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Microencapsulation of *Lactobacillus plantarum* FNCC 0026: Physical properties of microcapsules and antibacterial activity against multi-drug resistance organism (MDRO)

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ABSTRACT: This study aimed to evaluate the microparticle system of *Lactobacillus plantarum* FNCC 0026 in an optimal combination of a gelatin-alginate matrix. Four formulas with a variation of 2.5% alginate-gelatin polymer combination were encapsulated using the aerosolization technique. Microcapsule physical properties such as morphology, particle size distribution, swelling index, % mıoisture content, structural analysis by Fourier Transform Infrared (FTIR), as well as probiotic viability and antimicrobial activity in the matrix were investigated to evaluate the formulation. All formulations showed similarly uniform, spherical microparticles dispersed without agglomeration. The increased gelation concentration was accompanied by structural compactness as observed by scanning electron microscopy. Compared to free cells, the antibacterial activity increased against Methicillin Resistant *Staphylococcus aureus* (MRSA), and the inhibitory ability of microspheres against Extended Spectrum Beta Lactamase (ESBL) *Escherichia coli*. decreased. Under acidic conditions, gelatin can maintain the integrity of the capsule characterized by the absence of bacterial growth. Also, in alkaline conditions, the matrix swells well and has probiotic antibacterial activity. When stored, the microparticles could maintain probiotic function for up to 45 days. And from these studies, alginate gelatin matrix microspheres may form suitable capsules for *Lactobacillus plantarum* and probiotics could play an optimal role in inhibiting the growth of MRSA and ESBL E. coli.

KEYWORDS: Microencapsulation; Probiotic; *Lactobacillus plantarum;* Antibacterial activity

1. INTRODUCTION

Lactobacillus plantarum has been widely used to treat various infectious problems including preventing antibiotic resistance. In previous studies, *Lactobacillus plantarum* was known to be able to inhibit the two highest Multidrug Resistant Organism (MDROs) in Asia, such as Methicillin Resistant *Staphylococcus aureus* (MRSA) and Extended Spectrum Beta Lactamase (ESBL) *Escherichia coli* (E.coli) [1,2]. *Lactobacillus plantarum* exerts an anti-adhesion effect, which reducing the ability of pathogenic bacteria to adhere to host surfaces and impairing the persistence and resilience of bacterial infections [2]. Antibacterial activity of *Lactobacillus plantarum* is also triggered by its metabolites, such as lactic acid, acetic acid, and hydrogen peroxide, which creates an unfavorable environment for pathogens [1]. Plantaricin, a bacteriocin from *Lactobacillus plantarum*, also attack bacterial target membranes and cause leakage of intracellular compounds [3]. However, probiotics must be present in sufficient quantities to obtain optimum activity. In general, the viability of probiotics is 10^6 to 10^7 CFU/mL daily, but some decrease was found during digestion caused by exposure to acidic pH [4]. And microencapsulation was chosen as a delivery system to protect probiotics from extreme environments [5].

Probiotic microencapsulation is a physicochemical process to trap a probiotic into a suitable material to produce spherical particles with thin but strong semipermeable membranes ranging in diameter from nanometers to millimeters [6]. Microencapsulation has been successfully applied to various probiotics such as *Lactobacillus casei*, *Lactobacilus acidhophilus, Lactobacillus rhamnosus*, and *Lactobacillus plantarum* [7,8,9]. Polymers are important materials for forming probiotic microparticles. Alginate polymers have been thoroughly researched for their biocompatibility, non-toxicity, and low cost [5]. Sodium alginate forms hidrogel

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microparticles by cross-linking with Ca^{2+} ions [10]. These microparticles are used to protect active compounds, stable in acidic pH condition, and release active substances in the intestines [11].

However, these polymers have a porous structure which increases the risk of particle leakage [6]. Using mixed polymers such as alginate or gelatin can overcome this problem. NH^{3+} from gelatin forms electrostatic bonds with COO^- on M-monomers of alginate polymers, forming cohesive biopolymer networks [12,13]. The ratio of drug and polymer affects the characteristics of the microparticles. In previous research stated that a mixed polymer concentration of 2.5% will produce homogeneous beads and are not affected by the manufacturing process [13]. In the process of forming microparticles, the extrusion technique has advantages such as being simple, inexpensive, producing stable probiotics, and high viability. This method has undergone several technical developments, one of which is the aerosolization technique. This technique utilizes pressure to form a smaller, uniform, and smooth surface [14].

It is necessary to ensure that the preparation of probiotic microspheres is stable and can achieve the desired therapeutic effect. Therefore, it is necessary to evaluate the properties of microspheres as probiotic delivery systems. Evaluation of the physical properties of probiotic microspheres includes sensory stimulation, particle morphology, % moisture content, swelling index, and structural analysis [13]. To confirm the effectiveness of microspheres in protecting probiotics from the environment, it is necessary to study the viability and antimicrobial activity of probiotics in microspheres against storage conditions and digestive enzymes [9].

2. RESULTS

2.1. Pyhsical characterization of formulation

2.1.1. Organoleptic

Dry microsphere in the entire formula are in the form of a white and odorless powder. Data are shown on **Table 2.**

2.1.2. Determination swelling index and % moisture content

The moisture content (%MC) test was replicated three times for each formula with %MC values ranging from 11.3 to 12.5. And the whole formula expands well in solvents. The swelling process of the matrix was observed continuously. Data are shown on **Table 2.**

Table 2. Physical characteristic evaluation of *Lactobacillus plantarum* FNCC 0026 microsphere formula

Formula	Organoleptic	MC (%)	*Swelling index*			
			5 mins	30 mins	1 h	2 h
F1	White powder. odorless	12.32 ± 0.03	28.03	31.91	34.59	39.77
F2	White powder. odorless	12.52 ± 0.08	26.71	28.09	35.59	40.41
F3	White powder. odorless	11.55 ± 0.11	24.22	25.29	33.41	35.37
F4	White powder. odorless	11.37 ± 0.02	26.34	27.72	32.90	36.53

2.1.3. Structural analysis using Fourier Transform Infrared (FTIR) Spectroscopy Examination

The FTIR spectrum of each formulation is shown in **Figure 1**. All formula show a broad peak at 3300-3200 cm^{-1} (O-H vibration) due to high content of –OH groups. The increase in peak intensity at ~1600 cm^{-1} is characteristic of the presence of $-CONH_2$ groups, indicating that there was binding between the anions of alginate and the cations of gelatin.

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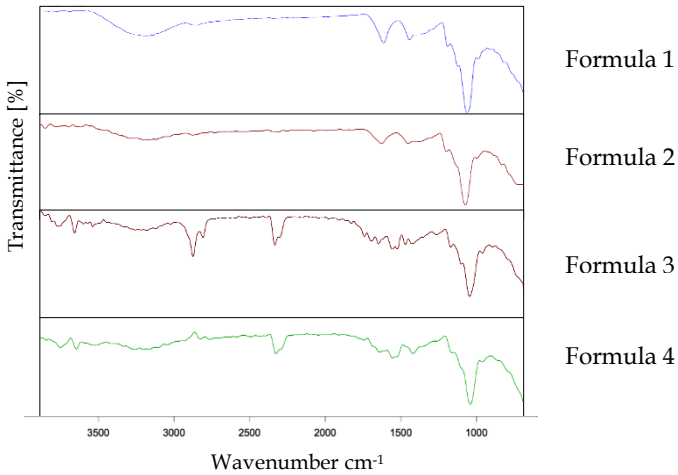

Figure 1. Fourier transform infrared spectroscopy (FTIR) spectra of each formula at a wavelength of 400 - 4000 cm^{-1}.

2.1.4. Shape and surface morphology of the microspheres

Microscopically, the beads are not uniform in shape but free from agglomeration. Formula 4 offers the smoothest, dense, and hardest microsphere surface appearance. **Figure 2** shows the shape of the microparticles in each formula. **Figure 3.** shows the surface comparison of each formulation by scanning electron microscope (SEM) at 500x and 5000x magnification.

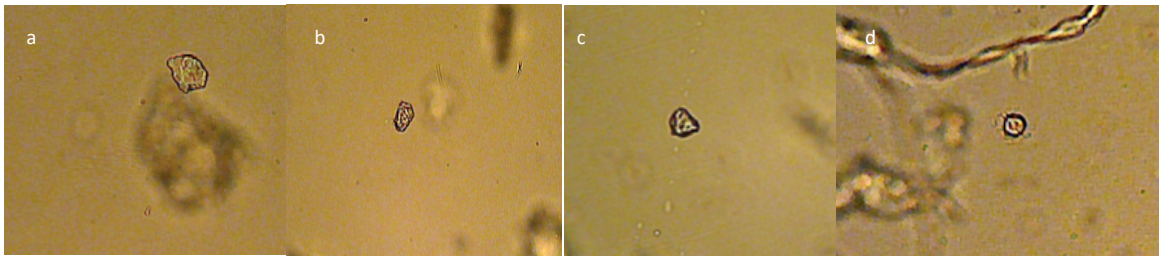

Figure 2. The shape of microsphere using optical microscopy with a magnification of 10x. a) F1; b) F2; c) F3; d) F4

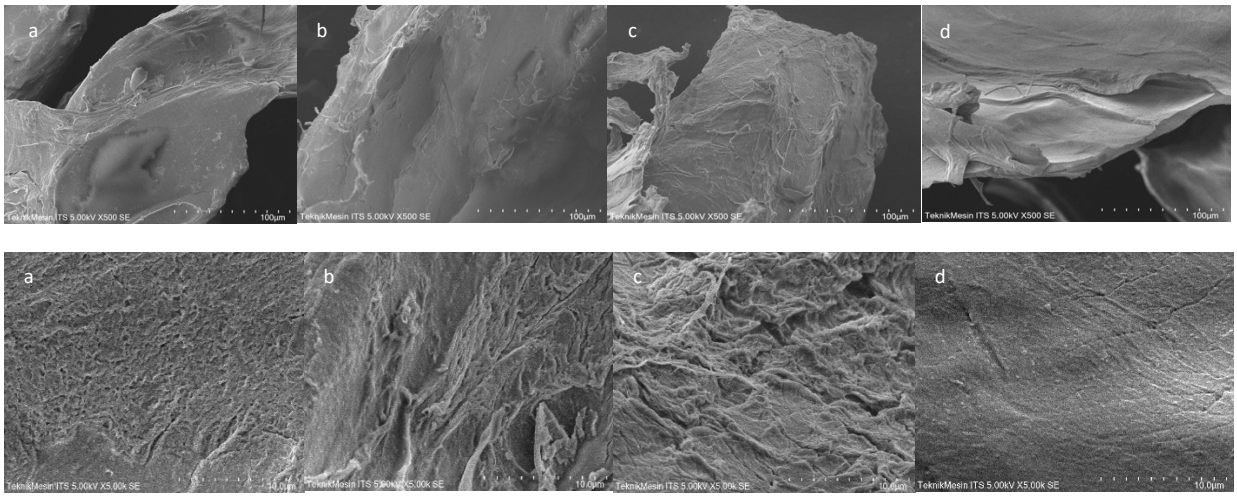

Figure 3. Microsphere surface appearance using SEM with a magnification of 500x (up) and 5000x (down). a) F1; b) F2; c) F3; d) F4.

2.2. Microbial assay of free and microencapsulated *Lactobacillus plantarum* FNCC 0026

Cell viability and antibacterial activity were compared to free cells and within the microspheres. The viable cell count in the probiotic suspension (free cells) used for microencapsulation was 1.2 x 10^{14} CFU/mL, and the inhibitory potencies against MRSA and ESBL E.coli were 12.33 mm and 12.13 mm, respectively. After encapsulation, the number of viable probiotic cells with formulas 2, 3, and 4 was significantly reduced. The inhibitory potency of all formulations against MRSA increased compared to free cells, but reduced inhibition of ESBL E.coli produced. Data are shown on **Table 3.**

Table 3. Cell viability and antibacterial activity of *Lactobacillus plantarum* FNCC 0026 microencapsulation

Formula	Viable cell count (CFU/mL)	Inhibition Zone (mm)	
		MRSA	ESBL *E.coli*
Free cells	1.2 x 10^{14}	12.33 ± 0.26	12.13 ± 0.12
F1	TNTC*	15.58 ± 0.76	10.60 ± 0.14
F2	1.5 x 10^{4}	16.27 ± 0.60	10.73 ± 0.30
F3	2.1 x 10^{9}	16.73 ± 1.40	10.72 ± 0.15
F4	5.1 x 10^{4}	17.33 ± 1.41	10.35 ± 0.52

*TNTC = too numerous too count

2.3. Survival assay of *Lactobacillus plantarum* FNCC 0026 microsphere in simulated GIT condition

This study was conducted to determine the ability of the matrix to protect against GIT conditions. There was no bacterial growth and activity under acidic conditions. On the other hand, under alkaline conditions, the number of living bacteria increased and showed antibacterial activity. The zones of inhibition formed against MRSA and ESBL E. coli were 8 mm and 9.5 mm, respectively. Data are shown on **Table 4.**

Table 4. Effect of pH on cell viability and antibacterial activity of microspheres *Lactobacillus plantarum* FNCC 0026. Acidic condition (pH 2.0; after 30 mins. 60 mins. 90 mins. and 120 mins); and alkaline condition (pH 7.4; after 150 mins)

Parameter	Formula			
	F1	F2	F3	F4
Cell viability (CFU/mL)				
30 mins	0	0	0	0
60 mins	0	0	0	0
90 mins	0	0	0	0
120 mins	0	0	0	0
150 mins	TNTC*	TNTC*	TNTC*	TNTC*
Antibacterial activity against MRSA (mm)				
30 mins	0	0	0	0
60 mins	0	0	0	0
90 mins	0	0	0	0
120 mins	0	0	0	0
150 mins	8.37 ± 1.61	7.70 ± 1.59	6.48 ± 0.22	7.93 ± 0.12
Antibacterial activity against ESBL *E.coli* (mm)				
30 mins	0	0	0	0
60 mins	0	0	0	0
90 mins	0	0	0	0
120 mins	0	0	0	0
150 mins	9.33 ± 0.68	9.80 ± 0.55	9.57 ± 1.04	9.30 ± 1.64

*TNTC = too numerous too count

2.4. Survival assay of *Lactobacillus plantarum* FNCC 0026 microsphere in storage

To determine the optimal matrix storage time to maintain probiotic viability, we investigated the stability of the microspheres during storage. Bacterial counts increased by day 7, but subsequent observations showed no bacterial growth. On the other hand, antibacterial activity increased to reach maximum capacity on its 7th and 15th day and gradually decreased with subsequent observations against MRSA and ESBL E.coli. Data are shown on **Table 5.**

Table 5. Cell viability and antibacterial activity of microspheres *Lactobacillus plantarum* FNCC 0026 during storage (2 – 8 °C)

Parameter	Formula			
	F1	F2	F3	F4
Cell viability (CFU/mL)				
Day 0	TNTC*	1.5×10^4	2.1×10^9	5.1×10^4
Day 7	TNTC*	TNTC*	TNTC*	TNTC*
Day 15	0	0	0	0
Day 30	0	0	0	0
Day 45	0	0	0	0
Day 60	0	0	0	0
Antibacterial activity against MRSA (mm)				
Day 0	15.58 ± 0.76	16.27 ± 0.60	16.73 ± 1.40	17.33 ± 1.41
Day 7	22.50 ± 1.22	19.23 ± 1.50	19.83 ± 2.01	20.07 ± 0.50
Day 15	20.60 ± 0.78	18.80 ± 0.00	18.63 ± 0.63	19.03 ±0.61
Day 30	17.33 ± 0.06	17.40 ± 0.42	16.45 ± 1.16	17.88 ±0.32
Day 45	15.05 ± 0.33	16.48 ± 0.21	15.78 ± 0.14	16.22 ± 0.13
Day 60	0	0	0	0
Antibacterial activity against ESBL *E.coli* (mm)				
Day 0	10.60 ± 0.14	10.73 ± 0.30	10.72 ± 0.15	10.35 ± 0.52
Day 7	11.92 ± 0.16	12.35 ± 0.62	13.22 ± 0.22	13.95 ± 0.18
Day 15	20.20 ± 0.86	20.45 ± 2.92	21.08 ± 1.64	23.03 ± 4.05
Day 30	16.72 ± 0.78	16.37 ± 1.51	16.80 ± 1.74	17.12 ± 2.29
Day 45	8.57 ± 0.42	8.53 ± 0.83	7.97 ± 0.84	7.38 ± 0.21
Day 60	8.00 ± 0.04	6.97 ± 0.33	6.52 ± 0.60	6.75 ± 0.54

*TNTC = too numerous too count

3. DISCUSSION

Microencapsulation is one of the appropriate technologies to maintain the stability and viability of probiotics under harsh conditions. Encapsulated probiotics are known to have a better survival ability than unencapsulated cells during storage and gastric transit [9]. The process of encapsulation of probiotics using polymers is strongly influenced by their concentration. From previous studies, the optimum concentration of sodium alginate ranged from 0.75% - 2% [15]. And the use of a polymer mixture of alginate and gelatin in a concentration of 2.5% is known to produce homogeneous beads and is not affected by the process [13]. From the results of the study, it was found that the size and shape of the microspheres was not uniform. Formula 1-3 obtained a rough surface, while in formula 4 the surface is smoother. Microsphere size is also strongly influenced by gelatin concentration. The higher the concentration of gelatin, the denser the microparticles. In formula 4, the particle size ranged from 0.6 - 2.3 μm and it gets bigger with decreasing concentration of gelatin. In formula 1, the largest particle size was found around 4.97 μm. This is due to the heterogenous gelation mechanism, where during gelation the polymer concentration on the surface is higher than in the center of the gel [15]. When the concentration between the two polymers is balanced, the surface formed will be smooth. In addition, this nonspherical shape can be caused by the consequences of high surface tension in crosslinking solutions [9].

To confirm that the biopolymer network is formed in the microparticles, it is necessary to analyze the structure using FTIR. Infrared spectroscopy results for all formulas show a broad absorption between 3300 and 3200 cm^{-1} wavenumbers, which is predicted to be the hydroxyl groups of sodium alginate and maltodextrin. The presence of absorption in the region around 1590 cm^{-1} indicates the presence of the carbonyl group of sodium alginate. Polymer-polymer interactions can be seen from the lack of absorption in the manuronate fingerprint region (850-810 cm^{-1}), and the gelatin C-N-H bend (1565-1500 cm^{-1}) [10]. In the spectra of formulas 3 and 4, there is an absorption at 1630 cm^{-1} which is the carbonyl group of gelatin. Due to the low gelatin concentration, there is no visible absorption of gelatin carbonyl groups from formulas 1 and 2. In formula 3 there is a strong absorption at 3000 - 2750 cm^{-1} which is an alkane group.

Probiotic microspheres must be fully swollen with target receptors to be therapeutically effective. This is influenced by the properties of each polymer material. Alginate expands to 10 times its original size when it absorbs water [16]. On the other hand, gelatin can prevent liquid from entering the beads for 2 hours [17]. Swelling index test results show that all formulations expand well. The bead continuously increases for up to 2 hours. Theoretically, gastric emptying time is 2 hours after oral phase. Microspheres are therefore expected to be fully expanded upon entering the intestine. And during storage, hydrogel beads should also be able to

maintain the water content within the matrix. A high moisture content risks the growth of unwanted microorganisms, and a low moisture content can make the beads brittle and easily wear the surface [13]. Moisture content also affects probiotic viability. Viability may drop to 4 log CFU/mL at a moisture content above 10% [9]. In this study, the average %MC for all formulas was 11.97% and cause a decrease of viable cell counts to 5-10 log CFU/mL for each formulation.

The viability of microspheres shows various values. That is inconsistent with previous studies that associated increased polymer concentration with decreased microsphere probiotic viability. Given that *Lactobacillus plantarum* requires complex conditions for optimal growth, the cause is still unknown. Antibacterial activity of *Lactobacillus plantarum* is due to its metabolites such as lactic acid and plantaricin (bacteriocin from *Lactobacillus plantarum*). Microencapsulation is known to increase plantaricin production. Bacteriocin production was dependent on bead size. Larger diameter beads produce more bacteriocin [18]. The mechanism of this metabolite is the binding of mannose phosphotransferase permease (Man-PTS) to his MptC and MptD subunits, which irreversibly opens endogenous channels and diffuses toxic ions [19]. Unfortunately, plantaricin is non-specific toxin so all bacteria are targets, including the probiotics themselves. This can explain the phenomenon of the absence and low of live bacteria but still having a same optimum antibacterial activity.

In general, the Lactobacillus group inhibits the growth of MRSA by actively penetrating and releasing metabolites that are toxic to the bacteria. In ESBL E.coli, on the other hand, probiotics form anti-biofilms, preventing bacteria from transmitting pathogenic agents to host cells. İn this study, the microsphere of *Lactobacillus plantarum* showed good antibacterial activity although the number decreased. Increased ability to inhibit MRSA growth and decreased ability of microspheres to inhibit ESBL E.coli across all formulas compared to free cell *Lactobacillus plantarum*. The high inhibitory capacity of microsphere in MRSA is related to the simpler structure of MRSA than ESBL E.coli and high content of *Lactobacillus plantarum* metabolites.

To confirm the effectiveness of microspheres in repelling probiotics from the environment, the resistance of probiotic microspheres during storage and in simulations of the digestive system has also been studied [9]. No probiotic growth or activity was observed under acidic conditions, indicating that the alginate-gelatin matrix can retain probiotics within the capsule. Meanwhile, under alkaline conditions, probiotics were able to get out of the matrix and had antibacterial activity against MRSA and ESBL E.coli of 6.5 - 8 mm and 9.3 - 9.8 mm, respectively. In general, probiotic microspheres lose potency with storage time. However, in this research, *Lactobacillus plantarum* was able to live during storage up to day 7. Then no bacterial colony growth was found on subsequent observations. Meanwhile, the antibacterial activity of *Lactobacillus plantarum* decreased in proportion to the length of storage time for both MRSA and ESBL E.coli.The antibacterial activity of microspheres against MRSA and ESBL E.coli was well observed with a decrease in potency at day 30. This is similar to the previous study that microencapsulated *Lactobacillus plantarum* was stable for 30 days [15].

4. CONCLUSION

The presented research shows that the formation of probiotic microspheres using a combination of alginate and gelatin matrix can form a good capsule for *Lactobacillus plantarum* FNCC 0026. The beads can protect probiotic life from acidic conditions and could expand well in alkaline conditions so that the antibacterial ability of the probiotic is within optimum conditions against MRSA and ESBL E.coli. Formula 4 gives the most satisfactory results. Evaluation of the physical characteristics of formula 4 has better results than other formulas. Its because the concentration of the two polymers is balanced so that a more rigid and smoother biopolymer network can be formed. Also, probiotics can be immobilized and protected from adverse conditions. Formula 4 showed good results in inhibiting the growth of MRSA and ESBL E.coli under various conditions. So from these studies, microspheres of *Lactobacillus plantarum* FNCC 0026 with an alginate-gelatin matrix can be an agent to overcome antibiotic resistance in Asia.

5. MATERIALS AND METHODS

5.1. Materials

Materials used for this research are *Lactobacillus plantarum* FNCC 0026 from Pusat Studi Pangan dan Gizi Universitas Gajah Mada Yogyakarta, Indonesia; De Man, Rogosa, Sharp (MRS) broth (MERCK Millipore); Nutrient agar (MERCK Millipore); Sodium alginate (Sigma-Aldrich Inc.); Gelatin bovine medium viscosity (Sigma-Aldrich Inc.), Calcium Chloride (MERCK Millipore); food-grade Maltodextrine; MRSA and ESBL E.coli were provided by RSUD Dr. Soetomo Surabaya, Indonesia.

5.2. Microbial preparation

Lactobacillus plantarum FNCC 0026 was grown in de Man, Rogosa, and Sharpe (MRS) broth at 37°C using a 100 mL elenmeyer flask with 50 mL MRS broth. Cultivation was conducted under optimal conditions (30 hours) and cultures were harvested by centrifugation at 1,000 rpm for 10 minutes. The pellet was resuspended in 50 mL saline solution and divided into 10 mL portions in vials. The culture]was stored at 8°C and each vial was used for one microsphere formula.

5.3. Microencapsulation of *Lactobacillus plantarum* FNCC 0026 by aerosolization technique

Four michrospheres formula were made with different matrix compositions, as shown in **Table 1**.

Table 1. Formula of *Lactobacillus plantarum* microsphere in alginate gelatin matrix

Ingredients	Function	F1	F2	F3	F4
L. plantarum FNCC 0026	Probiotic	1.2 x 10^{14} CFU/mL	1.2 x 10^{14} CFU/mL	1.2 x 10^{14} CFU/mL	1.2 x 10^{14} CFU/mL
Sodium alginat	Polymer	2.25% (w/v)	2% (w/v)	1.75% (w/v)	1.25% (w/v)
Maltodextrin	Substrate	5% (w/v)	5% (w/v)	5% (w/v)	5% (w/v)
$CaCl_2$	Cross linker	11.1 % (w/v)	11.1 % (w/v)	11.1 % (w/v)	11.1 % (w/v)
Gelatin	Polymer	0.25% (w/v)	0.5% (w/v)	0.75% (w/v)	1.25% (w/v)
Maltodextrin	Protectant	5 % (w/v)	5 % (w/v)	5 % (w/v)	5 % (w/v)

Sodium alginate and maltodextrin were weighed according to the formula, then dissolved in 90 mL of water, stirred at a speed of 1,000 rpm for 10 minutes. Calcium chloride dehydrate weighed as many as 11.1 grams, then dissolved in 100 mL of water, and stirred until dissolved. All solution were sterilized by autoclaving at 121°C for 15 minutes. After that, polymer solutions and 10 mL probiotics were sprayed using nozzle aerosolization into calcium chloride solution while stirred at a speed of 1,000 rpm, then left for 90 minutes.

The microspheres formed were separated using a Buchner funnel, while being washed until water free from calcium chloride. Microspheres that were free of calcium chloride were squeezed using filter paper until the amount of water was minimal, then weighed. After that, gelatin was weighed according to the formula, dispersed in 100 mL of 40°C sterile water, and stirred in a water bath at a speed of 1,000 rpm for 10 minutes. The solution was separated using a Buchner funnel. The microspheres were then dispersed in a 5% maltodextrin solution, as many as, ten times the weight of the microspheres. The microspheres were dried using a freeze dryer for 96 hours. After that, the characterizations of the microspheres and microbial assay were carried out.

5.4. Physical characterization of formulation

5.4.1. Organoleptic

The evaluation of organoleptic is performed visually by observing powder form, color, and odor.

5.4.2. Determination swelling index and % moisture content

Moisture content analysis was measured using a moisture analyzer (Mettler Toledo HB43 S) of 500 mg microspheres and replicated three times. And for swelling index, 50 mg of microspheres were weighed and added 20 mL of sterile water. Observations were made at 37°C for 5 minutes, 30 minutes, 60 minutes, and 120 minutes. The microspheres were dried using filter paper and weighed as the final weight [13]. Swelling index is calculated by the formula:

$$swelling\ index = \left|\frac{initial\ weight - final\ weight}{intial\ weight}\right| x100\%$$

5.4.3. Structural analysis using Fourier Transform Infrared (FTIR) spectroscopy examination

The evaluation of the occurrence of cross-linking reactions was carried out by infrared spectra examination using ALPHA II FTIR Spectrometer. The result of the examination was compared with the infrared spectrum of sodium alginate, gelatin, and microspheres.

5.4.4. Morphology of the microspheres

To see the shape and surface morphology of the microsphere, it was carried out using an optical binocular microscopy (Olympus) coupled to a digital camera at 10x magnification and a scanning electronic microscope (SEM) (Hitachi Flexsem 100) in 500-5000x magnification. The particle size distribution was determined by span factor equation and the results expressed as volüme weighted mean diameter (µm).

$$span = [(d\vartheta, 90)1 - (d\vartheta, 10)]/(d\vartheta, 50)$$

where $(d\vartheta,10)$,$(d\vartheta, 50)$, $(d\vartheta, 90)$ correspond to the diameter at which teh cumulative sample volüme were under 10%, 50%, and 90%, respectively.

5.5. Microbial assay of free and microencapsulated *Lactobacillus plantarum* FNCC 0026

Cell viability and antibacterial activity were performed by comparing free cells and microspheres. Cell viability was performed using the total plate count method in MRS medium up to 10^{20} dilution. Incubation was for 30 hours at 35°C. Colonies were counted using a colony counter (Fungke Gerber 8500). On the other hand, the antibacterial activity was measured using the diffusion well method using a modified medium according to the test bacteria. MRSA used a 25%: 75% (w/v) MRS-NA medium combination and ESBL E.coli used a 50%: 50% (w/v) MRS-NA medium combination. Incubation was at 37°C for 24 hours. The diameter of the growth inhibition zone was measured with a digital vernier caliper (Taffware JIGO-150).

5.6. Survival assay of *Lactobacillus plantarum* FNCC 0026 microsphere in simulated GIT condition

The simulated gastrointestinal track (GIT) condition can be divided into two states: simulated gastric juice and intestinal juice. Simulated gastric fluid (SGF) was prepared in saline (9 g/L NaCl) and adjusted to pH 2.5 (meal stomach pH) with 1N HCl. Meanwhile, simulated intestinal fluid (SIF) was prepared with 6.5 g/L NaCl, 0.835 g/L KCl, 0.22 g/L $CaCl_2$, and 1.386 g/L $NaHCO_3$ and adjusted to pH 7.5. Both solutions were then sterilized by autoclaving at 121°C for 15 minutes. The test procedure was performed using 10 mL of SGF inoculated with 10% microspheres and incubated at 35°C. Viable cell counts and antimicrobial activity were observed after 30, 60, 90, and 120 minutes. Each tube was then centrifuged for 6 minutes at 2500 rpm, and the pellet was inoculated with 10 mL SIF and observed for 30 minutes [9].

5.7. Survival assay of *Lactobacillus plantarum* FNCC 0026 microsphere in storage

Dried *Lactobacillus plantarum* Itmicrospheres were stored aseptically at 8 °C. Probiotic viability was assessed by measuring viable cell counts and antimicrobial activity immediately after bead formation and 7, 15, 30, 45, and 60 days after storage [16].

5.8. Statistical analysis

All results were subjected to analysis of variance (ANOVA) using a completely randomized design with three replications for all treatments. The differences between means were tested at a significance value of $p < 0.05$.

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