

Microencapsulated *Lactobacillus acidophilus* FNCC 0051 and *Streptococcus thermophilus* FNCC 0040 Technique Emulsion Using Gelatin and Sodium Alginate

ABSTRACT

The research was held from September 2021 until January 2022 in Laboratory Livestock Product Technology Universitas Brawijaya, Malang, Indonesia. Microencapsulation is a technique used to protect bacteria from harmful (extreme) environmental factors such as heating, freezing and low pH through a coating process or coating a core substance in this case LAB with a polymer wall layer. The purpose of this study was to obtain a combination of the use of gelatin and sodium alginate as a coating material in the encapsulation of *Lactobacillus acidophilus* FNCC 0051 and *Streptococcus thermophilus* FNCC 0040 using the emulsion technique based on its physical and microbiological properties. The research method used is a laboratory experiment using a completely randomized design (CRD) pattern with 3 treatments and 3 replications. The treatment using a combination of gelatin and sodium alginate consisted of T1 (1:1); T2 (1:2); T3 (1:3). Data were processed by Analysis of Variance (ANOVA); If the analysis happens to show a significant difference ($P < 0.05$) or a very highly significant difference ($P < 0.01$), then the Duncan's Multiple Range Test was applied. Data from the results of microstructure testing using Scanning Electron Microscopy which were qualitative were analyzed descriptively. The results of the analysis show that encapsulation using a combination of gelatin and sodium alginate gives a very significant difference ($P < 0.01$) to the value of encapsulation efficiency and does not give a significant difference ($P > 0.05$) on microcapsule particle size and microcapsule particle size distribution, with percentages T1 $97.43 \pm 0.31\%$, T2 $98.50 \pm 0.48\%$, T3 $99.00 \pm 0.44\%$; T1 $1.08 \pm 0.07 \mu\text{m}$; T2 $1.18 \pm 0.11 \mu\text{m}$; T3 $0.95 \pm 0.11 \mu\text{m}$; and T1 4.79 ± 1.04 ; T2 2.53 ± 2.16 ; T3 4.15 ± 3.13 and microcapsules using SEM showed the microcapsules were round and smooth. The combination of gelatin and sodium alginate T3 (1:3) as a microcapsule material is a good alternative to protect lactic acid bacteria so that it can be applied in food

1. INTRODUCTION

Currently, functional food products have played an important role in the food industry sector due to the increasing public awareness of the importance of eating healthier and higher nutritional value foods. The functional food sector has represented one of the most dynamic and innovative categories in the food industry. These foods not only contribute to basic nutrition but also contain active physiological components, which is one of the main

segments represented by probiotic-based food products.

Lactobacillus acidophilus and *Streptococcus thermophilus* are a group of probiotic bacteria known to provide health benefits to humans. Bacteria that are probiotics will be able to work optimally if they contain living cells that can survive during the heating, food processing and storage processes. The number of live microbes must be

sufficient to have a positive effect on health and be able to colonize so that they can reach the required number for a certain time. According to [1] the amount given is around 10^6 - 10^7 CFU g⁻¹/day. Meanwhile, to lead to health benefits, the recommended amount is 10^8 - 10^9 /day [2]. Therefore, the viability of probiotics in the product needs to be considered. One of the efforts to maintain the viability of probiotics is to apply microencapsulation techniques.

Microencapsulation with the emulsion technique is an appropriate method for producing water particles in oil emulsions [3]. One of the advantages of this method is that the resulting particles are smaller (less than 100 m) and do not change the sensory properties of the product. This method does not require special equipment and sophisticated techniques, the formulation is simple and low cost, this method has high cell viability and porous particles [4]. Materials commonly used as encapsulants are organic or inorganic polymers either derived from natural or artificial materials.

Sodium alginate is a natural polymer that is widely used in the microencapsulation process because of its biocompatibility, biodegradability, water-soluble, non-toxic and relatively low price. Sodium alginate can form a gel with two valence cations, including Ca²⁺ ions so in this study a CaCl₂ solution was used as a crosslinker, but alginate has a drawback, namely the microcapsules produced are too porous, so they are not optimal in protecting probiotics from environmental factors. The porous structure will also result in low probiotic trapping ability [5]. Therefore, other polymers are needed to minimize these deficiencies.

Gelatin is a polymer that is biocompatible, biodegradable, stable over a wide pH range, non-toxic, inexpensive, has good swellability, is water-soluble, is able to form films, has the ability as an emulsifier and can undergo cross-linking such as sodium alginate [6].

The application of the emulsion technique in the encapsulation of probiotics resulted in the size of the capsule in the form of micrometres. Capsule diameter has an important influence on probiotic cell viability, product sensory properties, metabolic rate and distribution quality of microcapsules [7]. Therefore, physical protection against probiotics is very necessary. The purpose of this study was to determine the combination of using gelatin and sodium alginate using emulsion techniques on the microencapsulation of *Lactobacillus acidophilus* FNCC 0051 and *Streptococcus thermophilus* FNCC 0040 based on the value of encapsulation efficiency, microcapsule particle size, microcapsule particle size distribution and microstructure using Scanning Electron Microscopy (SEM).

2. MATERIALS AND METHODS

2.1 Materials

The materials used in this study include biomass *Lactobacillus acidophilus* FNCC 0051 and *Streptococcus thermophilus* FNCC 0040, MRS agar (merck), MRS broth (oxoid), gelatin, sodium alginate, paraffin oil, span 80, NaCl 0.9% (braun), NaCl 0.85 % (braun), CaCl₂, 70% alcohol, aluminum foil, and aquades. The equipment used in this study included an autoclave (Hirayama), light microscope (Novex Holland), Laminar Air Flow (LAM), Particle size Analyzer (PSA) (microtrac), Scanning Electron Microscopy (SEM) (Hitachi TM 3000) analytical balance, centrifugator (jouan), vortex (labinco L 46), petri dish (duran), beaker glass (pyrex), erlenmeyer (pyrex), measuring cup (iwaki), magnetic stirrer (SH-2), incubator (memmert), wire ose, syringe, water bath (GFL), test tube (pyrex), test tube rack, glass funnel (pyrex), measuring flask (duran), micro pipette, blue tip, white tip, bunsen, bottle (duran), centrifuge tube (sakamed), dropper, portable stove, refrigerator (LG), whatman filter paper no. 1, thermometer, gloves, cover glass, object glass, tweezers and stirrer.

2.2 Methods

2.2.1 Probiotic Culture Preparation

Procedure for the propagation of *Lactobacillus acidophilus* FNCC 0051 and *Streptococcus thermophilus* FNCC 0040 [8]. Cultures *Lactobacillus acidophilus* FNCC 0051 and *Streptococcus thermophilus* FNCC 0040 in ampoules were taken 3 times using a wire loop inoculated into 10 ml of MRSB, incubated at 37°C for 40 hours. The cell suspension was then centrifuged at 4,500 rpm for 10 minutes at 4°C to obtain cell biomass. The cell biomass obtained was washed twice with sterile NaCl (0.85% w/v).

2.2.2 Encapsulation of *Lactobacillus acidophilus* FNCC 0051 and *Streptococcus thermophilus* FNCC 0040

The procedure for making LAB microcapsules through the emulsion technique in this study refers to [8] with some modifications. Sodium alginate solution was prepared by dissolving 1.5 grams of sodium alginate in 100 ml of distilled water and sterilized at 121°C for 15 minutes then cooled at room temperature. Gelatin solution was prepared by dissolving 5 grams of gelatin in 95 ml of distilled water at a temperature of 50-55 °C with a magnetic stirrer and then cooled at room temperature. Furthermore, 50 ml of paraffin oil was added with 0.5% span 80 emulsifier and sterilized at 121°C for 15 minutes and then cooled at room temperature. CaCl₂ was dissolved in distilled water with a concentration of 0.1 M, then sterilized and cooled at 4°C. After the solution was prepared, each of T1 gelatin:sodium alginate 1:1 (v/v), T2 gelatin: sodium alginate 1:2 (v/v) and T3 gelatin:sodium alginate 1:3 (v/v) were mixed with cell suspension of *Lactobacillus acidophilus* FNCC 0051 and *Streptococcus thermophilus* FNCC 0040 (1:1) totaling 7 ml with a concentration of 2.88×10^9 CFU/ml and homogenized until well mixed. The mixture of gelatin, sodium alginate and

cells was put into a syringe and then slowly dripped into 50 ml of paraffin oil containing 0.5% span 80 which was stirred at 450 rpm, added 10 ml of sterile 0.1 M CaCl₂ into the solution and stirred for 30 minutes to harden the formed microcapsules and break the emulsion. The microcapsules were harvested by centrifugation at 3,500 rpm for 15 min to obtain the supernatant (microcapsules). The microcapsules were washed twice with sterile distilled water to separate the microcapsules from the oil. The microcapsules were filtered using filter paper and then transferred to a sterile petri dish.

2.2.3. Encapsulation efficiency

Encapsulation efficiency was determined based on the method of [5] with modifications. One gram of microcapsules from different treatments was weighed and dissolved in 9 ml of sterile 0.9% (w/v) NaCl solution and stirred using a magnetic stirrer for 20 minutes until the microcapsules were completely dissolved. After that, a series of dilutions were made up to 10⁻⁹, the last 3 diluents (10⁻⁷, 10⁻⁸, 10⁻⁹) were poured into a petri dish containing bacteria and incubated for 48 hours at 37°C under anaerobic conditions.

The encapsulation efficiency (EE) was determined following a modified method from [9]. EE is calculated using the equation:

$$EE = \log N / \log N_0 \times 100\%$$

Where N is the number of cells trapped and viable to be released from the microcapsules and N₀ is the number of free cells not added to the biopolymer mixture.

2.3.4. Particle Size

The particle size of the microcapsules was determined based on the method [10]. The size of the microcapsules was determined based on the analysis of the particle size system using laser diffraction in which water was the dispersion medium. The SPAN value indicates the particle size dispersion.

$$\text{SPAN} = \frac{D(v,90) - D(v,10)}{D(v,50)}$$

Where $D(v,90)$, $D(v,50)$, and $D(v,10)$ are the mean particle diameters representing each microcapsule at 90, 50 as the cumulative volume.

2.3.5. Particle Size Distribution

Testing the particle size distribution of microcapsules using PSA aims to determine the distribution of the resulting particles based on the use of the encapsulation material used. The particle size distribution is characterized by the parameter $d_{0.5}$ (the diameter at which 50% of the distribution is below) and the parameter range (width of the particle size distribution) [11].

2.3.6. Microcapsule Microstructure Use Scanning Electron Microscopy

Microcapsule microstructure use Scanning Electron Microscopy was determined based on the method of [12] with modifications. *Lactobacillus acidophilus* FNCC 0051 and *Streptococcus thermophilus* FNCC0040 microcapsules were freeze-dried. The capsule sample was placed in an aluminium sample holder and coated with gold with a thickness of 10 nm, observed at various magnifications using SEM and examined for morphology at an intensity of 20 kV and a current of 12 mA with a magnification of 2.000 times.

2.3. Statistical Analysis

Data were processed by Analysis of Variance (ANOVA). If the analysis happens to show a significant difference ($P < 0.05$) or a very highly significant difference ($P < 0.01$), then the Duncan's Multiple Range Test was applied. Data from the results of microstructure testing using Scanning Electron Microscopy which were qualitative were analyzed descriptively.

3. RESULTS AND DISCUSSION

3.1 Encapsulation Efficiency

The results of the analysis showed that the microencapsulation of *Lactobacillus acidophilus* FNCC 0051 and *Streptococcus thermophilus* FNCC 0040 using a combination of gelatin and sodium alginate through emulsion technique gave a very significant difference ($P < 0.01$) to the average encapsulation efficiency. The higher the concentration of the use of encapsulation material, the higher the efficiency of the resulting encapsulation. The average value of encapsulation efficiency is listed in Table 1.

The average encapsulation efficiency increased along with the increase in the use of sodium alginate. The use of a combination of gelatin and sodium alginate (1:3) as T3 showed the best percentage of efficiency values, namely $99.00 \pm 0.44\%$ compared to the use of a combination of gelatin and sodium alginate at T1 (1:1) and T2 (1:2) respectively $97.43 \pm 0.31\%$ and $98.50 \pm 0.48\%$. This indicates that the amount of LAB released from T3 is less than that of T1 and T2. The bacteria in T3 is thought to have not been completely released due to the composition of gelatin and sodium alginate because it produces denser pores, tighter gel pores provide less chance of leakage. According to [13] sodium alginate is the lyophilized component (main polyelectrolyte), while gelatin acts as an inhibitory component (guest polyelectrolyte). So the use of sodium alginate is higher than gelatin. The higher concentration of sodium alginate causes more cross-links to occur, which results in the resulting pores becoming denser. The tighter pores of the gel provide less chance of leakage during immobilization so that it can hold LAB out of the matrix at the wrong time. Negatively charged sodium alginate tends to form a gel faster due to the presence of Ca^{2+} ions which can form a three-dimensional "egg-box" structure, while gelatin will form a "honeycomb" is a three-dimensional network structure under the dual action of cooling after heating and ion induction. Gelatin can also assist in increasing the strength and rigidity of the working tissue structure through stability and electrostatic interactions.

Intermolecular hydrogen bonds between the cationic amine group in gelatin (-NH^{3+}) and the carboxylate group in alginate (-COO-). This interaction is intended to create a safe environment so that the probiotics trapped in the microcapsules can be protected from the harmful external environment [14].

The LAB types used in this study were *Lactobacillus acidophilus* FNCC 0051 and *Streptococcus thermophilus* FNCC 0040, the results showed that the colony growth of *Lactobacillus acidophilus* FNCC 0051 after encapsulation was lower than that of *Streptococcus thermophilus* FNCC 0040. This is thought to occur because *Lactobacillus acidophilus* is LAB. low viability. This is supported by [15] that *Lactobacillus acidophilus* bacteria show a short stationary phase followed by a rapid loss of cell viability.

The encapsulation efficiency level test aims to measure the effectiveness of the encapsulation process. A high-efficiency value indicates a high number of encapsulated bacteria. The high value of encapsulation efficiency indicates that the encapsulation process is working optimally [16]. Encapsulation efficiency is influenced by the nature of the encapsulation material used. It has been observed that sodium alginate provides effective cross-linking and mechanical support for protection against probiotics. The use of sodium alginate as a probiotic coating material shows a high level of efficiency because it is a wall material that has good compatibility [17]. The emulsion method is one of the methods used to increase encapsulation efficiency which has low water solubility by producing microcapsules that can improve particle size control and distribution [18].

3.2 Particle Size

The results of the analysis showed that the microencapsulation of *Lactobacillus acidophilus* FNCC 0051 and *Streptococcus thermophilus* FNCC 0040 using a combination of gelatin and sodium alginate through the emulsion technique did not give a significant difference ($P>0.05$) to the particle size

of the microcapsules. The average size of microcapsules is shown in Figure 1. The smallest particle size was found in T3 and the largest size was found in T2. Microcapsule particle size is an important characteristic in microencapsulated products because it is related to the release and absorption of bacteria by the mucosa. Changes in microparticle process parameters can affect the properties and performance of microparticles such as mechanical strength, ease of filtration and brittleness [19].

The particle size of the encapsulated live bacterial cells is an important factor in the design of industrial applications to produce the required uniformity and precision level of microcapsules [20]. Smaller microcapsules showed poor encapsulation efficiency and the release of core material in unwanted places. Larger microparticles may also exhibit poor encapsulation efficiency due to prolonged release time and undesirable size [21]. Therefore, the microcapsule gel size should be in the appropriate range, not too large and not too small [22]. The LAB microcapsule measurement results obtained at T1, T2 and T3 have reached the expected target, the microcapsules formed fall into the microcapsules size range of 0.2 μm - 5000 μm . According to [23] the size of microencapsulation is divided into three sizes, namely macro ($>5.000 \mu\text{m}$), micro (0.2 μm - 5.000 μm) and nano ($<0.2 \mu\text{m}$). Capsules with the emulsion technique have a smaller diameter so that the resulting pore size is also smaller. This small pore size makes the transfer of fluid from outside the capsule into the capsule more limited [24]. The size of the microcapsules produced in this study was able to increase the encapsulation efficiency value up to 99.00%.

3.3 Particle Size Distribution

The results of the analysis showed that the microencapsulation of *Lactobacillus acidophilus* FNCC 0051 and *Streptococcus thermophilus* FNCC 0040 using a combination of gelatin and sodium alginate through the emulsion technique did not give a significant difference ($P>0.05$) to the mean particle

size distribution of microcapsules. The average particle size distribution of microcapsules is shown in Figure 1.

The size and size distribution of microcapsules has a great impact on the process mechanism and the release rate of the encapsulated core material. The particle size distribution shows that the higher the ratio of polymer used, the larger the particle size produced. The variation in the size of microcapsules is influenced by several factors, including polymer concentration, the pressure difference when forming microcapsules through a syringe, the distance between the syringe and the microcapsule-forming solution and the high and low position of the syringe when dropping the microcapsules. Stirring at a speed of 300 rpm to 2.000 rpm gave a significant effect on the size distribution of the microparticles. Microparticles made with higher stirring speed resulted in smaller microparticles while lower agitation resulted in larger microparticle sizes. The high agitation rate helps the large

droplets to be broken down into smaller droplets [25].

The different particle size distribution of microcapsules was indicated to occur due to differences in the stability of the emulsifier due to the unstable surface tension of the oil and water phases. [18] states that the smaller the water phase particles in the water-in-oil emulsion, the smaller the diameter of the resulting capsule will also be. Therefore, an emulsifier is used to reduce the surface tension between the water and oil phases so that the resulting droplet size becomes smaller. The particle size distribution containing anticancer drugs ranges from 1 to 30 μm by mixing larger and smaller microparticles, thereby achieving a release profile with an intermediate release rate [21]. The large and variable size of the capsule can cause uneven distribution of cells in the capsule. In addition, capsules with a diameter of 1.000-3.000 μm can have an unpleasant effect on the sensory assessment of the product [24].

Table I. Average Encapsulation efficiency of *Lactobacillus acidophilus* FNCC 0051 and *Streptococcus thermophilus* FNCC 0040 encapsulation using a combination of gelatin and sodium alginate through emulsion technique

Treatment	Encapsulation Efficiency (%)
T1	97.43 \pm 0.31 ^a
T2	98.50 \pm 0.48 ^{ab}
T3	99.00 \pm 0.44 ^b

Description: ^{a,b}different superscripts in the same column indicate the treatment using a combination of gelatin and sodium alginate through the emulsion technique gave a very significant difference ($P < 0.01$)

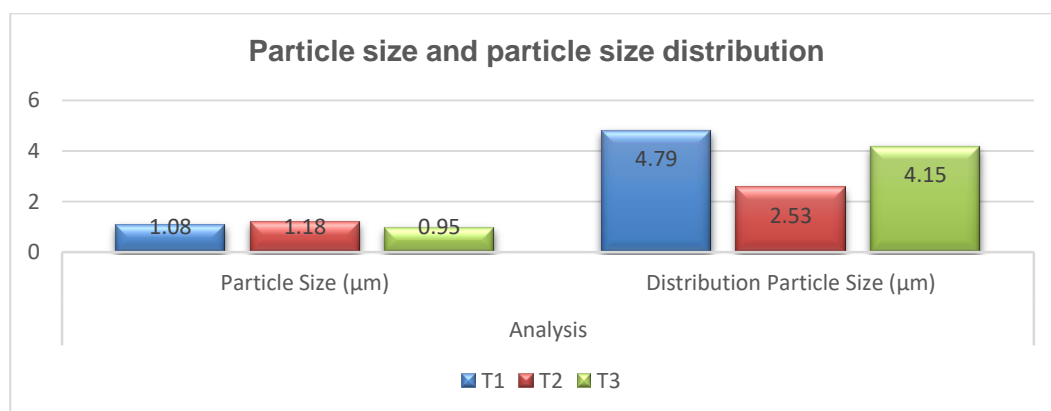


Figure 1. Analysis particle size and particle size distribution of *Lactobacillus acidophilus* FNCC 0051 and *Streptococcus thermophilus* FNCC 0040

3.4. Microstructure Microcapsules

The microstructure of LAB microcapsules made using a combination of gelatin and sodium alginate through emulsion techniques was carried out through microscopic analysis using Scanning Electron Microscopy (SEM) aimed at knowing the surface structure in protecting bacterial cells. The cross-section of the microcapsule (2.000x magnification) shows the integrity of the bacterial cells trapped inside the capsule (Figure 2). The presence of cells confirms that this

technique is effective for the microencapsulation of bacteria. Scanning images using SEM on microcapsules T1, T2 and T3 showed the capsules were round, smooth, thick although some were found to be irregularly shaped and shrivelled (Figure 2). Observation of microcapsules using a light microscope showed a spherical shape, but not evenly distributed in T1 and T2 while in T3 it was spherical, uniform distribution with the more uniform size is shown in Figure 3.

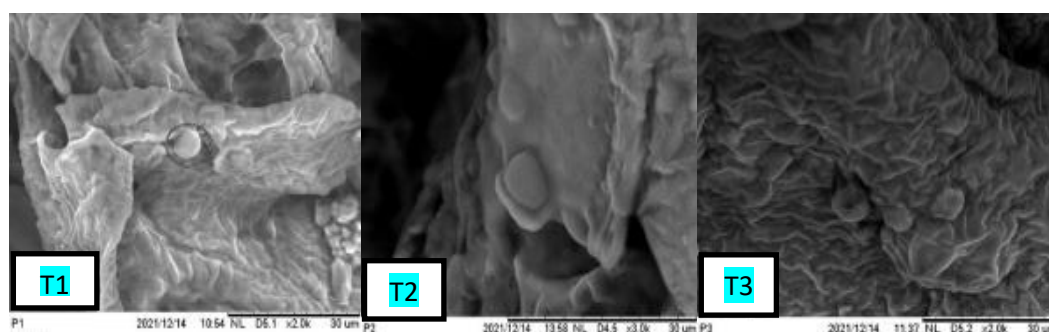


Figure 2. Microstructure of encapsulated LAB microcapsules using Scanning Electron Microscopy (SEM) with a magnification of 2.000x. T1 microcapsules using a combination of gelatin : sodium alginate (1:1); T2 microcapsules using a combination of gelatin : sodium alginate (1:2); T3 microcapsules using a combination of gelatin : sodium alginate (1:3).

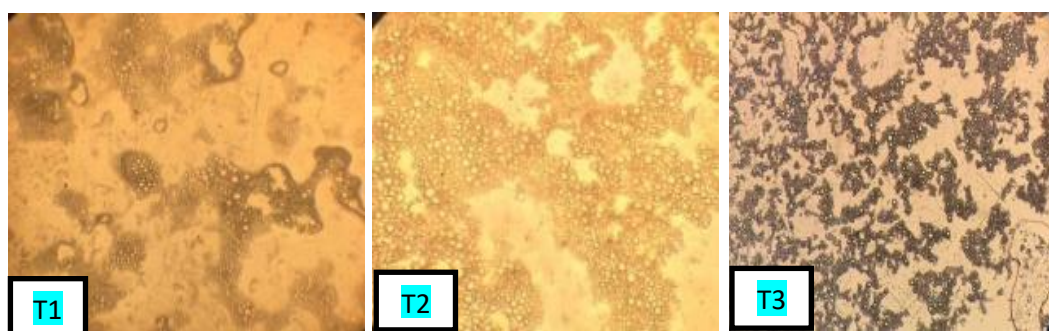


Figure 3. The shape of the encapsulated LAB microcapsules observed using a light microscope with a magnification of 10x. T1 microcapsules using a combination of gelatin : sodium alginate (1:1); T2 microcapsules using a combination of gelatin : sodium alginate (1:2); T3 microcapsules using a combination of gelatin : sodium alginate (1:3)

The Na alginate material used in encapsulation can produce round, small beads with a surface such that it can protect bacterial cells [26]. The emulsion technique used can protect bacterial cells from an unfavourable environment to increase the viability of bacterial cells. Small microcapsules can trap cells better when compared to larger ones. Generally, wrinkling of the microcapsules occurs due to mechanical stress caused by the non-uniform drying of the various parts of the liquid droplet at the initial stage of drying. High molecular weight polymers must dry quickly to prevent the release of internal vapours which can result in increased bubble formation in the wall material matrix, creating morevoids and

4. CONCLUSION

Based on the results of the research conducted, it can be concluded that the microcapsules of *Lactobacillus acidophilus* FNCC 0051 and *Streptococcus thermophilus* FNCC 0040 were produced through the emulsion technique using gelatin and sodium alginate coatings as microcapsules. The best treatment was obtained at T3 (1:3) with an encapsulation efficiency value of 99.00%, resulting in good particle size, particle distribution and morphological structure.

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expanding the internal space of the microcapsules. The kinetics of gelatinization can form a capsule-like structure on the alginate particles which is determined by the rate of Ca^{2+} penetration, the structure and concentration of the alginate, and the presence of Na^+ ions which prevent the gelatinization of the alginate. The presence of Ca^{2+} and Na^+ ions also supports accelerating the formation of a homogeneous gel. Inside the microcapsule consists a semi-porous network. The level of porosity of alginate is important to maintain because it is related to the viability of bacteria so that they can survive when passing through the digestive tract [27].

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COMPETING INTERESTS

Aauthors have declaret that no competing interests exist.

AUTHORS' CONTRIBUTION

Author, RDN wrote the draft of manuscript and performed the statistical analysis. Author MES and AM designed the study, managed the analysis study and the literature searches. All authors read and approved the final manuscript.

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