52 ± 0.14	6.16 ± 0.72	6.00 ± 0.14
20	6.64 ± 0.25	6.08 ± 0.27	5.86 ± 0.35	5.78 ± 0.58

Each value is the mean ± SD of experiments performed in triplicate.

Table 1: Numbers (log_{10}) of probiotic microorganisms in milk/carrot mix drinks over 20 days of storage at 4°C.

above the critical level of 10^6 cfu ml^{-1} in MCJ for 20 days. The changes of viable cell counts of all the strains during cold storage are insignificant (<0.05) but the viability of *L.Plantarum* decreased slightly and more than other probiotics in the same time. *B.lactis BB12* do not grow well in milk [17] and loss of bifidobacteria viability due to low proteolytic activities and low availability of nutrients, has also been reported by Kun et al. [14] and Martinez-villaluenga et al. [18] in dairy products. Kun et al. [14] reported that bifidobacteria were found to be capable of growing well on pure carrot juice without nutrient supplementation but its growth in milk is generally slow. The results showed viability of *B.lactis BB12* in milk and carrot juice mix drink is relatively high during refrigerated keeping for 3 weeks. *L. rhamnosus GG* is unable to ferment lactose and has been shown to survive well in dairy products [7,17,19]. Alegre et al. [20] reported *L.rhamnosus GG* remained viable in orange juice and apple wedges over 12 and 4 weeks of storage at 4°C, respectively. The MCJ was a good medium to keep viability of *L.rhamnosus GG* more than 90% during cold storage. It was shown that *L.acidophilus LA5* can grow and survive well in milk and dairy products [17]. *L. plantarum* has the coding capacity for the uptake and utilization of many different sugars, uptake of peptides, and formation of most amino acids. *L.plantarum* indicated the ability to adapt to many different conditions [21] and good viability was detected for different *L.plantarum* strains during cold storage of sterile skim milk until day 28 [22]. All the probiotic samples showed a longer shelf life in compare to controls except *L.rhamnosus* which was less. The international standard FIL/IDF describes that the probiotic products should be contained minimum of 10^6 viable probiotic bacteria per gram of product at the time of consumption for health and functional claiming [23,24], so the studied MCJ´s in this research could be probiotic after 20 days keeping in cold storage. Lactic acid bacteria not only improve health when consumed, but they can also play a protective role against pathogens in the product itself during storage by competing with pathogens for nutrients (vitamins, minerals, trace elements and peptides), producing organic acids and bacteriocins (antimicrobial peptides) [20].

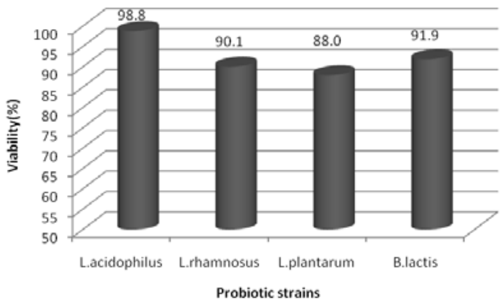

Figure 1: Viability of probiotic strains in milk/carrot juice drink during cold storage.

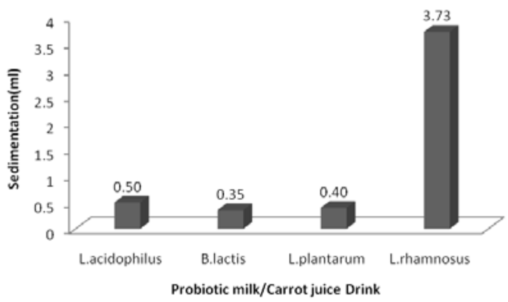

Figure 2: Comparison of sedimentation (ml/10 ml sample) in different probiotic noculated milk/carrot juice drinks after refrigerated storage for 20 days.

pH, acidity and sedimentation

The changes of pH and acidity of the different probiotic MCJ´s drinks studied in this work were shown in Table 2. The pH of carrot juice before adding to milk was about 5.7. After adding of 30% the carrot juice to low fat milk (control=without any probiotics), there was significant decrease in pH of milk from 6.7 to 6.48. During cold storage of control samples at 4°C, the pH did not show significant change in first week but it was decreased to 5.85 in 3rd week. This might be due to the degradation of lactose or produced galacturonic acid and other acids by enzymatic breakdown of pectin [15,16]. On the other hand, there is an insignificant increase ($p \leq 0.05$) in the pH of probiotic carrot flavored milk except *L.rhamnosus* inoculated sample in days of 5 and 10 in comparison with first day. LA5 showed a 0.13 decrease in pH of MCJ drink during storage time. Over the total storage period, the most changes in pH were seen in *L.rhamnosus* included drinks, which it decreased from 6.47 in day 1 to 5.33 in last day. The acidity of milk/carrot juice drinks was about 0.13 based on lactic acid percent, which is less than milk in production day. This is due to milk content of the drink that is about 63%. *L.rhamnosus* showed highest rate of change in acidity and after 10th day, the differences between this sample and other probiotic MCJ drinks became significant ($p \leq 0.05$).However, with increasing the acidity of *L.rhamnosus* included drink and control sample, they spoiled after 10 and 15 days, respectively. These results showed some strain of probiotic bacteria that can prolong the shelf-life of MCJ drinks in comparison with non-probiotic drinks. Although, in this study, fermentation was limited by keeping the samples in refrigerator but the MCJ drinks inoculated with *LA5*, *BB12* and *L.plantarum* kept their drinking capability until 20 days. Antimicrobial substances produced by probiotics such as bacteriocins or organic acids and competition with other microorganisms in product may be known as reasons of longer shelf life of probiotic samples than control [25].

The sedimentation values in end of storage time (20 days) ranged from 0.35 ml in *BB12* to 3.73 ml in *L.rhamnosus* (Figure 2). The sedimentation values for *L.plantarum* and *L.acidophilus* were 0.4 and 0.5, respectively. Charanjiv et al.[16] reported that the sediments of carrot flavoured milk ranged from 0.1 to 0.2 ml/10 ml in different

strains	pH					Acidity(% lactic acid)				
	Storage duration(Days)					Storage duration (Days)				
	1	5	10	15	20	1	5	10	15	20
L.acidophilus LA5	6.56 ± 0.07	6.62 ± 0.06	6.61 ± 0.1	6.54 ± 0.09	6.43 ± 0.22	0.127 ± 0.007	0.135 ± 0.009	0.135 ± 0.009	0.14 ± 0.000	0.153 ± 0.015
B.lactis Bb 12	6.54 ± 0.03	6.61 ± 0.03	6.66 ± 0.03	6.64 ± 0.07	6.63 ± 0.08	0.127 ± 0.007	0.133 ± 0.015	0.135 ± 0.010	0.135 ± 0.013	0.135 ± 0.013
L.plantarum	6.53 ± 0.02	6.63 ± 0.03	6.64 ± 0.06	6.60 ± 0.09	6.61 ± 0.04	0.127 ± 0.007	0.140 ± 0.017	0.133 ± 0.003	0.138 ± 0.012	0.145 ± 0.010
L.rhamnosus	6.47 ± 0.03	6.02 ± 0.46	5.95 ± 0.51	5.58 ± 0.64	5.33 ± 0.95	0.133 ± 0.015	0.195 ± 0.043	0.223 ± 0.081	0.263 ± 0.100	0.315 ± 0.130
Control (no bacteria)	6.48 ± 0.05	6.48 ± 0.04	6.30 ± 0.00	6.07 ± 0.03	5.85 ± 0.07	0.13 ± 0.000	0.132 ± 0.025	0.150 ± 0.002	0.180 ± 0.004	0.285 ± 0.015

Table 2: Changes in pH and acidity of milk/carrot juice drinks by probiotic strains during storage at 4°C.

samples during 4 days keeping at 4°C. The sedimentation value is related directly to acidity (or pH) and with increasing of acidity, the amount of sedimentation increase, too.

Conclusion

The present study was undertaken to identify suitable probiotic bacteria which can survive in milk/carrot juice drink during storage at 4°C. All four strains showed an acceptable viability with less than one log cfu ml^{-1} reduction at refrigerator temperature for 20 days. The MCJ´s drinks inoculated with *L.acidophilus LA5, L.plantarum* and *B.lactis Bb 12* showed a longer shelf life (3 weeks) in comparison with *L.rhamnosus GG* and non-probiotic MCJ with 1 and 2 weeks, respectively. So, after considering all the results it can be suggested that *L.acidophilus LA5, L.plantarum* and *B.lactis BB 12* are suitable probiotics for exploitation in fresh milk/carrot juice drink.

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