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Survival of free and microencapsulated *Lactobacillus acidophilus* and *Bifidobacterium lactis* in Doogh (a yogurt drink)

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ABSTRACT

The present study was investigated the survival of probiotic strains, namely *Lactobacillus acidophilus* (LAFTI-L10) and *Bifidobacterium lactis* (LAFTI-B94), in both microencapsulated and free forms in Doogh as a yogurt drink. The effects of probiotic strains on physicochemical and sensory properties, stability, and microstructures of Doogh were evaluated during storage for 45 days in a cold room at a temperature of 5±1 °C. The statistical analysis of the results showed that the number of *L. acidophilus* and *B. lactis* in free forms decreased about 1.1 and 2.2 log cycles, respectively. However, the number of the microencapsulated forms were remained relatively constant during the storage period. Both free and microencapsulated forms were successful in keeping the counts of *L. acidophilus* and *B. lactis* in Doogh high enough for the therapeutic minimum (10^6-10^7 $cfug^{-1}$) after 45 days. Nonetheless, the number of microencapsulated probiotics was higher than that of the free cells. The acidity, pH, and stability were significantly different between the samples. The microstructure of the samples was affected by the microencapsulation of bacteria because the sample distribution of the clusters and particles was more regular in the samples containing microencapsulated probiotics than in those containing free forms. According to the results of sensory evaluations, probiotics not only exerted no adverse effects on the physicochemical and sensory properties of Doogh but also improved the stability, microstructure, and flavor of this drink. Therefore, Doogh can be considered an effective food carrier for the delivery of probiotic organisms.

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Introduction

Doogh is an Iranian traditional fermented acidic dairy drink (1). This product that is usually consumed along with food has become a popular drink in Iran owing to its pleasurable sensory properties and healthy characteristics. Similar products of this drink are available with different names in various countries, such as Ayran in Turkey and Laban in Arabic countries (2). Doogh has high acceptability and demand in east European countries (3). Incorporation of probiotic bacteria into Doogh could greatly facilitate the improvement of the health characteristics of this product. Probiotics are live microorganisms that settle down in the colon after consumption and have beneficial effects on human health (4). *Lactobacillus acidophilus* and *Bifidobacterium lactis* are the most important probiotic bacteria that are used in food products (5). The results of some recent investigations on probiotic products have shown that probiotic organisms cannot resist in fermented dairy products, and also in gastrointestinal conditions (6).

Microencapsulation is a novel method through which a target compound is covered by a thin layer of polymeric material (7). In this technique, a variety of functional agents, including flavors, essential oils, enzymes, and microorganisms, are the most considered target substances. The coating material could be a protein, carbohydrate, or lipid. Microencapsulation technique has been investigated for enhancing the viability of probiotic microorganisms in both dairy products and the intestinal tract (8). There is limited literature regarding the application of microencapsulated probiotics in Doogh. In a study, probiotic and synbiotic Doogh (supplemented with free or encapsulated *L. plantarum* LS5 and *Helianthus tuberosus* inulin) was effectively produced (9). In the same vein, the present study investigated the microencapsulation of *L. acidophilus* and *B. lactis* with sodium alginate according to the extrusion method for incorporation into Doogh.

Mathews (7) showed that the survival of microencapsulated probiotics (i.e., *L. acidophilus* and *L. casei*) significantly increased in a simulated gastrointestinal condition. In addition, he indicated that the microencapsulation of prebiotics with alginate-gelatin could successfully and significantly protect *L. acidophilus* and *L. casei* against the adverse condition of the simulated human gastrointestinal condition. Shu *et al.* (10) prepared the microencapsulations of *L. acidophilus* in xanthan-chitosan and xanthan-chitosan-xanthan polyelectrolyte complex gels. Their results

showed that the application of the encapsulation system in yogurt led to great success in bacterial survival during the 21 days of storage at 4°C. Furthermore, in the mentioned study, the pH and acidity in yogurt were significantly influenced by the encapsulation system in comparison to free suspension during the storage. Mahdavi et al. (11) showed that in cocktail juice, the number of *L. casei* significantly decreased during the storage. In addition, they reported that the use of probiotics had no significant effect on the color and flavor of cocktail juice.

Mortazavian *et al.* (12) studied the effect of the microencapsulation of *L. acidophilus* LA-5 and *B. lactis* BB-12 by means of the emulsion method on cell survival over 42 days of refrigerated storage in Iranian yogurt drink (i.e., Doogh). They showed that the initial pH of product at the onset of storage was 4.52. Furthermore, the final pH values at the end of the storage time were 4.51 and 3.77 for the samples containing microencapsulated and free cells, respectively. At the end of the storage period, the viable counts of *L. acidophilus* and *B. lactis* in the samples containing microencapsulated cells were about 5.5 and 4.0 log cycles higher than in those containing free cells, respectively. In another study, Mortazavian *et al.* (13) showed that the viability of free probiotic cells respectively increased from 0.6% and 0.2% (for *L. acidophilus* and *B. lactis*, respectively) to 18.0% and 9.5% at reinforced gastrointestinal conditions after microencapsulation. At real-alleviated conditions, the cell survival rates were 16.1% and 21% for *L. acidophilus* and *B. lactis,* respectively, before microencapsulation. These amounts improved to 26.3% and 34.0% for *L. acidophilus* and *B. lactis*, respectively, after microencapsulation.

With this background in mind, the present study aimed to investigate the survival of *L. acidophilus* (LAFTI-L10) and *B. lactis* (LAFTI-B94) in both microencapsulated and free forms and their effects on physicochemical, sensory properties, and microstructures of Doogh under a refrigerated condition.

Materials and methods

For the purpose of the study, yogurt starter cultures YC-X11 (e.g., *Streptococcus thermophiles* and *L. delbrueckii* subsp. *bulgaricus*, Chr. Hansen, Denmark), *L. acidophilus* (LAFTI-L10), and *B. lactis* (LAFTI-B94; DSM, Australia), culture media de Man, Rogosa and Sharpe (MRS) agar (Scharlau, Spain), M17 and reconstituted Clostriadial agar (RCA, Liofilchem, Italy) and Sorbitol (Qulab, England), sodium alginate (Sigma-Aldrich Co., NSW, Australia), anaerobic gas pack, calcium chloride, and Rhodamine-B dye (Merck, Germany) were obtained.

Microencapsulation of probiotics

The extrusion technique was used to microencapsulate the bacteria (4). To this end, the cultures were suspended in 5 mL of sterile 0.1% peptone solution, mixed with 20 mL of 2% (w/v) sodium alginate solution and then sterilized at 12 °C for 15 min. The cell suspension was injected through a 0.11-mm needle into sterile 0.05 M $CaCl_2$. The beads were allowed to stand for 30 min for gelification, and then rinsed with sterile 0.1% peptone solution and kept in the same solution at 4°C until examined.

Preparation of probiotic Doogh

Yogurt was produced in the Food Laboratory of Agricultural Research Center, West Azerbaijan, Iran, using the conventional method. In this regard, the raw milk (containing 12.53 % total solids, 3.6% fat, 3.2% protein, and 0.14% titratable acidity at a pH of 6.7) was first standardized to 1.5% fat content using a lab model separator at 50 °C. Subsequently, it was pasteurized at 85°C for 15 min in a water bath and immediately cooled to the inoculation temperature of 42-44 °C.

The milk was inoculated with yogurt starter cultures according to the manufacturer's instructions (0.05 g kg^{-1} of milk) and incubated at the same temperature until reaching the pH value of 4.5. The samples were then immediately cooled to below 10°C. The Doogh samples were prepared by diluting the yogurt to the total solids of about 5% using pasteurized water containing 1.6% food grade NaCl. The samples were homogenized using a laboratory homogenizer (AVP 1000, Denmark) (1).

In this stage, the probiotic bacteria were first transferred into containers immediately before filling and capping. Five trials of Doogh were produced containing different adjunct culture compositions, including control (i.e., without probiotic), *L. acidophilus* in a free form, L. *acidophilus* in a microencapsulated form, *B. La*ctis as free form, and *B. Lactis* as microencapsulated form, in three replicates. The experimental samples were then transferred to a cold room (5±1 °C) and kept for 45 days for further analysis on days 0, 15, 30, and 45.

Physical and chemical analysis

The Doogh samples were analyzed for salt, fat content, moisture content, and density by Volhard method, Gerber's volumetric method, oven-drying at 102±3 °C (Memmert, Germany), and hydrometer, respectively. The pH of the samples was measured using a digital pH meter (Swiss, Metrohm 691) after calibration with the standard buffer solutions of pH 4.0 and 7.0. Titratable acidity was measured as the percent of lactic acid by titrating the Doogh samples with 0.1 N NaOH using phenolphthalein as an indicator (14).

Enumeration of probiotic bacteria

For the purpose of the enumeration of probiotic bacteria, 10 g from each Doogh samples was transferred into a sterile stomacher bag under aseptic conditions, and then homogenized in 90 mL of sterile trisodium citrate solution 2% for 2 min using a lab 400 stomacher (Seward Laboratory, London, UK). Serial dilutions were prepared by adding 1 mL to 9 mL 0.1 % sterile peptone water. Enumeration of *B. lactis* and *L. acidophilus* was carried out using pour plate method on RCA agar and MRS agar plus Sorbitol, respectively. After inoculation, the plates were transferred to an anaerobic jar and

incubated at that condition using the gas pack system at 37°C for 72 h (8).

Measurement of stability

In order to measure stability, 50 mL of the Doogh samples was poured into the sterile graduated cylinders, and then packed with aluminum foil and stored at 4°C. The stability was determined on experimental days using *Equation 1* (15):

$$Stability\% = \frac{Doogh\ initial\ volume - separated\ serum\ volume}{Doogh\ initial\ volume} \times 100 \quad (1)$$

Microstructure

The samples were analyzed in terms of their microstructure using a light microscope (Leterz, Watzlar, Germany) after staining the samples with Rhodamine-B dye (1).

Sensory evaluation

Sensorial characteristics of the samples were evaluated by a trained panel composed of 12 panelists consisting of staffs from the Agricultural Research Center of West Azerbaijan, Urmia, Iran, on day 45, together with a consumer sensory evaluation questionnaire. The samples were served in cups on a blind-labeled basis. Subsequently, the consumers were asked to evaluate the sensory characteristics of the samples, such as color, flavor, and overall acceptability, on a 5-point hedonic scale ranging from 1 (extremely dislike) to 5 (extremely like).

Statistical analysis

Statistical analysis was performed using the SAS System for Windows 7 (USA). Furthermore, the means were compared using the least significant difference test.

Results

Characteristics of Doogh

Table 1 shows the physiochemical properties of Doogh.

Survival of probiotic bacteria in Doogh

Variations in the counts of probiotic bacteria in Doogh either in free form or in microencapsulated form during a storage period of 45 days are depicted in Figure 1. As Figure 1 illustrates, the numbers of *L. acidophilus* and *B. lactis* used in free form were respectively about $\log_{10}$ 7.58 and $\log_{10}$ 8.1 CFU g^{-1} on day 1. Their number continuously decreased to $\log_{10}$ 6.53 and $\log_{10}$ 5.89 CFU g^{-1} on day 45 of storage, respectively.

Therefore, the number of both *L. acidophilus* and *B. lactis* used in the free form significantly decreased during the storage.

The reduction rates were 1 and 2.2 log cycles, respectively. Therefore, the reduction of *B. lactis* was 1.2-log cycle greater than that of *L. acidophilus*. Nonetheless, as it is clear in Figure 1, the number of both probiotic bacteria added as encapsulated form did not show a significant reduction during the storage period.

Changes in pH and acidity

The pH value and titratable acidity of the samples showed significant differences across different product types and storage durations (P<0.01).

The pH showed a significant reduction, while the acidity demonstrated a significant elevation during the 45 days of storage (P<0.01). At the end of the storage duration, the samples containing the free form of *L. acidophilus* showed the highest acidity and lowest pH value. However, there were no significant differences between the probiotic and control samples containing the capsulated forms in terms of acidity.

On the other hand, a significant difference was observed between the probiotic and control samples regarding pH. In this regard, the samples containing the capsulated forms of *B. lactis* and controls had the highest pH in comparison with the samples containing the capsulated forms of *L. acidophilus*.

Stability

The results revealed a significant difference between the different treatments of Doogh in terms of stability (P<0.01; Table 3). The stability in the samples containing *L. acidophilus* both in the free and encapsulated forms was higher than that in the capsulated form of *B. lactis*. Both bacteria in the encapsulation form were effective in the enhancement of the stability of Doogh in comparison to those in free forms.

Microstructures

Figure 2 depicts the microstructure of the Doogh samples. There were some clusters and large accumulations of protein particles separated with large pores containing water.

Sensorial properties

Figure 3 illustrates the spider diagram of the sensory attributes in the Doogh samples. The statistical analysis showed no significant difference between the samples in terms of color.

Nonetheless, the probiotic treatment significantly affected the flavor of Doogh (P<0.05). The addition of alginate capsules to Doogh mixture did not cause any changes in the color.

Table 1 Characteristics of Doogh used in this experiment

Total solid (%)	Salt (%)	Density (g cm^3)	pH	Protein (%)	Fat (%)	MSNF (%)
5.95±0.45	0.8±0.12	1.0201±0.001	4.42±0.02	1.57±0.08	0.8±0.0	4.35

MSNF: milk solids-not-fat

Table 2 Mean values of pH and acidity in Doogh during 45 days of storage

Parameter Test	Doogh	Storage duration (days)			
		1	15	30	45
pH SEM=0.03	C	4.30^{aA}	4.12^{bA}	4.02^{cA}	4.00^{cA}
	BL	4.21^{aB}	4.04^{bB}	3.83^{cC}	3.89^{dB}
	BLM	4.26^{aA}	4.11^{bA}	4.00^{cB}	3.92^{dA}
	LA	4.28^{aA}	4.15^{bA}	3.96^{cB}	3.70^{dC}
	LAC	4.23^{aB}	4.12^{bA}	4.06^{cA}	3.88^{dB}
Acidity (% lactic acid) SEM=0.08	C	0.54^{cA}	0.63^{bB}	0.64^{bB}	0.68^{aB}
	BL	0.56^{cA}	$0.66b^{AB}$	0.68^{bA}	0.73^{aAB}
	BLM	0.54^{cA}	0.62^{bB}	0.63^{bB}	0.71^{aB}
	LA	0.55^{cA}	0.68^{bA}	0.71^{bA}	0.80^{aA}
	LAM	0.54^{cA}	0.63^{bB}	0.65^{bB}	0.71^{aB}

C: control Doogh, BL: Doogh containing a free form of *B. lactis*, BLM: Doogh containing an encapsulated form of *B. lactis*, LA: Doogh containing a free form of *L. acidophilus*, LAM: Doogh containing an encapsulated form of *L. acidophilus,* SEM: standard error of mean. Superscript lowercase letters denote significant differences (P<0.01) between different storage periods. Superscript capital letters represent significant differences (P<0.01) between different Doogh samples.

Table 3 Stability of Doogh samples

Samples	C	BL	BLM	LA	LAM
Stability (%)	35.17 ± 1.09^{d}	39.50 ± 1.22^{c}	43.75 ± 0.93^{b}	42.03 ± 1.32^{b}	46.93 ± 1.12^{a}

C: control Doogh, BL: Doogh containing a free form of *B. lactis*, BLM: Doogh containing an encapsulated form of *B. lactis*, LA: Doogh containing a free form of *L. acidophilus*, LAM: Doogh containing an encapsulated form of *L. acidophilus* Mean values in with the same letters are not significantly different from each other (P<0.05).

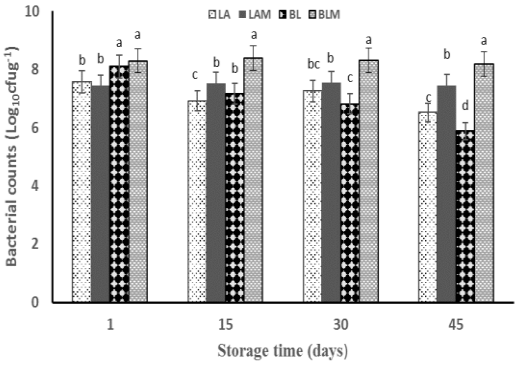

Figure 1 Survival of free and microencapsulated *Lactobacillus acidophilus* and *Bifidobacterium lactis* in Doogh during storage (The error bars represent the standard deviation of means [n=3]). BL: Doogh containing a free form of *B. lactis*, BLM: Doogh containing an encapsulated form of *B. lactis*, LA: Doogh containing a free form of *L. acidophilus*, LAM: Doogh containing an encapsulated form of *L. acidophilus,*

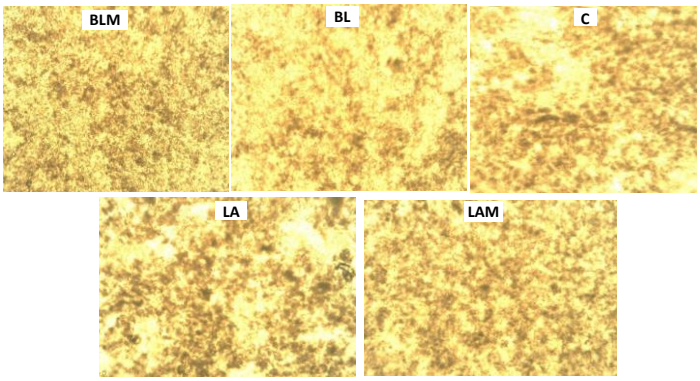

Figure 2 Microstructure of Doogh; C: control Doogh, BL: Doogh containing a free form of *B. lactis*, BLM: Doogh containing an encapsulated form of *B. lactis*, LA: Doogh containing a free form of *L. acidophilus*, LAM: Doogh containing an encapsulated form of *L. acidophilus*

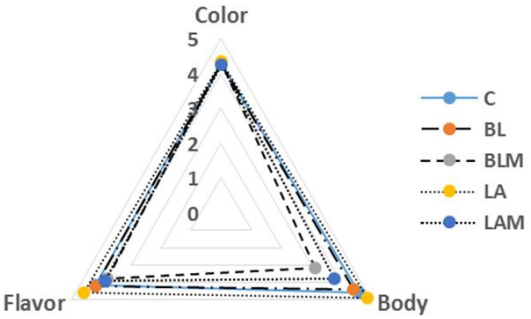

Figure 3 Spider diagram of descriptive sensory analysis on five Doogh samples (C: control Doogh, BL: Doogh containing a free form of *B. lactis*, BLM: Doogh containing an encapsulated form of *B. lactis*, LA: Doogh containing a free form of *L. acidophilus*, LAM: Doogh containing an encapsulated form of *L. acidophilus*)

However, the physical characteristics of the samples, including smoothness, showed significant differences between the Doogh samples containing the free and microencapsulated forms of bacteria. As the results in Figure 3 indicate, the *L. acidophilus* samples gained the highest scores of flavor and physical characteristics.

Discussion

According to the Codex, the milk solids-not-fat (MSNF) content of Doogh should not be lower than 3.2%, and the fat content of this product should not exceed the half amount of its MSNF content. In addition, the protein content of Doogh should not be lower than 1.2%, and the salt amount must be within the range of 0.2-1% (3). In this study, the physiochemical properties of Doogh were in a standard range.

The results showed that the number of *L. acidophilus* and *B. lactis* used in the free form continuously decreased to 14% and 27.28%, respectively, at the end of the storage period. Nevertheless, the number of *L. acidophilus* and *B. lactis* in the microencapsulated forms remained relatively constant during this period.

Previous studies have also shown that the counts of probiotic bacteria in the free form decreased in various probiotic-containing foods and yogurt during the storage. In this regard, yogurt starters can enhance the growth of probiotics via the production of desired substrates or reduction of oxygen pressure (16). Kök TAŞ and Güzel-Seydim (15) showed that the survival of *L. acidophilus* in Aryan was greater than that of *Bifidobacterium*. Our findings are in good agreement with those of previous studies carried out on various probiotics-containing foods and yogurt (6, 8, 9, 11, 14). In this respect, the results of a study carried out by Kailasapathy (17) indicated that encapsulation increased the survival of *L. acidophilus* and *B. lactis* 2 and 1 log cycles, respectively.

Mortazavian et al. (12) showed that at the end of the storage period (i.e., after 42 days), the viable counts of *L. acidophilus* and *B. lactis* in the samples containing encapsulated cells were respectively about 5.5 and 4.0 log cycles higher than those in the samples containing free cells. The difference in the number of viable counts can be related to the encapsulation method. They have used the emulsion method for capsulation, while we adopted the extrusion method.

Reduction of pH and elevation of acidity during storage are caused by acid formation through the conversion of lactose to lactic acid (18). The pH fall was greatly higher in the Doogh samples containing a free form of bacteria than in the probiotics-incorporated samples. This can be due to the negative effect of capsules surrounding the bacteria on their activity. In addition, encapsulation leads to lower metabolic activity of microorganisms (19, 20).

Similar results were reported by Zomorodi et al. (4), Mortazavian et al. (12), and Kailasapathy (17) for Iranian white cheese, Iranian yogurt drink (i.e., Doogh), and yogurt containing probiotic bacteria, respectively. The samples containing *L. acidophilus* either in free or microencapsulated forms were capable of producing a large amount of acid, causing a lower pH.

One of the most important problems with Doogh is its syneresis during storage, which happens due to its low viscosity and pH, and the effect of this phenomenon on the sedimentation of proteins. In such low-pH products, the protein reaches the isoelectric point and as a result starts aggregation and sediment. This phenomenon causes instability and syneresis during the storage (approximately 40-50% syneresis in a month). In normal conditions, the syneresis does not affect the nutritional value of Doogh; however, it gives an undesirable appearance to the final product (21, 22). According to the results, the most and the least stability belonged to microencapsulated *L. acidophilus* and control samples, respectively. According to the results, *L. acidophilus* and *B. lactis* increased the stability of Doogh. The encapsulated forms of the probiotics caused higher stability in Doogh than their free forms.

With regard to the microstructure of the samples, the distribution of clusters and particles were more regular in the samples containing encapsulated probiotics than in those containing the free forms of probiotics. It can be concluded that encapsulation led to the regular aggregation of casein micelles; in this regard, *L. acidophilus* resulted in higher aggregation than *B. lactis*. Furthermore, the regular distribution of particles all over the gel network promoted the stability of Doogh.

The sodium in the alginate hydrogels may be replaced with calcium ions, and this phenomenon may increase the grittiness. The results showed that the addition of alginate as microencapsulation material and also microencapsulated cultures could influence the smoothness of Doogh. This result is in line with those obtained by Kailasapathy regarding yogurt (17).

Given that *L. acidophilus* has high acidic activity, it could tolerate higher acidic condition than *B. lactis*. Accordingly, the *L. acidophilus* samples gained the highest scores of flavor and body. According to the sensory attributes of Iranians, sourness in Doogh is the most preferred taste; as a result, the treatment containing *L. acidophilus* gained a higher sensory score. However, the trap of probiotics in the capsules lowered their acidic activity; as a result, the sensory score of the samples containing microencapsulated bacteria was

lower than that of the samples containing the free form.

Conclusion

As the findings indicated, the survivability of microencapsulated probiotic bacteria was higher than that of the free forms. The number of probiotic bacteria used either in free or microencapsulated forms after 45 days of storage at 5±1°C was higher than the recommended number (10^6-10^7 CFU g^{-1}). Moreover, there was a significant difference between the samples in terms of pH, acidity, moisture, and stability. *Lactobacillus acidophilus* caused a more significant decrease in pH; consequently, they had more increase in acidity than *B. lactis*.

The probiotics used in free forms produced higher acidity than those in encapsulated forms. In addition, the stability of the samples containing probiotics was higher than that of the samples containing no probiotics. The probiotics not only did not cause an undesirable effect on the physicochemical and sensory properties of Doogh but also improved the stability and flavor of this drink. Consequently, it can be concluded that Doogh can be a good carrier of probiotic bacteria.

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Ethical approval and consent to participate

None.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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