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EC MICROBIOLOGY
Research Article

Effects of Coating Materials on Viability of Microencapsulated *Lactobacillus Plantarum* A7 under Stress Conditions

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Received: April 19, 2016; **Published:** October 18, 2016

Abstract

In the present study, whey proteins and sodium alginate were used as coating materials to enhance the viability of *Lactobacillus plantarum* A7 under stress conditions. Viability of encapsulated cells improved significantly ($P < 0.05$) in sodium chloride solution and in yoghurt compared to free cells. Although, free cells satisfied the recommended level for yoghurt during the given shelf life, coating enhanced viability over a long storage. Viability of cells in yoghurt was approximately 3-7 times higher than that of the cells in sodium chloride solution. Exposing to simulated gastrointestinal (SGI) conditions showed that viability was enhanced with significant difference ($P < 0.05$) between free and encapsulated cells. Viability of incorporated free and microencapsulated cells into yoghurt improved significantly ($P < 0.05$) compared to those that were transferred from sodium chloride solution to SGI conditions. Results showed that applying emulsion method along with coating with whey proteins and sodium alginate is an efficient way to enhance the viability of encapsulated probiotics under stress conditions.

Keywords: *Lactobacillus plantarum A7; Microencapsulation; Calcium Alginate Microcapsule; Coating; Whey Proteins; Viability*

Abbreviations

ALG: Calcium-alginate microcapsules; ALCW: Calcium-alginate whey protein-coated microcapsules; ALCA: Calcium-alginate alginate-coated microcapsules; Y1: Yoghurt supplemented with free cells; Y2: Yoghurt supplemented with ALG; Y3: Yoghurt supplemented with ALCW; Y4: Yoghurt supplemented with ALCA

Introduction

One of the major and adaptable species among lactobacilli is *Lactobacillus plantarum* which has been found to have a large genome, capability in metabolising different carbon sources, growth ability [1], and colonising the human gastrointestinal tract [2]. It has also been considered as a GRAS (Generally Regarded as Safe) microorganism [3]. *L. plantarum* A7 is a native strain which was isolated from faecal flora of some Iranian infants and was proved as a probiotic by [4]. Daily usage of 10^8 cells of this strain could be considered as safe regarding its translocation from the intestinal lumen to other organs [5].

The International Dairy Federation has recommended that probiotic bacteria must be active and present in the product to a dose of at least 10^7 cfu per g or mL to the date of minimum durability, in order to exert beneficial effects [6]. However, studies have shown that probiotic bacteria may not survive in enough numbers in dairy products and also during gastrointestinal transit [6-10]. Therefore, the protection of living probiotic cells has become a prominent issue. In this context, microencapsulation is the most promising technique for providing a protective environment for probiotics in acidic products such as yoghurt and in the human gastrointestinal tract [11-14].

Alongside, the capsules must be sufficiently small to avoid a negative impact on the sensorial properties of the functional food product they have been added to [15]. One of the commonly used methods to produce micron size capsules is the emulsion technique [16] considered in relation to other techniques which produce millimeter size capsules [14,17-18].

Alginate is the most commonly used biopolymer for encapsulation of lactic acid bacteria due to the benefits of its low cost, non-toxicity, simplicity, biocompatibility, insolubility in acidic media and release possibility of entrapped cells in the human gut [19-21]. However, the use of alginate is limited because of its low stability in the presence of Ca^{2+}, chelating agents, excess monovalent ions, and acidic conditions [22-23]. Calcium-alginate microcapsules also appeared to be porous when carefully examined with an electron microscope [24]. Hence coating calcium-alginate microcapsules can fill or cover the porous matrix, and improve the chemical and mechanical stability of microcapsules, and therefore increase the efficiency of microencapsulation [12,21,23,25]. Studies have reported that coating calcium-alginate microcapsules using various coating materials could significantly enhance the viability of probiotic bacteria in yoghurt and simulated gastric juice or simulated intestinal juice [11,14,16,22,26,27]. In this context, whey proteins are efficient agents for coating calcium-alginate microcapsules loaded with probiotics, since they are biodegradable, convenient, cheap, and resistant to gastric juice [2,27]. R. Rajam., *et a*l. [28] reported that whey protein isolate is an ideal carrier for probiotics alongside sustained and targeted release in gastrointestinal tract. They indicated that the combination of denatured whey protein isolate and sodium alginate wall matrix is able to deliver probiotics with enhanced survival rate and is suitable for controlled core release applications [28].

Based on this perception, the aim of the present study was to investigate the potential of whey proteins as coating materials in comparison with the potential of sodium alginate to enhance the viability of *L. plantarum* A7 in simulated gastrointestinal (SGI) conditions, refrigerated storage, and in yoghurt. In many studies whey proteins have been used in combination with different encapsulation methods; in this study whey proteins were used as coating agent to enhance the efficiency of emulsion method. Moreover, correlations between the ability of probiotic bacteria to survive in low acidic environment (long-term storage in yoghurt) and ability to survive a few hour exposure to SGI conditions were evaluated.

Materials and Methods

Preparation of concentrated cell suspension

Pure frozen cultures (in 15%, v/v, glycerol (Sigma-Aldrich, Germany)) of *L. plantarum* A7 were obtained from Culture Collection of Isfahan University of Technology, Iran. Fresh cell cultures were prepared by cultivation of probiotic cells in MRS (de Man, Rogosa, Sharpe) agar (Merck, Germany) (1.5%, w/v, agar in MRS broth, 10% inoculum) following the growth of frozen cells in MRS broth. Plates were incubated at 37 ± 1°C for 18 h, in an anaerobic incubator (Model NO. NUAIRE™-5500E, Nu Aire, USA) and three subcultures were performed in the appropriate medium [29]. In order to prepare the cell suspension, 10 mL of last sub-culture was transferred into 250 mL MRS broth and incubated under the same condition. The probiotic biomass was harvested at the beginning of the stationary phase (which corresponded to the time of 18h) by centrifugation (Model 6K15, Sigma, USA) at 4500 × g for 5 min at 4°C. The media was decanted and the cells were washed twice with 0.85% (w/v) sterile sodium chloride solution (Merck, Germany). The cell pellet was diluted with sterile sodium chloride solution (20 mL) and stored at 4°C.

The cell suspension was subsequently used either directly as free cells in assays or subjected to microencapsulation as described below [30].

Microencapsulation and coating procedures

Preparation of calcium-alginate microcapsules (ALG)

An emulsion method described by Sheu and Marshall [31] was used for encapsulation of *L. plantarum* A7 cells. Briefly, 10 mL of cell suspension was incorporated directly into 50 mL of 4% (w/v) sterile sodium alginate solution (Biochemika, Steinheim, Germany). The

bacterial-alginate mixture was subsequently added drop-wise to canola oil (Ladan, Behshahr Oil Industry, Behshahr, Iran) (250 mL in flasks of 1000 mL) containing 0.5 mL Tween 80. To reduce the particle size, the mixture was stirred using a magnetic stirrer (Model: HM21, Fan Azma Gostar, Iran), set at approximately 200 rpm for 20 min, until a uniform creamy emulsion was obtained. To break the water/oil emulsion, 250 mL of 0.1M calcium chloride solution was added quickly, but gently down the side of the flask. The calcium alginate microcapsules (ALG) were formed during continuous stirring for 5 min and 10 min at 100 rpm. Then the mixture was allowed to stand for 30 min for gelification. Finally, the oil layer was drained and the microcapsules were removed from the aqueous phase by low speed centrifugation (350 × g, 10 min, 4°C).

The microcapsules were washed twice and recovered with 0.85% (w/v) sodium chloride solution under the same centrifugation conditions. Microcapsules were filtered (Whatman No. 4, filter paper, Fisher Scientific, Loughborough, UK) and stored at 4°C.

Coating with sodium alginate

Coating with sodium alginate was carried out using the methodology described by Krasaekoopt., *et al*. [21] and Mokarram., *et al*. [16]. Briefly, 15g of washed calcium-alginate microcapsules were suspended in 100 mL of 0.17% (w/v) sterile sodium alginate solution. To disperse the microcapsules, suspension was stirred in an orbital shaker (Model S150, Stuart, UK) at 100 rpm, 37°C for 20 min. The alginate coated microcapsules (ALCA) were collected by centrifugation (350 × g, 10 min, 4°C). The pellet was rinsed twice with 0.85% (w/v) sodium chloride solution and kept at 4°C until further analysis.

Coating with whey proteins

Washed calcium-alginate microcapsules were coated by whey proteins solution using method described by Gbassi., *et al*. [2] as follows: whey proteins isolate (WPI) (R & D of Milei company, Tehran, Iran) (20g) was dissolved in 1000 mL of sterile deionised water. The pH was then adjusted to 7.0 ± 0.1 by adding 0.1M NaOH. Then 2g of calcium-alginate microcapsules were immersed in 100 mL of whey proteins solution and the vial was shaken at 100 rpm for 15 min. Whey coated microcapsules (ALCW) were recovered by filtration and kept at 4°C.

Determination of resistance to refrigerated storage

Evaluation of viability of free and encapsulated cells in sodium chloride solution

The stability of free cells and encapsulated cells in both coated- and uncoated-microcapsules was evaluated in sterile sodium chloride solution (0.85%, w/v) over 50-day storage at 4 ± 1°C. To enumerate the living cell numbers, aliquots of 1 mL of free cells and 1 g of either coated- or uncoated-microcapsules were taken on 0, 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 days of storage. Evaluation of total viable count was performed as described by [26].

Evaluation of viability of free and encapsulated cells in yoghurt

Five different batches of yoghurt were prepared, i.e., control, yoghurt supplemented with free cells (Y1), yoghurt supplemented with ALG (Y2), yoghurt supplemented with ALCW (Y3) and yoghurt supplemented with ALCA (Y4). Reconstitution of skim milk powder (SMP) was carried out by mixing 500 g skim milk with 6 L distilled water to make milk containing 12% (w/w) total solids. Milk was pasteurised by heating to 80 ± 5 °C with high-speed stirring and holding at this temperature for 10 min. It was then cooled to 42 ± 1°C and the yoghurt starter culture (Flex- YF-L812, Christian Hansen, Hoersholm, Denmark) was added. The probiotic cultures were incorporated as free or coated- and uncoated-encapsulated cultures. Each yoghurt mix was distributed into 100 mL capped plastic containers, sealed, and incubated at 45 ± 1°C until a pH of 4.6 was reached. The yoghurt was cooled in an ice water-bath and stored at 4 ± 1°C and the microbiological analysis was performed at time intervals [26-27]. The '0 day' analysis was carried out after overnight cold storage and the viability of probiotic cells was evaluated over 50-day storage.

The coated and uncoated-microcapsules containing probiotic cells were released according to the method of Sheu and Marshall [31] and Godward and Kailasapathy [11]. Briefly, 10g yoghurt was dissolved in 100 mL of 0.1M phosphate buffer at pH 7.0 in a plastic bag. Plastic bags were placed in a stomacher blender (Model 400, Seward, England) for 10 min to allow complete release of the bacteria from microcapsules. The yoghurt containing free cells was treated in a similar way to maintain the same treatment conditions. Subsequent dilutions were made in 0.85% sodium chloride solution. The total viable count was determined by plating in the selective differential medium for *L. plantarum* in yoghurt (MRS-mannitol basal agar medium) comprising [33]: proteose peptone (10 g L^{-1}), meat extract (8 g L^{-1}), yeast extract (4 g L^{-1}), D-glucose (20 g L^{-1}), sodium acetate (5 g L^{-1}), triammonium sulphate (2 g L^{-1}), dipotassium phosphate (2 g L^{-1}), manganese sulphate (0.05 g L^{-1}), polysorbate 80 (1 mL), mannitol (20 g L^{-1}) (Merck and Sigma, Germany); sterilised by filtration through a 0.22 µm membrane filter before adding to the cooled medium and bacteriological agar (15 g L^{-1}).

The pH of the medium was adjusted to 5.5 ± 0.2 using acetic acid glacial. Plates were incubated at 37 ± 1°C under anaerobic conditions for 48h. Under these growth conditions, it was possible to differentiate starters from adjuncts and thus enumerate adjuncts. Furthermore, the viability of free and coated- uncoated-microcapsules containing living cells was investigated in SGI conditions at the end of 50-day storage in yoghurt.

Survival in simulated gastrointestinal tract conditions

Survival analysis of free cells and coated or uncoated-microcapsules containing living cells under simulated gastrointestinal tract (SGI) conditions was performed based on an *in vitro* method developed by Picot and Lacroix [28] using a modified method proposed by Gauthier, Vachon, and Savoie [34].

Preparation of simulated gastric juice and simulated intestinal juice

Simulated gastric juice (SGJ) was prepared by dissolving pepsin (Sigma-Aldrich, Germany) 1: 60,000 in 0.1 M HCl. The pH was adjusted to 1.9 with 1M NaOH to achieve a final concentration in the digestion mixture of 0.26 g L^{-1}. The reason of selecting pH 1.9 for SGJ was the maximal activity of pepsin in a pH range of 1.7 to 3.0 [35].

Simulated pancreatic juice (SPJ) was prepared by dispersing pancreatin (Sigma-Aldrich, Germany) 5× in 0.02 M sterile sodium phosphate buffer at pH 7.5. The pH was adjusted to 7.5 with 1M NaOH to achieve a final concentration in the digestion mixture of 1.95 g L^{-1}. Concentrated bile salt solution (150 g L^{-1}) was prepared by dissolving bile bovine powder (Sigma-Aldrich, Germany) in distilled water. Resulting solutions were filtered for sterilisation through a 0.22 µm membrane [27].

Survival of free and encapsulated cells after sequential incubation in simulated gastric juice and simulated intestinal juice

Fresh cell suspension was prepared in the same way as described in section 2.1. SGJ survival of free cells was examined by adding 5 mL of the cell suspension into a 125 mL flask containing 75 mL of pepsin preparation (0.277 g L^{-1}). Incubation was performed at 37°C and 100 rpm agitation in an orbital shaker. After 30 min, pH was adjusted to 7.5 with 1M NaOH until the reaction stopped. SIJ survival of free cells was evaluated by exposing the cells to the small intestine as follows: supplementing 5 mL of 0.5M concentrated sodium phosphate buffer at pH 7.5, subsequently adding 2.0 mL of bile salt solution to the flask. After quickly adjusting the pH to 7.5 and volume to 90 mL with sterile distilled water, 10 mL of SPJ were supplemented for a final volume of 100 mL. Incubation in the simulated gastrointestinal model was performed at 37°C and 100 rpm agitation for 6h. For determination of total cell counts of free cells, an aliquot 100 µL of cell suspension was withdrawn after 30, 60, 120, 180 and 360 min. The reaction was stopped by placing samples in ice for 5 min [27].

Freshly prepared ALCW (8g) was placed into a flask containing 50 mL of 30 mM CaCl2 and kept at 4°C for 15 min to form the whey protein film. Similar treatment was performed for ALG and ALCA to determine the protective effect of different microcapsules on the cell viability at the same condition. The resulting dispersions were incubated at 37°C.

After quickly adjusting the pH to 1.9 with 1M HCl and the volume to 60 mL with sterile distilled water, 20 mL of pepsin preparation (1.04 g mL^{-1}) was added. The three vials were incubated at 37°C for 6 h under agitation (125 rpm). The rest of the procedure was identical to that described for free cells. Aliquots of 100 µL were removed at 30, 60, 120, 180 and 360 min (for all trials) to enumerate viable cells as described in section 2.3. The cell counts were corrected by considering the dilutions resulting from pH and volume adjustments of the digestion medium at the end of the gastric phase and before starting the intestinal phase [27].

Statistical Analysis

The collected data were analysed using SAS statistics software (Version 9.2). A completely randomised factorial design was used for all analysis. Analysis of variance was performed using a one-way analysis of variance (ANOVA). Significant differences between treatment means were verified using the least significant difference (LSD), with a probability level $P < 0.05$. Graphs were created with Excel (Microsoft Office, Excel 2013). Analysis was carried out in triplicate and values were expressed as means ± standard from at least two independent experiments.

Results and Discussion

Resistance to refrigerated storage

One of the important properties of a microorganism to be considered as probiotic is its ability to survive storage conditions as a formulated product [36]. In general, fermented products containing added probiotics should be stored under refrigeration at 4°C.

Evaluation of viability of free and encapsulated cells in sodium chloride solution

Survival of encapsulated *L. plantarum* A7 on coated- or uncoated-microcapsules was compared with that of free cells in sodium chloride solution under refrigeration storage for 50 days (Figure 1).

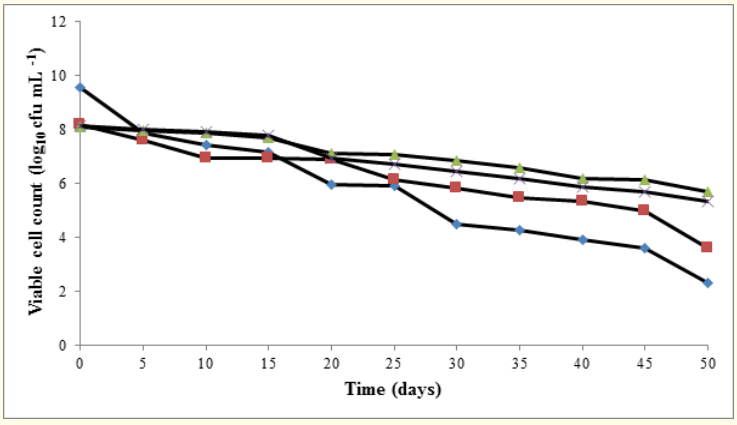

Figure 1: *The viability of L. plantarum A7 cells in sodium chloride solution (0.9%, w/v) under refrigeration (4±1°C): ◆, free L. plantarum A7 cells (FLP); ■, calcium-alginate microcapsules (ALG); ▲, calcium-alginate whey protein-coated microcapsules (ALCW); ×, calcium-alginate alginate-coated microcapsules (ALCA).*

According to the results, the number of free cells and encapsulated cells on ALG dropped considerably (about 7.28 and 4.57 log numbers, respectively) over a 50-day storage. However, at the 50th day, the loss of viability of encapsulated cells on ALCA and ALCW was 2.79 and 2.40 logs, respectively. There was a significant difference ($P < 0.05$) between four types of treatments at the end of refrigeration

storage. Even with the release of some cells during storage due to the collapse of microcapsules, ALCA and ALCW showed good stability and low loss of viability of cells. Brinques and Ayub [26] also reported that viability of encapsulated *L. plantarum* BL011 cells was greatly enhanced compared to free cells in sodium chloride solution and under refrigeration. Woraharn, Chaiyasut, Sirithunyalug, and Sirithunyalug [38] showed that the viability of encapsulated *L. plantarum* CMU-FP002 cells in calcium alginate beads improved in comparison with free cultures, and cell count of alginate beads maintained in high numbers (11 log cfu g^{-1}), when the beads were stored at 4°C for 5 days alternating with room temperature for 5 days, for a total of 2 months.

Cui, Goh, Kim, Choi, and Lee [39] found that the viability of Bifidobacteria-loaded alginate Poly-L-lysine microparticles was significantly improved under refrigeration storage. Hence, improving the stability of the microcapsules by coating could reduce the loss of cells to the medium and positively affect the viability of cells during storage.

Evaluation of viability of free and encapsulated cells in yoghurt

Sultana., *et al*. [14] suggested that there are two approaches for incorporating probiotic cultures into yoghurt; probiotics may be added after yoghurt production or may be inoculated in yoghurt as adjunct culture at the time of fermentation. In this study, free and encapsulated cultures were added to yoghurt as adjunct cultures. Results showing the viability of free and encapsulated *L. plantarum* A7 cells in Y1, Y2, Y3 and Y4 under refrigerated storage are illustrated in (Figure 2).

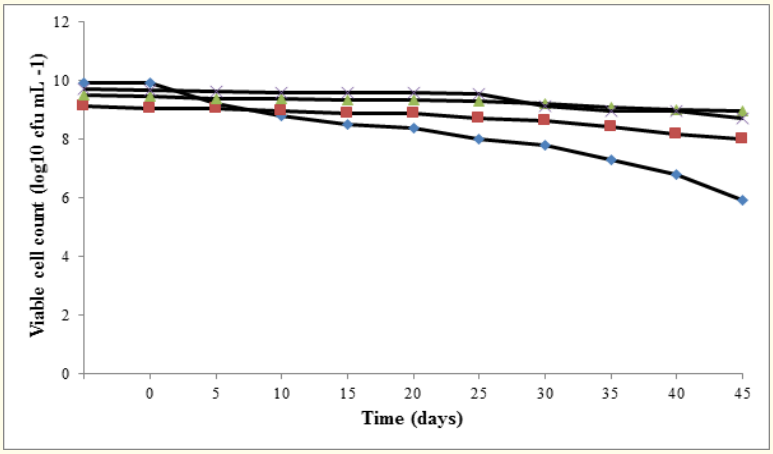

Figure 2: *The viability of L. plantarum A7 cells in yogurt under refrigeration (4±1°C): ◆, yoghurt supplemented with free cells (Y1); ■, yoghurt supplemented with ALG (calcium-alginate microcapsules) (Y2); ▲, yoghurt supplemented with ALCW (calcium-alginate whey protein-coated microcapsules) (Y3); ×, yoghurt supplemented with (calcium-alginate alginate-coated microcapsules) ALCA (Y4).*

Results showed that the viability loss of cells in Y3 and Y4 samples was only 0.51 and 1.01 logs, respectively, compared to that of in Y1 and Y2 with a sharp decline of 4.01 and 1.14 logs after 50 days. There was a significant difference ($P < 0.05$) between four types of yoghurt samples. The number of living cells in Y1, Y2, Y3 and Y4 decreased from 9.94, 9.13, 9.49 and 9.72 log cfu mL^{-1} to about 8.39, 8.88, 9.33 and 9.59 log cfu mL^{-1}, respectively, after a 25-day storage that is the normal shelf life for yoghurt at 4°C. Hence, free *L. plantarum* A7 cells can satisfy the recommended level of 10^6-10^9 cfu g^{-1} of yoghurt during the given shelf life [27]. Furthermore, cell count of Y3 and Y4 remained over 10^8 cfu mL^{-1} at the 50^{th} day of storage. Although the amounts of microcapsule used in this work were more than the acceptable quantities for real yoghurt formulations [27], these results suggest the effect of coating on enhancing the viability of Lactobacilli in yoghurt over a long storage.

Brinques and Ayub [26] reported that the loss of viability of encapsulated *L. plantarum* BL011 in alginate chitosan-coated microcapsules in yoghurt was of 0.55 log cycles during 38 days of storage. Krasaekoopt, Bhandari, and Deeth [40] showed an increase of 1 log in the viability of encapsulated *Lactobacillus acidophilus* 547 and *Lactobacillus casei* 01 in alginate chitosan-coated beads compared to free cells in yoghurt under refrigeration for 28 days. Kailasapathy [41] reported that there was an increase of 2 and 1 log in the viability of *L. acidophilus* and *Bifidobacterium lactis*, respectively, due to improving cell viability by microencapsulation in calcium-induced alginate-starch beads. Sultana., *et al*. [14] showed a slight decline of about 0.5 log numbers in the population of encapsulated *L. acidophilus* and *Bifidobacterium infantis* in yoghurt, while there was a decrease of about 1 log in free cells over a period of 8 weeks.

Results showed that coating was effective in preserving the stability of probiotic cells in sodium chloride solution and in yoghurt during long-period storage; however, the viability of probiotic cells in yoghurt was approximately three to seven times higher than that of the added cells in sodium chloride solution. Brinques and Ayub [26] also reported that when sodium alginate chitosan-coated microcapsules were used in yoghurt, the cell viability was approximately four times higher than when cells were kept in sodium chloride solution.

Survival of free and encapsulated cells after sequential incubation in simulated gastric juice and simulated intestinal juice

In general, probiotic bacteria must be alive in the product at the time of consumption and also capable of reaching the human gut in large population to facilitate colonisation and rapid multiplication to exert beneficial effects [42]. In summary, free and encapsulated cells were placed into SGJ (pH: 1.9) for 30 min followed by placing into SIJ (pH: 7.5) for 6 h at 37°C. The results of the viability of probiotics (before adding in yoghurt) in SGJ and SIJ are shown in (Figure 3).

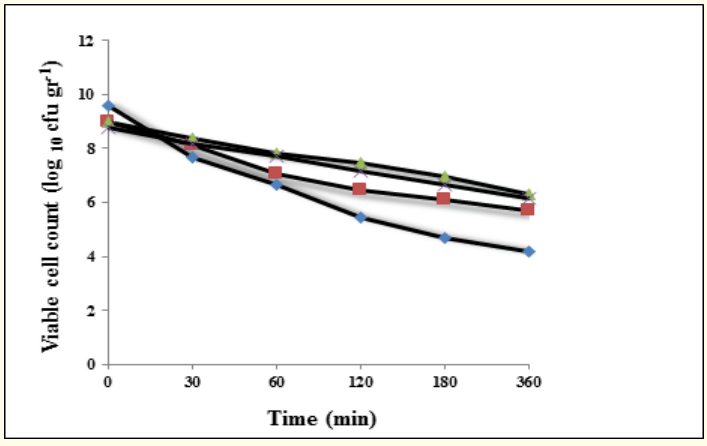

Figure 3: *The viability of free and encapsulated L. planterum A7 cells when exposed to SGJ and SIJ; before adding in yoghurt: ◆, free L. plantarum A7 cells (FLP); ■, calcium-alginate microcapsules (ALG); ▲, calcium-alginate whey protein-coated microcapsules (ALCW); ×, calcium-alginate alginate-coated microcapsules (ALCA).*

There was a reduction of 1.9, 0.84, 0.63, and 0.85 log in SGJ for free and encapsulated cells in ALG, ALCA and ALCW, respectively. There was no significant difference between ALG, ALCA and ALCW at 95% confidence. While the viability of free cells in SGJ was low, a large population remained, showing a good resistance of *L. plantarum* A7 to acidic conditions.

Exposure to SIJ drastically reduced the total viable counts of free and encapsulated cells in ALG, ALCW and ALCA to 3.51, 2.45, 2.09 and 2.03 logs, respectively. The viability of cells in both ALCA and ALCW was significantly ($P < 0.05$) better than that of the cells in ALG and free

cells. Therefore, coating was effective in enhancing the resistance of probiotic cells to SGI conditions with living cell numbers remaining above 10^6 cfu mL^{-1}, satisfied the accepted criterion of a minimum of 10^6 cfu mL^{-1} to acquire therapeutic benefits [43-44].

The resistance of incorporated free and encapsulated cells into yoghurt to SGJ and SIJ at the end of storage period are presented in (Figure 4).

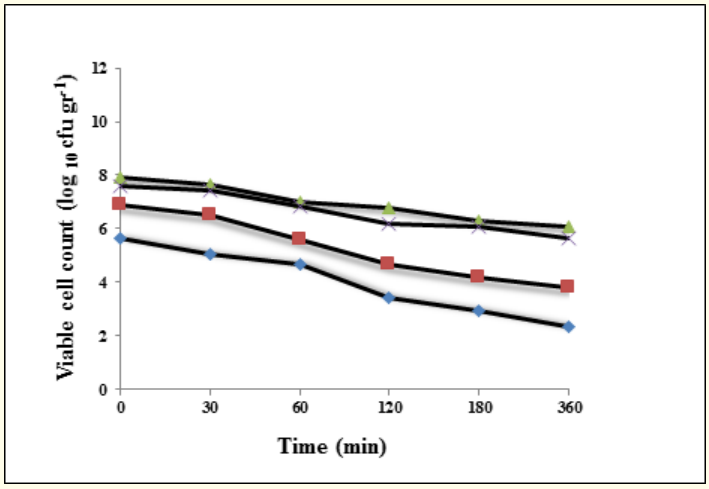

Figure 4: *The viability of free and encapsulated L. planterum A7 cells when exposed to SGJ and SIJ; after 50 days storage in yoghurt: ◆, free L. plantarum A7 cells (FLP); ■, calcium-alginate microcapsules (ALG); ▲, calcium-alginate whey protein-coated microcapsules (ALCW); ×, calcium-alginate alginate-coated microcapsules (ALCA).*

Exposing to SGJ caused linear decline of 0.55, 0.4, 0.19 and 0.25 logs in the viability of free and encapsulated cells in ALG, ALCA and ALCW, respectively. There was no significant difference between ALCA and ALCW at 95% confidence, while a significant difference ($P < 0.05$) was observed between encapsulated cells and free cells. Exposure to SIJ caused a decrease in the total number of survivors and there was a significant difference between all trials at 95% confidence. However, there was no significant difference ($P < 0.05$) between ALCA and ALCW.

The loss of living free and encapsulated cells in ALG, ALCA and ALCW after 6h incubation in SIJ was 2.72, 2.66, 1.77 and 1.58 logs, respectively. The population of living cells in ALCA and ALCW was remained more than 10^5 cfu mL^{-1} after 6h incubation in SGI conditions. According to the results, the viability of free and encapsulated cells in SIJ was lower than that of in SGJ. This can be explained with respect to the fact that the pH of bile salt solution (7.5) may not be suitable for *L. plantarum* as it grows and survives better in an acidic condition [1]. Mirlohi., *et al*. [1] reported a decline of 0.46 - 2.54 log cfu mL^{-1} for *L. plantarum* A7 cells by first exposure to acidic MRS and MRS containing bile. They showed that exposure to an acidic medium (pH 3) and subsequent exposure to the neutralised environment containing bile had a more adverse effect on the cell survival; however, using a stronger acidic medium (pH 2.5) and subsequent exposure to bile, cells did not show further reductions. They also reported that cell numbers of this strain declined from 8.36 log cfu mL^{-1} to 8.08 log cfu mL^{-1} after 2h incubation in SGJ. According to their research, the presence of pepsin in SGJ decreased the cell count only 0.5 logs compared with MRS medium without pepsin in which 2 logs loss was observed [1].

Gbassi., *et al*. [2] reported a loss of the viability of three different strains of *L. plantarum* encapsulated in calcium alginate beads after 90 min of incubation in SGJ; however, they showed an increase in survival using alginate beads coated with whey proteins. The same authors also reported that viable count in whey proteins-coated alginate beads was 10^6 and 10^7 cfu g^{-1}, respectively, after 120 min exposure to SGJ with and without pepsin; however, the uncoated beads did not protect probiotic cells at the same period [32]. Picot and Lacroix [27] reported that encapsulation of probiotic cultures in whey protein-based microcapsules can provide a protective envelope that acts as a barrier against both acidity and gastric enzymes and enhance the viability of living cells when subjected to SGJ and SIJ.

Studies have shown that cross-linked membrane can provide a physical barrier against the entry of detrimental components of the GI tract into the coated and uncoated microcapsules. Whey proteins can form a complex with the surface of alginate to obtain a semi-permeable membrane on the basis of their amphoteric character [32]. Furthermore, the alginate core limits the hostile effect caused by the low pH in the stomach by suggesting a buffering capacity [45]. Woraharn., *et al*. [38] reported that by increasing incubation time in gastric fluid, it is likely that gastric juice entered the microcapsules through the surface pinholes resulting in a loss of viability; however, they showed that compacting membrane might lower diffusion of SGJ and SIJ. Chandramouli., *et al*. [19] found that there was a correlation between increasing the cell load during encapsulation and increasing the number of bacterial survivors that reach the colon in adequate quantities and suggested that the initial cell load 10^9 cfu mL^{-1} gave maximum viable cell counts in SGJ.

The increased viability as a result of microencapsulation in SGJ and SIJ has previously reported by several researchers for various strains of *Lactobacillus*. The results of the present study corresponded to achievements of [13,26,38,45]. Brinques and Ayub [26] showed that exposure to SGJ drastically reduced the total number of *L. plantarum* BL011 under all tested conditions, and while resistance to SGJ was low, remainder viable population showed a good resistance to the acidic conditions. They also indicated that SIJ did not have any negative influence on the cell viability of both free and encapsulated cells in ALG and ALPE (microcapsules prepared with mixture of sodium alginate and citric pectin) compared to the controls, except for the free cells that there was a decrease of 0.2 and 0.4 logs for control and SIJ, respectively. Martoni., *et al*. [46] reported that encapsulation led to low loss of the viability of *L. plantarum* 80 BSH+ in simulated stomach conditions at pH 2.5 and 3.0, with 1.09 and 0.6 log cfu mL^{-1} reductions, respectively. They also reported a linear decrease in viability, when cells were exposed at pH 2.0, with 8.98 log cfu mL^{-1} reduction after 4h, while after 30 min of exposure at pH 1.5, cells completely lost their viability. They verified that in the simulated small intestine, viable count of microcapsules containing L. plantarum 80 BSH+ cells was maintained over 10^{10} cfu mL^{-1} after 3, 6, and 12 h in bile concentrations up to 1.0%. Mokarram., *et al*. [16] showed that *L. acidophilus* and *L. rhamnosus* exposed to SGJ had higher viability when encapsulated in calcium-alginate with double coating sodium alginate. Koo, Cho, Huh, Baek, and Park [48] also reported that the survival rate of encapsulated Bifidobacteria and *L. casei* in alginate-chitosan microcapsules was higher than that of in alginate microcapsules without chitosan; moreover, viability decreased relatively when cells were exposed to bile salt solutions.

Rajam and Anandharamakrishnan [13] used the prebiotic 'fructooligosaccharide (FOS)' in combination with whey protein isolate (WPI) or denatured whey protein isolate (DWPI) for encapsulating the probiotic bacteria *L. plantarum* (MTCC 5422). According to their results, microcapsules of 1:1 core-to-wall ratio showed higher encapsulation efficiency than 1:1.5; however, microcapsules of 1:1.5 core-to-wall ratios increased the storage stability and resistance of probiotic cells in the simulated gastric and intestinal conditions.

It has been proved that different strains of probiotics have a different response to the GI conditions [14]. Furthermore, Brinques and Ayub [26] reported that the juices used in simulated tests differ widely among literatures; for SGJ, most researchers have only used sodium chloride solution with adjusted pH to the desired value and several authors have added enzymes in it; and for SIJ, many researchers have used sodium chloride solution with different concentrations of bile salts, but seldom with the addition of pepsin and pancreatin to these solutions.

Results showed that the viability of free and microencapsulated cells which were kept in yoghurt for 50 days prior to inoculation in SGI conditions, improved significantly ($P < 0.05$) compared to those which were transferred to SGI conditions before adding in yoghurt.

Martoni., *et al*. [45] suggested that if microcapsules loaded probiotic bacteria ingested in a buffered system such as milk, yoghurt, or milk based foodstuffs, then oral microcapsules therapy would be best accomplished.

Our study demonstrates that coating microcapsules with whey proteins and sodium alginate solution can be an effective way to enhance the viability of probiotic cells in the dairy fermented products and also in the human GI tract allowing better colonisation of the large intestine.

Conclusion

Results showed that coating with whey proteins and sodium alginate solutions significantly improved the viability of probiotic bacteria in yoghurt and sodium chloride solution under refrigeration and simulated GI conditions compared to free cells. Although, free *L. plantarum* A7 cells can satisfy the recommended level of 10^6-10^9 cfu g^{-1} of yoghurt during the given shelf life, coating can enhance the viability of *Lactobacilli* in yoghurt over a long storage. The amount of microcapsules used in this work was more than the acceptable quantities for real yoghurt formulations. However, it is important to test the lower concentrations of probiotic cells to achieve a standard inoculation rate of microcapsules with the best sensorial aspects of the product. The viability of probiotic cells in yoghurt was approximately three to seven times higher than that of the added cells in sodium chloride solution. The population of living cells in ALCA and ALCW remained more than 10^5 cfu mL^{-1} when exposed to SGI conditions, either before adding them to yoghurt or after 50-day refrigeration in yoghurt, satisfying the accepted criterion of a minimum 10^6 cfu mL^{-1} to exert beneficial effects. Incorporating free and encapsulated cells into yoghurt increased their resistance to SGI conditions, because the high total solids level in yoghurt including the fat and milk solids can provide better protection for probiotic bacteria rather than sodium chloride solution. Furthermore, the viability of both free and encapsulated cells was affected by the SGJ less than SIJ regarding the fact that the pH of bile salt solution (pH 7.5) may not be suitable for *L. plantarum* as it grows and survives better in an acidic condition. These are interesting characteristics that must be met for the commercial implementation of encapsulated *L. plantarum* in dairy and other food products. Additional research is required particularly with regard to the evaluation of other microencapsulation techniques and using different coating materials which could provide a protective environment for probiotic cells during processing, storage and gastrointestinal transit.

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