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Investigating the survival of microencapsulated *Lactobacillus acidophilus* in double emulsion with Persian gum during storage and digestive simulating conditions

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ABSTRACT

Microencapsulation is a common method to improve the viability of probiotic bacteria against environmental stresses. In this research, by using double emulsion with Persian gum, emulsion stability, physicochemical properties, microcoating efficiency and microcoating viability were investigated during storage and in a simulated gastrointestinal conditions of the digestive system. Encapsulation of *Lactobacillus acidophilus* in double emulsion (W/O/W) with Persian gum improved the survival under storage conditions at 4 °C for 28 days, and encapsulation treatment only 0.58 log decreased and the treatment without capsules reached zero after 7 days. The microcoating efficiency was 88% and the emulsion stability was between 85% and 95.43%. The optical microscope image showed a distinct double emulsion. In the simulating conditions of the stomach, the number of bacteria for encapsulation treatment decreased by 1.97 log and the treatment without capsules 3.9 log, and in the simulating conditions of the intestine, for encapsulation treatment it decreased by 0.45 log and for the treatment without capsules 1.3 log decreased. The particle size, zeta potential and dispersion index was 525 nm, -44.68 mv and 0.33 respectively. The results showed that the use of Persian gum double emulsion improves the survival of *Lactobacillus acidophilus* against storage conditions and the simulated environment of the digestive system.

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1. Introduction

Probiotics are live microorganisms that have positive physiological effects on the microbial flora of the host intestine. In recent years, the use of products containing probiotic bacteria has attracted the attention of researchers [1]. According to the definition of most researchers, probiotic products should contain 10 CFU/g^{8}-10^{7} bacteria were to be useful for health. The beneficial effects of these strains on human health include improving intestinal health and function, reducing blood cholesterol, improving defense mechanisms, etc. [2]. Probiotics, which are mainly gram-positive bacteria, include a wide range of microorganisms. Lactic acid bacteria and yeasts can also be considered as probiotics [3]. The activity and survival of probiotics when they reach the intestine is a basic condition for the occurrence of medicinal effects, but factors such as production conditions, pH changes, mechanical stresses and acid-bile conditions of the digestive tract reduce the number of probiotic bacteria [1]. Microencapsulation is one of the useful methods to improve the viability of probiotics and reduce the destructive effects of the environment [4].

Microencapsulation of probiotics is done in order to stabilize and increase their viability in unfavorable environments [1]. In fact, microencapsulation is a protective technology to cover sensitive compounds inside an edible polymer. Probiotics are coated as the main material inside the coating polymer and are protected by reducing its reactivity with the external environment [3]. There are many studies on the use of polymers such as alginate, chitosan, carboxymethylcellulose, etc. as coating materials [3]. Savamin et al. the effect of alginate multilayer coating on survival*Lactobacillus plantarum* Enclosed in simulated stomach solution and during storage in pomegranate juice at 4 °C, the results showed high survival of multi-layered bacteria [5]. According to the studies of Amin et al., cheddar cheese containing*B. long* Microencapsulated in alzinate showed good survival with a reduction of 2 log CFU/mL after 21 days compared to free cells with a reduction of 4 log CFU/mL [6]. Various methods such as spray drying, emulsion and extrusion methods are used for microencapsulation of probiotic bacteria [1]. Artaki et al. Bacterial survival*Lactobacillus paracasei* microcoated by extrusion method during the cheese making process and reported high bacterial viability [7]. Homayoni et al., two types of synbiotic ice cream containing 1% bacteria-resistant starch*Lactobacillus casei* produced free and micro-encapsulated by emulsion method and the survival of bacteria was investigated for 180 days and the results showed a high survival rate of micro-encapsulated bacteria [8].

Persian gum or zadu is transparent and comes from the almond tree (*Amygdalus scoparia*Spach.) is obtained. This gum is secreted due to temperature, humidity and insect bites [9]. Due to its special structure, it has found wide applications in food and pharmaceutical industries. Farsi gum with a moisture content of 3-13% has a very good surface activity and emulsification compared to other gums. Farsi gum is an anionic hydrocolloid with low protein content and high water absorption capacity [10].

*Lactobacilli*They are regular gram-positive bacilli that have fermentation properties and are probiotics.*Lactobacillus Acidophilus* It belongs to this family and grows easily at low pH levels (below 5) and its optimal growth temperature is 37 °C. Antagonistic effects (substances that interfere with the physiological function of another substance or disturb it) on growth*Staphylococcus Oreos*، *Escherichia overall*، *salmonella Tiffi Morim* And*Clostridium Perfringent* has been studied [11]. Emulsions that are used in various industries are in the form of water in oil (W/O) or oil in water (O/W), which are mechanically or spontaneously formed. Double emulsions are droplets of one liquid dispersed in another liquid, and due to their distinct characteristics and structure, they have attracted the attention of researchers. There are two types of multiple emulsions with the structure of water in oil in water (W1/O/W2) or oil in water in oil (O1/W/O2), which are used in industrial applications such as food, medicine and cosmetics. Since in the food industry, many food emulsions have an aqueous continuous phase and more food-grade hydrophobic stabilizers are available, the preparation of water-in-oil-in-water (W1/O/W2) emulsions is more common. The usual method accepted in the preparation of double emulsions is to use emulsifiers with

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different amounts of hydrophilic-lipophilic balance (HLB) so that the desired double emulsions can be formed based on the type of material that is to be microcoated. The purpose of this research is to investigate the effect of micro coating with Persian gum using double emulsion technique on the viability of probiotic bacteria.*Lactobacillus acidophilus* During the period of maintenance and examination of the environment simulating digestion and some characteristics of the production emulsion.

2- Materials and methods

2-1- Materials

Pure culture of bacteria*Lactobacillus acidophilus*(LA-5) was obtained from Christine Hansen Company, Denmark and activated under sterile conditions. Lipophilic emulsifier PGPR[1] (Pisgaman Shimi, Mashhad, Iran) and DATEM hydrophilic emulsifier[2] (Danish Banyan Pars Behdoh Asia Company, Mashhad, Iran), Farsi gum (Rehan Gam Parsian, Isfahan, Iran) and other consumables were obtained from Merck, Germany.

2-2- Preparation of bacterial suspension

Lactobacillus acidophilus in 200 cc of MRS Broth culture medium[3] It was incubated at 37 °C in anaerobic conditions until reaching the logarithmic phase for 24 hours. After the bacteria reached their logarithmic phase, they were centrifuged at 3400 g at 10°C for 2 minutes, and then the bacterial suspension was washed by phosphate buffer. Finally, cell suspension was prepared using physiological serum with a concentration of 4 McFarland.

2-3- Preparation of double emulsion

The double emulsion of water in oil in water was prepared at room temperature (25 °C) using a two-step method. Primary water-in-oil emulsion by cell suspension containing 10 CFU/ml^{8}x6 was bacteria, it was prepared and corn oil was used for the oil phase. The total concentration of emulsifiers in the initial emulsion was 8% by weight (1 part of DATEM and 4 parts of PGPR). The volume ratio of the dispersed phase in the initial emulsion was 0.3. Preparation of emulsion was done by homogenizer at 9000 rpm for 5 minutes. The cell suspension was dropped into the oil by a sterile syringe. In the second step, 30 ml of the primary emulsion was added to 70 ml of 8% gum solution and the double emulsion was homogenized by a homogenizer at 4500 rpm for 5 minutes and kept at 4 °C [12].

2-4- Counting cells*Lactobacillus acidophilus* Micro-coated

The double emulsion containing Lactobacillus acidophilus cells encapsulated in g15800 was centrifuged for 10 minutes. As a result, the double emulsions were broken and the trapped cells were released. 1 ml of the broken emulsion was added to 9 ml of 0.2 M phosphate buffer and successive dilutions were prepared from it. The prepared dilutions were cultured on MRS agar medium in the form of purple plates and kept in a greenhouse at 37 °C in anaerobic conditions for 72 hours. After this period, the grown cells were counted. Bacterial counting was repeated during a period of 28 days and at intervals of 7 days.

2-5- micro-coating efficiency

Microencapsulation efficiency was calculated by the method of Zhang et al. (2016), where the number of bacteria in the initial suspension (N_0), the number of microencapsulated bacteria in double emulsion (N_1) and the total number of emulsion bacteria (N_2) was calculated according to the following formula [1].

$$\text{Encapsulation efficiency} = \frac{N1 - N2}{N0}$$

6-2- Emulsion stability

The stability of the emulsion during the storage period at room temperature after centrifugation at 5000 rpm for 10 minutes at room temperature was calculated using the following equation, where Vt (ml) is the total volume of the samples and Vs (ml) is the volume of the lower phase [3].

$$Emulsion\ stability = \frac{Vt - Vs}{Vt}$$

2-7- Determination of particle size and dispersion index

[1]. Polyglycerol polyricinoleate

[2]. Diacetyl tartaric acid ester of mono- and diglycerides

[3]. DE MAN, ROGOSA and SHARPE Broth

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The physical and chemical properties of the prepared microcoatings including particle size distribution and dispersion index (PDI) were evaluated. A dynamic light scattering (DLS) device was used for the average droplet size and dispersion index (PDI) of microcoatings. The light intensity was set at 90 degrees and the sample was diluted 1:1000 with deionized water and their pH was adjusted and then placed in the DLS measuring chamber [13].

2-8- Zeta potential

In order to determine the zeta potential of the samples, a zeta sizer (CAD, Zeta compact company - France) was used [13].

2-9- Morphology

The shape of the particles were examined by an optical microscope with a magnification of 40, so that a smear was prepared from the double emulsion and observed under an optical microscope [14].

2-10- Examination of the survival rate*Lactophilus acidophilus* In the conditions of the digestive tract simulator

The stomach simulating medium was prepared by dissolving 1.12 grams of potassium chloride, 2 grams of sodium chloride, 0.11 grams of calcium chloride and 0.4 grams of basic potassium phosphate in one liter of distilled water. 1 gram of bacterial suspension was mixed with 50 cc of gastric simulating medium and kept for 2 hours in a shaker incubator at 37°C, and the survival of the bacteria during 0, 60 and 120 minutes was examined by pore pellet culture in MRS agar medium. took For the intestinal simulating medium, 1.95 g/liter of pancreatin and 1.95 g/liter of bile salt were added to the container containing the stomach simulating medium, and its pH was brought to about 7 with the addition of 1 normal solution and kept in a shaker incubator for 4 hours. It was kept at a temperature of 37 °C. To check the viability of bacteria, pore plate culture was performed on MRS agar medium at 0, 60, 120, 180 and 240 minutes [15].

11-2- Statistical analysis

In this research, one-way analysis of variance (ANOVA) and comparison of means were performed using Duncan's multi-range test at the 95% probability level. SPSS version 20 statistical software (SPSS Inc. Chicago, USA) was used to analyze the data.

3. Results and Discussion

3-1- Bacteria survival*Lactobacillus acidophilus* During the maintenance period

survival rate *Lactobacillus acidophilus* During the storage period at 4 °C, it is shown in Figure 1. Number*Lactobacillus acidophilus* For all treatments after 4 weeks of storage significantly ($P< 0.05$(decreased, but the survival of probiotic bacteria significantly during the storage period)$P<0.05$) Increased.*Lactobacillus acidophilus* Azad, during the storage period in the refrigerator, after 7 days, their number decreased drastically, but*Lactobacillus acidophilus* Micro-encapsulated in Persian gum after 28 days had a decrease of only log 0.58, and until the 28th day of storage, their number was in accordance with the desired amount (log 6). The protective effect of Persian gum caused the amount of*Lactobacillus acidophilus* be higher than the desired amount. Brinks et al. (2011) and Gandami et al. (2016) according to the viability of free and microencapsulated bacterial cells.*Lactobacillus plantarum* in sodium alginate and pectin and microcoating*Lactobacillus ramensus*with alginate and chitosan in apple juice during 90 days of storage at 4°C and 25°C and showed that bacterial microcovering increases survival [16, 17].

3-2- Microcoating efficiency

Microencapsulation efficiency is one of the important factors in determining encapsulated compounds. The efficiency of microcoating was calculated to be 88%, this high efficiency of microcoating is related to the method of microcoating, i.e. double emulsion. In the double emulsion, the bacteria are first preserved inside the water droplets and then dispersed in the oil droplets and finally covered with persian gum, which can be a good barrier for the bacteria against external stresses. According to the obtained results, it can be said that microcoating with double emulsion method using Persian gum increases the viability of bacteria.*Lactobacillus acidophilus* It has been effective. Silva et al. (2018) in their research using gelatin and gum

arabic at pH 4 for bacterial microencapsulation*Bifidobacterium lactis*, they obtained a microcoating efficiency of 86%, which is consistent with the results obtained in this study and indicates the positive effect of microcoating [18]. Ararat et al. (2015) performed the microencapsulation of omega-3 and probiotic bacteria using whey protein and gum arabic, which declared the microencapsulation efficiency to be 84.95%, which is consistent with the results of the present study [19]. Double emulsion microcoating leads to high efficiency in microcoating and effectively protects the microcoated material against mechanical pressure and temperature changes [4].

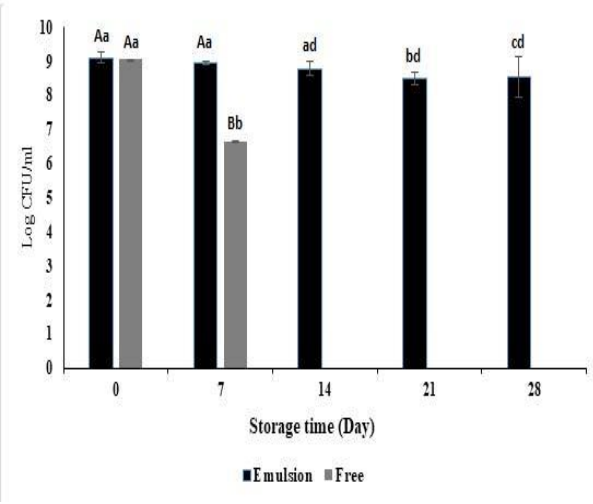

Fig 1 Bacterial survival during storage at 4°C

3-3- Emulsion stability

The term "emulsion stability" refers to the ability of an emulsion to resist changes in its properties over time. The more stable the emulsion, the slower its properties change. An emulsion may become unstable due to a variety of physical and chemical processes [20]. The results related to emulsion stability are shown in Figure 2, and according to the results on day zero, the stability rate was 95.43% and on day 28, it was 85%, which is consistent with the research findings of Jiang et al. (2006) for water-in-oil-in-water emulsion. has [21]. Increasing the concentration of gum increased the stability of the emulsion. This increase is probably because at higher concentrations, polysaccharide molecules can form a three-dimensional network to trap oil droplets and block their movement by forming complex polymer chains. Raisi et al. (2019) microcoated fish oil and garlic essential oil with chitosan and persian gum and the results showed that the highest stability in the ratio of gum:chitosan 1:2 with 100% stability and the lowest stability in the ratio of gum:chitosan 2:1 with The stability was 80%, which is in line with the results of our research [22].

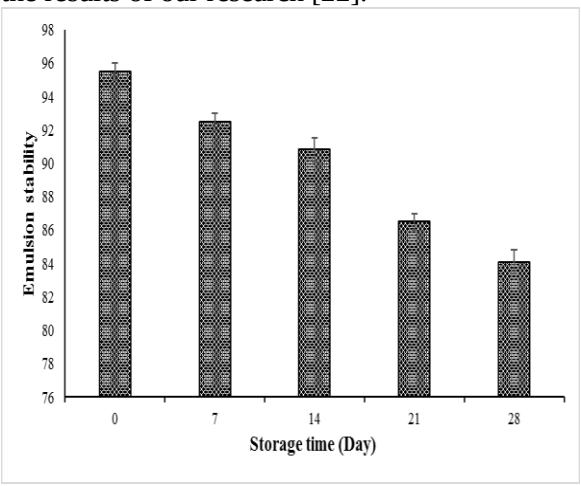

Fig 2 Emulsion stability during storage period

3-4- Morphology of capsules

One of the important factors in the microencapsulation of bacteria is the surface morphology of the capsule, because the more spherical the surface and the less gaps, the better. The morphology of the capsules is shown in Figure 3. According to the figure, it is clear that the internal water droplets (W1) are densely grouped and distributed inside the oil droplets (O) and dispersed in the external aqueous phase (W2), so they show the characteristic structure of double emulsions and with the results of Paula et al. Colleagues agree [4].

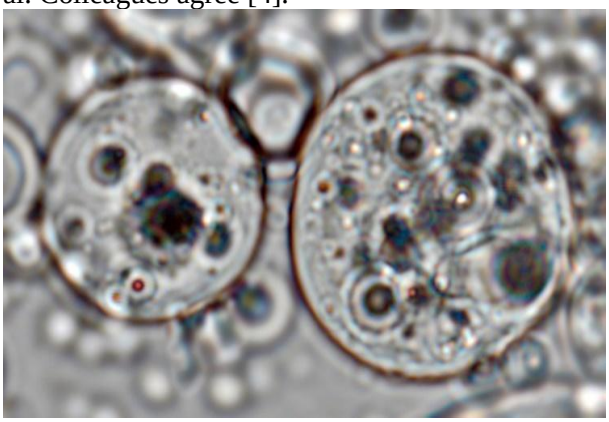

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Fig 3 The image from the light microscope (400x) of the microcapsules

5-3- Survival *Lactobacillus acidophilus* in the gastrointestinal tract simulator environment

In the preparation of foods containing probiotic bacteria, it is essential that these bacteria maintain their viability not only during the production process and storage conditions, but also during the digestive tract. Bacteria must withstand exposure to gastric acidity and bile salts in the small intestine. Intestinal flora in humans has a close relationship with health, and ensuring that a sufficient number of probiotic bacteria reaches the small intestine and large intestine ensures the supply of intestinal flora. The results of the survival of bacteria in the simulating environment of the digestive tract are presented in Figure 4. According to the presented results, the effect of time on different treatments is significant (*P<0.05*) Was. Bacterial numbers of both treatments in the digestive simulator environment decreased during the storage period. According to Figure 4, the number of bacteria in the microcoated and free treatment in the simulated environment of the stomach from 9.06±0.03 log and 8.8±0.2 log to 7.09±0.2 log and 0.05 log, respectively. 4.9±0.00 and in simulated intestinal conditions from 6.9±0.05 log and 4.9±0.07 log to 0.15±6.45 log and 0.4 log for microcoated and free treatment, respectively decreased by 3.6±0. The results showed that bacteria*Lactobacillus acidophilus* Microcoated in double emulsion, it resists the harsh conditions of the digestive tract and is more than the standard (10^6) reach the target organ to provide health benefits to the host. Gabassi et al. (2011) three strains*L. plants*Lp299v, Lp159 and Lp800) were encapsulated by calcium alginate granules and whey protein and subjected to the gastrointestinal tract simulation environment. The results showed that encapsulation maintained the bacteria in the gastrointestinal tract simulation environment, which was consistent with our results [23]. et al (2011) reported that no*Lactobacillus plantarum* encapsulated in alginate beads that were exposed to gastric simulating environment, increased from log 8.9 to log 2.5 during the storage period and showed that encapsulation of bacteria preserves the bacteria in the simulating environment of the digestive tract and in accordance with Our results were [16]. Rodriguez et al (2014) Bacterial viability*Lactobacillus plantarum* Encapsulated in a double emulsion during the making of Exaca cheese[4] and also investigated in the simulator environment of the digestive tract and the results showed that the cells*Lactobacillus plantarum* Encapsulated in double emulsions, they resisted harsh digestive conditions, which was in line with our results [12].

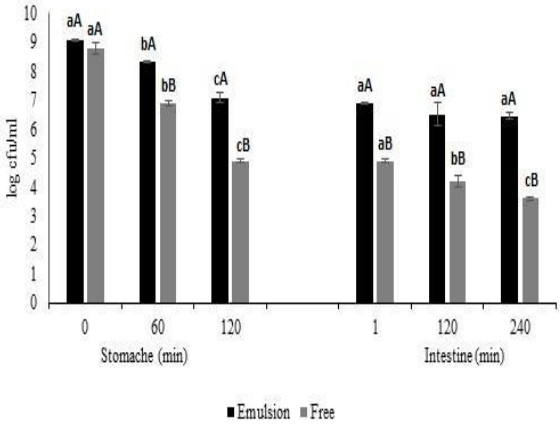

Fig 4 Survival of bacteria under conditions simulating digestion during storage

6-3- Particle size and dispersion index

Table 1 shows the particle size and dispersion index (PDI). Dynamic light scattering (DLS) analysis showed that the microcapsules*Lactobacillus acidophilus* with Persian gum, they had an average size of 525 nm. The performance of microcoatings (the ability to enclose, protect and deliver bioactive components) is directly influenced by its physical and chemical characteristics, such as size, morphology and surface electric charge [1]. Therefore, the size of the particles is an important parameter in terms of maintaining the structure of microcoats during the passage through the digestive system. Large particle size affects the viability of microencapsulated bacteria by producing large pores in hydrogels and allows small molecules such as oxygen, acids, bile salts

[4]. Oaxaca cheese

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or digestive enzymes to easily diffuse and inactivate the enclosed bacteria [24]. However, large grains lead to an unpleasant mouthfeel when consumed [25, 26]. Capsules larger than 100 µm in size should not be used due to gritty mouthfeel, and 30 µm in size does not cause any adverse mouthfeel [26]. The PDI value shows the size distribution of colloidal nanoparticles, and the lower the PDI value, the more uniform the particles are, and usually, the PDI value less than 0.3 indicates a relatively homogeneous colloidal suspension. In the present study, PDI was calculated as 0.33 [[27].

3-7- Zeta potential

Zeta potential is a parameter for the potential stability of the colloidal system. If all the particles in the suspension have a negative or positive charge, the particles tend to repel each other and cause no accumulation. The tendency of charged particles to repel each other has a direct relationship with the zeta potential. In general, the limit of stability and instability of suspension can be determined in terms of zeta potential [28]. According to the results of previous studies, a negative zeta potential higher than 30 mV is sufficient to prevent droplet coalescence [29]. The value of zeta potential obtained in this research (Table 1) was -44.68, which shows that the produced micro-coatings have a suitable colloidal balance. The low negative charge of the emulsion may be related to the uronic acid content of Persian gum. This stability is important in preventing build-up. According to the research results of Abd al-Khaleq et al. (2018), increasing the concentration of gum leads to an increase in the negative charge of the surface of the emulsion droplets, and as a result, the zeta potential with a negative charge, which stabilizes the emulsion system, and it was in accordance with our results [30].

Table 1 Properties of emulsion

Treatment	Zeta potential	PDI	Particle size
Emulsion	-44.68	0.33±0.35	525nm

4 - Conclusion

The results showed that the use of Persian gum to prepare w/o/w emulsions for microencapsulation of bacteria*Lactobacillus Acidophilus* It improved its survival. Particle size, zeta potential and dispersion index showed that the emulsion had good stability. Microencapsulated bacteria*Lactobacillus Acidophilus* Compared to free bacteria, they showed good resistance in the simulating environment of the digestive system. The results showed that the use of Persian gum double emulsion improves survival*Lactobacillus acidophilus* against the conditions of storage and the simulated environment of the digestive tract.

5- Resources

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بررسی زنده مانی *لاکتوباسیلوس اسیدوفیلوس* ریزپوشانی شده در امولسیون دوگانه با صمغ فارسی در طول انبارداری و شرایط شبیه سازدستگاه گوارش

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چکیده

ریز پوشانی روشی رایج برای بهبود قابلیت زنده مانی باکتری‌های پروبیوتیک در برابر تنش‌های محیطی است. در این پژوهش با استفاده از امولسیون دوگانه با صمغ فارسی، پایداری امولسیون، خصوصیات فیزیکوشیمیایی و مورفولوژیکی، راندمان ریز پوشانی و زنده مانی ریز پوشینه‌ها، در طول ذخیره‌سازی و محیط شبیه‌ساز دستگاه گوارش مورد بررسی قرار گرفت. اندازه ذرات، پتانسیل زتا و شاخص پراکندگی به ترتیب معادل ۵۲۵ nm، ٤٤/٦۸ mv- و ۰/۳۳ بود. راندمان ریز پوشانی ۸۸ ٪ و پایداری امولسیون بین ۸۵ ٪ تا ۹۵/٤۳ ٪ بود. ریزپوشانی *لاکتوباسیلوس اسیدوفیلوس* در امولسیون دوگانه (W/O/W) با صمغ فارسی باعث بهبود زنده مانی آن در شرایط نگهداری در دمای ٤ °C به مدت ۲۸ روز شد. جمعیت باکتریایی در تیمارهای ریز پوشانی نشده بعد از گذشت ۷ روز به صفر رسید درحالیکه در تیمارهای ریز پوشانی شده فقط log ۰/۵۸ کاهش داشت. در محیط شبیه‌ساز معده تعداد باکتری‌ها برای تیمار ریز پوشانی شده log ۱/۹۷ و برای تیمار حاوی باکتری آزاد log ۳/۹ کاهش یافت، و در محیط شبیه‌ساز روده نیز برای تیمار ریز پوشانی شده log ۰/٤۵ و برای تیمار حاوی باکتری آزاد log ۱/۳ کاهش یافت. نتایج نشان داد که استفاده از امولسیون دوگانه صمغ فارسی باعث بهبود زنده‌مانی *لاکتوباسیلوس اسیدوفیلوس* در شرایط نگهداری در دمای ٤ °C و محیط شبیه‌سازی‌شده دستگاه گوارش می‌شود.