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PROBIOTIC CHARACTERIZATION OF *LACTOBACILLUS* SP. IN VARIOUS ENCAPSULATION FORMULA

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Abstract

Research has been carried out on the characterization of the properties of L. paracasei and L. curvatus in several encapsulation formulas. This study aims to obtain the kind of encapsulation material formulation that maintains the viability and probiotic properties of both Lactobacillus. The method used in this research is a laboratory experiment using a completely randomized factorial design. The probiotic characteristics of the Lactobacillus sp. that have been encapsulated with several formulas were carried out by observing cell viability, acid resistance, bile salt resistance, and antimicrobial activity against pathogenic bacteria. The results showed that the bacteria L. paracasei in the cassava flour-alginate and L. curvatus in the alginate - skimmed milk had high viability for three weeks, same as the initial time, with the population reaching 3.52 x 10^{10} CFU/ml and 3,96 x 10^{10} CFU/ml, respectively. Furthermore, Lactobacillus bacteria encapsulated in alginate-skimmed milk formula have high resistance to acidic environments, high bile salt levels, and antimicrobial activity against E. coli and S. typhimurium. Therefore, alginate and skim milk as an encapsulant can protect probiotics survive longer and maintain their probiotics.

Key words*: encapsulation, Lactobacillus, probiotics characterization.*

INTRODUCTION

Probiotics are microorganisms that are beneficial to humans, especially in maintaining health and preventing disease. These microorganisms can enter the digestive tract and regulate the balance of microbes in the intestine, because they have special characteristics such as: non-pathogenic, resistant to gastric acid conditions, resistant to bile salt concentrations in the intestine, producing organic acids, and having antimicrobial properties against pathogenic intestinal bacteria (Markowiak & Śliżewska, 2017). One of the genera the probiotic found in cow colostrum is Lactobacillus. These bacteria have the ability to attach to host cells, remove or reduce pathogenic bacteria, produce acids, hydrogen peroxide, and bacteriocins that can inhibit the growth of pathogenic bacteria (Vieco-Saiz et al., 2019).

The viability of bacterial cells in probiotic products should be in the range of 10^8 – 10^9 CFU/g. The range will assure that probiotic bacteria survive the upper ingestion to exert their positive physiological functions in the human body. For instance, to ensure that it has been stated that the so-called "minimum therapeutic" level of viable probiotic microorganisms should be at least 10^6 CFU/g of viable cells throughout the product shelf-life because this viability will decrease during storage and while in the digestive tract (Terpou et al., 2019). Due to environmental factors that are less supportive to the survival of probiotic bacteria, namely a low gastric acid environment with a pH between 1.5-2.0 in an empty stomach and a pH between 4.5-5.0 in a filled stomach.

Also, the presence of the content of bile salts in the small intestine, which bacteria must pass while in the digestive tract. The concentration of bile salts equivalent to the physiological concentration of bile salts in the duodenum is 0.5% (Terpou et al., 2019; Oberoi et al., 2021; Puspawati, 2010). In addition, that all microbes that managed to live in a 0.3% bile salt content were declared to be resistant to bile salts in the small intestine (Wijayanto, 2009).

Among the several criteria for selecting candidate probiotic strains of Lactobacillus spp., bile salt resistance is one of the most important selective criteria, since bile salts are well known as strong surfactants and bile exposure in gastrointestinal tract is intensely toxic for probiotic Lactobacillus species to survive and retain activity in human intestine (Kusada et al., 2021). Microencapsulation is a technology of packing liquids, solids, and gaseous materials into tiny capsules that release those contents at controlled rates over long periods of time (Oberoi et al., 2021).

Encapsulation is the process of coating a core material, in this case, probiotic bacteria. This coating is carried out using certain encapsulating materials that are useful for maintaining viability, their characterization, and protecting probiotics from damage due to unfavorable environmental conditions. The viability of probiotic bacteria needs to be maintained during processing, food shelf life, supplements, and in the digestive tract. The purpose of encapsulation techniques is to stabilize cells increase the survival and stability of bacteria in production, storage, and digestion. Polysaccharides like alginate, gelan, carrageenan, chitosan, and starch are the most commonly used materials in the microencapsulation of bifidobacteria and lactobacilli. Techniques commonly applied for probiotic microencapsulation are emulsion, extrusion, spray drying, and adhesion to starch (Das et al., 2014).

Encapsulation materials often used are alginate, which is used as a protector or coating the core material; the advantage of using alginate is it is easy to form a gel matrix that coats bacteria. Most commonly employed polymer for immobilizing viable cells, due to its strong capacity to be cross-linked, easy to obtain, and release trapped cells, as a food additive, its lack of toxicity, low cost, simplicity, and biocompatibility. However, alginate is also weak and vulnerable in an acidic environment. Therefore, for the alginate encapsulation process to be carried out optimally, it is necessary to combine alginate with various other polymer compounds.

The addition of starch, skim milk, gelatine, and chitosan mixed with alginate can give better results after encapsulation. Blending alginate with other biopolymers could serve as a useful approach in strengthening the microcapsule structure (Mahmoud et al., 2020). Milk proteins are one of the candidates that can be combined with alginate to improve the structural characteristics of the microcapsules that envelope probiotic bacteria (Abd El-Salam & El-Shibiny, 2015). Milk proteins are caseins and whey proteins; whey protein induces gelation through heating (Abd El-Salam & El-Shibiny, 2015) and can be used as a coat or in combination with alginate for encapsulation probiotics (Mahmoud et al., 2020).

In this study, we aimed to characterize the probiotic properties of Lactobacillus which have been encapsulated with several types of encapsulation formulas against high acid and bile salt conditions, to determine the material formula that can be selected. The characteristics of the probiotic Lactobacillus sp. that has been encapsulated will be evaluated: cell viability, acid resistance, high bile salt resistance, and antimicrobial activity against pathogenic intestinal bacteria.

MATERIALS AND METHODS

The method used in this study was experimental in a laboratory with a completely randomized design (CRD) with a factorial pattern consisting of four tests. The first test was the storage of the viability of probiotic bacteria in an encapsulated formula with two factors consisting of *Lactobacillus paracasei* and *Lactobacillus curvatus* encapsulated in Alginate-skimmed, Tapioca Alginate. All treatments were stored for 1, 2, 3 and 4 weeks. The second test is the viability test of *Lactobacillus paracasei* and *Lactobacillus curvatus* which are encapsulated against the acidity of pH 2, 4, and 6. The third stage test is the viability of *Lactobacillus paracasei* and

Lactobacillus curvatus, encapsulated to bile salt concentrations of 0.3% and 0.5% (bile salt). The fourth stage of the test is a test of the antimicrobial activity of probiotic bacteria in the encapsulated formula against pathogenic digestive bacteria *E. coli* and *Salmonella typhimurium*. The data observed were the viability and the zone of inhibition.

Inoculum preparation

Lactobacillus paracasei and *Lactobacillus curvatus* were grown on MRS agar slant media for 24 hours at 37ºC. In sterile MRSB medium, 10% fresh bacterial culture was inoculated and incubated at 37ºC for 18-20 hours. The working cultures were then harvested and centrifuged at 5000 rpm for 10 minutes. The supernatant was separated from the filtrate to obtain biomass.

Alginate preparation

The bacterial biomass was suspended in physiological NaCl at MacFarland 3 turbidity, and TPC analyzed the population. Furthermore, 3 ml of the isolates of *Lactobacillus paracasei* and *Lactobacillus curvatus* were resuspended in 10 ml of sterile NaCl, then 60 ml of Sodium Alginate 3% (w/v) was added. After mixing, it was then dripped into a beaker glass containing 200 ml $CaCl_2$ 0.1 while stirring with a magnetic stirrer. Subsequently, it was washed with 0.8% sterile NaCl, then dried.

Skim alginate

Lactobacillus paracasei and *Lactobacillus curvatus* isolates that had been cultured were subcultured singly on 2.5 ml in MRSB and incubated for 9 hours. Then it was subcultured again in a consortium on 25 ml of skim milk and then set for 9 hours. Then it was subcultured again in 250 ml skim milk and then incubated again for 9 hours. After incubation, the bacterial inoculum in skim milk was centrifuged at 5000 rpm for 10 minutes at 4ºC. The obtained biomass was suspended in 100 ml of distilled water. A total of 100 ml of bacterial biomass suspension was then mixed with 100 ml of 3% alginate as a carrier. The mixture is then put into a syringe and dripped into a beaker glass containing 0.1 mol/l of $CaCl_2*2H_2O$ with a distance of 10 cm between the tip of the syringe to the surface of $CaCl_2*2H_2O$, then allowed to stand for 1 hour so that the encapsulated granules hardened while being stored on a shaker. Finally, the encapsulated granules were rinsed with sterile NaCl 0.8% and sterile distilled water, then dried.

Tapioca alginate

A sterile coating material, 1% (w/v) sodium alginate, dissolved in 90 ml of distilled water followed by 3% (w/v) tapioca flour, was prepared. First, inoculation of isolates biomass on the coating material was carried out by aseptically mixing 10% of the consortium biomass into 90 ml of the coating material formula solution until smooth. Then, the mixture of consortium biomass and the coating material is put into a dropper to be dripped on 0.1 M $CaCl_2*2H_2O$ solution, after which dripping Ca-alginate capsules will be formed. The capsules formed were separated from the $CaCl_2*2H_2O$ solution and washed twice using sterile distilled water. The washed Ca-alginate capsules were then put into NB medium and incubated for 24-30 hours in a rotary shaker at 150 rpm at room temperature to regrow the consortium of bacteria contained in the capsules (secondary multiplication) then dried.

Antimicrobial Activity Test

The method used to test the antimicrobial activity is the agar diffusion method, namely in a well. The tests for antimicrobial activity were carried out by taking 1 gram of microcapsules and putting it in 9 ml of NB, then vortexing. Then it was incubated for 24 hours at 37ºC. *Lactobacillus paracasei* and *Lactobacillus curvatus* cell-free supernatants were obtained using a centrifuge for 20 minutes and sterilized. A total of 20 L of cell-free supernatant and pellet were each put into wells on nutrient-dense agar (NA) media that had been inoculated with the test bacteria. The media was incubated for 24 hours at 37oC. The clear zone formed indicated an inhibition of the growth of the test bacteria by the supernatant. The diameter of inhibition (clear zone) (mm) around the hole was measured using a caliper. The provisions of the potential for bacteria can be seen by measuring the inhibition area, which is classified as Stout (2001).

Data analysis

The data obtained were analyzed using analysis of variance with a 95% confidence level and if there is a significant effect of treatment, it will be continued with Duncan's Multiple Distance test.

RESULTS AND DISCUSSIONS

Viability of *L. paracasei* and *L. curvatus* in the encapsulated formula at various storage times

Probiotic encapsulation is a process of coating probiotic bacterial cells using a protective material. The capsules formed immediately by contacting the cell-polymer droplet with the crosslinking solution. The formulations used as encapsulation materials in this study were alginate, skim alginate, and alginate cassava starch.

Tabel 1. Viability of *L. paracasei* and *L. curvatus* in Various Encapsulated Formulas Against Storage Time (CFU/ml)

Density (10^{10}) Cfu/ml						
Storage time	*L. paracasei* in Alginat	*L. paracasei* Alginate Skim	*L. paracasei* Alginat Cassava starch	*L. curvatus* Alginate	*L. curvatus* Alginate Skim	*L. curvatus* Alginat Cassava starch
t_0	1,06 D c	3,39 C b	3,72 C a	1,03 C c	3,73 C a	3,75 C a
t_1	3,17 A d	4,58 A c	4,91 A b	3,39 A d	4,91 A b	5,21 B a
t_2	3,00 AB e	3,86 B c	4,14 B b	3,26 A d	4,45 B b	5,55 A a
t_3	2,83 B c	3,65 B b	3,52 C b	2,82 B c	3,96 C a	3,75 C ab
t_4	2,13 C b	2,1 D b	2,98 D a	1,06 C d	2,25 D b	1,79 D c

Duncan's Multiple Range Test (MRT) (Table 1) shows that the number or density of bacteria until the third-week *Lactobacillus paracasei* and *Lactobacillus curvatus* encapsulated in the alginate skim matrix can maintain their viability with the bacterial cell density equal to the initial encapsulation time. The population reached 3.65 x 10^{10} and 3.96 x 10^{10} CFU/ml and began to decrease in the fourth week. The number of bacterial colonies that still reached 10^{10} CFU/ml showed that the alginate skimmed matrix as an effective encapsulation was used as a protective material.

Some works mention that alginate microbeads can protect probiotics during food storage, but not upon exposure to low-pH solutions, such as gastrointestinal conditions. However, mixing alginate with other polymers, such as chitosan and starch can enhance microcapsules' resistance to acidic media (Oberoi et al., 2021). An increase in microcapsule alginate resistance was also seen in the encapsulation of probiotics with alginate cassava starch which resulted in a higher probiotic population than only alginate.

Mahmoud et al. (2020) reported that Alginate-Skim proved to be the most promising encapsulating combination that maintains the survivability of *L. plantarum* to the recommended dose level under almost all the stress conditions. The results also revealed that microencapsulation does not affect the metabolic activity of the entrapped cells. Furthermore, there was no significant difference in the production of bioactive compounds between the encapsulated and the unencapsulated cells. Blending alginate with other biopolymers could strengthen the microcapsule structure. Milk proteins are also candidates for can be incorporated with alginate to improve the structure characteristic of the microcapsules as envelope probiotic bacteria. Milk proteins are caseins and whey proteins; whey protein induces gelation through heating (Abd El-Salam & El-Shibiny, 2015) and can use as a coat.

Tolerance of *L. paracasei* and *L. curvatus* in Encapsulated Formula Against Acidic

Duncan's Multiple Range Test (MRT) (Table 2) shows that the higher the pH the higher the number of probiotic in microencapsulated. At pH 6 *L. paracasei* and *L. curvatus* in all encapsulated formula of alginate, alginate-Skim, and alginate-cassava starch grew optimally, because the optimum growth of Lactobacillus bacteria was at the optimal pH range of 5.5-6.2.

Tabel 2. Tolerance of *L. paracasei* and *L. curvatus* in Various Formulas Against Acidic (CFU/ml)

Encapsulation Formula	Number of Population		
	pH 2 (10^2)	pH 4 (10^5)	pH 6 (10^8)
L. paracasei in Alginat	5.05 C b	7.5 B c	4.03 A A
L. paracasei Alginate Skim	4.33 C B	2.84 B Ab	3.93 A A
L. paracasei Alginat Cassava starch	6.12 C ab	1.86 B b	4.57 A a
L. curvatus Alginate	1.81 C c	2.17 B ab	3.26 A a
L. curvatus Alginate Skim	1.02 C a	3.69 B a	3.71 A a
L. curvatus Alginat Cassava starch	1.08 C d	2.08 B b	3.86 A a

The probiotic response in various formulas did not show a significant difference. However, up to pH 4 both *L paracasei* and *L. curvatus* which

were encapsulated by alginate skim had a higher bacterial population than the other formulas. Skim milk is composed of various complex ingredients such as lactose, casein protein, citrate, and phosphate which can act as a buffer so as to protect bacteria exposed to acids and bile salts.

Tolerance of *L. paracasei* and *L. curvatus* Encapsulated Against Bile Salt

Tolerance test of probiotic bacteria in the encapsulated formula to the concentration of bile salts was carried out to determine the ability of the bacteria to survive in an environment with high concentrations of bile salts, on 0.3% and 0.5% concentrations**.** One of criteria as candidate probiotic strains of Lactobacillus spp., is resistance bile salt since bile salts are well known as strong surfactants and bile exposure in gastrointestinal tract is intensely toxic for probiotic Lactobacillus to survive and retain activity in human intestine.

The analysis of variance showed that the bacteria *L. paracasei* and *L. curvatus* in the encapsulated formula had high resistance to bile salts of 0.3% and 0.5% after incubation for 6 hours. The number of colonies of probiotic bacteria in this encapsulated material shows a fairly high number, which is around 10^{10} CFU/ml. Although these results were not significantly different from the mean number of bacterial cells, the result showed that skim incorporated into alginate gave the population of bacteria higher than another formula. Lactobacillus resistance to high concentrations of bile salts is due to the presence of the enzyme bile salt hydrolase (BSH) which can hydrolyze bile salts. This enzyme is able to change the physico-chemical ability of bile salts to be non-toxic to lactic acid bacteria.

Antimicrobial Activity of *L. paracasei* and *L. curvatus* Encapsulated Against Digestive Pathogenic Bacteria

Based on the analysis of variance, there was no interaction between pathogenic bacteria and *L. paracasei* and *L. curvatus* encapsulated. However, the two pathogenic bacteria showed significantly different responses to each probiotic species. Based on Table 3, the pathogenic bacteria *E. coli* has a higher sensitivity than the bacteria *Salmonella typhimurium*.

Table 3. Duncan's Multiple Range test inhibition zone of *L. paracasei* and *L. curvatus* encapsulated against *E. coli* and *Salmonella typhimurium*

Pathogen	Zone Inhibition (mm)
E. coli	15.22 a
Salmonella	9.19 b

The antimicrobial activity test of probiotic bacteria in the encapsulated formula against pathogenic digestive bacteria to determine the ability of probiotics that have been encapsulated in the various coating in inhibiting pathogenic digestive bacteria such as *E. coli* and *Salmonella typhimurium*. Based on Duncan's Multiple Range test inhibition zone, it is known that *E. coli* has a higher sensitivity to *L. paracasei* and *L. curvatus* encapsulated than *Salmonella typhimurium*. Lactobacillus, including *L. paracasei*, produce bacteriocin, which is antimicrobial against Gram-negative bacteria *E. coli* and *Salmonella enterica* and Gram-positive bacteria *S. aureus* and *Bacillus thuringiensis*. However, bacteriocins from LAB with a broad antimicrobial spectrum against Gram-negative and Gram-positive bacteria and fungi are still not frequently reported. Bacteriocins are a group of antimicrobial compounds, which are ribosomally synthesized peptides produced by bacteria to inhibit the growth of similar or closely related bacterial strains either in the same species, or across genera (Todorov, 2019).

L. paracasei and *L. curvatus* bacteria produce organic acids in the form of short chain fatty acids which are antimicrobial against pathogenic bacteria, so that they can inhibit the growth of these two pathogenic bacteria. According to Brink et al. (2005), the antibacterial and inhibitory activity against *E. coli* and other enterobacteria was caused by the increased production of short-chain fatty acids and acetic acid, which caused a decrease in pH in the digestive tract.

The low pH condition is the optimum pH for the growth of lactic acid bacteria, resulting in an increase in the population of lactic acid bacteria that will compete with pathogens for nutrients. Short-chain fatty acids such as formic, acetic, propionic, butyric, and lactic acids are produced during the anaerobic metabolism of carbohydrates and have an important role in decreasing pH. The microbial

growth inhibition by organics may be due to the ability of these acids to pass across the cell membranes, dissociate in the more alkaline environment of the cell interior, and acidify the cytoplasm (Gilor et al., 2008). The sensitivity of each pathogenic bacteria to probiotic bacteria in the encapsulated material can be seen in Table 4.

Table 4. Sensitivity of Pathogenic Bacteria *E. coli* and *Salmonella typhimurium* to encapsulated *L. paracasei* and *L. curvatus*

Probiotic Bacteria	Supernatan / Pellet	Pathogenic Bacteria	Inhibition Diameter (mm)	Note
L. paracasei Alginat	Supernatan	*E. coli*	13.17	Sensitive
	Pellet		14.67	Sensitive
	Supernatan	*S. typhimurium*	9.67	Less Sensitive
	Pellet		7.17	Less Sensitive
L. paracasei Skim Alginate	Supernatan	*E. coli*	11.33	Sensitive
	Pellet		13.33	Sensitive
	Supernatan	*S. typhimurium*	8.00	Less Sensitive
	Pellet		8.83	Less Sensitive
L. paracasei Tapioca Alginate	Supernatan	*E. coli*	13.17	Sensitive
	Pellet		14.17	Sensitive
	Supernatan	*S. typhimurium*	7.50	Less Sensitive
	Pellet		9.83	Less Sensitive
L. curvatus Alginate	Supernatan	*E. coli*	18.83	Sensitive
	Pellet		18.33	Sensitive
	Supernatan	*S. typhimurium*	9.33	Less Sensitive
	Pellet		9.50	Less Sensitive
L. curvatus Skim Alginate	Supernatan	*E. coli*	20.83	Sensitive
	Pellet		14.83	Sensitive
	Supernatan	*S. typhimurium*	12.67	Sensitive
	Pellet		11.33	Sensitive
L. curvatus Tapioca Alginate	Supernatan	*E. coli*	15.00	Sensitive
	Pellet		15.00	Sensitive
	Supernatan	*S. typhimurium*	10.00	Sensitive
	Pellet		6.50	Less Sensitive

Based on the results in Table 4, supernatants and pellets of *L. paracasei* and *L. curvatus* bacteria in the encapsulated formula produced the same inhibitory power against *E. coli* and *S. typhimurium* bacteria. Both have high enough antimicrobial activity to inhibit the growth of pathogenic bacteria *E. coli* and *S. typhimurium*. The mean inhibition diameter of the supernatant and pellet of probiotic bacteria encapsulated against *E. coli* and *S. typhimurium* was the highest produced by *L. curvatus* in alginate-skim 14.83 mm 20.83 mm against *E. coli*, and the inhibition zone was 11.33 mm and 12.67 mm against *S. typhimurium*.

The results showed that using Alginate-skimmed milk as an encapsulant was effective as a coating so that the inhibitory activity was more optimum. The results of this antimicrobial activity test showed that *E. coli* was more sensitive to both *Lactobacillus paracasei* and *L. curvatus* species than *Salmonella typhimurium.* Meanwhile, alginate and alginate-skim as the best probiotic protectors and produce high sensitivity to pathogens, especially *E. coli.*

CONCLUSIONS

Based on the analysis results, the conclusion is as follows:

1. The bacteria *Lactobacillus paracasei* and *Lactobacillus curvatus* encapsulated in various formulas can withstand acidic conditions and high bile salt levels.
2. Skim alginate is the best encapsulation material that can maintain probiotic viability against acidic conditions and high bile salt levels.
3. The skim alginate is the encapsulation material that can maintain the characteristics of probiotics, having antimicrobial activity against pathogenic bacteria in the form of supernatants and pellets.

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