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Quality Evaluation of New Developed Symbiotic Yogurt over the Storage at Refrigerator

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ABSTRACT: Two probiotic bacteria (*Lb. acidophilus* and *B. lactis*) were immobilized on the alginate coat of strawberry pieces and were added to the prepared sweet non fat dietary stirred yogurt. Physico-chemical parameters namely pH value, titratable acidity and synersis along with the viability of probiotic bacteria of the samples were evaluated over the 8 days of storage at 5 ºC at 2 day intervals. A significant decrease in pH value occurred after 6 and 8 days and the titratable acidity significantly increased after 8 days. Synersis did not show significant changes probably due to the presence of applied hygroscopic nature of some ingredients in the samples. Some free probiotic cells were identified in yogurt matrix of the samples due to the migration phenomenon. The decrease trend of embedded cells happened slower than the free ones. This might be due to the protective effects of alginate coat on the survival of probiotic bacteria surrounded by yogurt.

Keywords: *Alginate Coat, Probiotic Bacteria, Stirred Yogurt, Strawberry.*

Introduction

Bifidobacteria and *Lactobacilli* might be considered as the most common probiotics (Farnworth *et al*., 2007) that are associated with maintaining optimum microbial balance in the digestive tract with a number of well documented health benefits (Akin *et al*., 2007). Thus, these organisms have been extensively incorporated into dairy foods over the last decade and yogurts containing *Lactobacillus acidophilus* and *Bifidobacterium* species are widely marketed (Gilliland, 2003; Shah, 2000).

Several reports have shown that the survival and viability of probiotic bacteria is often low in yogurt (Shah, 2000; Vinderola, Bailo & Reinheimer, 2000) and resulted in less than 10^7 – 10^8 cfu/g daily, the recommended intake for the health benefits (Lourens- Hattingh & Viljoen, 2001).This remains the concern of both manufacturers and consumers and might be solved to some extent, by applying probiotic growth promoting factors like prebiotics and/or encapsulation technique (Anal & Singh, 2007; Kim *et al*., 2008; Krasaekoopt *et al*., 2004; Lakkis, 2007; Özer *et al*., 2009). In the former case inulin is one of the most popular used prebiotics (Özer *et al*., 2005) in synbiotic (Holzapful & Schillinger, 2002) dairy products. Furthermore, the dietary properties of inulin justify its application in dietary low fat dairy products as a good fat replacer (Meyer *et al*., 2011).

On the other hands, as the functional properties of yogurt increases with added inulin and probiotic bacteria, the addition of fruit positively improves the nutritional value and the taste of this product. These elements play a considerable role in the consumption and sale of yogurt.

Most viability studies, have been conducted on natural or plain bio- yogurts and very few studies have investigated the

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yogurt containing fruit as its natural form (not fruit preparation form).

In this study we have mixed encapsulation knowledge and coating technique to immobilize the probiotic bacteria (*Lb. acidophilus* and *B. lactis*) in the fruit (strawberry) edible (alginate) coat to incorporate it into formulated dietary stirred yogurt. Prepared symbiotic fruit yogurt was examined for viability of probiotic bacteria and physico- chemical analysis were conducted throughout the shelf life (8 days) at refrigerated temperature (4ºC).

Materials and Methods

The following materials and ingredients were used:

fresh Strawberry (from local market), skim milk powder (Pegah Dairy Industries Co. Iran), inulin (Sigma- Aldrich Inc., Germany), food grade sodium alginate (Sigma- Aldrich Pte Ltd, Singapore), calcium chloride (Merck Co. Germany), sodium citrate (Merck Co. Germany), low methoxyl pectin (30, Provisco Ltd, Switzerland), gelatin (220-240, Avizheh, Iran), freeze dried starter cultures (Chr. Hansen, Denmark) consisted of La-5 (*Lactobacillus acidophilus*), Bb-12 (*Bifidobacterium lactis*) and YC-X11 (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*), MRS (de Man Rogosa, Sharp) broth and agar (Merck Co., Germany).

- *Preparation of probiotic-coated strawberry*

Exactly 3g of La-5 and Bb-12 starter cultures were individually inoculated into 50 mL MRS broth and then incubated at 37ºC for 24h under aerobic and anaerobic conditions respectively. Activated cells were harvested by centrifugation at 3000g for 5 min at 25 ºC and washed twice with sterile quarter strength Ringer solution (Özer *et al*., 2009)

Sodium alginate solution (2 % w/v) was prepared by stepwise addition of sodium alginate salt in distilled water, followed by heating at 70 ºC and stirring until the solution became clear (Olivas et al., 2007). Calcium chloride solution (2 % w/v) was prepared to cross-link the used carbohydrate polymer (sodium alginate) because divalent cations such as Ca^{2+} preferentially bind to polymer of L-glucronic acid of alginate that resulted in building of calcium alginate gel (Krasaekoopt *et al*., 2003). Sodium citrate solution (1 % w/v) was prepared for using as a chelating agent that shares affinity for calcium thus destabilizing the alginate gel that resulted the release of entrapped cells (Krasaekoopt *et al*., 2003). Before the onset of the coating process, washed activated probiotic cells were mixed with sterile sodium alginate solutions.

Fresh strawberries without any signs of mechanical damage or fungal decay were selected and sanitized by immersion in sodium hydrochloride solution (10 mg/L) for 4 min, rinsed and dried by natural air convection at 25 ºC prior to cutting them into small cube pieces. Coating process included dipping the fruit pieces into sodium alginate solution containing probiotic bacteria and allowing 1 min for clearing the residual solution and then submerging them for 2 min in the calcium chloride solution. All three mentioned steps were performed under perfectly sanitary conditions (Olivas *et al*., 2007). Final results of this step were L and B samples of probiotic coated strawberry pieces containing *Lb. acidophilus* and *B. lactis*, respectively.

- *Preparation of stirred yogurt*

The premix composition for yogurt is given in Table 1. Skim milk powder, inulin (Meyer et al., 2011), sucrose (Chandan & O'Rell, 2006), LMP(Kumar & Mishra, 2004) and gelatin were mixed with distilled water to reconstitute the milk base. Prepared milk base in a 1000 mL glass container was heated at 85 ºC for 30 min by circulating in a

hot water bath and subsequently cooled down to 43ºC and then at this point, 3 % (v/v) of YC-X16 starter culture, according to the manufacture's reference, was added and gently shaken. The inoculated mix was incubated at 43 ºC until the pH decreased to 4.60- 4.80 and then rapidly cooled down to 4 ºC in an ice-water bath. In the beginning of cooling step, the yogurt was manually stirred with a sterile spoon 6 times clockwise and 6 times anti clockwise.

Table 1. The composition of premix for yogurt

Ingredient	(%)
Skim milk powder	12
Sucrose	10
Inulin	2
Gelatin	0.4
LMP	0.2

- *Preparation of final probiotic strawberry yogurt*

20 g of L and B probiotic- coated strawberry samples were individually added to 180 g of prepared stirred yogurt for making the L and B final strawberry yogurt (L and B SY) samples, respectively.

The experiments were conducted in triplicate order and prepared samples were kept at 5°C for 8 days. Physico-chemical and microbiological analyses were performed throughout the refrigerated storage period at 2-day intervals.

- *Physico-chemical analysis*

The pH values of L and B SY samples were measured at room temperature with a digital pH meter (Hana Instruments, Amorim, Portugal). Titratable acidity of yogurt part (after separating the strawberry pieces) of sample was measured on Dornic degree (AOAC 2002: 947.05).

To determine synersis, 20 mL of yogurt part of the sample was placed in a graduated closed tube and centrifuged (Sigma 3K- 30, Germany) for 10 min at 669 × g and 20ºC and then the volume of expelled whey was determined. The synersis (%) is measured as mL of whey per 100 mL of initial sample (Singh & Muthukumarappan, 2007).

Microbiological analysis

- *Preparation of fruit and yogurt parts of samples*

Coated strawberry pieces were separated from L and B SY samples and rinsed with sterile quarter strength Ringer serum to remove the residual yogurt. 25 g of these fruit pieces, from each sample was separately, added to sterile stomacher bags and liquified with 225 mL of sterile sodium citrate solution (1 % w/v) in stomacher (Funke Gerber, Bag Mixer 400, Germany). Thus, the first dilution of the fruit part of the samples (10-1) was obtained that were used for preparation of other (serial) dilutions with sterile quarter strength Ringer solution.

1 g of yogurt part of each sample was diluted with 99 mL of sterile quarter strength Ringer solution. Subsequent 10-fold serial dilutions were made with sterile quarter strength Ringer solution.

- *Enumeration method*

Standard Plate Count (SPC) method with pour plate technique was used to quantify viable *Lb. acidophilus* and *B. lactis* cells. For enumeration of probiotic cells of fruit and yogurt parts of the samples, 1 mL of prepared dilutions were spread on MRS and MRS-bile (Mortazavian et al., 2007) agars respectively. Aerobic and anaerobic (GasPak system, Merck) incubations of relative *Lb. acidophilus* and *B. lactis* plates were respectively performed at 37 ºC for 72 h (Lankaputhra et al., 1996).

- *Statistical analysis*

One-way and two-way analysis of varianes (ANOVA) were used to analyze the physico-chemical (pH value, titratable acidity and WHC) and microbial (probiotic cell counts) changes of samples, respectively. Least significant difference

(LST) test was conducted to determine the significant
differences with mean values comparison. All statistical analyses were carried out using SPSS software (Version 12.0, SPSS Inc, US) at 5 % confidence ($p< 0.05$).

Results and Discussion

Table 2 shows the the mean values of physic-chemical characteristics of L and B SY samples during 8 days storage at 5ºC.

The pH values of the samples had a decreasing trend from day 0 to day 8 at 5ºC but the first significant fall ($p < 0.05$) was observed on the day 6. The pH decrease was not significant between the 6th and the 8th day of storage period. A significant decrease of 0.29 value, relative to the first day was observed at the end that was logical due to the retention of β-galactosidase activity of viable or nonviable cells of yogurt stater bacteria, specially *Lb. bolgaricus* upon refrigerated storage (Hughes & Hoover, 1995).

The titratable acidity of the samples over the storage time had increasing trend. Relative to the day 0, the significant increase of 14.16 value was obtained at the end of storage period and reached to 110.83 ºD ($P< 0.05$). This rise of titratable acidity along with the mentioned fall of pH value is fully explainable due to the activity of *Lb. bulgaricus* in yogurt. Although the mismatch of their significant changes might be attributed to the presence of an acidic fruit like strawberry in the samples. The activity of *Lb. bulgaricus* during the storage period of yogurt samples in the presence of sucrose (Gauueb et al., 1979) and stabilizers (Kumar & Mishra, 2004) has been explained.

The synersis values of the samples showed a decreasing trend over the storage period but they were not statistically significant ($p > 0.05$). This might be the result of (a) the nonsignificant changes of pH and titratible acidity values of the samples and (b) the hygroscopic natures of gelatin, and LMP in yogurt beside the calcium alginate of the strawberry coat. The rise of acidity and fall of the pH values caused the synersis in yogurt by the contraction of protein gel structure but in stirred yogurt samples, like ours, the presence of gelatin (Lal *et al*., 2006), inulin (Akinet *et al*., 2007), LMP and alginate in samples were the main causes of the absence of synersis over the 8 days of storage time (Everrett & Mc Leod, 2005).

Table 2. The mean values for physic-chemical characteristics of the samples over the storage period (n=3).

Storage time (day)	pH	Titratable value (ºD)	Synersis (%)
0	4.82 ± 0.15^{a}	96.83 ± 0.22^{a}	4.80 ± 0.14^{a}
2	4.72 ± 0.12^{a}	100.83 ± 0.27^{ab}	3.56 ± 0.20^{a}
4	4.69 ± 0.15^{ac}	104.66 ± 0.21^{ab}	2.88 ± 0.19^{a}
6	4.59 ± 0.18^{bc}	106.83 ± 23^{ab}	2.81 ± 0.21^{a}
8	4.53 ± 0.09^{b}	110.83 ± 28^{bc}	2.48 ± 0.17^{a}

[a-c] Means in the same column followed by different letters were significantly different ($p < 0.05$).

Table 3. The mean values for the counts of viable embedded probiotic bacteria in strawberry coat over the storage period (n= 3).

Probiotic bacteria (log cfu/ mL)	Storage time (day)				
	0	2	4	6	8
Lb. acidophilus	10.63 ± 0.25^{a}	10.39 ± 0.31^{ab}	10.26 ± 028^{ab}	9.91 ± 0.20^{abc}	9.56 ± 18^{c}
B. lactis	10.93 ± 0.35^{a}	9.93 ± 0.41^{b}	9.58 ± 0.25^{c}	9.27 ± 0.27^{d}	8.74 ± 0.37^{e}

[a-e]Means in the same row followed by different letters were significantly different ($p < 0.05$).

The viable counts of imbedded probiotics in alginate coat of strawberry pieces in L and B SY samples, over the 8 day storage period at refrigeration temperature, has been shown in table 3.

The viable counts of imbedded *Lb. acidophilus* showed a slight decline over the 8 days of storage at refrigerator. The first meaningful ($p < 0.05$) decrease was 0.72 log cfu/mL that was evaluated between the days 0 and 6. This significant fall reached 1.10 log cfu/ mL at day 8. It should be mentioned that the viable counts of this bacteria between the days 4 and 8 were statistically evaluated significant ($p < 0.05$).

The decline of the viability of *B. lactis* in fruit pieces coat in B SY sample, occurred in higher rate. The differences of viable counts of *B. lactis* between day 0 and days 2, 4, 6 and 8 were 1.00, 1.35, 1.66 and 2.19 log cfu/mL respectively, that all were evaluated statistically significant ($p < 0.05$). On the other hand, all the obtained results for each assessment day showed significant decrease in respect of their previous findings.

The results of this section (Fig. 1) indicates the partial protective role of calcium alginate to safeguard probiotic bacteria in acidic medium. This is due to the pores (< 17 nm) of calcium alginate gel (Krasaekoopt *et al*., 2004) that allows the diffusion of H^+ ions that subsequently affect viability of embedded probiotic bacteria (Trindade & Grosso, 2000; Sultana et al., 2000; Anal & Singh, 2007).

The probable migration of probiotic bacteria from calcium alginate coat of strawberry pieces were evaluated by enumeration of probiotics in yogurt part of the samples at days 2, 4, 6 and 8 of storage at refrigerator (table 4).

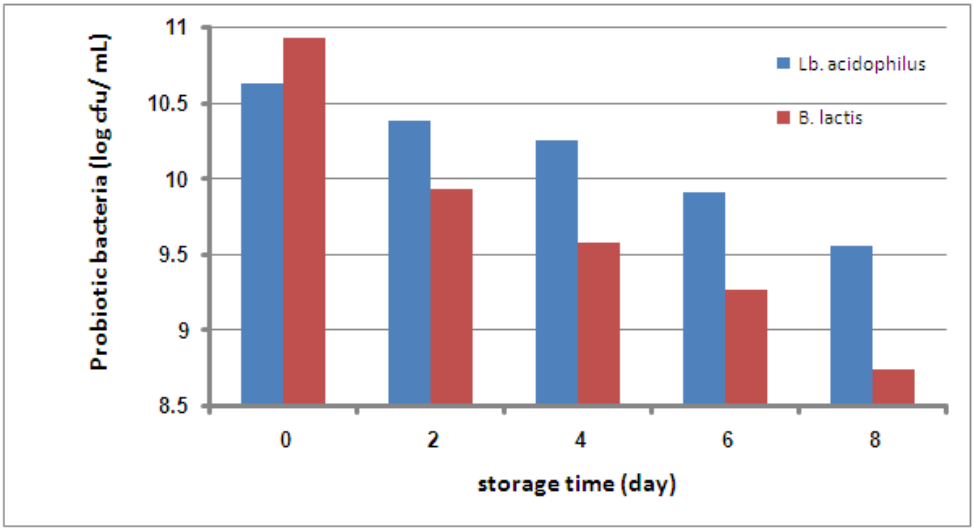

Fig. 1. The shifts of viable embedded probiotic bacteria in strawberry coat surrounded by yogurt throughout 8 days of storage at refrigerator.

Table 4. The mean values for the counts of viable released probiotic bacteria in yogurt matrix (n= 3).

Probiotic bacteria (log cfu/ mL)	**Storage time (day)**			
	2	**4**	**6**	**8**
Lb. acidophilus	4.29 ± 0.11^{a}	4.09 ± 0.20^{b}	3.70 ± 0.32^{c}	3.29 ± 0.13^{d}
B. lactis	4.52 ± 0.22^{a}	4.10 ± 0.10^{b}	3.72 ± 0.18^{c}	2.79 ± 0.08^{d}

[a-d]Means in the same row followed by different letters were significantly different ($p < 0.05$).

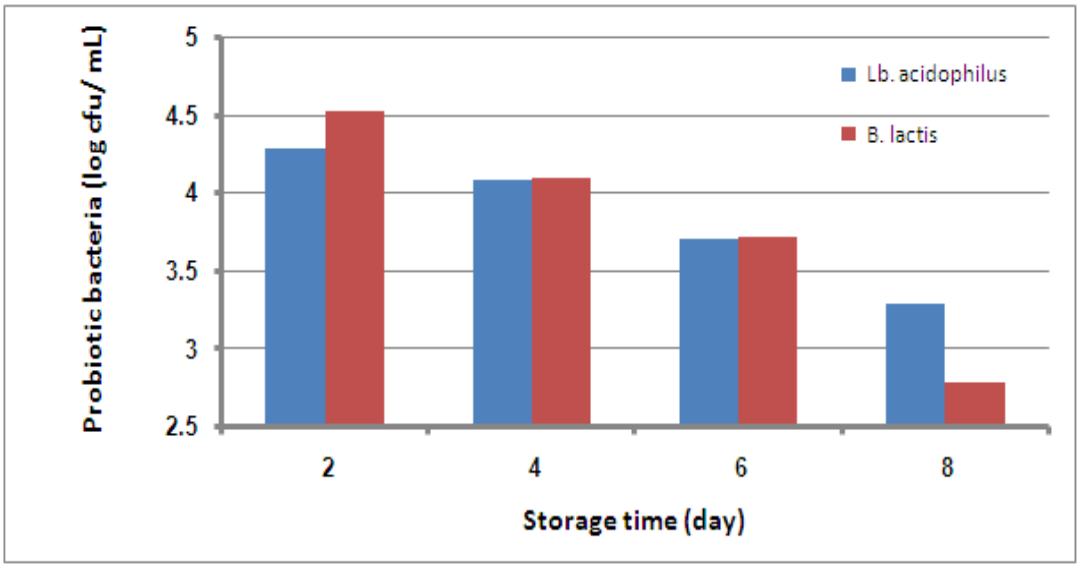

Fig.2. The shifts of viable released probiotic bacteria in yogurt matrix of samples throughout 8 day storage period at refrigerator.

The initial enumerations of free *Lb. acidophilus* and *B. lactis* in yogurt matrix of L and B SY samples, at day 2, were 4.29 and 4.52 log cfu/ mL, respectively. Viable counts of both bacteria showed significant decrease between two successive assessments in each sample ($p < 0.05$). The biggest significant falls were observed between days 2 and 8 that were 1.00 and 1.73 log cfu/ mL for *Lb. acidophilus* and *B. lactis* respectively (Fig. 2). Identification of viable free probiotic cells in yogurt matrix of samples show the permeability of calcium alginate bed that allows the migration of probiotic cells from its porous structure (Trindade & Grosso, 2000; Sultana et al., 2000). The acidic medium of yogurt helps the gradual deterioration of alginate bed that results the easer release of embedded probiotics (Chan & Zhang, 2005). The decrease of viable free probiotic bacteria in yogurt matrix of samples might be attributed to the presence of organic acids like lactic acid of yogurt and probably expelled citric acid of strawberry to the surrounding medium, high osmotic effects of medium because of the presence of sucrose, inulin, gelatin and pectin in the samples, the effect of induced cold shock during the refrigerated storage (Moayednia *et al.*, 2009) and insufficiency of free amino groups and growth factors in medium specially for *B. lactis* (Schlillinger et al., 1999).

Conclusion

This study is concerned with the new method of preparation of probiotic strawberry yogurt based on encapsulation knowledge and coating technique with the aim of helping the viability of probiotic bacteria in symbiotic yogurt. Beside the expected changes of pH values and the titratable acidity in yogurt products, the synersis didn't show significant changes because of the applied inulin, gelatin, LMP and alginate in the samples. The viable count of embedded probiotic cells decreased over the storage period due to the acidic matrix of yogurt medium and/ or the migration of cells from porous structure of calcium alginate coat to the surrounded yogurt. The decrease trends of free probiotic cells in yogurt were faster than the embedded ones perhaps because of the protective effect of alginate coat on the viability of embedded cells as compared with the exposed free cells. Considering the importance of the viability of probiotic cells in probiotic products, the

viable counts of *Lb. acidophilus* and *B. lactis* in the studied final products were maintained above the recommended minimum.

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