



**MICROENCAPSULATION IMPACT ON THE VIABILITY OF BACTERIA
LACTOBACILLUS RHAMNOSUS LC705 IN SIMULATED CONDITION OF GASTRIC
AND INTESTINAL**

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ABSTRACT

Probiotics are dietary components of living microbes' kinds that have beneficial effects on the health of their consumers. Microencapsulation of probiotic bacteria is a way that improves their performance in the food and digestive tract. In this study, bacteria *Lb. rhamnosus* LC705 isolated from breast milk were microencapsulated in the coverage of whey alginate-protein. To see the particle morphology and determine the capsule size, electron microscopy SEM and instrument of measuring the diameter of the particles were used and the survival of bacteria in free and encapsulated in the simulated condition of stomach and intestines was studied. Images taken from electron microscopy showed nearly spherical shaped microcapsules with a diameter of less than 100 micrometers. Based on data derived from particle distribution curve, diameter of 90 % particle (d90) is equal to or less than 95.973 micrometer. Results of live bacteria counts in the simulated condition of stomach and intestines revealed that the initial population of alive and encapsulated bacteria cells after 120 minutes of exposure to the environment is dropped respectively to $1.6 \pm 0.8 \times 10^2$ and $2 \pm 0.4 \times 10^6$. It can be understood from the results that the microencapsulation process helps to increase the survival of bacteria against the gastric acid,

enzyme, and bile alkaline salts and in this case, the proposed range by the International Dairy Federation is (10^6 - 10^7 cfu / g).

Keywords: Microencapsulation, *Lb. rhamnosus* and Probiotic

INTRODUCTION

Microencapsulation is a process in which a substance or mixture of substances covered by other material or are trapped in this case the prisoned material is called nuclear or active material and coating material is called as wall materials. In general, in this method an emulsion of nuclear materials and a concentrate layer of (protein or polysaccharide) are produced then the emulsion obtained will be condensed or dried by different methods. Today, this method is widely used in the food, pharmaceutical, cosmetic, chemical and printing industrials (7). Lactic acid bacteria, is a core group of Microbial flora in animal and human digestive tract who are involved in the metabolism of the host. Some of the *Lactobacillus* genus bacteria have been introduced as probiotic bacteria (8). The concept of probiotics is described by Fuller in 1989. According to this, the probiotics are live bacteria that improve the host intestinal microbial balance as a nutritional supplement in a good attitude (2). Low viability of probiotics in hard conditions of food products and also under the acid-bile condition of digestive tract has been

encouraging the researchers continue to find ways to improve this property. Microencapsulation as one of the most modern ones of these methods had a significant role in this field. In effect of the controlled release of the encapsulated probiotics, these bacteria will not face a food and gastrointestinal conditions in one place, but gradually released from the capsule and for a long time, the population of probiotics are continually updated in the gut (5). In this study Alginate cover-protein powder of whey (whey protein), as wall material is used in microencapsulation. Alginate is a linear hetero-polysaccharide composed of D-mannuronic and L-guluronic acid which can be diagnosed in the cell wall and intercellular spaces of brown algae. Calcium alginate has been widely used for microencapsulation of lactic acid bacteria due to the nature of the risk, ease of use and low cost (6). Whey is a by-product of cheese or casein production. Whey proteins are essential because of amino acids and their amounts are very favorable. Derivatives of whey protein are full of

sulfur amino acids. The amino acids released during the heating process and reduce the potential reduction (13). In this study strains isolated from breast milk, *Lactobacillus rhamnosus* LC705 which their probiotic properties are under study and investigation are used (14). *Lactobacillus rhamnosus* is one of the most common usual strains isolated from breast milk and ability of probiotic strains of this species have been identified in various studies (10 and 11). The survival of bacteria in the digestive tract (bile salt and acid resistance), is one of the most important factors in choosing a probiotic strain. The purpose of this research is to complete the identification of native bacteria to our country, for its industrial and medical applications, the study of the viability of free and microencapsulated bacteria in the simulated digestive tract.

MATERIALS AND METHODS

Preparations of microorganism

To activate the strain *Lactobacillus rhamnosus* LC705, which is prepared in lyophilized form of Mashhad (14), the amount of bacteria with sterile ANAS in a 5 ml environment of seminal fluid of MRS (Scharlau Chemie SA, Spain) and was amplified for the duration of 24-16 hours in C ° 37. Then the obtained sample in 95 ml

environment of seminal fluid of MRS and was produced under the above conditions. At the end the obtained biomass by centrifugation at 7800 ×g for 5 minutes with a 0.1 % sterile solution of peptone water in two stages (Quelab, Canada) was washed and stored at four degrees C (8).

Capsulation of bacteria in alginate and whey protein powder

The method of preparation of wall ingredients was performed based on the Chen and Subirade method (4). Action of microencapsulation was performed by using of foreign gelatinization method reported by Truelstrup et al (15) and Mokarram et al (12).

Counting bacteria trapped in the capsules

To determine the effectiveness of microencapsulation process of bacteria, 1 gram of microcapsules was dispersed into 99 ml of 1% weight-volume sodium citrate (Merck, KGaA, Germany) and for 10 minutes at room temperature by magnetic stirring, stirring operation was continued. The number of viable cells by dilution and production in the selective MRS Agar culture, after 48 hours of incubation at C ° 37, was counted. This was done by 3 repetitions (12).

Determination of the morphology of the capsules

Scanning of capsules was done with Kv15 electron radiation by electron microscope (SEM, VEGA, TESCAN-LMU and Razi Metallurgical Research Center).

Determination of the size of the capsules and their distributions

To determine the size of the capsule and the abundance of each of these, particle diameter measurement device (Analysette 22, Fritsch, Germany) was used. For this purpose alginate-encapsulated protein was dispersed in whey protein in 96 °ethanol and the results based on the particle average volume diameter \pm standard error and the mean diameter was analyzed on the basis of formula (12).

Assess survival of bacteria in simulated intestinal and stomach condition

For this purpose, according to the method proposed by Vizoso Pinto et al(16), for producing stomach latex, an electrolyte solution containing 6.23 grams per liter of NaCl, 2.29 grams per liter of potassium chloride, 0.229 grams per liter of calcium chloride and 1.2 gram per liter of sodium bicarbonate (Merck, Germany) was produced sterile and pH was reduced to 2 ± 0.2 . At the time of pepsin Enzyme testing to a final concentration of 3.0

percent (Merck, Germany) was added to the content. Simulated intestinal Latex was produced by using of sterile electrolyte solution containing 0.239 g/L of KCl, 1.28 g/L of NaCl, 4.6 g/L of calcium carbonate and 0.5 percent of bile salt (Sigma-Aldrich GmbH, Germany) (16).

Then, based on the Mokarram et al method (12), amount of 1 gram of bacterial capsules or its equivalent, 1 ml of suspension of free bacteria is poured in a glass vial containing 9 ml of gastric juice, for two hours is placed in the 37 C ° incubator during shaking, and then remove it, is transferred to another vial containing 10 ml of intestinal simulated environment and was incubated for 2 hours and during this time, in half-hour intervals (0, 30, 60, 90 and 120 minutes), the number of living cells with three replicates for each sample was counted.

Statistical analysis

In this study, a factorial experiment was used in a completely randomized design and average comparison using Duncan's test was performed by the Spss16.0 software and charts were drawn using software Excell 2010.

RESULTS AND DISCUSSION**Counting the number of trapped bacteria in the capsules**

The results showed that the number of viable bacteria cells before microencapsulation with sodium alginate and whey protein is equal to 12.54-13.17 Log cfu/ g that after the microencapsulation process of trapped live bacteria cells 11.04-11.06 Log cfu / g were reported. Few amounts of lost bacteria in the capsulation step, indicates high efficiency of emulsion method (foreign gelatinization) in trapping bacteria cells. In other words, based on these results, capsulation has no significant effect on the number of bacteria and capsulation efficiency is 88%.

The morphology and size of the capsules

Images from electron microscopy showed the spherical microcapsules production with a diameter less than 100 micrometers (Figure 1). Also figure 1 shows that walls cover of whey protein and sodium alginate surrounds the bacterial cells evenly. Thus, with this method can make uniform and micron-diameter capsule that creates a soft tissue in food supplements containing live bacteria.

Based on data derived from particle distribution curve, the mean diameter (VMD) of capsules is 8.49 micrometers and the standard deviation from the average is 0.897 microns. Diameter of 90 % of particles (d90) is equal to or less than

95.973 micrometers, 50% (d50) is equal to or less than 17.2 micrometers, 10 % of capsules (d10) have a diameter equal to or less than 0.589 micrometers dpeak is obtained equal to or less than 211.045 micrometers (Figure 2).

The method used in this study to generate microcapsules was emulsion method (foreign gelatinization). In this method can produce the capsules in the range of 25 μm -2 mm by changing of the stirring rate and the ratio of water to oil. Also in the emulsion method can improve the viability percent and organoleptic properties of microcapsules by applying a layer of secondary alike praised (3). In a research Mekkaram and colleagues reported that capsules ($75.339 \pm 0.209 \mu\text{m}$) with the structure composing of two different materials protected bacterial cells better against the gastrointestinal conditions (12).

Effects of simulated gastrointestinal conditions on bacteria survival

Effects of simulated gastric environment on the viability of free *Lb. rhamnosus* LC705 and microencapsulated is shown in Table 1. The initial number of bacteria in free and microencapsulated condition was 3.5×10^{12} cfu / g and was $1.1 \pm 0.3 \times 10^{11}$ respectively. But the average number of free and microencapsulated live cells, after

2 hours of exposure to gastric simulated environment reached the $1.3 \pm 0.1 \times 10^5$ cfu / g and $1.9 \pm 0.2 \times 10^8$. In other words, the survival of free and microencapsulated *Lb. rhamnosus* LC705 was reduced 6 and 4 logarithmic cycles. D-values of cells in microencapsulated Bacteria were more than the D-values of the free cells and these two D-values statistically have significant differences ($p < 0.05$).

In Figure 3 the viability of free and encapsulated bacteria cells percent is simulated in stomach environment.

The number of these for encapsulated and free bacteria cells are 70.9 and 40.7 respectively and the differences are statistically significant at the 5% level ($p < 0.05$). In fact, the sodium alginate coating and whey protein in encapsulated cells protect them against the acidic and enzymic condition of stomach.

The effect of intestinal simulated environment on survival of free and encapsulated *Lb. rhamnosus* LC705 is shown in table 2.

The results showed that initial population of free bacteria cells is reduced after 120 minutes exposure in the intestine $1.6 \pm 0.8 \times 10^2$ cfu / g. While the number of microencapsulated bacteria after 2 hours of exposure was reduced from $1.6 \pm 0.8 \times 10^2$

to $2 \pm 0.4 \times 10^6$ cfu / g. Thus, the microencapsulated bacteria (D-values 27.10 min) compared to free bacteria (D-values 13.38 min) had higher survival against bile salts. Survival percent of bacteria cells (free and microencapsulated) in intestinal simulated environment are shown in figure 4.

This amount for the encapsulated and free bacterial cells is 57.06 and 17.54 percent, respectively. In this environment, encapsulated cells survived more compared to uncovered cells. Komnan et al (2009) reported that sodium alginate coating and whey protein compared with alginate coating alone, has increased the survival of bacteria in stomach simulated environment at a rate of 5-7 log cycle (9). Also the results of microencapsulation of probiotic bacteria *Lb. rhamnosus* in a coating of sodium alginate and whey protein showed improved survival of bacteria in the acidic conditions of the stomach and duodenum's bile environment (1). Yang et al (2013) reported that microencapsulation of bacteria *Lb. rhamnosus* GG in whey protein or whey and modified resistant starch protein coverage compared with the capsule consists of a modified resistant starch, has increased the viability of the bacteria in apple juice or citrate buffer (pH

= 3.5) (17). According to Rajam and colleagues (2012) found that coated bacterial cells with denatured whey protein

and alginate, could well withstand the conditions of simulated gastrointestinal (13).

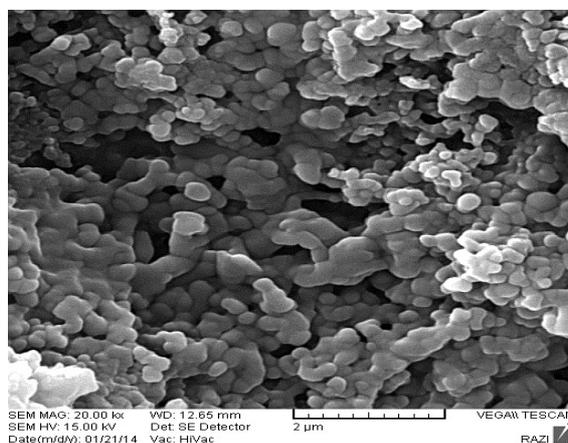


Figure1: SEM image of microcapsules containing *Lb. rhamnosus LC705*

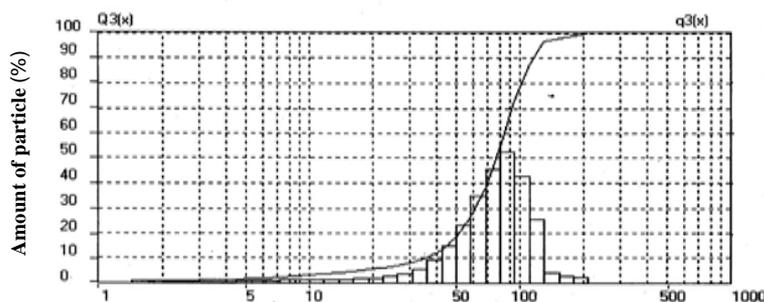


Figure 2. Particle size analysis of microcapsules.

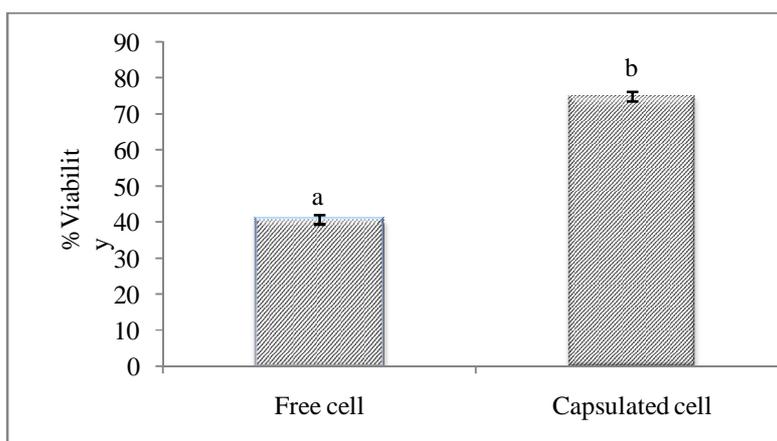


Figure 3. Effect of coating on survival of *Lb. rhamnosus LC705*. under simulated gastric conditions. Values with the same letters are not significantly different ($P > 0.05$).

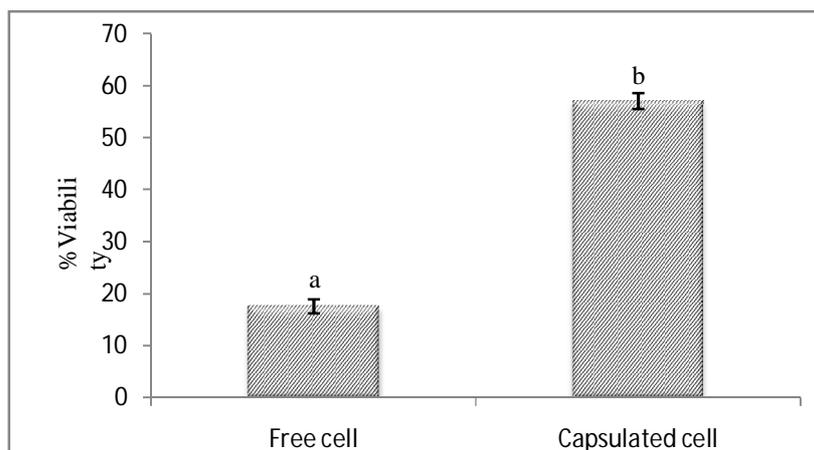


Figure 4: Effect of coating on survival of *Lb. rhamnosus* LC705. after incubation in simulated gastric and intestinal juice. Values with the same letters are not significantly different ($p < 0.05$).

Table 1: Survival of *Lb. rhamnosus* LC705 (log cfu/g) after exposure to a pH 2.5 solution at different time intervals

Cell type	Time (min)					D-value (min)
	0	30	60	90	120	
Free	$3.5 \pm 0.1 \times 10^{12}$	$4 \pm 0.2 \times 10^{10}$	$4 \pm 0.3 \times 10^7$	$4 \pm 0.1 \times 10^5$	$1.3 \pm 0.1 \times 10^5$	15.10 ^a
Encapsulated	$1.1 \pm 0.3 \times 10^{11}$	$5.5 \pm 0.1 \times 10^{10}$	$6 \pm 0.2 \times 10^9$	$4.75 \pm 0.2 \times 10^8$	$1.9 \pm 0.2 \times 10^8$	39.52 ^b

^{a, b} Values with the different letters show significant difference ($p < 0.05$); Values are average \pm standard error (n = 2).

Table 2: Survival (log cfu/g) and D-values of free and microencapsulated *Lb. rhamnosus* LC705 in simulated intestinal juice

Cell type	Time (min)					D-value (min)
	0	30	60	90	120	
Free	$3.5 \pm 0.1 \times 10^{12}$	$1 \pm 0.4 \times 10^4$	$6.1 \pm 0.2 \times 10^3$	$1.9 \pm 0.1 \times 10^2$	$1.6 \pm 0.8 \times 10^2$	13.38 ^a
Encapsulated	$1.1 \pm 0.3 \times 10^{11}$	$4.4 \pm 0.7 \times 10^7$	$3.2 \pm 0.3 \times 10^7$	$2 \pm 0.2 \times 10^6$	$1.1 \pm 0.4 \times 10^6$	27.10 ^b

^{a, b} Values with the different letters show significant difference ($p < 0.05$); Values are average \pm standard error (n = 2).

CONCLUSION

In this study, the survival of micro-organisms *Lb. rhamnosus* LC 705 isolated from breast milk of Iranian mothers was evaluated in simulated gastrointestinal tract. The results of these studies showed that the microencapsulation of bacteria in whey protein and sodium alginate coating can increase the viability of bacterial cells in the simulated condition of digestive tract. When bacteria was placed in the simulated environment of gastrointestinal tract, number

of free bacterial cells after two hours of exposure was reduced by 10 logarithmic cycle. While the encapsulated cells in such a situation fell 5 log cycles. This level of cell numbers in encapsulated condition is in the scope of the proposed levels of the International Dairy Federation (10^6 - 10^7 cfu / ml). Since the bacteria have human origin, it can be introduced as a native of probiotic strains when having other characteristics of probiotic bacteria.

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