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The Behavior of *Lactobacillus Casei* as a Potential Probiotic in Food Carrier and Simulated Gastric Juice

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Abstract

To supply health benefits, a sufficient number of viable bacteria as probiotics must be present throughout the entire shelf life of food carriers, high viable survival amount during delivery through the gastrointestinal tract to permit enough live-cell arrival to the human gut. The offering study was undertaken to check the behavior of *Lactobacillus casei* as a probiotic in food carrier and pH determination during cold storage and assessment of the capability of probiotic bacteria to tolerance the gastrointestinal environment. Samples of the yogurt were stored for 14 days at 4 ℃ until the time of analysis, which involves microbiological, pH determination, and tolerance to acid. A gradual decline in pH was noticed throughout the cold storage. Counting of starter cultures decreased by 0.87 log cycles cycle, and the probiotic's viability decreased by 1.30 log cycle at the end of storage. Whereas probiotic's viability in samples subjected to re-pasteurization exhibited good stability until the end of the storage period. Counting of probiotics decreased by 2.71, 2.59 log cycles after the incubation period (3 h) at 37° C in simulated gastric juice pH 2.0 and 3.0, respectively. Counting of probiotics remained viable at levels above the recommended 10^6 CFU/g after 14 d in the refrigerated storage, which refers that the yogurt would be a suitable carrier for probiotics. According to these characteristics, *Lactobacillus casei* showed adequate properties for probiotic applications.

Keywords: viability, gastric juice, cold storage, *Lactobacillus casei*, probiotic

Introduction

Over the last few decades, detailed information on the effect of food on human health has increased seriously, and populations across the world have become aware of the need for a so -called ˊhealthy food ˋ (Awaisheh *et al.*, 2005). The consumption of dairy products having bacterial strains claimed to promote well-being has been steadily increasing during the last period (Morelli,2007). The raising request for protected food has encouraged the attention in changing chemical additives by biological products, excluding damaging the host or nature. Lactic acid bacteria are industrially essential organisms known for their fermentative capability as well as their health and nutritional assistance (Rattanachaikunsopon and Phumkhachorn, 2010). Lactic acid bacteria (LAB) are discovered in many fermented foods and digestive tracts of humans and animals and these have been accorded the GRAS (Generally Recognized As Safe). Their growth and metabolism prevent the normal spoilage of flora and bacterial pathogens over both bacteriostatic and bactericidal action (Waites *et al*., 2001). Antimicrobial activity by lactic acid bacteria arises from pH depression, organic acids, bacteriocins, carbon dioxide, hydrogen peroxide, diacetyl, and ethanol, as well as nutrient reduction and overpopulation (Adams,2001).

Lactic acid bacteria are commonly used in probiotic formulations. These are established in large numbers in the healthy gastrointestinal tract. Probiotics are positively related to dairy products and yogurt is one of the best-known products that comprise probiotics. Yogurt is a coagulated milk product that is manufactured by the fermentation of milk by lactic acid bacteria (Parvez *et al*., 2006). Available of a sum

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of definitions for probiotics, the most extensively used and accepted definition is that suggested by a joint FAO/WHO (2002) panel, "live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host". General criteria for choosing a good probiotic strain have been listed by several researchers and include being of human origin, non-pathogenic, survive passage through stomach transit, maintain its viability and metabolic activity in the intestine, tolerance to bile salt, the capability to adhere to the intestinal mucosa, to colonize the digestive tract of human tissue, competitive of pathogenic bacteria, and resistance of antibiotic (De Vries *et al*., 2006)**.** The functional effects related to probiotic bacteria involve the reconstruction of normal flora after disorders, a decrease of cholesterol level in the blood, supporting of immune functions, control of bacterial infections, elimination of carcinogens, enhancement of calcium absorption as well as the reduction of lactose – intolerance (Holzapfel and Schillinger,2002). For most of these advantages, sufficient numbers of viable cells of probiotics need to be consumed. Thus, it is significant that the probiotics continue viable throughout the storage of products containing those (Nighswonger *et al*., 1996).

Lactobacillus is the major genus within the lactic acid bacteria (LAB) have a good hygienic quality of fermented products, especially in the dairy industry. *Lactobacillus* species are either naturally existent in raw milk and dairy products or combined intentionally for technological causes or to create health promotes. Amongst lactobacilli, *Lactobacillus casei* strains are extensively used in the dairy industry and fermentation practices. Also, some of the *Lactobacillus casei* strains show probiotic effects and act as health-promoting live cultures, specifically is yogurt and fermented drinks (Hosseini *et al*., 2015). Viability and activity of the probiotic are significant attentions, in sufficient numbers of at least 1×10^6 $CFU.ml^{-1}$or.g^{-1} of product at the time of consumption because the probiotic bacteria requisite survive in the food throughout shelf life, in transit through the acidic conditions of the stomach, withstand degradation by hydrolytic enzymes and bile salts in the small intestine (Kailasapathy and Chin, 2000).

However, studies have displayed the low viability of probiotics in market formulations. The need to check the survival of probiotic microorganisms in fermented products has frequently been neglected, with the result that some products reach the market containing a low concentration of viable bacteria (Ashraf and Shah, 2011). Therefore, the objective of this study was to use dairy products (yogurt) as a food carrier for probiotic (*Lactobacillus casei*) in the gastrointestinal tract, to evaluate the behavior of probiotic bacteria in dairy products during the storage period, and the effect of gastric juice on probiotic bacteria.

Materials and methods

Preparation of inocula probiotic

L. casei as probiotic bacteria (ATCC 393) was supplied by the Department of Food Science, engineering and Technology, Faculty of Agricultural Engineering and Technology, University of Tehran. It was maintained at (-80°C) in 40% v/v glycerol, when requisite, then; it was sub-cultured three times in de Man Rogosa Sharp (OXOID, CM0359 LTD., BASINGSTOKE, HAMPSHIRE, ENGLAND) broth under anaerobic conditions at (37°C) overnight for routine analysis. The bacterium was characterized by cell morphology and biochemical methods. After (17–22 h of incubation) cells were harvested by centrifugation at 6000 ×g, 4°C for 10 min. The pellet was washed twice in 0.85% sterile saline solution (pH 7.0) before suspension in UHT milk (1.5% fat). The purity of cultures was noticed continually and at the start of each experiment by Gram staining (Mishra and Prasad,2005).

Yogurt production

Yogurt production carried out at the plant in the Department of Food Science, Engineering and Technology, Faculty of Agricultural Engineering and Technology, University of Tehran. Yogurt manufacture initiates by boiling raw milk to 85-90 °C for 30 min. to destroy any undesirable bacteria, such as those that can generate spoil milk or are pathogenic. After pasteurization, milk is cooled to the favored incubation temperature, generally between 40°C and 43°C. Starter cultures for yogurt as soon as were added, and involvement of two organisms, *Lactobacillus delbrueckii* subsp. *Bulgaricus* and *Streptococcus* which to be suggested in what can be defined as "Yogurt" (FDA,1996). The mixture temperature was retained for three hours at 42°C until the pH attains approximately " 4.5". Probiotic

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culture (*L. casei*) was added immediately after the end of yogurt fermentation, just before transferring the samples to cold storage at a concentration of 5% (v/v) which was equivalent to more than 1×10^6 CFU/ g at the time of inoculation (Ng *et al*., 2011). Probiotic was also added to other samples of yogurt that were undergone re-pasteurization by heating at 75°C for 15 min to kill all starter cultures at the end of fermentation just before transferring the samples to cold storage at a concentration of 5% (v/v).

Storage and sampling intervals

Samples of the yogurt were stored for 14 days at 4 °C until the time of analysis which involve microbiological (probiotics and starter cultures counts) and physicochemical parameter (pH) of the samples were done in triplicate in time 0 at the end of fermentation. Subsequently, analyses were carried out after 1, 3, 7, 10, and 14 days of refrigerated storage.

Media Preparation

Diluent of peptone and water

Bacteriological peptone-saline water and water diluent (0.15%, w/v peptone; 0.85%, w/v saline) were prepared by dissolving 1.5g, 8.5g of bacteriological peptone medium (Hi-Media, Mumbai, India), pure Sodium chloride respectively in 1 L of distilled water. The pH was regulated to 7.0 ± 0.2, followed by sterilizing by autoclaving at 121 °C for 15 min (Tharmaraj and Shah, 2003) .

Selective media

Yogurt, as well as fermented dairy products, are examples of foods that continuously comprise combinations of different lactic acid bacteria, therefore the existence of many kinds of lactic acid bacteria that strongly associated species in dairy products render the differential, enumeration, and isolation of probiotic and yogurt starter culture very difficult to complete due to similarity in growth requests, morphology, and biochemical reaction (Tabasco *et al*., 2007).

Reference Medium

MRS medium to be considered free selective chemical agents was dependent as a reference medium because MRS medium supported optimum growth for generally lactic acid bacteria (De Man,1960) .

Counting of starter cultures

Counting of starter cultures (*Lactobacillus delbrueckii* subsp. *Bulgaricus* and *Streptococcus thermophiles*) can be completed by subtracting practice which depended on the number of colonies counted on the selective medium for the probiotic bacteria was subtracted from the total counts obtained using reference medium (MRS) (Ong and Shah,2009a).

Enumeration of Lactobacillus casei in combination with other cultures by selective media.

According to Ong and Shah (2009b), *L. casei* was enumerated by MRS-vancomycin agar and anaerobic incubation at 37 ºC for 72 h. The media was equipped by adding 2 mL of 0.5 mg /mL vancomycin solution to 1 L of fluid MRS agar just before pouring to get 1 mg/ L of ending concentration. *L. casei* can grow in MRS – NaCl (4%) (Tharmaraj,2004). Forty grams of NaCl/ L were added for MRS-NaCl agar (4% end concentration). Agar powder was added to each broth at the rate of 1.8% and the media were autoclaved at 121 °C for 15 min.

Microbiological analysis and pH determination.

One gram of yogurt sample was diluted aseptically with 9 ml of 0.15%, w/v sterile peptone water (Hi-Media, Mumbai, India) containing 0.85%, w/v saline, and mixed homogeneously by a vortex mixer. Following serial dilutions were organized and viable numbers enumerated using the pour plate technique. Plates containing 25 to 250 colonies were counted and recognized as colony forming units (CFU) per gram of yogurt. The viable cell counts were expressed as log values. All experiments and analyses were

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replicated at least twice. The results offered are average of triplicates. Counts of *L. casei* were enumerated on MRS-vancomycin agar, NaCl (4%) agar. The pH value of the yogurt was determined by the pH meter (GLp22, CRISON. EEC.) by inserting the electrode directly into a sample of yogurt after calibrating with fresh pH 4.0 and pH 7.0 standard buffers.

Preparation of simulated gastric juice.

To control the transit tolerance over simulated gastric juice the method of Vinderola and Reinheimer (2003) was dependent on slight modifications. Simulated gastric juice consisted of filter-sterilized (0.22 μm membranes) pepsin (SIGMA-ALDRICH, GERMANY) (0.3% w/v) and NaCl (0.5% w/v) adjusted to pH 2.0 and 3.0. Overnight cultures (30 mL) were centrifuged (6000 x g, 20 min, 4 °C) and the pellets were washed twice with 0.85% sterile saline solution (pH 7.0) to eliminate the media and resuspended in 3 ml of the same solution. One milliliter of washed cell suspension was harvested by centrifugation (12.000-x g, 20 min, 4 °C) and suspended in 10 mL of gastric solution pH 2.0 and 3.0. Total viable counts of *L. casei* were done on MRS agar, before and after an incubation period of 3 h at 37 °C.

Statistical Analysis

The data were assessed using analysis of variance (ANOVA) with a level of significance at $p < 0.05$. All statistical analysis was performed using SPSS software (version 22) and the means of treatments were compared using Duncan's test.

Results and discussion

Changes in pH during the production of yogurt and cold storage

Figure 1. displays changes in the pH for bio-yogurt (yogurt that contains live probiotic microorganisms) and reveals changes in pH for conventional yogurt without probiotic as control. Also, the changes in the pH for other samples of yogurt were subjected to re-pasteurization at the end of the fermentation processes. Monitoring was made after the ending of the yogurt fermentation (0 h), during 14 day's storage of bio-yogurt. A gradual decrease in the pH was observed throughout the storage period for all the formulations. Significant differences ($p < 0.05$) in the pH of bio-yogurt through storage and the initial pH (4.12 at 0 h) decreased to (3.71 at 14 d). There were no significant differences ($p > 0.05$) in the mean of samples were subjected to re-pasteurization before inoculating with probiotics and the initial pH (4.14 at 0 h) decreased to (4.05 at 14 d). Significant differences ($p < 0.05$) in the pH of control yogurt (without probiotic) and the initial pH (4.13 at 0 h) decreased to (3.68 at 14 d) (Figure 1).

Yogurts were made after the symbiotic relationship growth of the two bacteria: *Streptococcus thermophiles* and *Lactobacillus delbrueckii* ssp. *Bulgaricus* is the lactic acid starter during the processing of yogurt. (Shah,2000). The present investigation illustrated that a slight change obtained in the pH values can be noticed in the samples that were undergone to re-pasteurization before adding the culture of *L.casei* to the yogurt at the end of fermentation during cold storage where the growth of *Lactobacillus delbrueckii* ssp. *Bulgaricus* in control of 'over- acidification was stopped by re-pasteurization (Figure 1). According to Sarvari *et al*. (2014)**,** the substantial slow decline in pH at the start of fermentation was attributed to the buffering capacity of yogurt. Mani-Lopez *et al.* (2014). reported that pH values of yogurts and fermented milk reduced in storage due to over-acidification. Shah (2000) showed that declining pH and acid production during refrigerated storage due to the remaining activity of microorganisms. Beal *et al*. (1999) illustrated that the yogurt could undertake an appearance called over-acidification that is the reduction of pH during the storage period because of persisting the biological activity of the lactic acid starter added to the milk which prepares for yogurt production (primarily *L. delbrueckii* subsp. *bulgaricus*). Our findings

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are similar to these results, supporting the residual acidification in cold storage.

The behavior of probiotics and lactic acid starter at the end of fermentation and storage time

Table 1. illustrates changes in counts of *L. casei* during storage period in selective medium, lactic acid starter of yogurt along with *L.casei* in reference medium (MRS) , and the counts of lactic acid starter of yogurt without *L. casei* (yogurt bacteria). The average number of the viable cell of *L. casei* significantly decreased from 7.52 ± 0.07 log CFU/ g on time 0 to 6..22 ± 0.24 log CFU/ g on the day14 while that of Lactic acid starter plus *L. casei* significantly decreased from 8.21 ± 0.02 log CFU/ g to 7.28 ± 0.08 log CFU/ g during same time, and lactic acid starter without *L. casei* (yogurt bacteria) significantly decreased from 8.11 ± 0.02 log CFU/ g on time 0 to 7.24 ± 0.07 log CFU/ g on the day 14. The viable cell counts of lactic acid starter culturs yogurt bacteria can be found by subtraction the average value of *L. casei* in selective medium from the viable cell counts of lactic acid starter along with *L. casei* in reference medium (MRS) (Table 1). The viable counts of all these groupings bacteria grow up to the day 1 , and then decline during the successive storage. There were significant differences ($P < 0.05$) among the viable counts of the bacteria mentioned above during cold storage period of bio yogurt. The viability loss of all groupings bacteria in Table 1 at 7 d. The viability loss of all groupings bacteria were gradual and stable during the storage. The viable count of the probiotic (*L. casei*) were found to be well above than the standard limit for probiotic foods (6-log CFU/ g or mL) until the ending of the storage period.

Table 2 explains changes in average values of *L. casei* in selective media, and in reference medium (MRS) for the probiotic yogurt was subjected to the re-pasteurization before inoculating with *L. casei.* The ended product was re-pasteurized by heating at 75°C for 15 min to destroy all starter cultures. The average viable cell counts of *L. casei* declined from 7.23 ± 0.06 log CFU/ g on time 0 to 7.17± 0.08 log CFU/ g on the day14 in selective media while the average viable cell counts of *L. casei* did not change at the end of the storage period in reference medium(MRS) in comparison with time zero. There were significant differences ($P < 0.05$) among the viable counts of *L. casei* in selective as well as reference media during cold storage of bio- yogurt. The viable cell counts of all groupings bacteria raised up to day 7. *L. casei* as probiotic in selective and reference media displayed good constancy during cold storage and the viability loss of *L. casei* bacteria at day 14 in selective media only (Table 2).

Many studies have shown the low viability of probiotics in yogurt (Dave and Shah,1997; Shah,2000). Our results found, however, that *L. casei* retained an acceptable degree of viability during refrigerated storage of bio- yogurt, these are in agreement with studies by Dave and Shah (1997) who described that lactic acid production by *L. delbrueckii* subsp. *Bulgaricus* throughout storage of yoghurt, which is so-called post-acidification, is one of the influences identified to affect the viability of probiotic in these products . Ng *et al*.(2011) reported that many factors could affect the viability of probiotics in yogurts: strain variation, acid accumulation, interaction with starter cultures, the concentration of dissolved oxygen, and hydrogen peroxide (H_2O_2), and storage condition. Previous studies have described that the most important contributing reasons for loss of cell viability are decreasing pH throughout product storage (post-acidification) and the accumulation of organic acids as a consequence of growth and fermentation. The low pH of fermented foods is one of the most substantial factors creating evident viability loss of probiotics(Beal *et al*.,1999; Shah,2000; Sarvari *et al*.,2014).

As pH, decreases in fermented milk might produce raise in the concentration of undissociated organic acids in them and, consequently, increases the bactericidal effect of these acids. The above-mentioned effect of organic acids results from their lipophilic nature. They can be transmitted through the microbial cells and dissociate within them, altering the intracellular pH. Which can involve damage of activity of the comparatively acid-sensitive glycolytic enzymes (which severely affects the capability to generate ATP) and structural damage to the cell membrane and macromolecules such as DNA and proteins. Moreover,

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organic acids might bind to several intracellular compounds. Both of these occurrences disrupt cell metabolism (Cotter and Hill, 2003, Korbekandi *et al*., 2005).

Also, because of the absence of *Lactobacillus delbrueckii* ssp. *bulgaricus* that responsible for over-acidification by re-pasteurization, probiotics are more stable throughout the refrigerated storage (Table 2). Over- acidification is found to cause loss of viability of probiotic bacteria. *Lactobacillus delbrueckii* ssp. *bulgaricus* produces sufficient hydrogen peroxide to display inhibition of probiotics. Accumulation of hydrogen peroxide in growth media can happen because lactobacilli do not have a catalase enzyme. Hydrogen peroxide can react with other components to form inhibitory components(Shah, 2000). In the current work, the starter yogurt bacteria that included *Streptococcus thermophiles, L. delbrueckii* ssp *.bulgaricus*, it is common to observe reducing counts at the end of storage. It is possible that attributed to the secretion of inhibition metabolites (e.g., bacteriocins) created by probiotics may reduce the numbers of any sensitive organisms that may be existent in product or starter cultures that could affect species of the same genus. Yang *et al*.(1992) reported that a decrease of pH could bring about reduced adsorption of the bacteriocin molecules to the producer cells, and subsequently in an elevation biological availability. Mani-Lopez *et al*.(2014) reported that *L. casei* maintaining viability for 7 d, the population of *S. thermophilus* declined by 1.8 to 3.5 log cycles, and the population of *L. delbrueckii* ssp. *bulgaricus* as well reduced in probiotic yogurts and varied from 30 to 50% of the initial population through 35 d at 5°C respectively, and who claimed that a gradual decrease in the probiotic population, which was independent of pH due to the end of metabolic activity of bacteria because of long storage.

Vinderola *et al.*(2002) described that probiotic bacteria delay the growth of starter cultures. They noticed that *L. casei* reduces the growth of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* in milk. Our findings are in consistent with previous studies. Generally, at the end of the storage of probiotic yogurt (bio-yogurt), there was a decrease of all groupings probably caused by opposing conditions, such as insufficiency of nutrients, pH depression, and low temperature.

The behavior of probiotic bacteria after exposure to simulated gastric juice

The effect of simulated gastric juice on the viability of *L. casei* was shown in (Table 3). The average number of the viable cell of *L. casei* decreased from 9.23 ± 0.02 log CFU/ mL on time 0 (before incubation period) in pH 2.0 to 6.52 ± 0.17 log CFU/ mL after the incubation period (3h) at 37 ° C in pH 2.0. Whereas the average viable cell counts of *L.casei* decreased from 10.41 ± 0.09 log CFU/ mL on time 0 in pH 3.0 to 7.82 ± 0.04 log CFU/ mL after incubation period (3 h) at 37 ° C in pH 3.0. There were significant differences ($P< 0.05$) among the viable cell counts of *L.casei* during incubation period (3 h) at 37 ° C in pH 2.0 and 3.0 (Table 3). Based on the results obtained (Table 3), the viability of *L. casei* has established to be successful to meet the minimum criterion of 1×10^6 viable probiotic cells per ml at pH 2.0 and 3.0 after exposure to simulated gastric juice for 3 hours.

To reach the intestine, bacteria must first pass through the stomach, which offers a powerful barrier to the entrance into the gut (Morell,2007). Probiotics survive in the environment and conditions of the intestinal tract critical to recent effects on probiotics in the digestive tract (Horáčková *et al*., 2011) . Around 2.5 L of gastric juice at a pH of nearly 2.0 is excreted per day in the stomach, which destroys the majority of microorganisms consumed (Vinderola and Reinheimer,2003). Therefore, resistance to human gastric transit is a prerequisite selection criterion for probiotics, however, during the digestive process, the pH rises to almost 3.0 due to the existence of food (Charteris *et al*., 1998). Maragkoudakis *et al*.(2006) demonstrated that a good probiotic should survive at pH 3.0. They claimed that the pH of the stomach in individuals ranges from 1.0 during fasting, to 4.5 after the meal, and food ingestion occurs during 3 h. Mishra and Prasad (2005) investigated characteristics of seven *L. casei* strains, and showed that all strains were able to resist pH 3.0 for 3 h whereas four strains only displayed resistance for pH 2.0. Generally, in the current study there are a reduction in probiotics counts , as they were exposed to pH 2.0 and pH 3.0 after 3 hours of incubation at 37 ° C. Our findings are in consistent with those obtained from

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prior similar studies (Dave and Shah , 1997; Mishra and Prasad ,2005 ; Maragkoudakis *et al*., 2006) where *Lactobacillus* strains were capable to retain their viability when exposed to pH around 3.0 but exhibited loss of viability at lower pH values . Probiotic lactobacilli strains are exposed to extreme acid stress when they arrive at the gut where hydrochloric acid is existent. Some mechanisms control the homeostasis of internal pH. The F0F1- ATPase is of the greatest importance for fermentative bacteria (Hutchins,2006) . This enzyme was categorized from *L. casei* and its activity was revealed to be optimum at pH values (5.0–5.5). The over-all proton permeability of the cell membrane also contributes to the regulation of the internal pH. Least membrane permeability of *L. casei was* documented at pH 4.0, while that in the acid-sensitive organism was discovered at pH 6.0. It seems that F0F1- ATPase shows the main role in moving protons out of cells and in reducing their net permeability to protons (McDonald *et al*.,1990). Wu *et al*.(2011) showed that several metabolic pathways were involved in the response of *L. casei* in acid stress; they demonstrated that glycolytic enzymes were included in the production of adequate energy for the cell through growth under acidic conditions. Hosseini Nezhad *et al*.(2015) reported that the survival of probiotic lactobacilli in acidic condition was improved in the existence of metabolized sugar to provide ATP to F0F1- ATPase; also, they showed that growth of *L. casei* in acidic conditions created molecular changes in the cell surface to develop an adaptive approach to allow growth at low pH. Corcoran *et al.* (2005) demonstrated that F0F1-ATPase demands ATP for removal of H^+ from the cell, consequently maintaining pH homeostasis and cell viability. They claimed that the accumulation of ATP is a result of energy-generating factories, such as the glycolytic system. McDonald *et al*.(1990) referred that since fermentative bacteria gain less energy from substrates than do respiratory bacteria, fermentative bacteria adapt to low pH through mechanisms that are not energy-consuming.

Coclusions

This study has shown the behavior of *L. casei* as a potential probiotic. Probiotic bacteria have a long history of relationship with dairy products; this study has demonstrated that yogurt is a good carrier for the delivery of probiotic into the gastrointestinal tract as well as the survival of probiotic strains to proteolytic and acidic stresses could be substantially improved by the protective action of yogurt components. Culture of probiotic remained viable at levels above the recommended 10^6 CFU/g after 14 d in the cold storage, therefore the concentration of initial inoculums of probiotic an important factor.*L. casei* showed promising results for acid tolerance.

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CAPTIONS

Figure 1. Changes in the pH during storage of probiotic yogurt (*L. casei)*; pH 1 illustrates the mean of pH values for yogurt along with *L. casei,* pH 2 illustrates the mean of pH values for yogurt was undergone to re-pasteurization before inoculating with *L. casei*, and pH 3 illustrates the mean of pH values for yogurt without *L. casei* as control. 0-14 d: Observations were made after completion of the yogurt fermentation; during 14- day storage of probiotic yogurt respectively.

Figure 1.

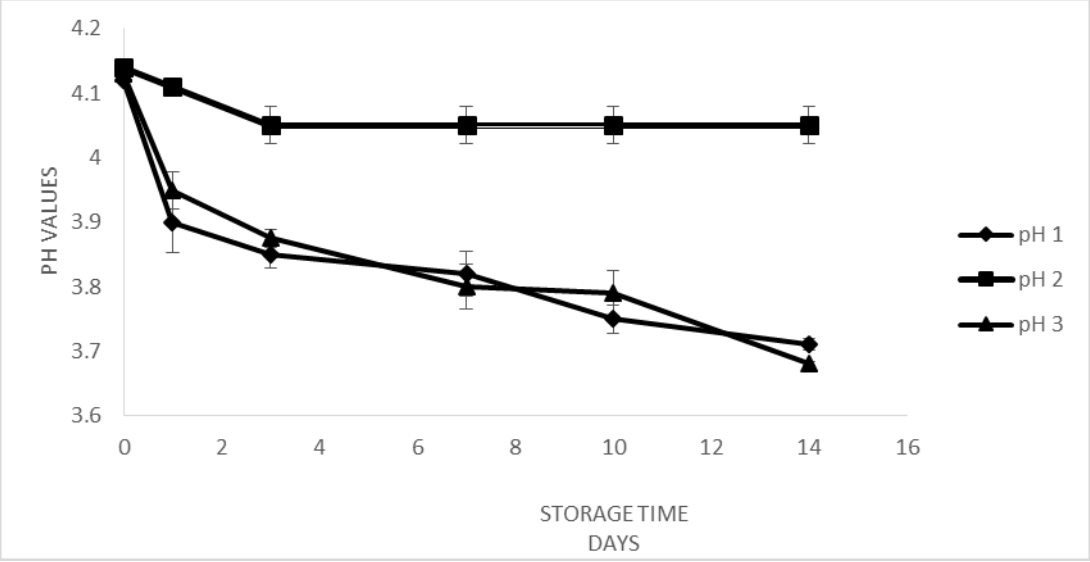

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Table 1. Changes in the viable counts of *Lactobacillus casei* in selective medium, starter cultures of yogurt in reference medium along with *Lactobacillus casei*, and the viable counts of starter cultures of yogurt without *Lactobacillus casei* (conventional yogurt bacteria) as found by subtraction technique during the cold storage of bio yogurt.

Period	Average values of *L.casei* in selective mediium*.	Average values of Lactic acid starter along with *L.casei* in reference medium (MRS).	Average values of Lactic acid starter without *L.casei* (yogurt bacteria).
[0]Zero time	7.52 ± 0.07^{bc}	8.21 ± 0.02^{bc}	8.11 ± 0.02^{b}
1 d	8.61 ± 0.04^{a}	8.76 ± 0.03^{a}	8.20 ± 0.03^{a}
3 d	7.73 ± 0.06^{b}	8.27 ± 0.02^{b}	8.11 ± 0.01^{b}
7 d	7.49 ± 0.08^{c}	8.19 ± 0.01^{c}	8.10 ± 0.02^{b}
10 d	7.03 ± 0.12^{d}	7.94 ± 0.01^{d}	7.88 ± 0.03^{c}
14 d	6.22 ± 0.24^{e}	7.28 ± 0.08^{e}	7.24 ± 0.07^{d}

Legend: Any two means at the same column followed by different superscript letters represent significant differences ($p < 0.05$). Average values (log mean CFU /g ± SD) from three independent repetitions are offered.

* MRS-vancomycin agar, NaCl (4%) agar.

Table 2. Changes in the viable counts of *Lactobacillus casei* during cold storage in a selective medium as well in reference medium for the bio-yogurt samples were undergone to re-pasteurization process before inoculating with *Lactobacillus casei*

Period	Average values of *L.casei* in selective media*.	Average values of *L. casei* in the reference medium (MRS).
[0]Zero time	7.23 ± 0.06^{c}	7.24 ± 0.06^{c}
1 d	8.64 ± 0.02^{b}	8.63 ± 0.02^{b}
3 d	8.71 ± 0.02^{ab}	8.75 ± 0.02^{a}
7 d	8.78 ± 0.01^{a}	8.76 ± 0.01^{a}
10 d	8.72 ± 0.02^{ab}	8.71 ± 0.02^{a}
14 d	7.17 ± 0.08^{c}	7.24 ± 0.05^{c}

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Legend: Any two means at the same column followed by different superscript letters represent significant differences ($P < 0.05$). Average values (log mean CFU /g ± SD) from three independent repetitions are offered.

* MRS-vancomycin agar, NaCl (4%) agar.

Table 3: Resistance of *Lactobacillus casei* to low pH during (3h) at 37 °C

Probiotic bacteria	pH	Average values of *L. casei* (Log mean CFU ml^{-1} ± SD).	
		Before the incubation period. [0]Zero time	After an incubation period (3 h) at 37 °C.
L.casei	2.0	9.23 ± 0.02^{a}	6.52 ± 0.17^{b}
	3.0	10.41 ± 0.09^{a}	7.82 ± 0.04^{b}

Legend: Any two means at the same row followed by different superscript letters are significant differences ($P < 0.05$). Average values (mean ± SD) from three independent repetitions are offered.