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Effect of Microencapsulation on the Viability of *Lactobacillus Acidophilus* and *Saccharomycess Boulardii* Probiotics at Different Storage Temperatures in Doogh

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ABSTRACT

Background: Encapsulation of probiotics in a carrier material is a useful tool to improve the viability, release, delivery of probiotic cells and extend food shelf by holding extra multiplying of probiotic cells to prevent changing the sensory characteristic of supplemented food. This study aimed to determine the effects of microencapsulation on the viability of *Lactobacillus acidophilus* and *Saccharomycess boulardii* probiotics in Doogh samples stored at different temperatures and their combined effect on pH, stability, and sensory attributes of Doogh samples.
Methods: Micro-encapsulation of probiotics was done using the emulsion method under sterile conditions. Enumeration of probiotics and physical analysis (pH, stability, and organoleptic characteristics) of 120 heated Doogh samples containing free and encapsulated *L.acidophilus* and *S.boulardii* probiotics were conducted for 28 days at 7-day intervals (0, 7, 14, 21, 28 days) at three temperatures (4, 20, 35 °C).
Results: The survival of probiotics decreased continuously at all temperatures during storage time except for the encapsulated forms of *L.acidophilus* at 4 and 20 °C and the free forms of *S.boulardii* at 4 °C remained constant significantly ($P < 0.05$). The treatments containing free *S.boulardii* had the least significant decrease in pH on the last day of storage at 20 and 35 ºC temperatures. The sensory evaluation shows acceptable scores in all treatments stored at 4 °C.
Conclusion: Our results show synergistic effects of encapsulated probiotics on extending the shelf life of Doogh.

1. Introduction

Probiotics are live bacteria and yeasts which have several health benefits as a dietary supplement when ingested in adequate amounts [1,2]. Several studies have shown health benefits and potential effects of probiotics, including improving gastrointestinal (GI) disorders (diarrheal infections, lactose intolerance, inflammatory bowel disease), reducing cholesterolemia, antimicrobial activity, anti-carcinogenic and antimutagenic attributes, and stimulating the ability of immune system [2, 3, 4, 5]. Several foods have been used as probiotic carriers, categorized as functional foods that should have at least 10^7 cfu/g or ml probiotic cells [2,4]. Probiotics need to survive at the time of ingestion. Adverse environments such as acid media of stomach or carrier food, bile salts of the intestine, storage time, oxygen, and high probiotic temperatures can threaten these

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beneficial microorganisms' viability [5]. Different methods are available to improve the viability of probiotics in adverse environments [4]. Encapsulation or entrapping of probiotic cells or active agents in a carrier material is a useful tool to improve the viability of probiotic cells, extend food shelf life, controlled release, and optimized delivery by holding extra multiplying of probiotic cells to prevent changing the sensory characteristic of supplemented food [5]. Several studies have been conducted to develop the survival of various probiotics by encapsulating different natural materials such as calcium alginate, Chitosan, whey proteins, and sodium alginate [4, 6, 7, 8]. Doogh is a mixture of water and yogurt (1/1 V/W), which contains salt < 2% and flavoring agents (mint, oregano extracts, or a mixture of them). It is an indigenous, traditional, and widely consumed fermented dairy beverage in Iran [9]. This product is one of the most common acidic dairy drinks produced in Iran and other countries, including Afghanistan, Azerbaijan, Armenia, Iraq, Syria, Bulgaria, Turkey, and Balkan islands. It is known as Ayran in Iran, Azerbaijan, Turkey, and Laban in Arabic countries [8]. Lactic acid bacteria (LAB) are the main microorganisms with probiotic potential in raw milk and dairy products, including lactobacilli, bifidobacteria, etc.[10]. They are used in food fermentations and enhance taste and texture in fermented food products as starter cultures [10]. A probiotic microorganism that probably exists in typical yogurt is *Lactobacillus acidophilus*. Their growth optimum is commonly at pH 5.5–5.8, with complex nutrient requirements such as carbohydrates, lipids, proteins, vitamins, and minerals [11]. Their diversity and environmental growth conditions determine cultured dairy products' characteristics and shelf life [11*]. Saccharomycess boulardii* is a well-recognized probiotic yeast used as a preventive and therapeutic agent for GI disorders. It is a eukaryotic cell and differs from prokaryotes (probiotic bacteria) in cell size, cell wall, probiotic implication, and optimal growth condition [12]. Probiotics' health benefits and effects are strain-related, which is why determining the genus and species of probiotics is important [13]. Ever-expanding of functional foods and products containing probiotics in the world market shows that the acceptability of probiotic products has been widespread globally, especially in Europe, the United States, and Japan [3]. Despite high consumer demands for functional foods and fermented dairy products, the manufacture of probiotic-containing dairy yogurt drinks is currently hindered because of the lack of probiotic viability at different temperatures, stability of texture, and undesirable sensory attributes. The survival of different probiotics was investigated by researchers in other foods [8, 4, 14]; however, there is no study about the survival of free and encapsulated probiotic *Saccharomycess boulardii* in combination with *Lactobacillus acidophilus* and their effect on Doogh pH, stability and organoleptic characteristics. Therefore, this study aimed to investigate the effects of microencapsulation on the viability of *Lactobacillus acidophilus* and *Saccharomycess boulardii* probiotics in Doogh samples stored at different temperatures and their combined effect on pH, stability, and sensory attributes of Doogh.

2. Materials and Methods

2.1 Materials

Probiotic strains were provided from the microbial cluster collection of Pasteur Institute, Iran, and The American microbial cluster collection (ATCC) for *Lactobacillus acidophilus* ATCC 314 and *Saccharomycess boulardii* ATCC 74012, respectively, with established probiotic functionality. All culture media (de Man, Rogosa and Sharpe (MRS) agar, Yeast Extract Peptone Dextrose (YPD) Agar, Pepton water) anaerobic gas pack A and calcium chloride were provided from Merck Company (Merck, Dramstadt, Germany). Sodium alginate, Tween 80 and Starch were provided by Sigma-Aldrich Company (Sigma-Aldrich, St. Louis, MO, USA). Heated Doogh samples were gathered from a dairy plant located in Ghazvin province, Iran.

2.2 Samples Preparation

In this study, microbial and physical analyses of 120 heated Doogh samples were conducted for 28 days at 7-day intervals (0, 7, 14, 21, 28 days) at three temperatures (4, 20, 35 °C). Prepared treatments were Doogh samples containing free and encapsulated probiotic *L. acidophilus*, free and encapsulated probiotic *Saccharomycess boulardii*, plain Doogh samples without probiotic bacteria, and Doogh samples containing free forms of both probiotics, and Doogh samples with encapsulated forms of both probiotics.

2.3 Stock culture preparation and growth conditions of L. acidophilus and S.boulardii

First, 0.5 ml of Brain Heart Infusion (BHI) broth (Merck, Dramstadt, Germany) was added to a vial and incubated at 37 ºC to activate the bacteria, according to the Pasteur institute guideline. „ After 0.5 h, all vials were inoculated to (de Man, Rogosa, and Sharpe) MRS broth medium (Merck, Dramstadt, Germany) and incubated in anaerobic condition at 35 ºC. After 3-5 days, the cell culture was centrifuged (3000 rpm) for 10 min at 25 ºC. The supernatant was discarded, and the harvested bacterial cells were washed twice with phosphate buffer saline (Oxoid Ltd, Basingstoke, UK)[15]. According to the guideline of ATCC , 1 ml of a culture medium containing yeast extract (0.1%), Glucose (5%), and K2HPO4 (1%) with pH=5.8 was added to a stock vial and incubated at 28 °C for 72 h in a shaker incubator at 130 rpm speed rate to activate the probiotic yeast. After yeasts' growth, it was centrifuged at a 3000 rpm speed rate. Yeast biomass was washed twice with sterile normal saline. After that, a dilution containing 10^{12} CFU/ml of probiotics was prepared. All prepared stock cultures were directly subjected to the stress factors such as low pH and bile salts to demonstrate the tolerance and viability of probiotics used in

this study under simulated gastrointestinal conditions according to the described methods by Hassanzadazar et al. (2012) [16].

2.4 Encapsulation of probiotic L.acidophilus and S.boulardii

Micro-encapsulation of probiotics was done according to the described method by Khosravi et al. (2013) using the emulsion method under sterile conditions with some modification [14]. At first, a mixture of 3 g alginate sodium and 2 g of heat-resistant starch were dissolved slowly in 100 ml of distilled water and sterilized in an autoclave. After cooling, the alginate solution was mixed with microbial suspension (0.1%) for 5 min. A homogeneous emulsion was added to 500 ml corn oil containing 0.2 % tween 80 and mixed for 30 min using a magnetic stirrer (500 rpm). To form capsules, calcium chloride 0.1 mol was added to the mixture. After 30 min, the mixture was centrifuged at 3500 rpm for 10 min to isolate precipitated capsules. The supernatant was discarded, and remained mass was washed with 0.1% phosphate buffer saline and stored at 4 °C until use [14]. Agitation of the mixture continued for 6 h at determined temperatures (4, 20, 35 °C). To confirm encapsulation of the probiotic, gram stain of the mixture without direct fixation was provided and observed microscopically. The main point was that calcium chloride should be added to the solution during mixing and precipitating for 30 min, increasing trapping and obtaining the appropriate concentration of probiotic bacteria.

2.5 Probiotic encapsulation efficiency (EE)

The encapsulation efficiency was determined based on the enumeration of viable cells in the solutions before and after the microencapsulation of the probiotics. The efficiency rate (percentage) of the encapsulation was determined using the following equation: $EE\ (\%) = Xt/Xi \times 100$ [9] . Xt is the enumeration of cells in the microcapsules, and Xi shows the primary count of cells initially added to the alginate solutions [9].

2.6 Preparation Doogh samples containing free and encapsulated probiotics

In a sterile condition, prepared stock solutions of each free and encapsulated form of probiotics were incorporated into Doogh samples (15 % V/V) at determined temperatures (4, 20, 35 °C) separately after the pasteurization step of Doogh samples to protect any harmful effect of temperature on probiotic cells. It should be considered that the prepared samples (the mixture of Doogh and probiotic) must be agitated for 72 h for free containing and at least 6 h for encapsulated containing samples at 28-30 °C to prevent clumping and well growth of probiotic bacteria, otherwise the main population of probiotic decline due to the competition to acquire nutrients between the cells [17]. To prepare Doogh samples containing combined forms of free and encapsulated strains, equal volumes of prepared stocks were mixed and then added to Doogh at a 15 % rate (V/V).

2.7 Enumeration of free and encapsulated probiotic L. acidophilus and S.boulardii

Free *L.acidophilus* and *S.boulardii* count were enumerated according to the described method by Krasaekoopts et al. (2003) [18]. To determine the population of viable free probiotics in Doogh samples, 10 ml of each Doogh sample was added to 90 ml of sterile trisodium citrate solution 2% in a sterile stomacher bag and homogenized for 2 min using a lab 400 stomacher (Interscience, France). A decimal serial dilution was prepared, and 1 ml of each tube containing L. acidophilus was pour plated on MRS agar (Merck, Dramstadt, Germany). Following that, YPD agar (Merck, Dramstadt, Germany) incubated in an anaerobic jar at 37 °C for 72 h, and 1 ml of each tube containing *S.boulardii* was pour plated on YPD (YEPD) agar and incubated at 28-30 °C for 72 h [19]. The enumeration of encapsulated probiotics was conducted after incubating the mixture bags (containing supplemented Doogh with encapsulated probiotics and trisodium citrate solution 2%) for 10 min at ambient temperature and before homogenization with stomacher to dissolve microcapsules and release the probiotic cells [14].

2.8 PH measurement

The pH of the Doogh samples was measured using a digital pH meter (Swiss, Metrohm 691) according to Iran National Standard NO.2852. pH meter calibration was done using standard buffer solutions with pH 4.0 and 7.0 [20].

2.9 Evaluation of Doogh *Stability*

Doogh is a diluted beverage of yogurt with water. Therefore, its stability as a physical characteristic can be influenced by the primary composition during the storage time. To determine the stability of Doogh, 10 ml of Doogh was poured into 15 ml sterilized tubes and then tested at different temperatures during storage time intervals. The stability was determined in percent based on the following equation [21]. Doogh stability % = (Initial volume of Doogh – Supernatant Serum Volume) / (Initial Doogh Volume) × 100

2.10 Sensory evaluation

The sensory attributes of the Doogh samples were evaluated using a questionnaire including flavor, taste, color, texture, and overall acceptability by 10 trained panelists from the department of microbiology of the School of medicine in Zanjan University of microbiology medical science, Zanjan, Iran. Blind-labeled samples were served in cups. Questions were asked based on a 5-point hedonic scale ranging from 1 (Dislike extremely), 2 (Like slightly), 3 (Acceptable), 4 (Good), and 5 (Like extremely) during the experimental interval days [22].

2.11 Statistical analysis

All tests were performed in triplicate. The results were compared to determine the viability of probiotic strains using one-way ANOVA test followed by the Tukey test with

95% confidence level by SPSS software Ver. 20 (SPSS Inc, IBM, Chicago, USA).

3. Results and Discussion

3.1 Viability of probiotic bacteria in Doogh

Evaluation of encapsulation efficacy (EE) showed that alginate capsules containing *L.acidophilus* had a higher EE percentage (95.7±1.2 %) than the *S.boulardii* containing capsules (94.6± 2.5 %). The population of the viable free and microencapsulated probiotic L. acidophilus and *S.boulardii* (log cfu⁄ml) in heated Doogh samples over prolonged storage at different temperatures are shown in Table 1. As is shown in Table 1, the numbers of free and encapsulated probiotics decreased continuously at all temperatures (4, 20, and 35 °C) on the last day compared to the first day of storage except for encapsulated *L.acidophilus* at 4 and 20 °C and free forms of *S.boulardii* at 4 °C that remained constant significantly ($P < 0.05$). Several studies have reported a higher survival rate of encapsulated cells than free forms of probiotics [18,21,23-25]. In our study, this viability is a strain and temperature-dependent character. Encapsulation increased the viability of free *L.acidophilus* 3 log cycles at 4 °C, but the survival of *S.boulardii* in encapsulated form decreased significantly near 6 log cycles at 4 °C. The most important factors influencing the survival of probiotics are the type of probiotics, percentage of inoculation, using glass containers instead of plastic types during storage, competing microorganisms, adding prebiotics, solids content and nutrient availability in food products, incubation and storage temperature, fermentation time, pH and chemical composition and buffer properties, presence of inhibitory and microbial agents such as bacteriocin or hydrogen peroxide in food product and encapsulation [26]. The possible reason for the decrease in the number of encapsulated probiotics at 35 ºC can be related to the effect of temperature on the persistence of probiotics and the inability of probiotics to compete with other competing microbes. The average numbers of free and encapsulated probiotics in combined forms were constant at all temperatures (4, 20, and 35 °C) during the prolonged storage period ($P < 0.05$) (Table 2). The synergistic effect between free and encapsulated forms of probiotics was shown better at 20 and 35 °C temperatures. It could be due to the biological assistance of Saccharomyces yeast and *L.acidophilus* through Proteolysis, β-galactosidase activity, pH increase, molecular oxygen removal, and carbon dioxide production. As a result, co-existing of *S.boulardii* and *L.acidophilus* improved and stimulated the growth of each other. It is consistent with the results of Moktari et al. (2017) that Bifidobacterium bifidum and *L.acidophilus* facilitate each other in grape juice [27]. The encapsulation of probiotics used to cover and enclose them with a layer of microscopic hydrocholloid to separate them from the environment, resulting in a targeted release that involves them at the right place and time. It makes them resistant to pH changes, mechanical stresses, temperature changes, severe freezing or freezing drying, enzymatic activity, bile compounds and molecular oxygen, osmotic pressure, antimicrobial compounds, and slow release of moisture through the capsule, providing the necessary nutrients (If oligosaccharides are used in encapsulation) and prevents cell damage or loss. Calcium alginate blending with high corn starch not only creates uniform capsules or coatings but also increases the survival of both probiotics because of the mentioned characteristics and prebiotic properties of this coating [28, 29].

3.2 Changes in pH

The results showed that the treatments containing free *S.boulardii* had the least significant decrease in pH on the last day of storage compared to the first day at 20 and 35 ºC temperatures (Table 3). This condition was also seen in the Doogh samples containing encapsulated *S.boulardii* and the combination of the free form of both probiotics at 4 ºC. The significant effect of encapsulation on pH was seen in treatment containing encapsulated *L.acidophilus* than its free form at 20 and 35 ºC temperatures. A significant decrease in pH was seen in the treatment containing free form of *L.acidophilus* and *S.boullardii* compared to the treatment containing just free *L.acidophilus* on the last day at all storage temperatures (Table 3). These findings are in line with reported results by Amineslami et al. (2014) and Khosravi Zanjani et al. (2013) [14,28]. As a result of producing organic acids and other metabolites, the pH of the food product decreases during the storage period. Free forms of L. acidophilus are sensitive to pH changes, and the growth of this probiotic is significantly reduced or stopped at $pH < 4$ (Table 3). However, encapsulation of probiotics results in their less acidic activity, and therefore the pH of these samples is higher than treatments containing free forms of probiotics [28]. One way to deal with the problem of pH reduction and thus reducing the survival of probiotics in the product is to combine probiotics and their synergistic cooperation. The present study used the *S.boulardii* and the *L.acidophilus* freely and encapsulated in the respective treatments. As a result, probiotic yeast reduces the acidity of the product by consuming organic acids, increasing the pH of the product and the viability of probiotic bacteria during the storage period [29].

3.3 Stability (Phase separation changes)

The average rates of texture stability in Doogh treatments at different temperatures (4, 20, 35 °C) during refrigerated storage (28 days) are demonstrated in Table 4. The highest tissue stability was seen in the treatment containing free *L.acidophilus* and the combination of encapsulated *L.acidophilus* and *S.boulardii* than the other treatments, respectively. A significant effect of encapsulation on tissue stability and reduction of phase separation was observed in the treatment containing encapsulated *S.boulardii* and treatments including the combination of encapsulated L .

Table1: The mean±standard deviation of free and encapsulated *Lactobacillus acidophilus* and *Saccharomyces boulardii* in heated *Doogh* (log cfu/ml) during 28 days storage period at 4, 20, and 35 °C) per 7 days interval

Temp (°C)	Time (Day)	Free L. acidophilus	Encapsuled L. acidophilus	Free S. boulardii	Encapsuled S. boulardii
4	0	11.8 ±0.1^{Aa}	11±0.07^{Aa}	12± 0.67^{Ca}	**11.6±0.16^{Ca}**
	7	8.1±0.16^{Ba}	11± 0.67^{Ab}	11.9±0.06^{Ca}	**7± 0.39^{Db}**
	14	7.7±0.0^{Ba}	10.9±0.00^{Ab}	11.1±0.72^{Ca}	**6± 0.19^{Db}**
	21	7.4±0.13^{Ba}	10.8±0.08^{Ab}	10.7±0.07^{Ca}	**5.9± 0.02^{Db}**
	28	7.1±0.22^{Ba}	10.7±0.1^{Ab}	10.3±0.21^{Ca}	**5.7± 0.03^{Db}**
20	0	11.7±0.1^{Aa}	11.2±0.42^{Aa}	12.1±0.2^{Ca}	**11.5±0.17^{Ca}**
	7	10.9±0.05^{Bb}	11.1±0.72^{Aa}	9.3±0.19^{Da}	**10.6±0.14^{Ca}**
	14	10.6±0.14^{Ba}	11±0.04^{Aa}	9.1±0.29^{Da}	**9.7±0.08^{Da}**
	21	9.9±0.04^{Ba}	10.9±0.04^{Ab}	9.3±0.21^{Da}	**9.5±0.1^{Da}**
	28	9.5±0.11^{Ba}	10.8±0.07^{Ab}	9.2±0.22^{Da}	**9.4±0.18^{Da}**
35	0	11.1±0.72^{Aa}	11.6±0.13^{Aa}	11.5±0.2^{Ca}	**11±0.67^{Ca}**
	7	10.7±0.1^{Aa}	9.6±0.1^{Ba}	7.2±0.12^{Da}	**10±0.49^{Cb}**
	14	10±0.04^{Ba}	9.2±0.22^{Ba}	7±0.24^{Da}	**9.1±0.36^{Db}**
	21	9.2±0.31^{Ba}	9.1±0.74^{Ba}	6.2±0.11^{Da}	**8.8±0.04^{Db}**
	28	8.7±0.04^{Ba}	9±0.49^{Ba}	6±0.19^{Da}	**8±0.19^{Db}**

* The non-similar uppercase letters in each column show a significant difference in a treatment at the same temperature during storage days (P <0.05). The non-similar lower case letters in each row indicate a significant difference between treatments on the same day at mentioned temperatures (P <0.05).

Table 2: The mean±standard deviation of free and encapsulated *Lactobacillus acidophilus* and *Saccharomyces boulardii* in the treatments containing both probiotics (log cfu/ml) during 28 days of storage at 4, 20and 35 °C.

Independent variables		Different treatments in 28 days			
Temp (°C)	**Time (Day)**	**Containing free L. acidophilus & S. boulardii**		**Containing encasulated L. acidophilus& S.boulardii**	
		L. acidophilus	S. boulardii	L.acidophilus	**S. boulardii**
4	0	12.5. ±.017 ^{Aa}	12.5±.024^{a}	12.5±.02^{Aa}	12.3±0.31^{Aa}
	7	11.5±.019^{Aa}	12±.067^{Aa}	11.7±.009^{Aa}	12.1 ±.056^{Aa}
	14	10.7±.008^{Aa}	11.6±.012^{Aa}	11±.067^{Aa}	11.9±.006^{Aa}
	21	10±.052^{Ba}	10.2±.045^{Aa}	10.9±.008^{Aa}	11.4±.021^{Aa}
	28	9.9±.004^{Ba}	10±.067^{Aa}	10.9±.007^{Aa}	11±.007^{Aa}
20	0	12.1±0.72^{Aa}	12.5±0.19^{Aa}	12±0.67^{Aa}	12.4±0.32^{Aa}
	7	11.3±0.24^{Aa}	12.1±0.72^{Aa}	11.6±0.11^{Aa}	12.1±0.48^{Aa}
	14	10.8±0.08^{Aa}	11.7±0.11^{Aa}	11.3±0.36^{Aa}	11.9±0.06^{Aa}
	21	10.2±0.34^{Aa}	10.3±0.29^{Aa}	10.8±0.06^{Aa}	11.5±0.16^{Aa}
	28	10±0.04^{Aa}	10.1±0.76^{Aa}	10.8±0.07^{Aa}	11.3±0.27^{Aa}
35	0	12.1±0.76^{Aa}	12.1±0.76^{Aa}	12.1±0.72^{Aa}	12±0.67^{Aa}
	7	10.9±0.07^{Aa}	11.2±0.37^{Aa}	10.9±0.06^{Ba}	11.2±0.78^{Aa}
	14	9.5±0.13^{Ba}	10.2±0.26^{Aa}	10.7±0.11^{Ba}	10±0.67^{Aa}
	21	9.3±0.19^{Ba}	10±0.49^{Ba}	10.1±0.72^{Ba}	9.9±0.05^{Aa}
	28	9±0.49^{Ba}	10±0.04^{Ba}	10.1±0.72^{Ba}	9.7±0.08^{Aa}

*The data values are the average ± standard deviation. The large non-similar letters in each column and at different temperatures and lower case letters in each row indicate a significant difference at the 5% level (P <0.05).

Table 3: pH changes at different temperatures (4, 20, 35 °C) during refrigerated storage (28 days)

Independent variables		pH in different treatments (Mean±SD)						
Temp (°C)	Time (Day)	Free La	Encapsuled La	Free Sb	Encapsuled Sb	Free La&Sb	Encapsuled La&Sb	Control
4	0	3.75±0.04^{Aa}	3.75±0.03^{Aa}	3.75±0.05^{Aa}	3.76±0.03^{Aa}	3.74±0.04^{Aa}	3.75±0.03^{Aa}	**3.76±0.01^{Aa}**
	7	3.66±0.03^{Aa}	3.74±.004^{Aab}	3.7±0.04^{Aab}	3.67±0.03^{Ba}	3.7±0.03^{Aab}	3.71±0.03^{ABab}	**3.7±0.02^{Bab}**
	14	3.68±0.03^{Aa}	3.71±.004^{Aa}	3.7±0.03^{Aa}	3.69±0.04^{ABa}	3.71±0.03^{Aa}	3.72±0.03^{Aa}	**3.74±.001^{Aa}**
	21	3.68±0.04^{Aabc}	3.69±0.03^{Aabc}	3.72±0.03^{Abc}	3.75±0.03^{ABc}	3.73±0.03^{Abc}	3.63±0.03^{BCa}	**3.66±0.01^{Cab}**
	28	3.66±0.03^{Abcd}	3.66±0.04^{Abcd}	3.71±0.04^{Acde}	3.77±0.03^{Ae}	3.74±0.04^{Ade}	3.6±0.04^{Cb}	**3.57±0.01^{Da}**
20	0	3.75±0.02^{Aa}	3.76±0.03^{Aa}	3.76±0.04^{Aa}	3.74±0.04^{Aa}	3.75±0.03^{Aa}	3.76±0.04^{Aa}	**3.77±0.02^{Aa}**
	7	3.65±0.04^{Ba}	3.74±0.04^{Ab}	3.72±0.03^{Aab}	3.73±0.03^{Aab}	3.71±0.03^{Aab}	3.65±0.03^{Ba}	**3.73±0.01^{Bab}**
	14	3.62±0.04^{BCa}	3.73±0.03^{Ab}	3.72±0.04^{Ab}	3.7±0.04^{Abc}	3.68±0.04^{Aab}	3.7±0.04^{ABab}	**3.72±0.01^{Bb}**
	21	3.56±0.03^{CDa}	3.74±0.04^{Ac}	3.75±0.03^{Ac}	3.71±0.03^{Abc}	3.69±0.03^{Abc}	3.69±0.04^{ABbc}	**3.63±0^{Cab}**
	28	3.53±0.03^{Da}	3.73±0.04^{Ab}	3.76±0.04^{Ab}	3.72±0.03^{Ab}	3.69±0.04^{Ab}	3.67±0.04^{ABb}	**3.53±0.02^{Da}**
35	0	3.74±0.04^{Aa}	3.77±0.03^{Aa}	3.75±0.04^{Aa}	3.77±0.04^{Aa}	3.76±0.05^{Aa}	3.76±0.04^{Aa}	**3.77±0.01^{Aa}**
	7	3.19±0.04^{Ba}	3.26±0.04^{Bab}	3.76±0.03^{Ac}	3.27±0.03^{Bab}	3.25±0.04^{Bab}	3.3±0.04^{Bb}	**3.72±0.01^{Bc}**
	14	3.15±0.03^{Ba}	3.25±0.02^{Cab}	3.82±0.02^{ABd}	3.24±0.03^{Bb}	3.18±0.04^{Bab}	3.2±0.03^{Cab}	**3.71±0.01^{Bc}**
	21	3.17±0.04^{Ba}	3.22±0.03^{BCab}	3.86±0.03^{Be}	3.26±0.03^{Bbc}	3.23±0.03^{Bab}	3.31±0.03^{Bc}	**3.61±0.01^{Cd}**
	28	3.15±0.03^{Ba}	3.24±0.03^{BCb}	3.89±0.03^{Bd}	3.26±0.04^{Bb}	3.24±0.04^{Bb}	3.32±0.04^{Bb}	**2.52±0.02^{Dc}**

*The non-similar lowercase letters in each row show a significant difference between treatments -during storage days (P <0.05). The non-similar uppercase letters in each column show a significant difference in treatment during storage days (P <0.05). **La.** *Lactobacillus acidophilus*; **Sb.** *Saccharomyces boulardii*; **La&Sb.** Mixture of *Lactobacillus acidophilus* and *Saccharomyces boulardii*.

Table 4: Stability rate of *Doogh* treatments at different temperatures (4, 20, 35 °C) during refrigerated storage (28 days)

Independent variables		Stability rate (%) in different treatments (Mean±SD)						
Temp (°C)	Time (Day)	Free La	Encapsuled La	Free Sb	Encapsuled Sb	Free L.a&S.b	Encapsuled L.a&S.b	Control
4	0	78.4±2.2^{Ac}	60.66±2.33^{ABb}	48±1.9^{Aa}	63.1±2.1^{Ab}	63.2±2.2^{Ab}	74±1.9^{Ac}	**60±1.4^{Ab}**
	7	70±2.1^{Bd}	54.5±2.4^{Cc}	36.9±2.8^{Ba}	42.2±2.3^{Aab}	37±2.5^{Ba}	54.5±2.4^{Bc}	**45.8±1.1^{Bb}**
	14	69±2.3^{Bc}	63.6±1.7^{Ac}	40.9±2.5^{Ba}	57.1±2.5^{Bb}	40.8±2.6^{Ba}	68.6±2.3^{Ac}	**38.6±1.3^{Ca}**
	21	62.1±2.1^{Cd}	56±2.4^{BCc}	37.5±2.3^{Ba}	44.4±2.3^{Bb}	37.7±2.4^{Ba}	57.6±2.2^{Bd}	**37.5±1.1^{Ca}**
	28	57.1±1.9^{Cd}	54±2.1^{Cd}	36±2.2^{Bb}	43.5±2.2^{Bc}	36±2.7^{Bb}	56.5±2^{Bd}	**36.5±1.3^{Cb}**
20	0	69.2±2.2^{Ad}	50±2.3^{Ab}	42.1±2.1^{Aa}	53.5±2.5^{Ab}	55.6±2.3^{Abc}	59.6±1.9^{Ac}	**53.8±1.3^{Ab}**
	7	62.7±2.2^{Bd}	40.8±2.2^{Bbc}	36.5±2.2^{ABab}	40.4±2^{Cbc}	34.6±2.2^{Ba}	43.4±2.3^{Bc}	**40±1.6^{Babc}**
	14	63.6±2.3^{ABf}	40.1±2.5^{Bc}	32.6±2.4^{Bab}	48.7±2.3^{ABd}	39.2±2.2^{Bc}	57.5±2.3^{Ae}	**31.1±1.2^{Ca}**
	21	68.4±2.4^{ABd}	35.7±2.3^{BCa}	33.3±2.3^{Ba}	46.8±2.4^{Bb}	37.2±2.3^{Ba}	57.1±2.4^{Ac}	**32.5±1.3^{Ca}**
	28	68.8±2.2^{Ad}	31.5±2.4^{Ca}	33±2.8^{Ba}	46±2.1^{Bcb}	36.5±2.4^{Ba}	56.8±2.6^{Ac}	**31±1^{Ca}**
35	0	74±2.6^{Ad}	44.4±2.2^{Aa}	56±2.1^{Ab}	59.2±2.3^{Abc}	65±2.4^{Ac}	72.7±2.1^{Ad}	**45±1.1^{Ba}**
	7	64.5±2.5^{Bf}	48.8±2.3^{Acd}	38.7±2.3^{Ba}	54.3±2.2^{ABde}	42.5±2.3^{Bab}	58.5±2.5^{Ce}	**52.1±1.1^{Acd}**
	14	43.1±2^{Cab}	44.1±2.1^{Ab}	37.7±2.5^{BCa}	54±2.2^{ABc}	42.5±2.2^{Bab}	61.1±2.1^{BCd}	**41.8±1.2^{Bab}**
	21	78.9±2.5^{Ae}	46.1±2.5^{Abc}	32.4±2.5^{CDa}	50±2.6^{Bc}	36.3±2.1^{BCa}	66.6±2.6^{ABd}	**50±1.6^{Ac}**
	28	78±2.3^{Ad}	45±2^{Ab}	30±1.9^{Da}	49±2.3^{Bb}	35±2.7^{Ca}	67±2.1^{ABc}	**45±1.4^{Bb}**

*The non-similar lowercase letters in each row show a significant difference between treatments during storage days (P<0.05). The non-similar Uppercase letters in each column show a significant difference in treatment during storage days (P <0.05). La. *Lactobacillus acidophilus*; Sb. *Saccharomyces boulardii*; La&Sb. The mixture of *Lactobacillus acidophilus* and *Saccharomyces boulardii*

acidophilus and *S.boulardii* at all temperatures compared to their free form. While significant synergistic effects were seen on the last day in the treatment of combined encapsulated *L.acidophilus* and *S.boulardii* compared to the treatments containing just encapsulated *S.boulardii* at all temperatures and encapsulated *L.acidophilus* at 20 and 35 °C. Taheri et al. (2009) reported non-significant effects of probiotic *L.acidophilus* on phase separation at 4 °C, which is inconsistent with our results [30]. Accumulation and deposition of casein particles, acidity rate, pasteurization, storage conditions, the effect of mechanical factors such as homogenization, percentage of dry matter and fat content, primary raw material, starter type and using stabilizers and emulsifiers can be effective factors on separation phase and Doogh stability [22]. Adding probiotics is one of the ways to increase stability and prevent serum phase separation and the absorption of proteins in fermented dairy drinks. Amineslami et al. (2014) reported that producing polysaccharides by probiotics can improve the viscosity and texture stability of the food product. Exopolysaccharides produced by lactic acid bacteria strengthen the casein network. Sodium alginate that used in this study to encapsulate probiotics can increase the stability of the product due to the replacement of sodium ions in the alginate gel with calcium ions. Swelling the capsules can also help increase the product′s viscosity [17, 31].

3.4 Sensory attributes

The overall acceptability of sensory evaluation in the heated Doogh samples treated with probiotics is shown in Figure 1. There was a sharp decline in taste and odor of all treated samples except for the control sample and the treatments containing free *L.acidophilus* that had the higher score and overall acceptance. The treatments containing free *L.acidophilus* and a combination of encapsulated *L.acidophilus* and *S.boulardii* also had the best tissue texture. There was a little sandy and roughness sense in the taste of the treatments containing encapsulated probiotics. In addition, in treatments containing probiotics, especially encapsulated treatments, the creamy color has been reported by evaluators in the last days of storage. According to the results, the treatments containing free forms of probiotics had better taste compared to other treatments, consistent with the results reported by Pourjafar et al. 2020[4]. The higher scores in overall acceptance are related to treatments stored at 4 ºC, which is consistent with Taheri et al. (2009) [30]. They confirmed the positive effects of cold storage on the acceptance of Doogh samples containing *L. acidophilus* stored at 4 ºC. Our results revealed a little sandy taste in the treatments containing encapsulated probiotics which is inconsistent with the results reported by Amineslami et al. (2014) [28]. Probiotics containing fermented food products have a weak aroma and taste. Improving the flavor quality of such probiotic dairy products controls the aromatic and flavor defects by adjusting the initial raw ingredients. For example, adding threonine to milk fortified with whey protein increases the acetaldehyde production by *L.acidophilus*, which results in better and more desirable products[23]. However, the best way to enhance and improve the aroma of L.acidophilic-containing products is using a subsidiary microbial culture such as yogurt starter culture to enhance the aroma and flavor by producing effective compounds. Co-administration *L.acidophilic* with *S.boulardii* decreases glucose and fructose concentrations and, as a result, increases alcohol and gas production that creates an unpleasant taste. However, it increases the bioavailability of probiotics. Another defect probably seen in probiotics containing fermented products is the possibility of forming a relatively high amount of Co_2 gas, which depends on the fermentation path chosen by the probiotic microorganism. Free *L.acidophilus* is one of the homophermentative probiotics that produce small amounts of Co_2 gas, unlike *S.boulardii* [14]. The sour taste in the treatments can be due to the conversion of simple sugars into acidic metabolites such as lactic acid. Although encapsulation often improves the product′s sensory properties and stabilizes it, especially during storage, the probiotics can produce small peptides that cause bitterness in the product. The unwanted sandy taste is due to the granules′ diameter increase and thus thickening the capsule larger than one millimeter[14, 23]. The color change in encapsulated probiotic-containing treatments could be due to the production and accumulation of various metabolites in the product, which intensified at 35 ºC.

4. Conclusion

According to the findings, the encapsulation technique improves the survival and viability of probiotic strains of *L.acidophilus* and *S.boulardii* compared to their free forms. Particularly, *L.acidophilus* stabilized pH and prevented its decrease, which in turn caused the increase in the survival of probiotics. In addition, the significant effect of encapsulation on tissue stability was seen. Using *S.boulardii* in Doogh stabilized and even increased the pH of the product, which can provide conditions for the survival of *L.acidophilus*.

Also, the concomitant use of probiotic strains of *L.acidophilus* and *S.boulardii* in free and encapsulated treatments stabilized pH and improved the tissue stability of Doogh. The sensory evaluation scores were higher in the treatments containing free forms of *L.acidophilus* stored at 4 °C. Other treatments showed some degree of odor and discoloration, particularly on the last days of the storage time and at the higher temperatures. In general, it can be concluded that the combination of probiotics can be applicable in Doogh as a suitable carrier and shows synergistic effects on the shelflife of this dairy product.

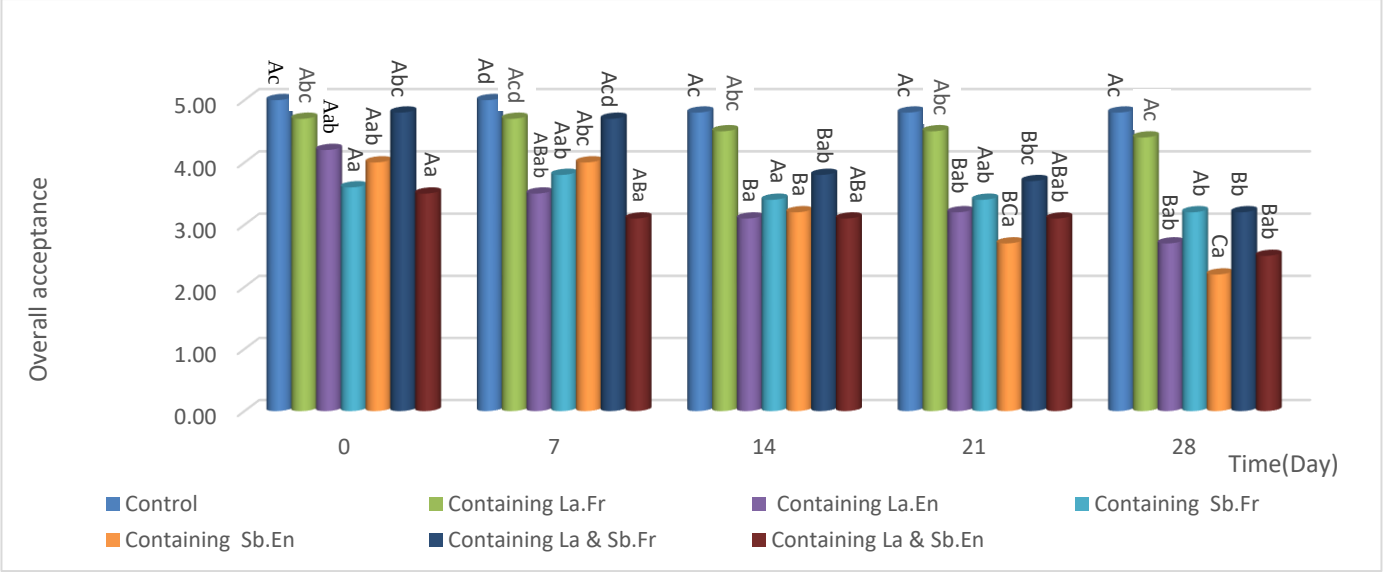

Figure 1: Sensory evaluation and overall acceptability of Heated Doogh treated with free and encapsulated probiotics
*La. Fr: Free *Lactobacillus acidophilus*; Sb. Fr: Free *Saccharomyces boulardii*; La&Sb. Fr: Free mixture of *Lactobacillus acidophilus* and *Saccharomyces boulardii*; La. En: Encapsulated *Lactobacillus acidophilus*; Sb. En: Encapsulated *Saccharomyces boulardii*; La&Sb. En: Encapsulated mixture of *Lactobacillus acidophilus* and *Saccharomyces boulardii*.

Authors' Contributions

Hamidreza Halimi :Conceptualization; investigation; resources; data curation; writing–original draft preparation; writing–review and editing; project administration. Habib Zighami :Conceptualization; visualization; supervision. Majid Aminzare :writing–review and editing. Hassan Hassanzadazar :methodology; formal analysis; resources; writing–original draft preparation; writing–review and editing; supervision; visualization .

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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