ARTICLE

CHARACTERISTICS OF BEADS FROM LACTOBACILLUS ACIDOPHILUS PROBIOTIC MICROENCAPSULATION WITH CALCIUM ALGINATE AND RESISTANT STARCH

Fereshteh Ansari[1], Hadi Pourjafar[2]*

[1]*Iranian Evidence Based Center of Excellence, Tabriz University of Medical Sciences, IRAN*

[2]*Department of Basic Sciences, Maragheh University of Medical Sciences, Maragheh, IRAN*

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ABSTRACT

Background: *Lactobacillus acidophilus has large beneficial impacts as a probiotic and the low viability of the bacteria and other probiotics in the extreme acidic- biliary conditions of gastrointestinal tract and food products have always encouraged researchers to seek approaches for improvement of these indicators.* ***Material and method:*** *Microencapsulation as one of the newest methods has revealed sub stantial out comes in this respect. The aim of this study was to evaluate the morphological and protective features of beads obtained from the microencapsulation of L. acidophilus probiotic. Microencapsulation by calcium alginate and resistant starch was carried out through extrusion.* ***Result and discussion:*** *The strength of beads was determined within 12 h and the survival rate of encapsulated bacteria was as certained in the hydrochloric acid solution, 0.1 M phosphate buffer, and a solution containing digestive powder during 120 min.* ***conclusion:*** *The results showed that the stability levels of beads were different in various media, whit physical tension playing an important role. Under adverse environmental conditions, microencapsulation with calcium alginate and resistant starch plays a critical role in the protection of L. acidophilus probiotic. The survival rate of microencapsulated bacteria in all conditions was significantly higher than free bacteria [p < 0.05].*

KEY WORDS
Microencapsulation, probiotic, Lactobacillus acidophilus, calciumalginate, resistant starch

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***Corresponding Author**
Email:
drhpsglad@yahoo.com

INTRODUCTION

Probiotics are live microorganisms that are found in the gastrointestinal tract with a specific number and exert one or more beneficial effects on the health of the host. Some notable beneficial effects are increased food digestion, boosted immune system, and increased resistance to infection, reduced blood cholesterol, as well as anti-mutagenic and anticancer properties [1]. In addition, probiotics are today introduced as viable alternatives to antibiotics to deal with pathogens in humans and animals leading to considerable acceptance and consumption of probiotic foodstuffs and drugs [2]. The foods containing such bacteria are classified as functional foods. According to the recommendation by the International Dairy Federation [IDF], such foods should contain 107 cfu/g probiotic bacteria and the consumer should use at least 100 g/day of the food to acquire the beneficial effects of the foods in this category [1 &3].

The low viability of the bacteria and other probiotics in the extreme acidic- biliary conditions of gastrointestinal tract and food products have always encouraged researchers to seek approaches for improvement of these indicators. Microencapsulation as one of the newest methods has revealed substantial outcomes in this respect [3,4]. From a microbiology perspective, microencapsulation includes covering a layer of hydrocolloid around living cells that protects them from adverse conditions of surrounding environment and raises the survival of these cells [5]. Different materials such as gelatin, chitosan, etc. have been used for the encapsulation of probiotic bacteria. However, microencapsulation with calcium alginate has largely been applied for this purpose, particularly on lactic acid bacteria, due to numerous advantages such as non-toxicity, safety to the human body, reasonable prices, and convenient handling [6, 4, 3].Alginate combined with starch and in particular resistant starch has shown better results because it leads to the formation of an additional capsule around the beads raising the wall stability and subsequently the viability of bacteria therein[7].

L. acidophilus is the predominant natural intestinal flora of humans and animals. It is also found in the usual fermentation products and possess many beneficial effects listed above as the most important and most widely used probiotic [8]. It is, therefore, of particular importance to examine and enhance the survival of this bacterial species within food products through a fitting and strong microencapsulation and probiotic drugs and also to enable the transfer and safe release of the bacteria in appropriate locations of the gastrointestinal tract. Moreover, the translocation of a variety of other probiotic bacteria such *as L. casei* was studied in this respect [9].

On the one hand, it is of paramount importance to study the morphology of beads obtained from microencapsulation such as shape, size, numbers of wall layers and beads capsules lying as a buffer between the bacteria and the exterior space, bacterial distribution within the beads, matrix density surrounding bacteria, and finally bacterial release from the beads [7, 10, 11].Furthermore, several studies have been conducted on the stability and protective capacity of the beads [maintaining a high viability of probiotic bacteria] in the unfavorable conditions of gastrointestinal tract and internal milieu of some food products including cheese, yogurt, and honey [12, 13, 14]. The strengthening effect of using UV radiation has recently been examined for the proper inherent stability of beads [15]. The aim of this study was to evaluate the morphological and protective characteristics of beads from encapsulated *L. acidophilus* probiotic as the prevailing normal flora of human gut by calcium alginate and resistant starch.

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MATERIALS AND METHODS

This is an in vitro experimental study conducted on the beads obtained from microencapsulation *of L. acidophilus* and assessing the survival rates of both free and encapsulated bacteria.

Activation of L. acid ophilus Freeze-dried starter culture is usually released in the market. The starter culture package for *L. acidophilus* La5 [CHR-Hansen, Horsholm, Denmark] was opened with care in sterile conditions. One gram of starter was uniformly mixed in 100 ml of MRS broth [de Man-Rog as a-Sharpe] and incubated at 37 °C for 24 h in order for the starter to completed or mancy with the absorption of water and enter the logarithmic growth phase. The next day, one ml of the culture obtained was diluted again with 99 ml of fresh medium [1%] and incubated at 37 °C. During the week, the culture was transferred to the fresh medium three times and after incubation for 24 h, stored at 4 °C in order to permanently access to probiotic bacteria in the logarithmic growth phase [7].

Purification of bacteria

In this study, almost 2 ml of medium prepared in the starter culture activation and stored at 4 °C was transferred to ca. 200 ml of fresh medium and incubated at 37 °C for 18 h, which was used for the purification of the target bacteria. For this purpose, the medium was thoroughly stirred to a homogeneous state and centrifuged [Centurion centrifuge, Model 2010, West Sussex, BNI8OHY, UK] in 10000 rpm for 10 min. After centrifugation, the supernatant was discarded and the bacterial pellets in the micro tubes were centrifuged twice by normal saline [0.09%] to be thoroughly washed [16]. The bacterial emulsion was used for microencapsulation process.

Microencapsulation

In this study, the probiotic was microencapsulated by calcium alginate and resistant starch by extrusion method using a multi-nozzle extruder micro encapsulated device [17]. To do this, a mixture of sodium alginate [Sigma, USA] and corn resistant starch, Hi-maize [Merk, Darmstadt, Germany] with a purity of 99.9% and a significant amount of the bacteria was prepared by purification from the medium in sterile distilled water.

Sodium alginate [20 g] was added to 200 ml of distilled water followed by sterilization. Alginate solution was then refrigerated overnight in order for alginate particles to adequately absorb water. Afterwards, alginate solution was transferred to the laboratory at the same temperature. The resistant starch [20 g] was gently added to the alginate solution and stirred by a magnet at regular rounds on a hot plate unit [I KA Labortechnik, Model 79219 staufen, KG, Germany]. Afterwards, 10 micro tubes containing the bacterial emulsion [totally 10 ml] prepared in the previous step were evacuated into the alginate/starch mixture followed by the addition of circa 0.5 mltween 80 [Merk, Hohenbrunn, Germany]. The resulting mixture was slowly placed in the micro encapsulated tank to complete the microencapsulation process. After injecting the mixture into 1.0 M calcium chloride solution, the capsule walls were perfectly formed as a result of alginate contact with calcium ions. The beads were deposited as droplets in calcium chloride solution [7, 16, 18]. The beads obtained were finally collected from the tank outlet.

Release of bacteria from beads

To release *L. acidophilus* bacteria from the beads, one g of beads was stirred in 1.0 M phosphate buffer [9 ml, pH=7] on a clipped shaker [IKA- Model Janke & Kunkel GMBH. Type VX5-Germany] for 30 min to dissolve the beads as a homogenate [7 and 16].

Assessing the stability of beads

The stability of microencapsulated beads was evaluated through the production of beads [1 g] with a diameter of approx. 50- 200 µ affected by the following processes at each stage:

1. Hydrochloric acid [9 ml, pH = 2] with and without mechanical stress [conducted by magnets at 400rpm for each experiment];
2. Phosphate buffer solution [1.0 M, 9 ml, pH = 7] with and without physical tension;
3.Distilled water [9 ml] containing digestive powder [including 4500units of amylase, 6000 units of lipase, 50 ml of hemicellulose, 25 ml of bovine bile extract, and pH = 8.3] with and without physical tension incubated at 37^{0}C.

The stability of beads and viability of the containing bacteria were evaluated under the influence of the above conditions at different times [from 30 min up to 12 h]. In case the beads were destructed, one ml of the solution in which the beads were dissolved was harvested, transferred to MRS broth medium, and incubated for 24 h to detect the survival of bacteria released to the medium. If the beads were preserved within the maximum time [12 h], the bacteria were initially released, transferred to MRS broth medium, and incubated for 24 h to realize bacterial growth/lack of growth within the beads.

Evaluation of encapsulated bacterial survival in hydrochloric acid solution, phosphate buffer solution, and a solution containing digestive powder, with and without physical tension were carried out.

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For this purpose, the beaded microencapsulated bacteria [1 g] and 1 ml of the free bacteria [one of the tubes containing bacterial pellet emulsified by saline] were added under similar conditions to hydrochloric acid solution [10 ml, pH= 2], 1.0 M phosphate buffer solution [10 ml, pH = 7], and the solution containing digestive powder [10 ml, including 4500 units of amylase, 6000 units of lipase, 50 ml of hemicellulose, 25 ml of bovine bile extract, and pH = 8.30], all of which were already autoclaved [121°C, 15 min] and incubated at 37° C in two conditions of with and without mechanical stress. In order to evaluate the survival rate of microencapsulated cells at zero, 30, 60, 90, and 120 min, each medium was diluted by peptone water [0.1 percent] and incubated in MRS- Salicin-agar medium as a mixed culture at 37°C for 72 h [19]. Independent t-test [α = 0.05] was used to compare the number of live bacterial cells at each of the time periods stated above.

RESULTS

Assessing the stability of beads in hydrochloric acid solution [Fig. 1] revealed that the structure of beads was maintained within 12 h without physical tension and the containing bacteria were capable of growing after release and transfer to the medium. The structure of beads was completely dissolved with physical tension after3 h, after which the bacteria were immediately able to grow in the medium.

Evaluating the stability of beads in phosphate buffer solution [Fig. 1] showed that the structure of beads was maintained within 12 h without physical tension and the containing bacteria were capable of growing after release and transfer to the medium. The structure of beads was completely dissolved with physical tension after 30 min, after which the bacteria were able to grow in the medium.

Examining the stability of beads in digestive powder solution [Fig. 1] clarified that the structure of beads was entirely dissolved within 10 h without mechanical tension and the containing bacteria were capable of growing after release and transfer to the medium. The structure of beads was completely dissolved with physical tension after 1 h, after which the bacteria were able to grow in the medium.

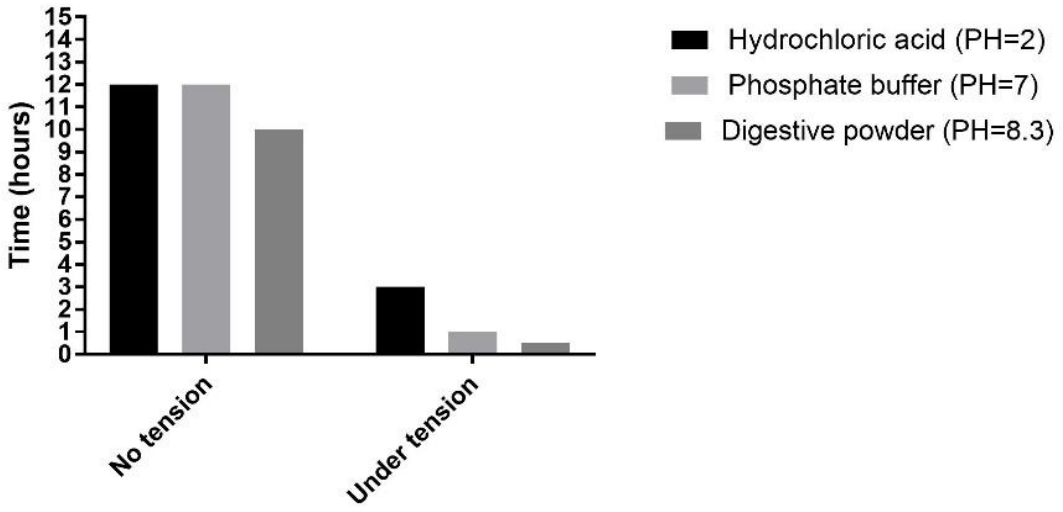

Fig. 1: Stability of beads in hydrochloric acid [pH = 2], phosphate buffer [pH = 7] and digestive powder [pH = 8.3] in two physical states [no physical tension and under physical tension].

[Fig. 2] shows the survival rates of free and encapsulated cells of *L. acidophilus* La5 after incubation in hydrochloric acid, phosphate buffer, and digestive powder solutions with and without physical tension, respectively, at 37 °C within 2 h. Independent t-test [α = 0.05] compared the number of live bacterial cells at each time period stated. In all circumstances, the number of microencapsulated live bacterial cells was significantly greater than free forms [$p < 0.05$]. Additionally, the number of free and microencapsulated live cells under no physical tension was significantly higher than those under physical tension [$p < 0.05$].

DISCUSSION

Several studies conducted on the survival of *L. acidophilus* indicate the particular importance of the bacteria as a natural and important human gut flora as well as an important bacterial species in most fermentation products [20, 21, 22, 23]. Micro encapsulation is considered one of the newest methods to increase the viability of probiotic bacteria. In the present study, the morphological features of beads obtained from micro encapsulated *L. Acidophilus* probiotic was shown by calcium alginate and resistant starch using extrusion method. In numerous studies, morphological characteristics of beads from encapsulated probiotic bacteria have been studied via different methods and using light and electron microscopy [24, 11, 10, 6]. Our results showed that the stability of beads was different under the influence

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of diverse media, with physical tension playing an important role. In a study by Simpson et al. [2004], it was concluded that calcium chloride increases alginate gel stability, and that greater levels of Ca+2 resulted in elevated capsule thickness, hence, the stability [25]. Anselmi et al. [2002] used a new technology to improve the microencapsulation characteristics. They applied the strengthening effect of UV radiation and discussed the proper inherent stability of beads, low toxicity, a better resistibility, and a simple cheap formulation as the main goals of the technology [15].A number of recent and ongoing investigations conducted in this regard suggests the importance of increasing the stability of beads containing probiotics both in the product until use and during passage through the digestive tract until it reaches the target area of probiotics' function, i.e. the colon [26, 30].

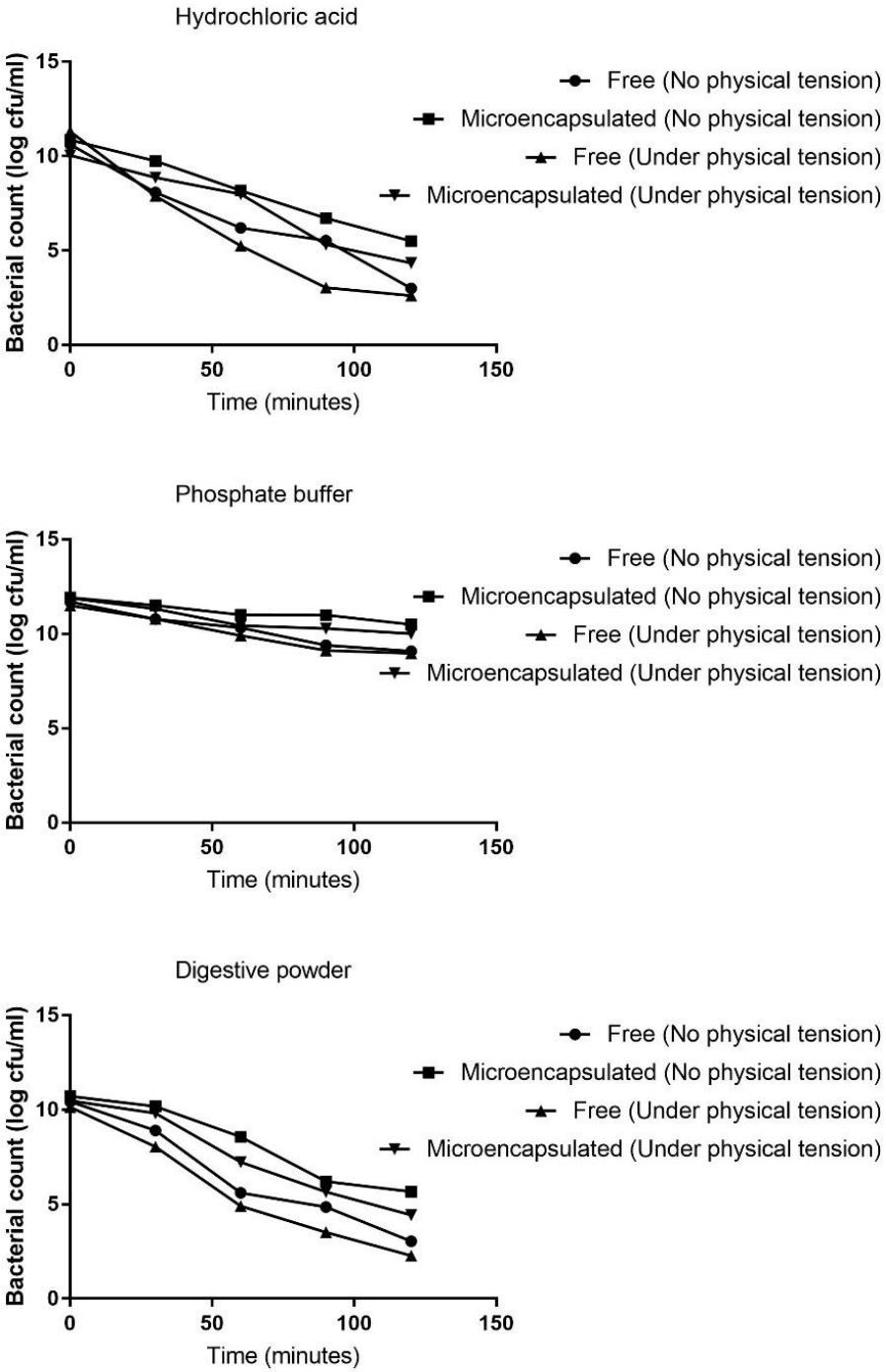

Fig. 2: Free and microencapsulated *L. Acidophilus* viability during 2 h incubation at 37°C in three chemical treatments [hydrochloric acid, buffer phosphate, and digestive powder] and two physical sates [no physical tension and under physical tension].

Note: In each graph, the viability of microencapsulated bacteria is significantly higher than free forms and viability significantly decreased under physical tension in both free and microencapsulated bacteria [P <0.005].

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The results of bacterial counts at zero, 30, 60, 90, and 120 min of incubation at 37 °C in hydrochloric acid, phosphate buffer, and digestive powder solutions with and without physical tension showed that microencapsulation with calcium alginate and resistant starch plays a crucial role in protecting *L. acidophilus* probiotic. The survival rate of microencapsulated bacteria was significantly higher than free forms in all conditions. This means that microencapsulation as a barrier reduces the adverse effects of unfavorable conditions on the bacteria leading to their extended survival. This is consistent with the findings of Krasaekoopt et al. [2004] [10]. Likewise, Sultana et al. [2000] encapsulated probiotic bacteria with alginate-starch and studied the survival of bacteria in conditions similar to gastrointestinal fluid and in yogurt. Their results presented evidence on the improvement oflive bacterial encapsulation using high-amylose starch or Hi-maize [as a prebiotic] in comparison with no starch application. They further observed that the survivability of *L. acidophilus* and encapsulated Bifi do bacteria strains decreased as much as 0.5 log and 1 log in free cells within 8 weeks of incubation in yogurt. The combined effect of alginate-starch in increased survival of probiotic bacteria atadverse conditions corresponds our observations [24].

Kailasapathy et al. [2002] [30] studied encapsulated probiotic bacteria, probiotic products, and increased survival of these organisms in products, especially in the gastrointestinal tract of human. They noted that encapsulation is carried out with the aim to provide a physical barrier to protect probiotics against adverse environmental conditions as well as immobilization of probiotic bacteria in biotechnology. Different micro encapsulation methods were also discussed in their study stating that calcium alginate is the major part of this technology indicating the importance of this substance. Other materials other than alginate with the highest usage were introduced as Kappa-carrageenan, gellan gum, gelatin, and starch. Research needs in this area include the design of small-sized beads at micro and nano scales with high stabilities and many commercial applications. Food carriers reported include yogurt, cheese, ice cream, and mayonnaise [6].

CONCLUSION

The overall results of this study show that the microencapsulation of *L. acidophilus* probiotic, known as important flora of the human gut and fermented products, is of particular importance resulting in significant increased survival of the bacteria at difficult environmental conditions. It is recommended to investigate other substances such as gelatin, chitosan and poly-L-lysine for microencapsulation of *L. acidophilus* and other beneficial intestinal bacteria.

CONFLICT OF INTEREST
There is no conflict of interest.

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