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ORIGINAL ARTICLE

Preparation of Alginate Beads Containing *Lactobacillus acidophilus* Using $AlCl_3$ as a Cross Linker

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ABSTRACT

Beads of alginate-containing probiotic bacteria of *Lactobacillus acidophilus* were prepared by an extrusion method using $AlCl_3$ as a cross linker and characterized in terms of size, morphology and encapsulation efficiency. The optimum formulation was selected, and the viability of encapsulated *L. acidophilus* after acid treatment (pH = 2, duration = 2 hours) was investigated. Spherical beads ranging from 1.45 mm to 1.65 mm in size were achieved. The shape and size of the optimum beads (F9) prepared using 3% alginate and 3.5% $AlCl_3$ as a cross linker were not significantly different than those of beads prepared using 3% alginate and 3.5% $CaCl_2$ as a cross linker (F10). However, substitution of $AlCl_3$ for $CaCl_2$ significantly increased the acid viability of the encapsulated *L. acidophilus* in F9. The increased acid viability is probably due to the greater crosslinking within the structure of the produced coat, which is permitted by the extra linkage sites in $AlCl_3$. Additional experiments to evaluate the physicochemical and morphological structure of the prepared coat should be performed to explain the exact mechanism of protection. *Biomed. Int.* 2013; 4: 87-93.

Key words: Alginate, bead, cross linker, *Lactobacillus acidophilus*

INTRODUCTION

As a global trend towards naturally originating materials, there has been a special attention to "probiotics." Probiotics are defined as "live microorganisms (bacteria or yeasts), which when ingested or locally applied in sufficient numbers confer one or more specified demonstrated health benefits for the host".[1] These benefits can mainly be categorized as maintenance of normal intestinal microflora, immunomodulation, and metabolic effects.[2,3] Among the diverse category of probiotics, including bacteria, moulds, and yeasts, lactic acid bacteria (LAB), typically associated with the human gastrointestinal tract, are the well-known probiotic microorganisms. LAB possess various degrees of intrinsic resistance against harsh conditions of gastrointestinal track. However, to efficiently use the beneficial specifications of the probiotics, they should transit safely the gastric tract and colonize and grow on the epithelium of colon in sufficient population (a minimum count of 10^{6-7} CFU/g or ml of viable probiotic bacteria).[1,5] Various techniques have been utilized to appendage this intrinsic resistance so far that the most applicable one is the encapsulation of probiotics in wide variety of polymers like alginates.[6] Alginates belong to a family of unbranched

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binary copolymers of (1 → 4)-linked β-D-mannuronic acid and α-L-guluronic acid residues.[7] The ion binding properties of alginates for multivalent cations is the basis for their gelling properties. Preparation of alginate bead, with well-retained bacteria in their matrix, can be easily achieved by simple techniques like extrusion or emulsion methods. Calcium chloride is the frequently and widely used cross linker agent for the preparation of alginate beads in order to encapsulate the probiotic bacteria.[4]

Aluminum chloride is a trivalent cation which is expected to form a three dimensional valent bonding structure with the alginate and consequently it has the potential to produce stable and stiff beads.[8] In spite of the potential merits of $AlCl_3$, it has not been applied as crosslinking agent to prepare alginate beads in probiotic coating strategies, according to the published literatures. As a result, the objective of the present work is to investigate the potential of $AlCl_3$ as cross-linker in the preparation of alginate beads. Different beads of alginate containing *Lactobacillus acidophilus* were prepared using $CaCl_2$ or $AlCl_3$ as cross-linker via extrusion technique and their sizes, morphologies and bacterial viabilities in acid (pH 2, 2 h) conditions were evaluated.

MATERIALS AND METHODS

Materials

L. acidophilus DSMZ20079 was obtained from DSMZ (Germany), Sodium alginate, oxgall from Sigma-Aldrich (Germany), MRS broth and MRS agar, Peptone water, Sodium hydrogen phosphate, Calcium Chloride, Aluminum Chloride, Sodium Chloride, and hydrochloric acid from Merck (Germany).

Methods

L. acidophilus was cultured in MRS broth at 37°C for 24 h. Culture was harvested by centrifugation at 700 g at 4°C for 7 min and washed twice with saline and collected by centrifugation as above. The washed bacterial cells were resuspended in 7 ml saline and the cell count was determined using pour plate technique in MRS agar in triplicate. The cell suspension divided in some equal parts and consequently was used to prepare different formulations.

The extrusion technique was used to prepare alginate beads.[9] Sodium alginate and other solutions sterilized at 121°C for 15 min. The cooled alginate solutions (25 ml) were mixed with bacterial inoculums (5 ml) and gently stirred for 30 min to obtain a homogeneous suspension. This suspension was extruded drop wise through a 27 gage nozzle into sterile hardening solution ($CaCl_2$ or $AlCl_3$ with different concentration). The beads were washed twice with sterile water and kept in 0.1 % peptone solution at 4°C. The studied formulations were represented in *Table* 1.

Table 1: Composition of the studied formulations.

Formulation	%Alginate	%Cross linker
F1	1	2%$AlCl_3$
F2	2	2%$AlCl_3$
F3	3	2%$AlCl_3$
F4	1	3% $AlCl_3$
F5	2	3% $AlCl_3$
F6	3	3% $AlCl_3$
F7	1	3.5% $AlCl_3$
F8	2	3.5% $AlCl_3$
F9	3	3.5% $AlCl_3$
F10	3	3.5%$CaCl_2$

The particle size of beads was assessed using optical microscopy (Dino-lite, Taiwan) by Scion image analyzer software. Data were collected from 20 beads in each sample and mean particle size was reported as the average of maximum and minimum feret diameter. Also, to determine the shape factor of the beads, aspect ratio was calculated as the ratio of the width to the height of the beads.

The topographical properties of selected beads were investigated by scanning electron microscopy (SEM) (Philipse XL30, Holland) at an accelerating voltage of 20 KV. Prior to examination, samples were prepared on aluminum stubs and coated with gold under argon atmosphere by means of a sputter coater.

To determine the encapsulation efficiency, firstly 10 beads were mechanically disintegrated in 5 ml of phosphate buffer (pH =6.8), then the number of entrapped cells was measured by pour plate method and counts were expressed as number of colony forming units (CFU). Encapsulation efficiency was calculated as: EE= (Log $_{10}$N /Log $_{10}$N$_0$) ×100, where N is the number of viable entrapped cells released from the beads, and N$_0$ is the number of free cells added to the alginate mixture immediately before the production procedure.

A low pH condition was produced using hydrochloric acid 0.01 M and pH was adjusted to 2. 30 beads or 100 µL of cell suspension were entered into 20 ml of the acid solution and incubated for 120 min at 37°C. After incubation, 1m aliquot of free cells and 10 beads were collected. The beads firstly were disintegrated in 5 ml phosphate buffer (pH =6.8), then 1.0 ml of the mixture removed and assayed using pour plate method. The aliquots of free cells were neutralized by adding 1 M NaOH and their counts were determined as pour plate method.

The survival (%) of the bacteria was calculated as follows: %Survival = (log CFU/g beads after 2 h exposure to acidic condition/ log CFU/g beads initial count) × 100.

RESULTS AND DISCUSSION

Characterization of prepared beads: size, morphology and encapsulation efficiency

In the preliminary experiments, different concentrations of alginate solution (1 to 3% w/v) and $AlCl_3$ solution (2 to 3.5% w/v) were examined. $CaCl_2$ in the optimum concentration of 3.5% w/v was used as a control according to the results of our previous work.[10] According to the results of the preliminary experiments; it was found that regardless of the type of hardening solution ($AlCl_3$ or $CaCl_2$), bead formation by alginate concentrations less than 1% w/v was quite difficult. It was in good agreement with majority of previous reports in this regard. It can be due to the decreased viscosity of the alginate solution and concomitantly less ion sites for the cross-linkage.[11,12] On the other hand, construction of the beads by alginate concentrations more than 3% also was also impossible, using our method, as a result of the high viscosity of the resultant alginate solution. In fact, high viscose gel did not passage easily through the nozzle in order to prepare the beads.[11] Nevertheless, source of alginate and consequently its chemical structure also has a dominant role in the optimization of the gel concentration for bead construction.[4] As a result, bead formation using the extrusion technique and $AlCl_3$ as cross linker was achieved in the alginate concentrations between 1-3%.

Table 2: Size and aspect ratio of the studied formulations

Formulation	Mean size (mm) ±SD	Aspect Ratio
F1	1.61±0.04	0.49
F2	1.57±0.05	0.55
F3	1.52±0.04	0.63
F4	1.58±0.03	0.53
F5	1.60±0.08	0.66
F6	1.49±0.06	0.92
F7	1.59±0.05	0.55
F8	1.52±0.06	0.66
F9	1.46±0.07	0.99
F10	1.45±.005	0.99

In the next step, the morphological characteristics of the prepared beads were investigated (*Figure* 1). The results showed that the acceptable morphology (e.g. uniformity and sphericity of beads) could not obtain when the concentration of alginate is less than 2% w/v. Indeed, as can be seen from the shape factors in *Table* 2, in the concentrations of 1-2%, the prepared beads were irregular and un-spherical regardless of the concentration of $AlCl_3$. Consequently, alginate concentration of 3% w/v was selected for optimum bead preparation.

On the other hand, a slight decrease in beads size was observed by increasing the $AlCl_3$ concentration from 2% to 3.5%. As can be seen from the results in *Table* 1, the size of 1.52 mm in the case of F3 decreased to 1.46 mm in F9. Moreover, based on our results the increase in the $AlCl_3$ concentration from 2% to 3% led to increase in aspect ratios and consequently the shape factors of the obtained beads ($p<0.05$). However, there were no significant differences between the aspect ratios of beads prepared using 3% and 3.5% of $AlCl_3$. Overall, in the alginate concentration of 3% and $AlCl_3$ concentration of 3.5% our optimum beads were produced and these beads were selected for further investigations.

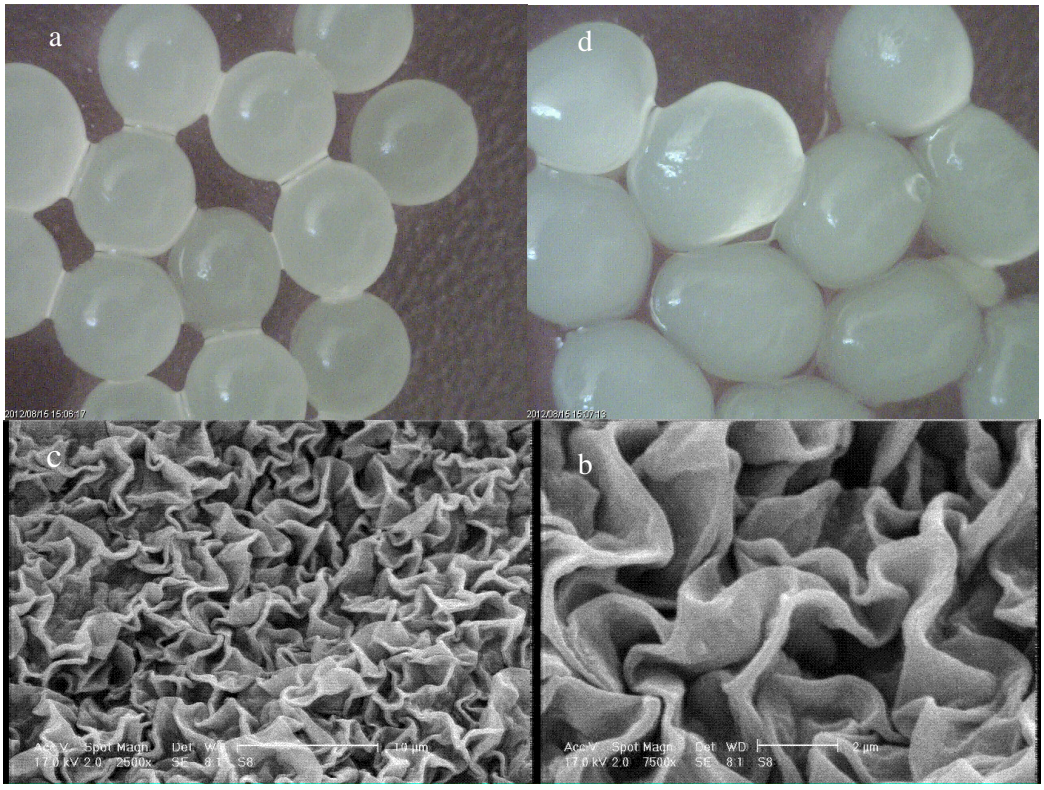

Figure 1: Optical microscope images of F9 and F10 (a and b) and electron microscope images of F9 (c and d).

Table 3: Encapsulation efficiency and % survival in acid condition of selected formulations (n=6)

Formulation	%EE±SD	%Survival in acid condition ±SD
F9	99.8±0.9	64.0±0.5
F10	99.1±0.5	59.6±0.9
Untreated Cells [a]	-	39.1 ± 0.8

[a]inoculum count: 8.23 ± 0.04 CFU/ml

Encapsulation efficiency (%EE) of the optimum beads was 97%, which was significantly higher than the corresponding bead's EE prepared using $CaCl_2$ ($p<0.05$) which was in line with our expectations and can probably due to the quantity of the bonding sites in $AlCl_3$ in comparison with that in $CaCl_2$. Since $CaCl_2$ is a divalent cation, its bonding to alginate was probably occurred in a two dimensional structure. On the other hand, trivalent aluminum cation, with three bonding opportunity was expected to form three dimensional valent bonding structure with the alginate.[8,12] The schematic representation for the possible cross linkage of $AlCl_3$ and $CaCl_2$ with alginate was depicted in *Figure* 2.

Evaluation of the viability of encapsulated bacteria in prepared beads in acid conditions

The effect of 2 h exposure to acid conditions (pH=2) on the viability of *L. acidophilus* was evaluated and the results were expressed in *Table* 3. Overall, the viabilities of bacteria after acid exposure, in prepared beads of $CaCl_2$ and $AlCl_3$ were 59.6% and 64% respectively, that were significantly ($P< 0.05$) higher than those of untreated cells (39%). In fact, the initial count of 8.23 ± 0.04 CFU/ml in the inoculum decreased to 3.2± 0.07 CFU/ml after 2 h exposure to acid condition, which was indicated around 5 log reductions in the bacterial population. Whereas, by encapsulation of *L. acidophilus* in alginate beads ($AlCl_3$ or $CaCl_2$) less than 3.5 log reduction in bacterial population was observed. It can be concluded that coating of the bacteria as alginates beads, regardless of the type of cross linker ($AlCl_3$ or $CaCl_2$), could improve the viability of *L. acidophilus* in acid condition. Protection of probiotics by encapsulation in alginates beads has been the subject of several studies so far[16] and majority of these investigations support our finding about the ability of alginate coat in protection of bacteria in acid condition.[9,14,15,16]

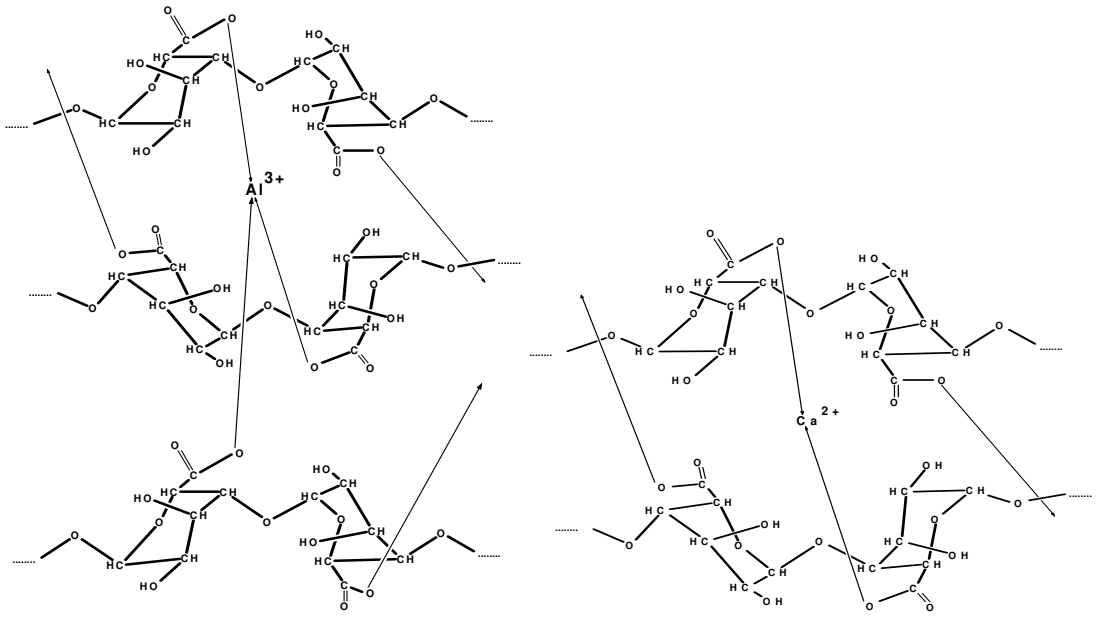

Figure 2: Expected mechanism of reaction between calcium and aluminum cations and sodium alginate matrices

On the other hand, the survival of *L. acidophilus* after 2 h exposure to acid condition in beads prepared with $AlCl_3$ is significantly higher than that in beads prepared using $CaCl_2$ ($p<0.05$). Indeed, the count of 8.18 ± 0.04 CFU/ml loaded in F9 lessened to 5.2± 0.2 CFU/ml (2.9 log reduction) in comparison with the count of 8.12 ± 0.08 CFU/ml loaded in F10 which was declined to 4.7± 0.09 CFU/ml (3.4log reduction) after 2 h exposure to acid condition.

This finding can probably explained by the structure of the obtained layers using $AlCl_3$ or $CaCl_2$. The main mechanism for bacterial protection inside alginate beads can be the protective effect of the surrounding layer and its physicochemical structure. Extra cross linked layer could result in more firm coat and therefore better protection of the encapsulated bacteria. The trivalent aluminum cation with three bonding site for alginate could produce more cross linked three dimensional structure. It could protect the bacteria more efficient than two dimensional structure produced with divalent $CaCl_2$.[8]

CONCLUSION

The probiotic bacterium, *L. acidophilus*, was encapsulated by extrusion method in alginate using $AlCl_3$ as cross linker. Morphological characterizations of the selected beads prepared using $AlCl_3$, in terms of shape and size were not significantly different than those of beads prepared by $CaCl_2$. However, substitution of $AlCl_3$ for $CaCl_2$ significantly increased the acid viability of the encapsulated *L. acidophilus* that was probably due to the more cross linked structure of the produced coat as a result of extra cross linkage site in $AlCl_3$. Additional experiments to evaluate the physicochemical and morphological structure of the prepared coat should be performed to explain the exact protection mechanism.

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