

Effect of refrigerated storage on the probiotic survival and sensory properties of milk/carrot juice mix drink

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Abstract

Background: There is a genuine interest in the development of probiotic milk and juice based beverages because they are a good-vehicle to deliver probiotic microorganisms to consumers. For this purpose, the viability and metabolism of four probiotic strains (*Lactobacillus acidophilus* LA5, *Bifidobacterium lactis* BB12, *L. rhamnosus* and *L. plantarum*) were studied in non-fermented milk and carrot juice mix drink. The drinks were evaluated in 5 days interval for viable cell count, pH, acidity, sedimentation and sensory quality during refrigerated storage at $4 \pm 2^\circ\text{C}$ for up to 20 days.

Results: The results showed that all strains had good viability in milk/carrot juice drink (88-98%), but *L. acidophilus* LA5 seemed more stable than three other strains. The levels of pH and acidity were ranged 5.33-6.6 and 0.13-0.31%, respectively. The drinks inoculated with *L. rhamnosus* and control (non-probiotic) showed more variation in pH and acidity. The most sedimentation was detected in drinks inoculated with *L. rhamnosus*, reaching 3.73 mL/10 mL sample. Sensory assessment indicated lowest acceptability in control and milk/carrot juice drink inoculated with *L. rhamnosus*, respectively.

Conclusion: This study indicated that some probiotic bacteria can be applied by food producers to produce functional drinks with an increased shelf-life.

Keywords: carrot juice, functional beverage, non-fermented, probiotic, viability.

INTRODUCTION

There is a major trend for consumers to purchase foods which provide excellent nutrition and health benefits, especially those that can prevent disease and/or maintain health, so development of foods that promote health and well-being is one of the key research priorities of food industry (Yoon et al. 2004). In the worldwide functional foods market, dairy products are key products and, among those dairy-based products, functional beverages account for an important fraction of this sector (Rodrigues et al. 2012). This trend has favoured consumption of functional drinks that enriched with physiologically active components such as prebiotics, probiotics, vitamins, minerals, dietary fiber, plant sterol and other functional ingredients (Betoret et al. 2011). Probiotics are defined as "Live microorganisms which when administered in adequate amounts confer a health benefit on the host beyond inherent general nutrition including the improvement of the intestinal microbial balance" (Fuller, 1989; FAO/WHO, 2002). Micro-organisms most commonly used as probiotics belong to the heterogeneous group of lactic acid bacteria (*Lactobacillus*, *Enterococcus*) and to the genus *Bifidobacterium*. These bacteria have been used widely in dairy and non dairy products (Holzapfel and Schillinger, 2002). Milk is a good-vehicle to deliver probiotic microorganisms to consumers.

Traditionally, probiotics have been added to yoghurt and other fermented dairy products (Ranadheera et al. 2010; Rivera-Espinoza and Gallardo-Navarro, 2010). On the other hand, fruits and vegetables have been suggested as ideal media for probiotic growth because they inherently contain essential nutrients, they are good-looking and have good taste (Luckow and Delahunty, 2004; Sheehan et al. 2007). 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 (Tuorila and Cardello, 2002; Yoon et al. 2004; Sheehan et al. 2007). 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 (Sánchez-Moreno et al. 2006; Zulueta et al. 2007), and phenolic compounds, which recent studies have shown to be good contributors to the total antioxidant capacity of the foods that contain them (Dillard and German, 2000; Vinson et al. 2001; Zulueta et al. 2007).

Carrots (*Daucus carota* L.) as one of the most popularly consumed vegetables are rich in functional food components such as vitamins (A, D, B, E, C, and K) and minerals (calcium, potassium, phosphorus, sodium, and iron). In carrots, β -carotene is present in a high concentration and can be considered as one of the most essential micronutrients because of its antioxidant activity and its property to act as a provitamin A (Knockaert et al. 2012). It has been noted that 100 g of carrot contains between 6 mg and 15 mg of carotenoids, mainly β -carotene (2-10 mg) (Bandyopadhyay et al. 2008; Kun et al. 2008). Thus, an increased intake of carrot may favour 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 (Bandyopadhyay et al. 2008; Kun et al. 2008). Therefore, carrot may protect humans against certain types of cancer and cardiovascular diseases. Additionally, the allergenic effect of carrot is very low or lacking (Bandyopadhyay et al. 2008).

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 flavourings 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 (Charanjiv et al. 2006). However, carrot juice is very sensitive to microbial spoilage and 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.

Studies dealing with the probiotic carrot juice can be found in literature but to our knowledge, this is the first report on the viability of probiotic bacteria in non-fermented milk/carrot juice mix beverage. Also the present work studies the changes of pH, acidity, sedimentation and sensory quality of the product during cold storage. The next aim is determining of suitability of this beverage as a 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. Physicochemical characteristics of the used milk are given in Table 1.

Carrot juice

Carrots (*D. carota* L. var Nantes) 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 particles 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 next using. The chemical composition of treated carrot juice used in this study is also shown in Table 1.

Table 1. Chemical composition of milk and centrifuged carrot juice used in this study.

Parameters	Unit	Milk	Treated carrot juice (centrifuged)
Fat	%	1	0.12
Dry Matter	%	9.50	8.05
Brix	%	8.10	7.50
pH	-	6.65	5.70
Density	gr/cm ³	1.032	1.023

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 (HMP, 0.2%) (Orana, Denmark) was added to it through a home blender with continuous stirring. Then the centrifugated carrot juice (30%) 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 to increase efficiency of next homogenization process. Homogenization process was conducted at 160/40 bar by a 2-stage homogenizer (APV, Denmark). The milk/carrot juice (MCJ) mixed drink then pasteurized at 80°C for 5 min and cooled down to 5 ± 1°C for next stage. Our preliminary investigations showed that the addition of the concentration levels studied for sugar, HMP and carrot juice resulted in desirable sensory attributes.

Cultures

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

Preparation of probiotic cultures

The *Lactobacillus* bacteria in the frozen cultures were activated by spread plating on Man Rogosa Sharpe (MRS) agar (MERCK, Germany), after incubation anaerobically for 48 hrs at 37°C (GASPAK, Darmstadt, Germany) but for *B. lactis* BB 12, spread plating was done on MRS + 0.5% cysteine hydrochloride (CyHcl). Then the colonies were inoculated in MRS-broth or MRS+CYHCL (MERCK, Germany) and incubated at 37°C for 72 hrs anaerobically and the cell mass were harvested by centrifugation at 5000 x 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 >10⁶ cfu mL⁻¹), each probiotic MCJ drink 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. Samples were taken at 5 days intervals for microbiological and chemical analysis.

Counts of viable bacteria

Samples were diluted in sterile Ringer's solution and viable probiotic strains were determined by pour plate counting in duplicate on MRS agar (MERCK, Germany). Subsequently *Lactobacillus* and *Bifidobacterium* were plated into 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 hrs of incubation at 37°C, after which they were expressed as log cfu mL⁻¹.

Measurement of acidity and pH

Acidity of samples was determined according to the general titration method and based on lactic acid percentage. 10 mL of sample was titrated against 0.1 N NaOH in presence of phenolphthalein. The values of pH were measured by a digital pH meter (Model METTLER TOLEDO, Switzerland). The pH meter was calibrated using standard buffer solutions (Merck) at pH 4.0 and 7.0.

Determination of sedimentation

The sediment content was determined according to the standard procedure based on centrifugation. Samples (10 mL) were centrifuged at 1250 rpm for 15 min (SIGMA, model 2-16 k, Germany). The supernatant was decanted and the volume of sediment was determined (ISIRI, 2007).

Sensory analysis

Sensory quality evaluation of samples was performed by trained panel of assessors on a 9-point Hedonic scale (Amerine et al. 1965). Samples were served in plastic cups at temperature of 20°C as recommended for sensory evaluation of fermented dairy products (IDF, 1997). Evaluation was based on colour, taste, flavour, consistency and overall acceptability. Each panellist scores were reflected on a hedonic scale of 1 to 9 where 1 = dislike extremely and 9 = like extremely.

Statistical analysis

Results were expressed as mean \pm SD. Values 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 (SPSS Inc., Chicago, IL, USA). Differences at $p < 0.05$ were considered to be significant.

RESULTS AND DISCUSSION

Viability of probiotic bacteria

Viable counts (log cfu mL⁻¹) of 4 probiotic strains in MCJ drink during storage at 4°C over 20 days are presented in Table 2. All strains attained viable cell numbers 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* 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 above the critical level of 10⁶ cfu mL⁻¹ in MCJ drink for 20 days. The changes of viable cell counts of all the strains during cold storage are insignificant ($p < 0.05$) but the viability of *L. plantarum* decreased slightly and more than other probiotics in the same time. The results also showed viability of *B. lactis* BB12 in milk and carrot juice mix drink is relatively high during refrigerated keeping for 3 weeks. All the probiotic samples showed a longer shelf life in compare with controls except *L. rhamnosus* which was less.

Based on existing standards and from a health view-point, it is very important that probiotic strains retain their viability and functional activity throughout the shelf life of product. Some probiotic strains do not grow well in milk. In such cases the presence of plant-based ingredients may improve the growth of probiotic cultures in milk such as tomato juice, peanut milk, soy milk, carrot and cabbage juice (Nadal et al. 2010). Today, most available dairy products with probiotic bacteria are fermented milks such as yoghurt. However, little is known of the stability of probiotics in non-fermented products. One of the main advantages of using non-fermented milk products as carriers of probiotics is the absence of fermentation end-products. Organic acids and flavour compounds have a negative impact on the survival of probiotic LAB. Sweet acidophilus milk is an example of a sweet milk product with added probiotic *L. acidophilus* (Mattila-Sandholm and Saarela, 2003). In this research, the viable cell counts of the four probiotic bacteria in the non-fermented MCJ drink ranged from 6.75 to 5.78 log cfu mL⁻¹ after 20 days of cold storage at 4°C. The *L. plantarum*, *L. rhamnosus* and *B. lactis* showed slightly reduction in cell count whereas *L. acidophilus* counts remained stable at 6.6 log cfu mL⁻¹.

Table 2. Viable counts [$\log \text{cfu mL}^{-1}$] of probiotic microorganisms, inoculated in milk/carrot juice drinks, during the storage period at 4°C for 20 days.

Storage days	<i>L. acidophilus</i> LA5	<i>L. rhamnosus</i>	<i>L. plantarum</i>	<i>B. lactis</i> BB12
1	6.72 ± 0.13 ^{ab}	6.75 ± 0.15 ^{ab}	6.66 ± 0.13 ^{ab}	6.29 ± 0.26 ^b
5	6.57 ± 0.27 ^{ab}	6.61 ± 0.16 ^{ab}	6.39 ± 0.58 ^{ab}	6.02 ± 0.47 ^b
10	6.48 ± 0.26 ^a	6.65 ± 0.15 ^a	6.28 ± 0.53 ^a	5.96 ± 0.19 ^a
15	6.66 ± 0.24 ^a	6.52 ± 0.14 ^a	6.16 ± 0.72 ^a	6.00 ± 0.14 ^a
20	6.64 ± 0.25 ^a	6.08 ± 0.27 ^a	5.86 ± 0.35 ^a	5.78 ± 0.58 ^a

^{a,b}Means in the same column followed by different letters were significantly different ($P < 0.05$). Each value is the mean ± SD of experiments performed in triplicate.

It was shown that *L. acidophilus* LA5 is a slow-growing organism and survives well in milk and dairy products (Ostlie et al. 2003). The *L. acidophilus* LA5 strain ferments lactose to DL-lactic acid and it is very stable and has a high resistance towards acids in fermented dairy products. The optimum pH for its growth is between 5.5-6 (Chr. Hansen A/S, 2008). In this research, during the storage time, pH values of three probiotic MCJ drinks were almost constant (except of *L. rhamnosus*), whereas the initial pH value was about 6.5, it did not changed significantly after 20 days of cold storage at 4°C. The main factors for loss of viability of probiotic organisms have been attributed to the decrease in the pH of the medium and accumulation of organic acid as a result of growth and fermentation (Yoon et al. 2004). So, high viability of the probiotics in this study might be due to constant pH throughout the 3 week - storage period and/or nutrient content of carrot juice-supplemented milk. Whilst there are a few researches about non-fermented probiotic foods, the effect of refrigerated storage on the viability of probiotics has been studied by many researchers. Mortazavian et al. (2007) investigated the effect of cold storage temperature on the viability of probiotics in yogurt and reported that the highest viability of *L. acidophilus* after 20 days, was observed in less cold storage temperature (2°C), whereas for *B. lactis* the highest viability was obtained when yogurt was stored at 8°C. Saccaro et al. (2009) noted a reduction of more than 2 log 10 cycles /mL in the counts of *L. acidophilus* at the end of the storage period in yogurt. *L. acidophilus* has no adverse effects on the taste, appearance, or palatability of the product. Furthermore, it is able to survive in the product until consumption (Hoppe and Larsen, 2008).

The optimum pH for growth of *Bifidobacterium* is 6-7. Therefore, the neutral pH of the non-fermented MCJ drink can prevent the decline of bifidobacteria populations. In this study, the highest viability after *L. acidophilus* LA5 was observed in *B. lactis* BB12 (Figure 1). Hughes and Hoover (1995) suggested the possibility to enhance the survivability of bifidobacteria in skim milk at refrigerated temperature with keep the pH unchanged. On the other hand, *B. lactis* BB12 do not grow well in milk (Ostlie et al. 2003) and loss of bifidobacteria viability due to low proteolytic activities, low betagalactosidase activity and low availability of nutrients, has also been reported by Martínez-Villaluenga et al. (2006), Kun et al. (2008), and Charalampopoulos and Pandiella (2010) in dairy products. Since bifidobacteria strictly anaerobic, so the dissolved oxygen levels in the product have also been cited to be important factors affecting the survival of probiotic bacteria in dairy products (Shimamura et al. 1992). Kun et al. (2008) 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. Bifidobacteria produce acetic and lactic acids under the fermentation process where two moles of glucose result of three moles of acetate and two moles of lactate. They can ferment galactose, lactose and fructose because of possessing Fructose 6 phosphate phosphoketolase (F6PPK) (Shah, 1997). The growth and activity of bacteria in non-fermented probiotic foods have been studied by some researcher. Zareba et al. (2011) demonstrated that the differences in the volatile profiles of probiotic supplemented non-fermented milk and milk fermented by *B. animalis* subsp. *lactis* BB-12 indicated the formation of volatile compounds not only during the fermentation process, but also during storage at low temperature. In this research, the MCJ drink was a good medium to keep viability of *L. rhamnosus* more than 90% during cold storage, attaining viable cell numbers of 6.75-6.08 log cfu mL⁻¹ after 20 days. After storage for 15 days at 4°C a faster reduction in viable cells was observed for *L. rhamnosus*. However, after 20 days, the viable cell count of *L. Rhamnosus* was still above 6 log cfu mL⁻¹. Unlike the *L. acidophilus*, *L. plantarum* and *B. lactis* that showed a stable pH in the storage period, *L. rhamnosus* showed a further decrease in pH from 6.47 to 5.33. *L. rhamnosus* is a facultative heterofermentative bacterium that ferments hexoses such as lactose and fructose to lactic acid, and also pentoses to a mixture of lactic and acetic acids (Oliveira et al. 2012). It has been shown that *L. rhamnosus* survive well in dairy products (Ostlie et al. 2003; Chr. Hansen A/S, 2008; Rivera-Espinoza and Gallardo-Navarro, 2010). Alegre et al. (2011) reported *L. rhamnosus* remained viable in orange juice and apple wedges over 12 and 4 weeks of storage at 4°C, respectively. The survival rate of this organism in probiotic yoghurts was found to be

more stable than *L. acidophilus* (Saccaro et al. 2009). *L. plantarum* is an important species in the fermentation of various plant and some animal products and it is known to produce antimicrobial substances, e.g. plantaricin, that are active against certain bacteria and pathogens (Cebeci and Gürakan, 2003). *L. plantarum* has the coding capacity for the uptake and utilization of many different sugars, uptake of peptides, and formation of most amino acids. The high galactosidase and glucosidase activities detected, and relatively low activities toward other carbon sources, suggest that *L. plantarum* strains prefer glucose and lactose for their carbon and energy requirement (Georgieva et al. 2009). *L. plantarum* indicated the ability to adapt to many different conditions (De Vries et al. 2006) but in the present study, the lowest cell survival was observed for *L. plantarum* with a reduction of 0.8 log cfu mL⁻¹ after 20 days in cold storage.

The international standard FIL/IDF describe that the probiotic products should be contained minimum of 10⁶ viable probiotic bacteria per gram of product at the time of consumption for health and functional claiming (Samona and Robinson, 1991; Roy, 2005), so the studied MCJ drinks in this research could be probiotic after 20 days keeping in cold storage if the provided initial cell counts in the product should be increased to 10⁷-10⁸ cfu mL⁻¹. The present results showed that some strain of probiotic bacteria can prolong the shelf-life of MCJ drinks in comparison with non-probiotic drinks. In this study, fermentation was limited by keeping the samples in refrigerator but it was reported that bacteria cells have some fermentative activity even during storage at 6°C (Nighswonger et al. 1996). 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, producing organic acids and bacteriocins (biopreservation). Biopreservation is the extension of storage life and enhancing of safety of foods using the natural or controlled microflora and/or their antimicrobial products (Rodgers, 2001; Alegre et al. 2011).

pH, acidity and sedimentation

The changes of pH and acidity of the different probiotic MCJ drinks studied in this work were shown in Figure 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, there was significant decrease in pH of milk from 6.7 to 6.48. During cold storage of control samples (without any probiotics) at 4°C, the pH did not show significant change in first week but it is decreased to 5.85 in 3rd week. On the other hand, there is an insignificant increase ($p \leq 0.05$) in the pH of probiotic carrot flavoured milk except *L. rhamnosus* inoculated sample in days of 5 and 10 in comparison with first day. *L. acidophilus* LA5 showed a 0.13 decrease in pH of MCJ drink during storage time. Despite the slight increase in the acidity during storage, the pH of the drink remained unchanged over the 3 week storage, indicating that the buffering capacity of the beverage was high. A similar result was reported by Nualkaekul and Charalampopoulos (2011) for *Lactobacillus plantarum* in fruit juices stored under refrigeration. 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 which it is less than milk in production day. *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.

The sedimentation values ranged from 0.35 in *B. lactis* BB12 to 3.73 mL/10 mL in *L. rhamnosus*, after keeping in cold storage for 20 days (Figure 3). The sedimentation values for *L. plantarum* and *L. acidophilus* were 0.4 and 0.5 mL/10 mL, respectively. The results indicated a higher amount of sediments in MCJ inoculated with *L. rhamnosus* compared to other MCJ drinks. The sedimentation value is related directly to acidity and with increasing of acidity, the amount of sedimentation increase, too (Figure 3). Charanjiv et al. (2006) reported that the sediments of carrot flavoured milk ranged from 0.1 to 0.2 mL/10 mL in different samples during 4 days keeping at 4°C.

Sensory evaluation

The results of sensory evaluation are given in Table 3. The scores allocated for colour, flavour, taste and consistency showed that during the first 5 days of the storage period all samples had the highest sensory acceptability. The sensory properties declined in the samples of control and *L. rhamnosus* added MCJ after 5th day as acidic or spoiled flavour and taste increased. However, after 15 days of storage, three other samples showed good sensory evaluation with gaining more than 70% of total

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Figures

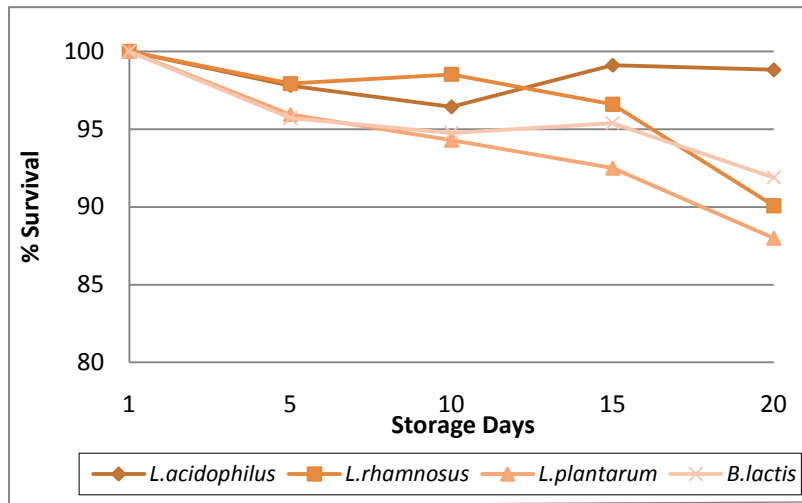


Fig. 1 Survival of probiotic strains in milk/carrot juice drink during cold storage.

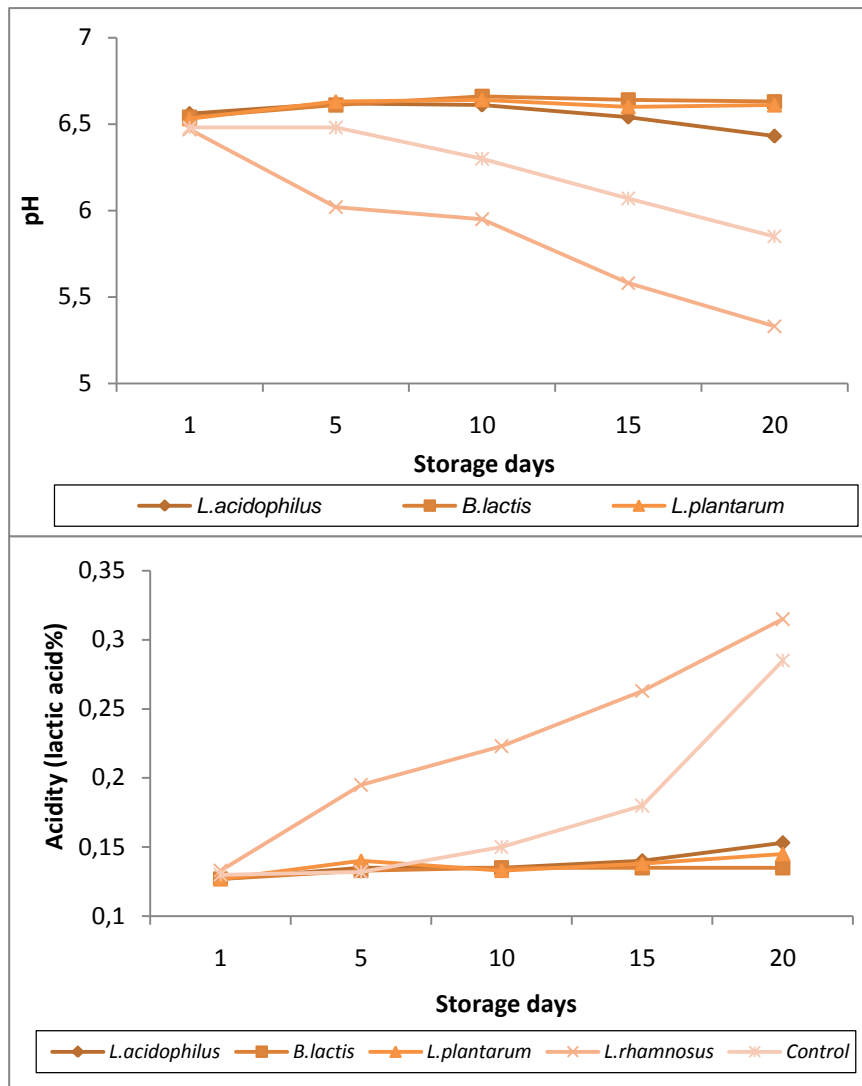


Fig. 2 Changes in pH and acidity of milk/carrot juice drink, inoculated with probiotic strains, and control (without probiotics) over 20 days at 4°C.

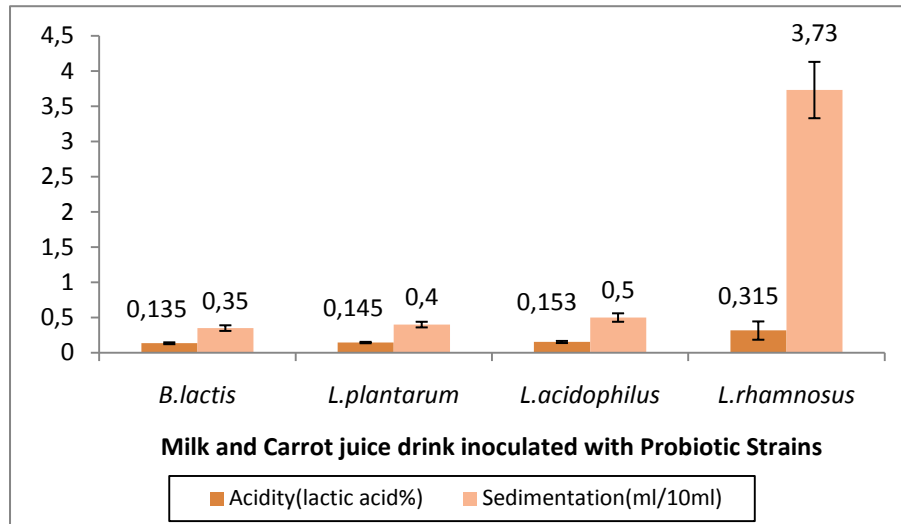


Fig. 3 Comparison of acidity (lactic acid %) and sedimentation (mL/ 10 mL sample) in different probiotic inoculated milk/carrot juice drinks after refrigerated storage for 20 days. Vertical lines represent standard deviations.

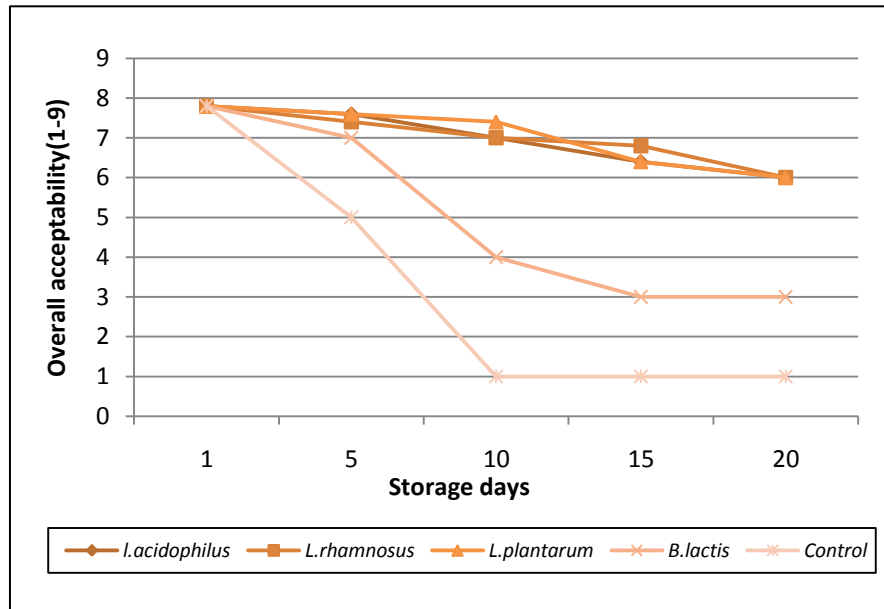


Fig. 4 Average overall acceptability scores for inoculated milk/carrot juice drink during cold storage time.