The Effect of Alginate, Chitosan, and Nano Chitin as Encapsulation Materials of *L. Casei* Probiotic Bacteria

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ABSTRACT--- ***Alginate, nano chitin, and chitosan polymers can be used to protect the Lactobacillus casei from gastric conditions. The goal of this study was to determine the effect of alginate, nano chitin, and chitosan as encapsulation materials of L. casei on their survivability in simulated gastric fluid (SGF). The encapsulation process in this study was carried out by the extrusion method. The resulted beads were soaked in SGF (pH of 1.2 and 3) for 1 and 60 min at 37°C. In SGF pH 1.2 for 60 min, the survivability of L.casei in all variations of the experiment was 0% except those encapsulated from alginate (1%), nano chitin (0.2%), and chitosan (0.2) % of 75.35%. In SGF pH 3 for 60 min, the survivability of L.casei was 0% for beads unencapsulation and encapsulation made from alginate, while the highest survivability of L.casei was 81.22% obtained in various encapsulation experiments using alginate (1%), nano chitin (0.2%), and chitosan (0.2%). The addition of nano chitin or chitosan to L.casei encapsulation material can increase the survivability of L.casei, also showed that the combination of alginate, nano chitin, and chitosan in the encapsulated material significantly increased the survivability of L.casei at SGF pH 1.2 and 3.***

Keywords--- Encapsulation, *L.casei*, alginate, nano chitin, chitosan

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1. INTRODUCTION

Alginate Based Encapsulation Material

Alginate is the most frequently used matrix for immobilizing probiotics, this is due to its economic efficiency and biocompatibility [1]. Some of the advantages of alginate beads are their resistance to acidic environments [2]. However, alginate porosity can be a drawback in its application as a probiotic drink. The presence of pores provides a way for gastric acid to enter the microbeads so that it has a great chance to kill the probiotics encapsulated by the alginate. Therefore, the combination of alginate with polymers or other foodstuffs is needed to overcome this porosity[3, 4].

Alginate is a natural polymer that has been successfully applied as a pH-sensitive material for the encapsulation of probiotic bacteria [5]. Alginate is a polysaccharide extracted from algae consisting of various quantities and sequential distributions of β-D-mannuronic (M) and α-L - guluronic acid (G) (copolymers containing MM, GG, and irregular sequences of M units. and G) which can affect the functional properties of alginate as the encapsulation material [6]. When sodium alginate solution containing a cell suspension is poured into a calcium solution, the bound ions interact with other GG blocks to form a complex leading to gel formation and possible release of cells residing in the intestinal tract [1].

Chitosan-Based Encapsulation Material

Treatment of chitin with an alkaline solution converts it to either completely or partially deacetylated chitosan. Chitosan can be defined as a natural, non-toxic biopolymer and a linear polysaccharide consisting of β-1,4-GlcNAc and β-1,4-GlcN. Chitosan is insoluble in water but dissolves in aqueous organic acid solutions [7]. Unlike chitin, chitosan is not a component in animal species and is rarely found in nature. Natural sources of chitin, including crab and shrimp shells, squid bone plates, and cuttlefish do not contain chitosan, however, fungi synthesize chitin and chitosan in their cell walls [7]. Chitosan is an important component of the cell wall of *Zygomycetes* [7]. Chitosan is also naturally found in the mycelia, stalks, and spores of *Basidiomycetes, Ascomycetes*, and *Phycomycetes* [8]. Chitosan from crustacean sources has a high molecular weight (MW) with low polydispersity, degrees of N-acetylation (DA) below 20%, and a solution viscosity of 1% from 500-1,700 cps. While mushroom chitosan has low MW with high polydispersity, DA lower than 15%, and 1% solution viscosity than 10-15 cps[7]. Annually, approximately 150,000 tonnes of industrially usable chitosan is derived from the conversion of chitin which is obtained as a byproduct of seafood processing. Most of the chitosan is used in cosmetics, organic fertilizers, and food supplements [9]. Chitosan can be distinguished based on the number of acetylation units of D-glucosamine. Chitin contains more than 70% acetate units, while chitosan has less than 30% acetylation. With organic acids such as formic acid, acetic acid, and ascorbic acid, chitosan forms salts and consequently becomes water-

soluble [7]. Chitosan contains three reactive functional groups, an amino or N-acetamide group along with two primary and secondary hydroxyl groups at positions C-2, C-3, and C-6 respectively. The main difference between the structure and physicochemical properties of different chitosan is the amino- or N-acetamide group [7]. The classification of chitosan can be carried out according to the residual N-acetate fraction (FA), DA, polymerization level (DP), MW, MW distribution (PD or Polydispersity), and N-acetylation pattern (PA) or sequence [10]. Chitosan offers great potential for applications in various industries due to its distinctive physicochemical characteristics such as biocompatibility, biodegradability, and low toxicity [7]. Figure 1 below showed the chemical structure of chitosan.

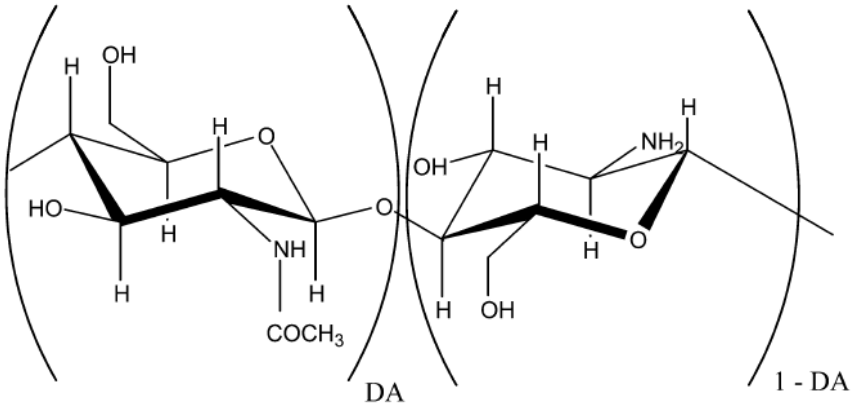


Figure 1. The structure of chitosan with a degree of acetylation (DA) [11]

Chitosan can form a gel with sodium alginate (non-toxic multivalent anion contraction) by ionic cross-linking [12]. The alginate beads coating and its efficiency in protecting probiotics have been extensively studied for several years. Previous studies have found that coating the alginate beads with chitosan has a significant effect on alginate stability, thereby increasing the viability of the encapsulated probiotics [4].

The researchers have encapsulated *L. casei* probiotics with extrusion techniques and carried out microbeads testing in SGF at 37ºC [13, 14]. The results of the research showed that at pH 6.5 (control), the viability of *L. casei* cells in SGF remained above $8Log_{10}$ CFU/g after 2 hours of incubation at 37ºC whether encapsulated or not. At pH 2, no free cells survived after 2 hours incubation in SGF and the results showed that *L. casei* was sensitive to SGF (pH 2). Under the same conditions (pH 2, 30 min), the number of live cells of *L. casei* in alginate beads, and alginate-chitosan beads were 7.97, and $8.09Log_{10}$CFU/g, and with increasing times of up to 120 min, the reduction of the two types of beads were $0.86Log_{10}$CFU/g, and $0.71Log_{10}$CFU/g, respectively. The viability of *L. casei* under acidic conditions indicated that there was a decrease in CFU/g similar to bacteria at pH 2 and 3. It was clear that the viability of encapsulated cells was significantly better than that of free cells after exposure to SGF (pH 2) and alginate beads [13]. The survival of probiotics was lower in gastric juice and decreased further as the incubation period increased. After 120 min, the survival of free *L. casei* decreased from 2.51×10^{11} to 1.1×10^{3} CFU/mL. However, the cell number of coated and uncoated encapsulated *L. casei* decreased to 6.3×10^{7} and 6.2×10^{6} CFU/mL respectively after 120 min. In the case of *L. casei*, the survival of cells in both coated and uncoated beads was significantly ($P < 0.05$) better than that of free cells and alginate-gelatinized starch with chitosan coating provided the best protection for both probiotics [14].

The alginate matrix, which consisted of an open lattice structure, forms porous beads and it was used to modify chitosan in a polyelectrolyte complex [15]. This approach was therefore employed here. The structure of chitosan was modified by cross-linking with alginate via ionic interaction between the carboxyl residues of alginate and the amino terminals of chitosan [15]. A conceptual representation of this was depicted in Figure 2. This complexation reduced the porosity of the alginate beads and decreases the leakage of the encapsulated substances.

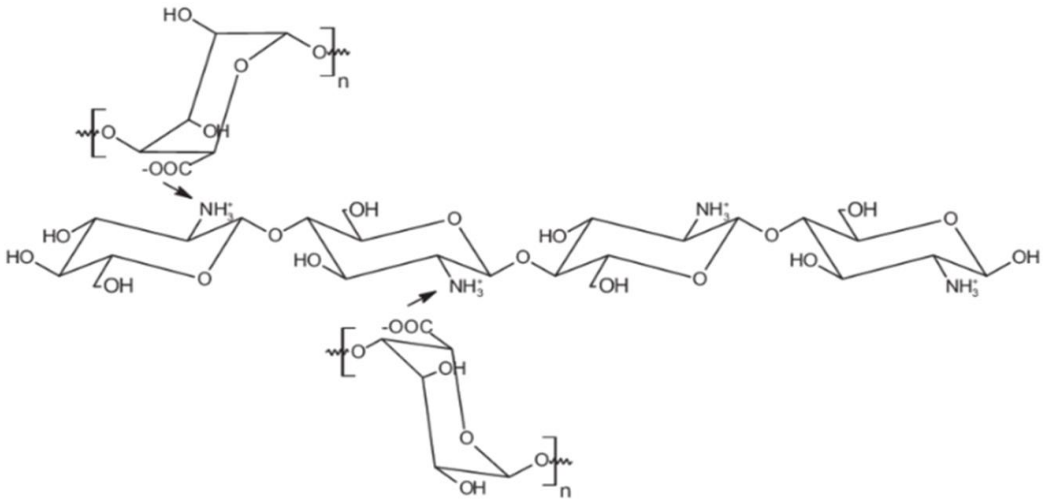


Figure 2. Chitosan-alginate cross-linking interaction [15]

Nano chitin

Chitin is a very important and abundant natural polysaccharide after cellulose. Chitin was first identified in 1884. Chitin occurs in nature as microfibril crystals to form compound structures in the arthropod exoskeleton or the cell walls of fungi and yeast. Although chitin is widespread until recently the main sources of chitin used commercially were crab shells and shrimp [7]. Chitin is tasteless, odorless, and insoluble in water, generally organic solvents, inorganic acids, and dilute bases. Chitin has antitumor, antimicrobial, antifungal properties, and can increase immune power[7]. Also, its biodegradable and nontoxic properties are considered to be used as a nutrient delivery system in the digestive system or encapsulation that can be applied with nanotechnology. The excess of food material at the nanometer scale affects its bioavailability and nutritional value [16].

2. EXPERIMENTAL SECTION

Equipments

Scanning Electron Microscopy (SEM) (JEOL JSM-IT300), Petri dishes (Normax), *ose* needles, beaker glass (Pyrex), measuring flask (Pyrex), Erlenmeyer, test tubes, stirring rods, funnels, spatulas, volume pipettes, micropipettes (Effendorf), watch glass, shaker incubator (CERTOMAT® BS-1), autoclaves (Tomy SX- 300), incubator (Memmert 854 Schwabach), Laminar Air Flow (ETL), refrigerators, wrap, aluminum foil, plastic vortex (Thermo), analytical balance, glass preparations, microscopes, pH meter (Horiba, Japan), hot plates, magnetic stirrers, syringe (Therumo) 22 G (inner diameter 0.394 mm), syringe 60 mL, filter paper Whatman no. 40.

Materials

The chemicals used were Na-alginate 1% (HIMEDIA® REF MB-114-100G), chitosan 0.2% (Sigma Aldrich PCode: 101729402), $CaCl_2$ Solution 32 g/L, NaCl 0.2% pH 1.5, Na-Citrate 1%, MRSA (de Man Rogosa Sharpe Agar) (Merck, KGaA) and MRSB (de Man Rogosa Sharpe Broth) (Merck, KGaA).

Procedure

Making sodium alginate solution containing the bacterial suspension

The preparation of sodium alginate solution was first made of alginate solution 40 mL with a concentration of 1% (w/v) with distilled water. Then sterilized by autoclaving at 121ºC for 15 min. After the sodium alginate solution has been cooled to room temperature then 10 mL *L. casei* suspension was added.

Making chitosan solution

The preparation of the chitosan solutions was made with a concentration of chitosan 0.2% (w/v). 0.2 g chitosan was dissolved in a 100 mL acetic acid solution (1% (v/v); pH 3.1). pH chitosan solution was adjusted to pH 6 using 1 M NaOH, then the solution was sterilized by autoclaving at 121°C for 15 min.

Encapsulation of L. casei using alginate-chitosan mixture

The *L. casei* probiotic encapsulation method chosen was the extrusion method and the ionic gelation method. The extrusion method was chosen because besides using a simple tool in the form of a syringe, this method was also chosen to avoid extreme temperatures and pressures and from unfavorable environments such as low temperatures in the freeze-

drying method, which can result in reduced bacterial viability[17]. While the choice of ionic gelation method was due to a simple process, did not use organic solvents, and can be controlled easily. While the principle of particle formation in the ionic gelation method was the occurrence of ionic interactions between the carboxylic anion (COO^-) of the alginate monomer and the divalent cation (Ca^{+2}). Cross-linking occurs because a calcium ion replaced two sodium ions in alginate. This crosslinking structure caused limited molecular motion and inhibits the development of polymers in a medium [18]. The extrusion technique of microencapsulation was used [17]. Alginate beads were prepared as follows: sodium alginate was dissolved in distilled water (1%, w/v) and sterilized at 121°C for 15 min. After cooling the alginate mixture, the cell suspension (10 mL) was mixed with the alginate mixture (40 mL) homogeneously and injected through a syringe into a sterilized 125 mL $CaCl_2$ (32 g/L) solution that was stirred continuously to form beads. The beads were allowed to harden for about 30 min in the $CaCl_2$ solution and then washed with the distilled water. The beads were dried under controlled air flow and temperature (4°C). Alginate–chitosan beads were prepared as follows: the wet state of alginate coated beads was soaked in 50 mL of chitosan solution (0,2%, w/v) and stirred for 15 min using a magnetic stirrer. The beads were dried under controlled air flow and temperature (4°C) [20].

Encapsulation of L. casei using alginate-nano chitin mixture

Alginate beads were prepared as follows: sodium alginate was dissolved in distilled water (1%, w/v) and sterilized at 121°C for 15 min. After cooling the alginate mixture, the *L. casei* cell suspension (5 mL) and the nano chitin (5 mL; 0.2% (w/v)) suspension were mixed with the alginate mixture (40 mL) homogeneously and injected through a syringe into sterilized 125 mL $CaCl_2$ (32 g/L) solution that was stirred continuously to form beads. The beads were allowed to harden for about 30 min in the $CaCl_2$ solution and then washed with the distilled water. The beads were dried under controlled air flow and temperature (4°C) [20].

Encapsulation of L. casei using alginate-nano chitin-chitosan mixture

Alginate beads were prepared as follows: sodium alginate was dissolved in distilled water (1%, w/v) and sterilized at 121°C for 15 min. After cooling the alginate mixture, the *L. casei* cell suspension (5 mL) and the nano chitin (5 mL) suspension were mixed with the alginate mixture (40 mL) homogeneously and injected through a syringe into sterilized 125 mL $CaCl_2$ (32 g/L) solution that was stirred continuously to form beads. The beads were allowed to harden for about 30 min in the $CaCl_2$ solution and then washed with the distilled water. The beads were dried under controlled air flow and temperature (4°C). Alginate-nano chitin-chitosan beads were prepared as follows: the wet state of alginate-nano chitin coated beads was soaked in 50 mL of chitosan solution 0,2% (w/v) and stirred for 15 min using a magnetic stirrer. The beads were dried under controlled air flow and temperature (4°C) [20].

Surface morphoLogy and bead size determination

The shape and surface characteristics were determined by scanning electron microscopy (SEM) using a gold sputter technique. The beads were vacuum-dried, coated with gold-palladium, and observed microscopically. The size of the *L. casei* loaded beads was measured with SEM.

Viability of L. casei loaded in beads

To determine the viable counts of the encapsulated *L. casei*, 1 g of beads were resuspended in 9 mL of sodium citrate (1 %) and stirred for 120 min using a magnetic stirrer. The complete release of bacteria from the beads in 120 min was previously assured by comparing the released number of cells from the beads. The colony-forming units (CFU/g) were determined by anaerobic plating on MRS agar plate and incubating at 37°C for 48 h. The plating procedures were carried out in triplicates. Non-encapsulated *L. casei* was enumerated in the MRS agar as control.

Viability and survivability testing of L. casei probiotic processes

Simulation gastric fluid (SGF) consisted of 0.2% Sodium chloride with a pH of 1.2 and 3 (adjusting the pH by adding 0.5 M hydrochloric acid). 1 g beads (extrusion flow rate of 1 mL/min with extruder voltage of 0 kilo Volt (kV)) were soaked in 9 mL SGF, incubated for 1, and 60 min at a temperature of 37°C. After that, it was filtered using Wattman 40 filter paper then soaked in 9 mL (1 g/100 mL) sterile sodium citrate solution with slow stirring at room temperature. Then sequential dilution was prepared to reach the number of cells that can be calculated by pouring a suspension technique that was spread on MRS Agar media. After that, it was incubated for 48 hours at 37°C [21]. The total colony can be calculated by the Total Plate Count (TPC) method.

3. RESULTS AND DISCUSSION

The results of *L. casei* observations using SEM with a magnification of 10,000 times were shown in Figure 3 below.

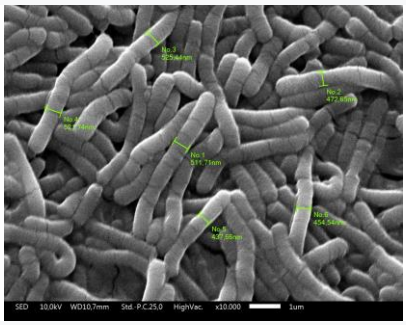
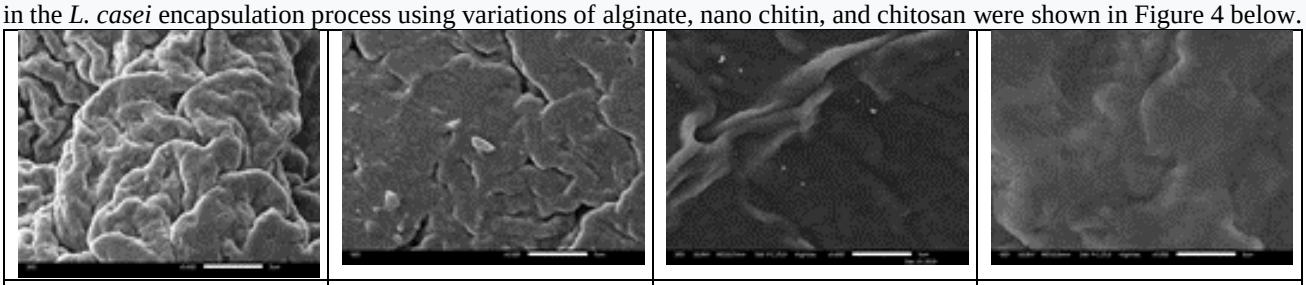


Figure 3. Observations of *L. casei* using SEM with a magnification of 10,000 times

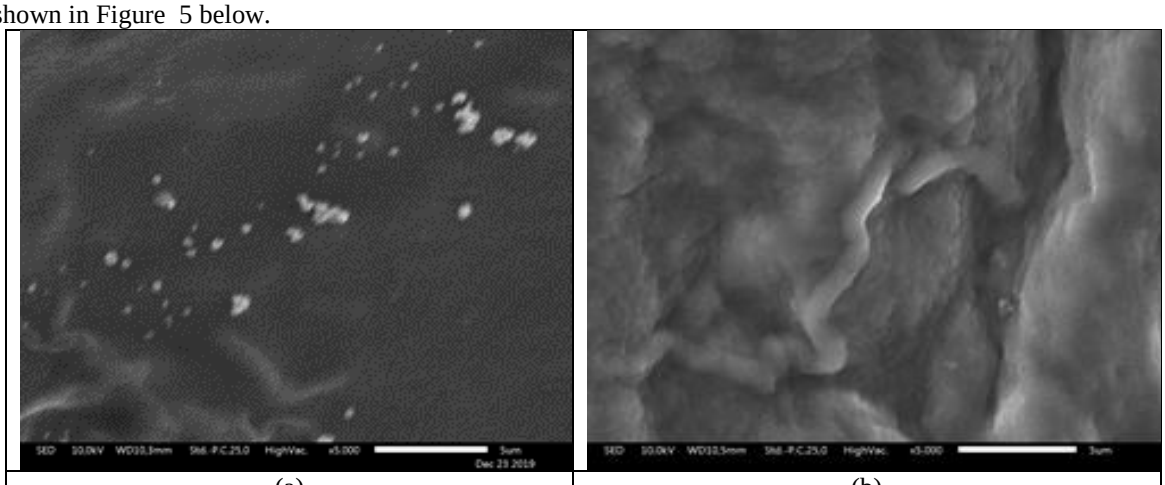
Figure 3 showed that *L. casei* was a rod with a width of about 0.5 µm and a length of 4 µm. The beads produced in the *L. casei* encapsulation process using variations of alginate, nano chitin, and chitosan were shown in Figure 4 below.



(a)	(b)	(c)	(d)

Figure 4. SEM analysis results of probiotics and beads were encapsulated before soaking in SGF, **(a)** ((Na-alginate 1% + *L.casei*) + $CaCl_2$); **(b)** ((Na-Alginate 1% + *L.casei*) + $CaCl_2$ + chitosan 0,2 %); **(c)** ((Na-alginate (1%) + *L.casei* + nano chitin 0,2%)+ $CaCl_2$) ; **(d)** ((Na-alginate 1% + nano chitin 0,2% + *L.casei*) + $CaCl_2$ + chitosan 0,2%)

Figure 4(a). showed that the beads formed by the alginate material produced a pore-rich surface. Meanwhile, the alginate beads coated with chitosan tend to have fewer pores, as shown in Figure 4(b). Meanwhile, the nano chitin mixed directly with alginate in the syringe tube produced a much smoother surface as shown in Figure 4(c). Figure 4(d) showed the surface of the beads made of nano chitin mixed directly with alginate in the syringe tube, then after forming the beads again coated with chitosan, the surface of the beads was much smoother and denser than Figure 4(a), (b) and (c).). The beads morphology was shown in Figure 4(d) was the best beads. The beads that have been soaked in SGF pH 1.2 liquid were shown in Figure 5 below.



(a)	(b)

Figure 5. SEM analysis results of probiotics and encapsulated beads after soaking in SGF pH 1.2, (a) ((Na-alginate (1%) + *L.casei* + nano chitin 0,2%)+ $CaCl_2$) ; (b) ((Na-alginate 1% + nano chitin 0,2% + *L.casei*) + $CaCl_2$ + chitosan 0,2%)

Figure 5 showed that the beads soaked in SGF pH 1.2 caused *L.casei* in the beads to start to push out the surface of the beads. The viability of A (free cell/unencapsulated), B (encapsulated using alginate 1%), C (encapsulated using alginate 1%, chitosan 0.2%), D (encapsulated using alginate 1%, nano chtin 0.2%), E (encapsulated using alginate 1%, nano chtin 0.2%, and chitosan 0.2%) at an extrusion voltage of 0 kV and the extrusion flow rate of 1 mL/min was shown in Figure 6 below.

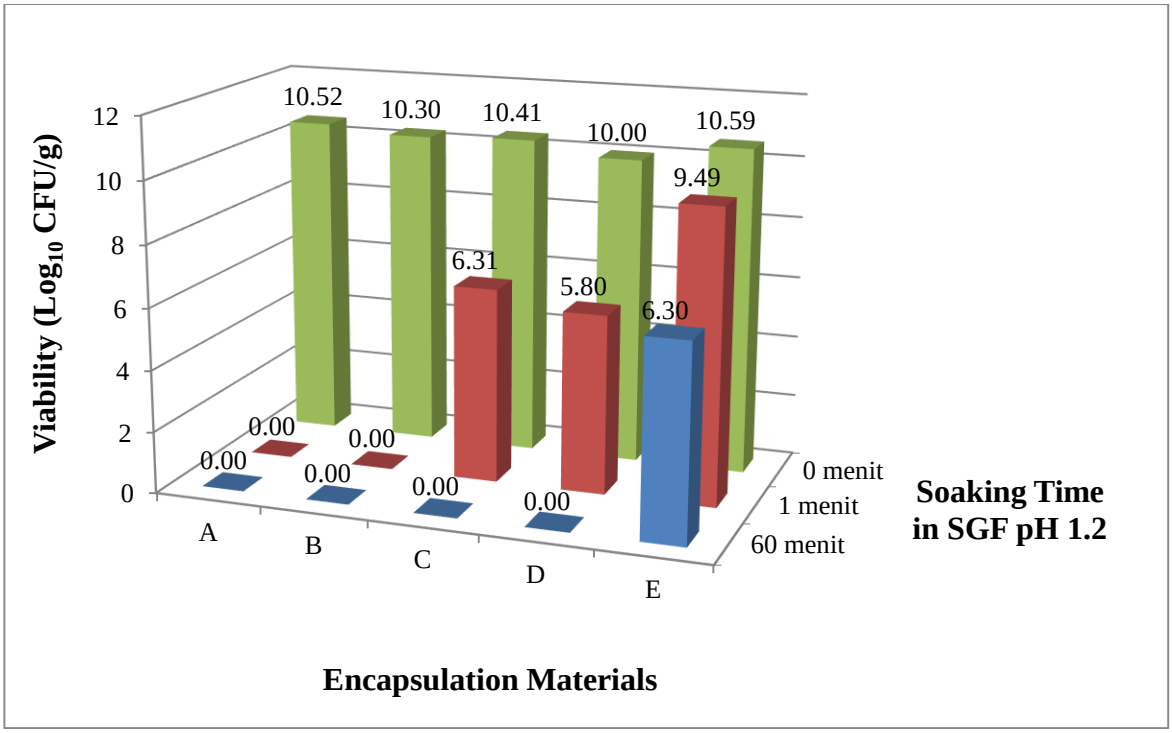


Figure 6. Viability of *L. casei* before and after soaking in SGF pH 1.2

Figure 6 showed that the initial viability value of *L. casei* ranges from 10 to 10.59Log_{10} CFU/g. This value showed the viability that was quite feasible for the soaking process in SGF. Figure 6 showed that the viability value of *L. casei* was 0 CFU/g, after soaking in SGF pH 1.2 for 1 min. Meanwhile, the highest viability was achieved by beads made of a mixture of alginate-nano chitin-chitosan which was 9.49Log_{10}CFU/g. This indicated that *L.casei* cannot survive in SGF pH 1.2, either unencapsulated or encapsulated with alginate. The surface of the beads which were formed from alginate material still had a lot of pores so that SGF liquid can easily penetrate the inside of the beads and came into direct contact with *L. casei* so that it was killed. The beads that were added with nano chitin and chitosan to the alginate beads tended to have a much tighter surface, thereby reducing the SGF liquid penetrating the inside of the beads. This happened because the nano chitin was mixed directly with the alginate in the syringe so that when the encapsulation process was formed, the nano chitin was physically trapped in the alginate which resulted in reduced alginate pores. While chitosan was added after the beads were formed by soaking the beads in a solution of chitosan, so that the surface of the beads was coated with chitosan which was an ionic bond between the amino group $-NH_3^+$ of chitosan with the COO^- carboxyl group of alginate [12]. This results in the pores of the alginate on the surface of the beads closed finely [22].

The viability value of *L. casei* was 0 Log_{10}CFU/g, after soaking in SGF pH 1.2 for 60 min. This showed that *L.casei* cannot survive in SGF pH 1.2 for 60, both unencapsulated and encapsulated with alginate, alginate-nano chitin, and alginate-chitosan. Meanwhile, beads made from a mixture of alginate-nano chitin and coated with chitosan had a viability of 6.3Log_{10}CFU/g. This showed that the beads formed with alginate-nano chitin-chitosan showed advantages over alginate, alginate-nano chitin, and alginate-chitosan. The survivability of *L.casei* unencapsulation and encapsulation that had been soaked in SGF pH 1.2 for 1 min and 60 min was shown in Figure 7 below.

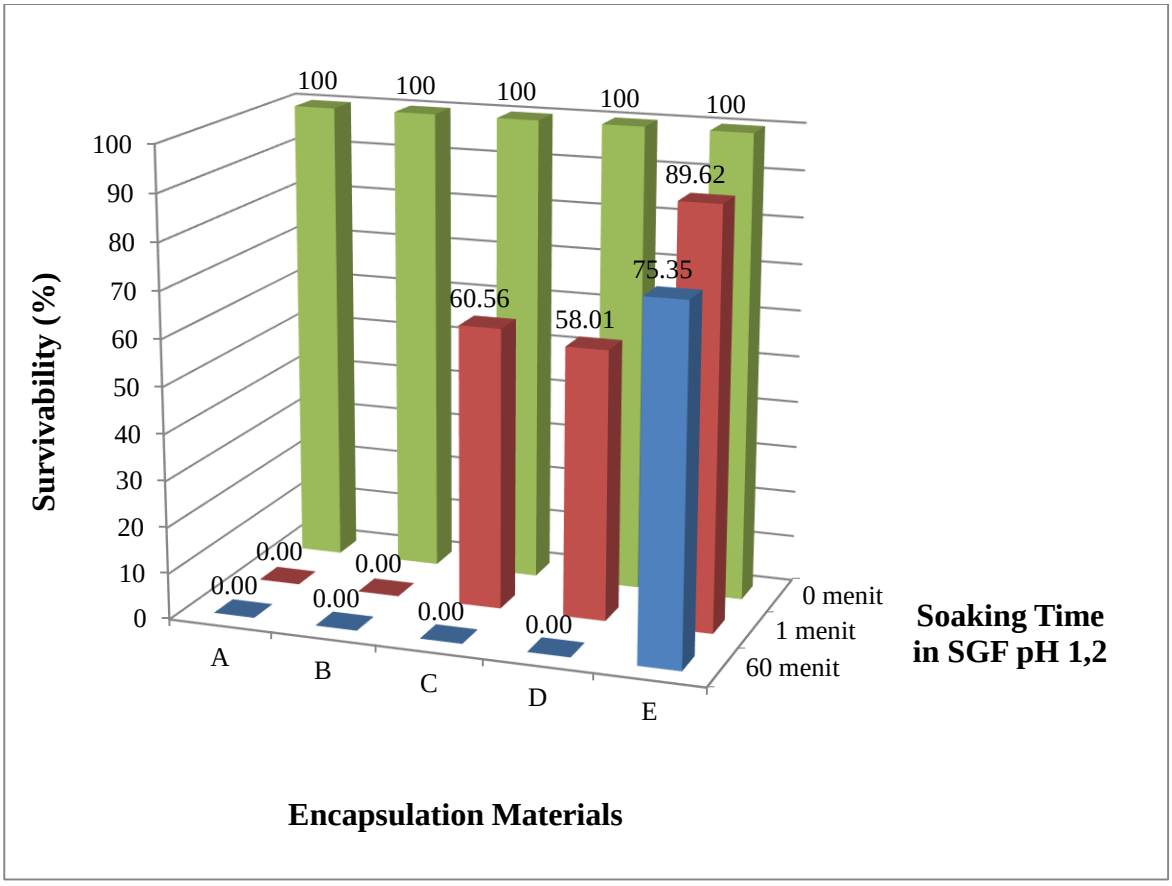


Figure 7. Survivability of *L. casei* before and after soaking in SGF pH 1.2

Figure 7 showed that the survivability value of *L. casei* was 89.62% owned by the beads encapsulated using a mixture of alginate (1%), nano chitin (0.2%) then coated with chitosan (0.2%) on the surface. The results of these experiments also showed that the combination of nano chitin and chitosan in the encapsulation material significantly increased the viability of *L.casei*.

In soaking the beads in SGF pH 1.2 for 60 min, Figure 10 showed the survivability of *L.casei* in all variations of the experiment of 0% except those made from alginate (1%), nano chitin (0.2%), and chitosan (0.2%) of 75.35%. The results of these experiments also showed that the combination of nano chitin and chitosan in the encapsulation material significantly increased the viability of *L.casei*. The viability of *L.casei* unencapsulation and encapsulation that had been soaked in SGF pH 3 for 1 min and 60 min was shown in Figure 8 below.

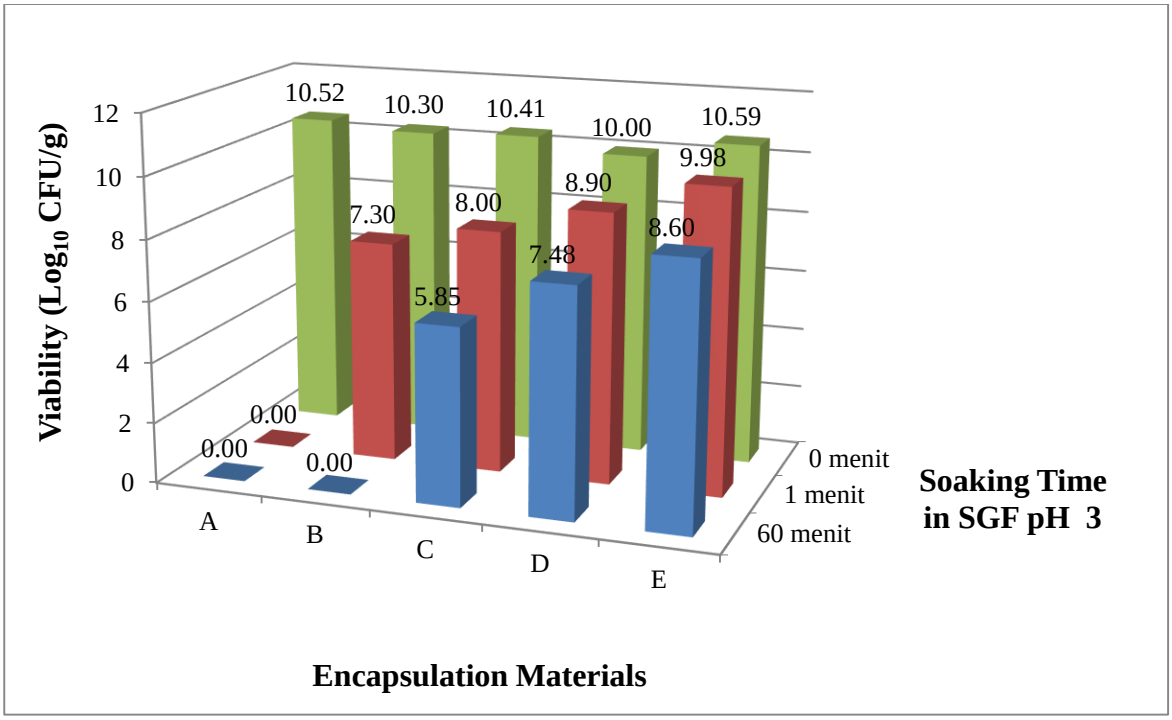


Figure 8. Viability of *L. casei* before and after soaking in SGF pH 3

Figure 8 showed that the viability value of *L. casei* was 0 CFU/g, after soaking in SGF pH 3 for 1 min. This showed that unencapsulated *L.casei* cannot survive in SGF pH 3, while *L. casei* which had been encapsulated by alginate or a mixture of alginate, nano chitin, and chitosan still have high viability (above 8Log_{10} CFU/g) after experience soaking in SGF pH 3 for 1 min.

Figure 8 showed that the viability value of *L. casei* was 0 CFU/g, after soaking in SGF pH 3 for 60 min. This indicated that *L. casei* cannot survive in SGF pH 3 for 60 min, either unencapsulated or encapsulated with alginate. The surface of the beads which was formed from alginate material still has a lot of pores so that SGF liquid can easily penetrate the inside of the beads and come into direct contact with *L. casei* so that it was killed. The beads that were added with nano chitin and chitosan to the alginate beads tended to have a much tighter surface, thereby reducing the SGF liquid penetrating the inside of the beads. This happened because the nano chitin was mixed directly with the alginate in the syringe so that when the encapsulation process was formed, the nano chitin was physically trapped in the alginate which results in reduced alginate pores. Meanwhile, chitosan was added after the beads are formed by soaking the beads in a solution of chitosan so that the surface of the beads were coated with chitosan, which was an ionic bond between the $-NH_3^+$ functional group of chitosan and the COO^- functional group from alginate. This results in the pores of the alginate on the surface of the beads closed finely. The highest viability after soaking in SGF pH 3 for 60 min was achieved by beads made of a mixture of alginate-nano chitin-chitosan, which was 8.6Log_{10}CFU/g. The survivability of *L.casei* unencapsulation and encapsulation that has been soaked in SGF pH 3 for 1 min and 60 min was shown in Figure 9 below.

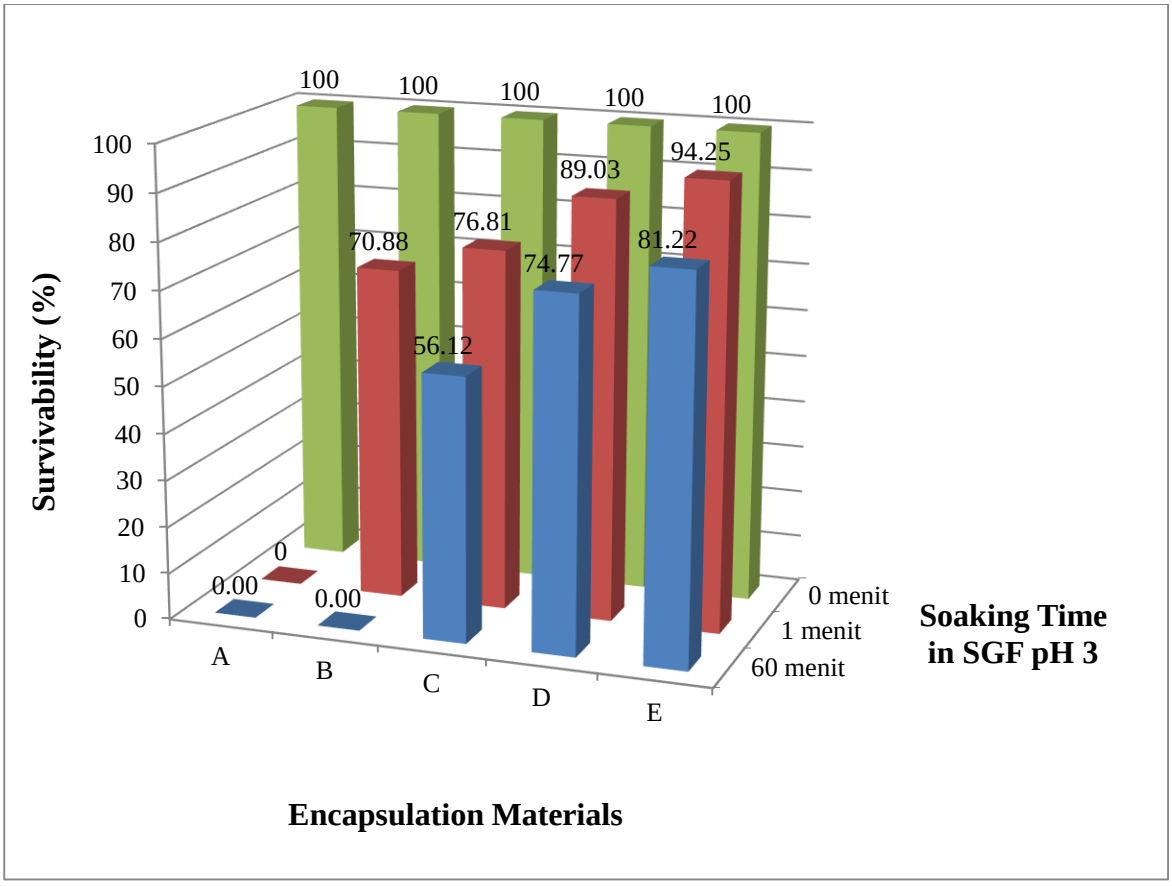


Figure 9. Survivability of *L. casei* before and after soaking in SGF pH 3

When soaking the beads in SGF pH 3 for 1 min, Figure 9 showed the survivability of *L.casei* unencapsulation of 0%. Meanwhile, encapsulation by adding alginate, nano chitin, or chitosan can increase the survivability of *L.casei*. While the highest survivability of *L.casei* was 94.25% obtained in the variation of the encapsulation experiment using alginate (1%), nano chitin (0.2%), and chitosan (0.2%). When soaking the beads in SGF pH 3 fluid for 60 min, Figure 9 showed the survivability of *L.casei* was 0% unencapsulation and encapsulation made from alginate. Meanwhile, encapsulation by adding alginate plus nano chitin or chitosan can increase the survivability of *L.casei*. While the highest survivability of *L.casei*, which was 81.22%, was obtained in a variety of encapsulation experiments using alginate (1%), nano chitin (0.2%), and chitosan (0.2%). The results of these experiments indicated that the combination of chitosan and nano chitin in the encapsulated material can significantly increase the viability of *L.casei*. In SGF pH 3, the survivability of *L.casei* encapsulated using alginate (1%), nano chitin (0.2%), and chitosan (0.2%) was higher than pH 1.2. When nano chitin was mixed with alginate as an encapsulation material, when it was soaked in acid it shrank while the nano chitin was more bound to the alginate and can close the alginate pores so that the viability of *L.casei* was higher than without nano chitin. Chitosan can bind to the alginate chemically so that it can cover the pores of the alginate, but chitosan dissolved in acid so that when soaked in acid it caused chitosan to erode, but when the amount of chitosan was large it caused greater protective power so that it can increase the viability of *L.casei*.

4. CONCLUSION

By soaking the beads in SGF pH 1.2 for 60 min, the survivability of *L.casei* in all variations of the experiment was 0% except those made from alginate (1%), nano chitin (0.2%), and chitosan (0.2%) of 75.35%. At the soaking of the beads in SGF pH 3 for 60 min, the survivability of *L.casei* was 0% for beads unencapsulation and encapsulation made from alginate, while the highest survivability of *L.casei* was 81.22% obtained in various encapsulation experiments using alginate. (1%), nano chitin (0.2%), and chitosan (0.2%). The results showed that the addition of chitosan or nano chitin to *L.casei* encapsulation material increased the survivability of *L.casei*, also showed that the combination of alginate, nano chitin, and chitosan in the encapsulated material significantly increased the survivability of *L.casei* at SGF pH 1.2 and 3. In SGF pH 3, the survivability of *L.casei* was higher than pH 1.2.

5. ACKNOWLEDGEMENT

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6. CONFLICT OF INTEREST

The authors declare that we have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

7. AUTHOR CONTRIBUTIONS

Djaenudin conducted the experiment, the calculations, wrote and revised the manuscript. Endang Saepudin, and Muhamad Nasir conducted supervision. All authors agreed to the final version of this manuscript.

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