

Immobilization and microencapsulation of *Lactobacillus casei* and *Lactobacillus plantarum* using zeolite base and evaluating their viability in gastroesophageal-intestine simulated condition

Inmovilización y microencapsulación de *Lactobacillus casei* y *Lactobacillus plantarum* usando base de zeolita y evaluando su viabilidad en condiciones simuladas de gastroesofágico-intestino

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ABSTRACT

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Aims: In order to improve survival in gastrointestinal conditions, probiotic bacteria, i.e. we developed a new encapsulation method of probiotics based on zeolite and by calcium-alginate zeolite-starch as an effective method for safer delivery of probiotics.

Material and Methods: *Lactobacillus casei* (ATCC 39392) and *Lactobacillus plantarum* (ATCC 29521) were used as probiotics. After microorganism's immobilization on zeolite base, encapsulation was done by calcium-alginate zeolite-starch with the extrusion method. Afterwards, they were incubated in the simulated gastric and intestinal condition. Viability of these bacteria was calculated on the basis of time required to reduce the logarithmic base in the microbial population. SEM techniques were used to study the appearance of the capsules.

Results: after incubation for 120 min under simulated gastric condition and 180 min in intestinal juice conditions (pH=8.2), the count of alive *L. casei* and *L. plantarum* cells were 6.3 log cfu/ml and 7.3 log cfu/ml. However, for controlling the cells, this value was zero and 2.1 log cfu/ml respectively.

Conclusion: According to the results, we had an increase of the survival rate of *L. casei* and *L. plantarum* in gastroesophageal-intestine simulated condition. These results indicated good efficacy and high performance of zeolite for immobilization and microencapsulation of probiotics.

Keywords: Probiotic, *Lactobacillus casei*, *Lactobacillus plantarum*, zeolite, Micro encapsulation.

RESUMEN

Objetivos: Para mejorar la supervivencia en condiciones gastrointestinales de las bacterias probióticas, hemos desarrollado un nuevo método de encapsulación de probióticos a base de zeolita y almidón de zeolita de alginato de calcio como método eficaz para la administración más segura de probióticos.

Material y métodos: se utilizaron *Lactobacillus casei* (ATCC 39392) y *Lactobacillus plantarum* (ATCC 29521) como probióticos. Después de la inmovilización del microorganismo en la base de zeolita, la encapsulación se realizó mediante zeolita-almidón de alginato de calcio con el método de extrusión. Posteriormente, se incubaron en la condición gástrica e intestinal simulada. La viabilidad de estas bacterias se calculó sobre la base del tiempo requerido para reducir la base logarítmica en la población microbiana. Las técnicas SEM se usaron para estudiar la apariencia de las cápsulas.

Resultados: después de la incubación durante 120 minutos bajo condición gástrica simulada y 180 minutos en condiciones de jugo intestinal (pH = 8,2), el recuento de células vivas de *L. casei* y *L. plantarum* fue de 6,3 log ufc / ml y de 7,3 log ufc / ml. Sin embargo, para controlar las células, este valor era cero y 2,1 log cfu / ml respectivamente.



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Conclusión: De acuerdo con los resultados, tuvimos un aumento de la tasa de supervivencia de *L. casei* y *L. plantarum* en condiciones simuladas de gastroesofágico-intestino. Estos resultados indicaron buena eficacia y alto rendimiento de zeolita para inmovilización y microencapsulación de probióticos.

Palabras clave: Probiótico, *Lactobacillus casei*, *Lactobacillus plantarum*, zeolita, Micro encapsulación.

1. INTRODUCTION

Probiotics are known as live microorganisms which have health promoting features and prevent from diseases by improving the microbial balance of the gut flora with the concentration of higher than 10^7 CFU/ml^{1,2}. Probiotic bacteria species are mainly consist of *Bifidobacterium* spp. and *Lactobacillus* spp. which are the important intestinal microbiota of the human gut in terms of their health³.

A large number of probiotic food products are available on the market today. Probiotics have been reported as a potential treatment for some clinical disorders such as diarrhea, gastroenteritis (diarrhea and vomiting), irritable bowel syndrome, inflammatory bowel disease (IBD), gastrointestinal tract cancer, poor digestion of lactose or allergies lipid (blood fats)^{4,5}.

The main problem of bacteria is being fragile under inappropriate environmental conditions, which could lead to the loss of their functional life. So, the best strategy to overcome this problem is immobilizing and trapping the bacteria cells⁶. The meaning of immobilization is trapping or physical establishment of healthy cells in specific areas of a carrier that maintains their desired activity⁷. Indeed, in the immobilization process the cells that normally grow on the surface or are trapped inside a building are modeled, because many of the microorganisms are able to adhere a variety of natural surfaces⁸.

Microorganisms can be stabilized on different matrices methods such as trapping within a porous material, adsorption or adhesion, inhibition of cells around a dam, and natural flocculation or artificial connection with the material, and covalent links and ionic connections^{9,10}.

Zeolite is a substance which can be admitted to stabilize due to its unique features. Zeolites are aluminous silicate crystalline which consist of AlO_4 and SiO_4 (four aspects) structural units¹¹. This tetragonal structure is connected by oxygen bridges and creates a network of canals and cavities with different sizes and shapes. Zeolites are actually created by the substitution of some Si^{+4} ions by Al^{+3} in silicates¹². Perch of aluminum ions instead of silicon ions causes a negative charge in the zeolite network, which could be balanced by metal cations. The metal cations are removable

and can be replaced with the solutions containing other cations when they are in contact¹³. The main physical and chemical properties of zeolites are related to their chemical composition and crystalline structure¹⁴. In other words, their features depend on the nature of the chemical and physical properties of water in the zeolite structure and also how water is positioned in the molecular networks. The presence of alkali and alkaline earth metals and the multiple interconnected voids and minerals are the other reasons for the development of the different zeolite properties. The application of zeolite in different industries depends on its physical and chemical properties. Some of these characteristics include density, size, shape, porosity, stiffness, adsorption, and cation exchange^{15,16}. Many researches are done for zeolite's usage as biomaterials and biosensors. Undoubtedly, usage of zeolite for creating pharmaceutical systems is one of the great and new medical applications. Because of zeolite's physical structure, it can be used for drug carrier, absorption and release control. Drugs which their reagent cause stomach problems, absorption happen in the gut and are sensitive to the digestive system, need zeolite.

Clinical and pharmacy studies prove that zeolite does not cause damage to health. Zeolite as a raw material has been used in the pharmaceutical industry in different pharmaceutical forms. Its current application is in children drugs such as chocolate form, confection and biscuit¹⁷.

Microencapsulation technology is a process in which the droplets of liquid, solid, or gaseous substances are immobilized inside the protective seed¹⁸. The general concept is that probiotic bacterial cells can be stabilized by microencapsulation and protected against harsh environmental conditions, such as osmotic pressure, bile salts, and enzymes in the digestive system. Thus, reliable and safe release of bacteria in the colon, which will be freed, will be possible^{19,20}.

The main component used in the encapsulation of probiotics is sodium alginate. This gum is a linear hetero polysaccharide, which is composed of building blocks of D-mannuronic acid and L-glucuronic acid. When the gel created, the diameter of the pores is about 17 nm. This gel can trap micron-sized bacteria in their voids²¹.

Compared with free bacteria, immobilized and encapsulated bacteria have some benefits including greater stability, continuous application, better control, higher efficiency in reactions, better recovery, higher purity of production, reduced environmental pollution and economic efficiency. So, they have found wide applications in a variety of bioreactors^{22,23}.

In this study, the probiotic bacteria including *Lactobacillus casei* and *Lactobacillus plantarum*, which were stabilized free in the zeolite form, encapsulated form with alginate and merge of stabilized and encapsulated forms coated with alginate-zeolite-starch. The core contained stabilized bacteria on the zeolite and their ability for the survival rate of bacteria was assessed in the simulated stomach-intestine conditions. In this study, we want to show the performance of alginate-zeolite-starch that has not been done for increasing the survival rate of bacteria. The future research will continue to evaluate another novel combination and help to the survival rate of bacteria.

2. MATERIALS AND METHODS

2.1. Bacterial preparation

L. casei (ATCC39392) and *L. plantarum* (ATCC 29521) were purchased as a lyophilized powder from the microbial collection of Iranian Research Organization for Science and Technology. For activation, lyophilized bacteria were grown in De Man, Rogosa and Sharpe (MRS), (Merck, Germany) broth medium²⁴. Lyophilized bacteria were poured into 5 ml MRS broth medium and incubated for 48 h at 37°C under anaerobic condition.²⁵ Biochemical tests were used for confirmation of strains species²⁶. The obtained biomass was separated by centrifugation at 4000×g for 5 min at 4°C and then washed with sterile solution of 0.1 % peptone water. The bacteria obtained from the broth medium was cultured on MRS agar medium to achieve fresh strains in logarithmic phase with the cell density of about 10⁹- 10¹⁰cfu/ml and incubated in anaerobic conditions using Gas Pak Plus (Becton Dickinson, USA) at 37°C for 18h²⁷.

2.2 Immobilization of bacteria in the zeolite base

200 ml MRS broth medium was prepared. Then, 2% (v/w) of the zeolite powder was added to this medium and autoclaved at 121°C for 15 min. The prepared suspension was placed in an ultrasonic bath for 30 min to have a uniform mixture.²⁸ The activated bacteria were added to the MRS-zeolite mixture and were incubated at 37°C by shaking. After 20h of incubation, bacteria-zeolite suspension was centrifuged for 2 min at 4000×g to stabilize the bacteria and free bacteria. For washing free bacteria, 0.1% peptone water was used and biomass was washed twice to cells that are not removed from the medium.²⁹⁻³¹ Stabilized bacteria on zeolite kept in good condition and temperature in order to be used in the case of osmotic stress.

2.3 Microencapsulation of bacteria coated with sodium alginate

Two grams of sodium alginate (Sigma Aldrich, UK) powder with medium viscosity is poured into 80 ml of distilled water and mixed. To dissolve the alginate powder and ster-

ilized, the mixture was autoclaved at 121°C for 15 min. After cooling of sodium alginate solution, active *L. casei* and *L. plantarum* obtained from the centrifuge and were added to the solution.²⁸

To produce the capsule seeds, alginate containing bacteria was extruded by the extrusion method to the firming sterile solution of 0.2M calcium chloride at 25°C and was then stirred with the magnetic stirrer at 150 rpm. After 1h, the seeds were removed from the firming medium and rinse twice with buffer solution to wash the encapsulated bacteria. The produced seeds are collected for use at the later stages and stored at 4°C in the buffer solution^{8,32}.

2.4 Formulating microcapsules with a stabilized bacterial core on the zeolite base and Na Alg-zeolite-starch coating

To prepare the capsule core, the bacteria were stabilized on the zeolite. To prepare the capsule coating, zeolite powder and starch powder are added with the ratio of 1: 1 in 20 ml of distilled water and mixed. Then, they are autoclaved to be sterile and placed in an ultrasonic bath for 30 min. They are held for 4 h on a shaker.

The amount of 2 g sodium alginate powder with medium viscosity is poured into 60 ml of deionized water and autoclaved to complete dissolution and sterilization. The starch-zeolite sterile suspension is mixed with sterile alginate and then the immobilized bacteria to the substrate are added to this mixture.

To produce capsule seeds, core and cover materials are mixed. As described, chloride firming solution was poured into calcium by the extrusion method. The prepared capsules are collected and stored after washing.

2.5 Enumerating the bacteria trapped inside the capsules

One gram of the prepared capsule samples was dispersed in 99 ml of 1% (v/w) sterile sodium citrate at pH=6 and stirred for 10 min at room temperature to dissolve the capsules completely and finally release the bacteria. Serial dilution was done in 0.9% saline for three fold and then, using MRS solid media, incubation was performed at 37°C for 24 h under anaerobic conditions and the number of bacteria was counted in triplicate²⁰.

2.6 Size and morphology of the microcapsules

Size of the capsules for each treatment and their frequency were determined using the particle diameter-measuring device (SALD-2101, Shimadzu, Japan). For this purpose, the capsules were dispersed in deionized water with the conductivity of 0.054µs and the results were reported based on volume mean diameter (VMD) particle ± standard er-

ror, d_{peak} , d_{10} , d_{50} and d_{90} . To determine the morphology and appearance of the particles, scanning electron microscope (SEM) techniques were used. Therefore, the capsules with double-sided adhesive were stabilized on the coater and covered by gold and palladium for 2 min. Observation of the capsules was performed with an electron microscope (MIRA3 FEG-SEM, Czech Republic) with 10kv electron irradiation.

2.7 Preparing gastro-intestinal tract simulated liquid

The simulated gastric liquid was prepared by dissolving 3.2 gram per liter (g/l) of pepsin and was adjusted pH to 2.1 using 0.1 M hydrochloric acid and 0.5% sodium chloride. The simulated intestinal liquid contained 10 g/l of glucose, 5 g/l of yeast extract, 10 g/l of pancreatin, and 8 g/l of bile salts; then, PH was adjusted to 8.2 using Na OH ^{33,34}.

2.8 Ability of *L. casei* and *L. plantarum* to viability in stomach simulator condition

The simulated gastric environment was sterilized using a 0.2 μ m filter. *L. casei* and *L. plantarum* were incubated in four different forms, including: free-bacteria, bacteria immobilized on zeolite, bacteria encapsulated with sodium alginate and capsulation with immobilized bacteria core coated with Na Alg-zeolite-starch.

Incubation of different microorganism forms was performed by transferring 1 ml or 1 g of bacteria in 10 ml of sterile environment of the gastric simulator.

After incubating the bacteria under the mentioned conditions, the prepared samples at 30, 60, 90, and 120 min were cultured on MRS agar medium for assessing the effect on survival of the bacteria under gastric environment. Finally, the prepared samples were incubated at 37°C for 48h under anaerobic conditions ³⁴.

2.9 Ability of *L. casei* and *L. plantarum* to viability in the small intestine simulator

Simulated liquid was passed from 0.2 μ m filter for sterilization. Four forms of bacteria after incubation under simulated stomach, according to the condition described above, were incubated in the intestinal environment at 30, 60, 90, 120, 150 and 180 min.

Then, to analyze the viability of bacteria, effects of simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) environment on the bacteria, it was pouring plate cultured on MRS agar medium and incubated at 37°C for 48 h under anaerobic condition. The initial count of bacteria in 4 forms was calculated and the loss of viability was determined after incubation in the intestinal condition ³⁴.

2.10 Statistical analysis

The ability of probiotic bacteria to viability was established based on a completely randomized design with 4 treatments of different immobilized and encapsulated forms of bacteria. Statistical Package for the Social Sciences (IBM Corporation, USA), statistical software, and Microsoft Excel 2010 were used for data analysis. ANOVA test and comparison test of Duncan at 5% were utilized for analysis of four treatments of different immobilized and encapsulated forms of bacteria. All the tests were performed with three replications.

3. RESULTS

3.1 Number of bacterial trapped in the capsule and determining particle size and dispersion

The initial number of viable cells before encapsulation was measured as 9.3- 10.01 log cfu/g. According to the data, the count of the cells trapped in the capsules was reported at 9- 9.2 log cfu/g level. The observed photos using SEM showed that the capsules were perfectly spherical. The average diameter of the capsules (VMD) 123.758 ± 0.161 μ m was measured (Figure 1). D_{peak} was also equivalent to 126.241.

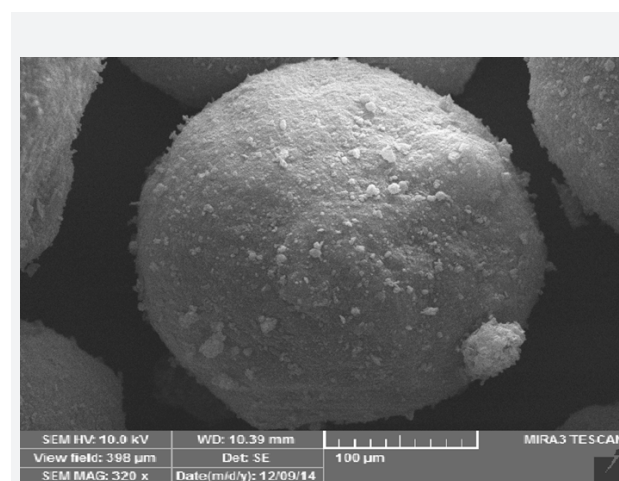


Figure 1: Scanning electron microscopy showing microencapsulation of *L. Casei* with sodium alginate-zeolite-starch

3.2 Infrared spectroscopy (FTIR)

In this research, infrared spectrum was used to study the interaction between alginate and zeolite in the bead structure. The obtained spectra and their description are given in below. Infrared spectra of alginate capsules and alginate-zeolite capsules are shown in Figure 2.

The obtained zeolite spectra, characteristic peaks were shown at 3836 1/cm (Si-O-H stretching), 3234 1/cm (OH-

stretching), 1030 and 1007 1/cm (Si-O-Si stretching), 912 1/cm (Al-O-H stretching), and 750 1/cm (Si-O stretching). In alginate beads, the appeared peaks at 1615 and 1417 1/cm could refer to symmetric and asymmetric stretching is COO^- , 3270 1/cm shows (tensile OH^-) and 1084 and 1027 1/cm shows (C-O-C tensile). When zeolite was added to alginate, it was observed that the symmetrical and asymmetrical portions of COO^- were transferred to 1621 and 1419 1/cm. That was because of the electrostatic interaction between the negative charge of carboxyl group in alginate and the positive charge of zeolite. In addition, cyanol groups (Si-OH stretching) of zeolite in the zeolite-alginate crystals disappeared and stretching OH^- moved to a higher wave number.

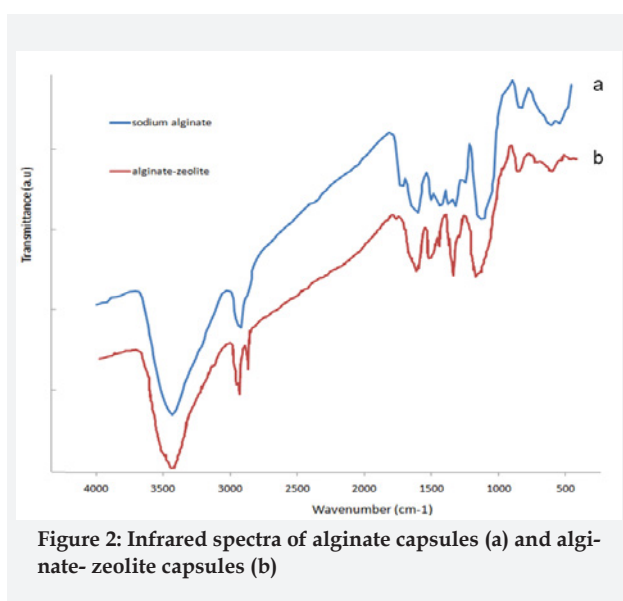


Figure 2: Infrared spectra of alginate capsules (a) and alginate-zeolite capsules (b)

3.3 Survival rate of bacteria in simulated stomach conditions

For *L. casei* with the initial population of $2.4 \times 10^9 \pm 0.1$ and *L. plantarum* with the primary population of $4 \times 10^9 \pm 0.05$ at probability level $P < 0.05$, encapsulation of alginate-zeolite-starch could provide better conditions for survival compared with other immobilization methods and free-form bacteria. After 120 min of immersion in the stomach condition, free-form of bacteria largely lost survival ability. No *L. casei* remained alive after 2 h of immersion in the stomach condition. But, at the same time, survival of *L. plantarum* was better. According to the data immobilization of bacteria on the zeolite increased survival ability about 5 logarithmic cycles in comparison with free-form of bacteria.

Effect of different models of immobilization and Microencapsulation of bacteria in the stomach simulator over time is shown in the following diagram (Figure 3 and 4).

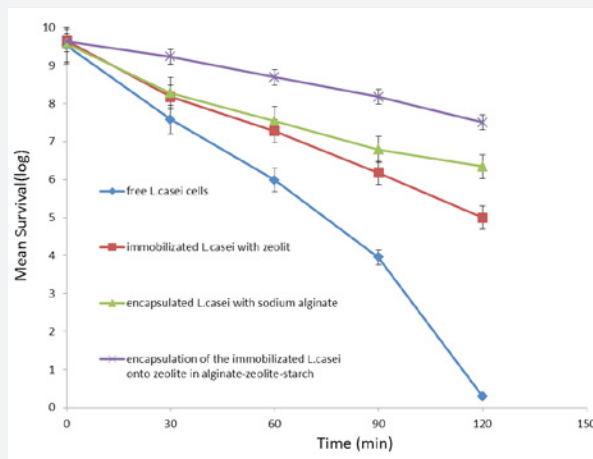


Figure 3: Survival of free, Immobilized onto zeolite, Microencapsulation of *L. casei* with alginate and encapsulation of the immobilization *L. casei* onto zeolite with alginate-zeolite-starch beads in simulated gastric juice.

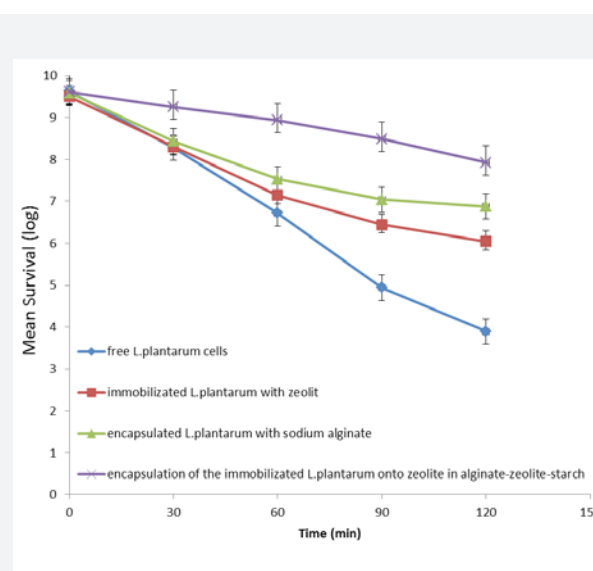


Figure 4: Survival of free, Immobilized onto zeolite, Microencapsulation of *L. plantarum* with alginate and encapsulation of the immobilization *L. plantarum* onto zeolite with alginate-zeolite-starch beads in simulated gastric juice.

3.4 Survival of bacteria in the gastrointestinal simulated conditions

Analysis of the data showed that the survival rate of all the bacteria and immobilized forms were decreased. By analyzing the average data, we concluded that the highest reduction appeared in the free form of bacteria and the lowest reduction occurred in the encapsulated and immobilized forms of the bacteria with alginate-zeolite. It was shown that the survival rate of bacteria in alginate-zeolite capsules had statistically significant difference from the other forms

($p < 0.05$). The results showed that *L. casei* could not grow after stomach environment. By the immobilization of bacteria on the zeolite base, zeolite demonstrated the ability to maintain bacteria after reducing 6 logarithmic cycles and presented better results in the SGF-SIF environment than the alginate capsules. According to the results for *L. casei*, 9.65 amounts to 3.39 log cfu/g and 63.9 amount to 3 log cfu/g was reported for the cells immobilized on zeolite and encapsulated cells with the alginate, respectively (Figure 5). In addition, for *L. plantarum*, the values of 9.5 to 5.4 log cfu/g and 9.6 to 5.6 log cfu/g were recorded in the cells immobilized on zeolite and encapsulated cells with the alginate, respectively (Figure 6).

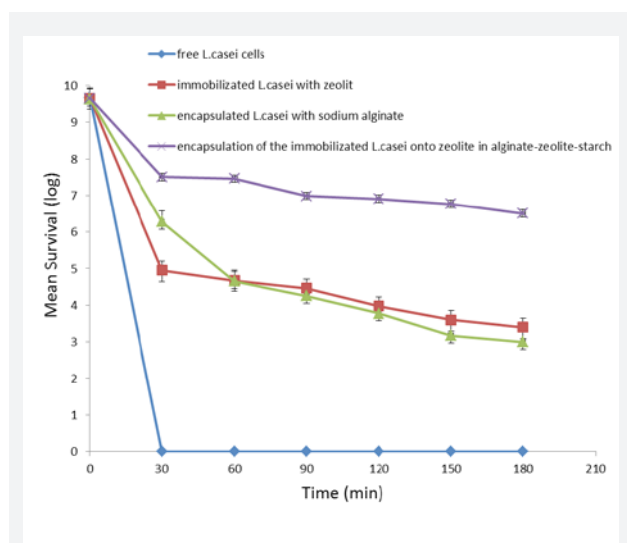


Figure 5: Survival of free, Immobilized onto zeolite, Micro-encapsulation of *L. casei* with alginate and encapsulation of the immobilization *L. casei* onto zeolite with alginate-zeolite-starch beads in simulated intestinal juice after incubation in simulated gastric juice.

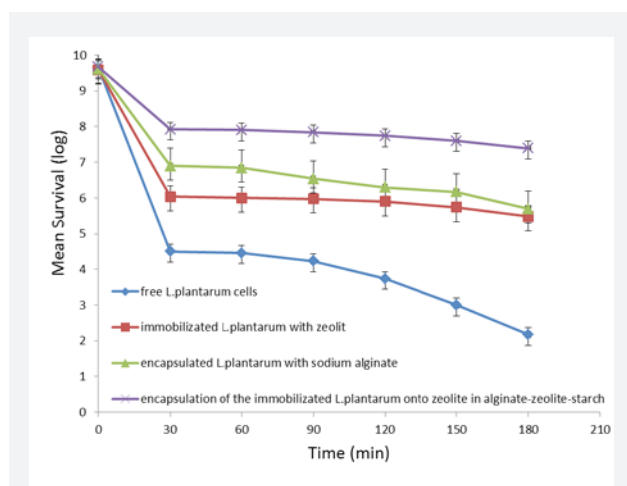


Figure 6: Survival of free, immobilized onto zeolite, micro-encapsulation of *L. plantarum* with alginate and encapsulation of the immobilization *L. plantarum* onto zeolite with alginate-zeolite-starch beads in simulated intestinal juice after incubation in simulated gastric juice.

4. DISCUSSION

The minimum loss in bacterial count showed the accuracy of capsulation stage. In other words, based on these results, capsulation had no effect on the initial count of bacteria. The results of particle size indicated that we could produce micron-sized capsules by this method, which could create a softer texture in food. This result corresponded with the previous research published about softer texture and size of microcapsules.^{22,35}

FTIR data revealed intermolecular hydrogen bonds and electrostatic forces between alginate and zeolite. These results found that alginate-zeolite beads could be used in the process of encapsulation. Obtained result from the immobilization of bacteria on zeolite base showed the increasing rate of bacteria survival. It seems that due to its unique structure protects the bacteria from gastric environmental stress and led it to milder mortality slope and increase survival by 5 logarithmic cycles than the free form of bacteria. In addition, the results showed that the performance of the alginate capsule to deal with acidic conditions was better than zeolite due to the compression of capsules. Therefore, it disallowed greater penetration of the stomach environment in to the capsules. The results were in conflict with those of other researchers who announced that capsulation did not have any protective role of bacteria in the stomach condition³². But, in accordance with the results of researchers about the capsulation of *Lactobacillus acidophilus*, it could be found that alginate capsules after entering the intestinal environment due to changes in pH and presence of enzymes begin to swell and become more permeable.^{36,37} In addition, the bacteria without proper protective could be exposed in the intestinal environment and have less protective features toward immobilized bacteria on zeolite.

According to the initial count of bacteria and obtained results, significant differences were observed between the mentioned forms of bacteria in survival and the best models of immobilized and encapsulated bacteria by zeolite. Because of its unique structure and the protective layer of hydrogel that caused delay in the penetration of gastric juice and effect of the intestinal tract on bacteria. These results are consistent with the findings by other researchers.^{36,37}

It should be noted that in vitro bacterial resistance to bile does not indicate their actual behavior, because of like other physiological shocks, their realistic simulation is difficult. It is observed that environmental factors may strengthen or weaken the resistance of microorganisms to the special agent. Pre-treatment of bacteria with acid, as well as different temperatures and atmospheric conditions effects on bacterial resistance to bile and can become more resistant. In addition, unlike the experimental conditions, the amount

of bile acids in the intestine and against different people with different ages is not constant; until the consumption of high-fat foods, the amount of this combination in the intestine will be very low. This is a factor to adaptation of bacteria and increase their resistance to bile is considered. The presence of food in the intestine can cause a protective shield for the microorganisms and some probiotics without contact with bile operate in the intestine. The in vitro activity of bile salts in the intestinal tract may be much higher than their real action, , therefore, in the intestine it is possible to combine these salts with phospholipids ¹⁹.

5. CONCLUSION

In this study, the viability of immobilized bacteria on the zeolite base and encapsulated in alginate-zeolite-starch beads were reviewed and was compared with the free form of bacteria in the gastrointestinal simulated environment. In addition, high performance of alginate-zeolite-starch was confirmed in terms of increasing the survival rate of bacteria. Observations by an electron microscope using SEM methods confirmed the presence of fully spherical and uniform capsules. Compared with the encapsulated cells and uncovered cells, the covered cells had better biological capability. In the stomach condition, this kind of coating caused an increase by about 7.2 (log cfu/g) for *L. casei* and 4.1 (log cfu/g) for *L. plantarum*. Also, incubated in succession in gastrointestinal, capsulation increased survival rate 6.5 (log cfu/g) for *L. casei* and 5.2 (log cfu/g) for the *L. plantarum*. According to the results of the present study, high performance of alginate-zeolite-starch was confirmed in terms of increasing the survival rate of probiotics.

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