Viability microcapsules *Lactobacillus plantarum* Mar 8 and KMar C2 in chocolate (*Theobroma cacao* L) for probiotic purposes

R D Mulyaningsih1*, N Nurhidayat2 & W Mangunwardoyo1

1Department of Biology, Faculty of Mathematics and Natural Science, Universitas Indonesia, Depok, 16424, Indonesia
2Research Center for Biology, Indonesia Science Center, Cibinong, Bogor, 16911, Indonesia

*Corresponding author: rdiannm74@gmail.com

Abstract. *Lactobacillus plantarum* Mar 8 and KMar C2 is a lactic acid bacteria can be used as probiotic. Chocolate have served as antioxidant and beneficial for health, it contains: flavonoids, theobromine, epicatechin, and catechin. Combination between chocolate and bacteria can increased chocolate benefit as probiotic chocolate. Probiotic used to decrease uric acid level in blood if contains $10^6 - 10^9$ cfu/g living bacteria. The research purpose study of viability probiotic in chocolate. The assay used in viability research is Total Plate Count. The process encapsulation of Mar 8 dan KMar C2 use 10% milk skim and 3% chocolate fat made into microcapsules with spray drying method. The result revealed microcapsule viability before mix with chocolate $1,925 \times 10^9$ cfu/g. After mixing 20 g microcapsules in chocolate, viability decreased 1 log to $1.7 \times 10^9$ cfu/g and concentration probiotic is $3.4 \times 10^7$ cfu/g. Chocholate can be used as a probiotic chocolate. Probiotic chocolate will be use to decrease the level of uric acid in blood.

1. Introduction

Bacteria *L. plantarum* produces lactic acid in the digestive tract, helps reduce flatulence, removes toxic components from food, helps absorption of vitamins and antioxidants [1]. It is very effective in inhibiting pathogenic microorganisms that cause disease by producing organic acids, hydrogen peroxide (H2O2), diacyl and bacteriocin, and can increase the secretion of proteolytic enzymes used to breakdown proteins into amino acids so that they can be absorbed more quickly by the intestine [2]. Bacteria *L. plantarum* has a zone of inhibition against gram negative bacteria, known as bacteriocin. Inhibitory zones are in the form of clear zones which show bactericidal properties (inhibit bacteria) and pseudo zones show bacteriostatic properties (inhibit microbial growth) [3].

Probiotics are lived microorganisms symbiosis with food ingredients, consuming the probiotic in sufficient quantities is able to provide the beneficial effects on health [4]. Probiotic characteristics among are antimicrobial, anti-carcinogenic, stimulation of the immune system resistant to acid, resistant to bile salt, stick to digestion, can colonize, safe for food and health [5]. The standard probiotic for health food has a concentration microorganism approximately $10^6 - 10^9$ cfu/g [6].

The feasibility of probiotic cells must be maintained during manufacture, storage and can be through the digestive tract of the host. The current constraint is that the probiotic bacteria are easily damaged
due to storage and transportation factors, so needed to management of spread as functional food ingredients to consumers. Technology development in preserving probiotic bacteria by using a dry powder system, as to provide better cell stability [6].

Viability is the ability or life cells to grow normally under optimal conditions. The amount of living microbes must be sufficient to provide a positive effect on health and be able to colony can reach the amount needed in a certain time, this give probiotic culture always refers to the dose consumed and the amount of microbes living [7].

Encapsulation is a technique for coating an active ingredient (probiotic bacteria) with a layer of polymer walls to produce micro or nano small particles. This coating can protect active ingredients from surrounding environmental conditions such as light, temperature, humidity, chemicals, stomach acid, bile salts and interactions with other substances, that prevent viability can be maintained [8]. Encapsulated probiotic a higher viability than without encapsulation and the success of the process encapsulation is effected by the coating material used [5]. Material with carbohydrates and proteins can proved good protection for microcapsules [8].

The encapsulation technique is the most used spray drying. The advantage of spray drying can be applied to hydrophilic conditions and hydrophobic polymer solutions, it can handle labile material and relatively cheaper, simple also faster, the resulting particle size is 2 - 1200 µm[5]. Spray drying is the safest and cheapest method for food industry. It involves atomization into the hot air drying chamber. The advantages of spray drying are the production of flowable powders, ability to control particle size, rapid drying, easy scale up and continuous production. Production results are easily packaged so as to reduce production costs [9].

Chocolate rich of antioxidants, anti-free radical and anti-carcinogenic because polyphenol contains flavonoid, epicatechin, catechin, theobromine [10], also contains vitamin and mineral. Polyphenol in chocolate can be use cholesterol risk reduce, heart attack, stroke prevented, hypertension [11], preeclampsia prevented, triglyceride reduce [12], preventing leukocyte adhesion, lowering coagulatory and inflammatory factors, reduced the postprandial impairment of vascular function after a glucose tolerance test, increased of thrombocytes, lymphocytes, monocytes, neutrophils, insulin [13].

*Lactobacillus plantarum* Mar8 and KMar C2 is bacteriocin, improved immune system, the detection of toxic compounds, is able to growth at low pH, tolerating bile salts, producing organic acids, survive the preservation process and during storage, showed uricase activity can degrade uric acid, *L. plantarum* Mar 8 and KMar C2 can be use as probiotic. *L. plantarum* Mar 8 and KMar C2 are encapsulated with 10% skim milk and 3% chocolate fat can provide optimal protection against excessive heat during the encapsulation process and survive in the digestive system. The mixed chocolate and *L. plantarum* able to add value to chocolate for reduce uric acid level in blood [14]. The research purpose study of to analyze microcapsules process and the viability microcapsules *L. plantarum* powder in probiotic chocolate.

2. Methods

2.1. Subculture *Lactobacillus plantarum* Mar 8 and KMar C2

Isolate *Lactobacillus plantarum* Mar 8 and KMar C2 obtained from Collection of Microbiology Laboratory LIPI Cibinong, subculture in the media of Pepton Yeast Glucosa (GYP). Inoculated of 1 ose bacterial isolate in GYP, incubated 48 hours at 37°C [15].

2.2. The essay activity of *L. plantarum* Mar 8 and KMar C2 in degradation uric acid

The subculture of bacteria isolate, was inoculated in to GYP medium of uric acid, incubated 48 hours at 37°C. The clear zone results indicated the ability degradation of uric acid by *Lactobacillus plantarum* Mar 8 and KMar C2 [3].

2.3. Encapsulation process of *L. plantarum* Mar 8 and KMar C2
To prepare culture starter *L. plantarum* Mar 8 and KMar C2 inoculated in 5 ml of liquid GYP, incubated 48 hours at 37°C and mixed *L. plantarum* Mar 8 and K Mar C2.

2.4. **Inoculated isolate on lining and solvent media**

Amount of 100 µL from culture starter, inoculated in 5 mL lining sterile media of 10% milk skim and 3% chocolate fat, incubated 24 hours, follow all culture are inoculated in 45 mL lining sterile media, incubated 24 hours, and further inoculated in 450 mL lining sterile media, was incubated 24 hours, after that it was centrifuged at 9500 rpm for10 minute at 4 °C, the supernatant was removed and pellet dissolved with solvent at 12% skim milk and 3% chocolate fat [14].

2.5. **Total cell before encapsulated**

Amount of 1 mL was from solvent media isolated to count amount of bacteria cell population with *Total Plate Count* (TPC) method, and dilution solvent media at 10-5, 10-6, and 10-7 using NaCl 0,85%, observation was observed 48 hours at 37 °C [15].

2.6. **Encapsulation of L. plantarum Mar 8 and KMar C2**

The isolated was applied in spray drying equipment, at outlet temperature 125 °C, inlet temperature 50 – 70 °C, air flow rate 1 m2/min, and pressure 20 x 10 kPa. The results spray drying is microcapsule powder [14].

2.7. **Viability test after the encapsulation**

Microcapsule powder is divided into 3 different places and stored in 3 different temperatures is 4 °C, 27 – 30 °C and 37 °C. Viability testing is carried out in 3 stages, after the encapsulation process, after storage for 7 days and after storage for 14 days. It was taken 1 g of microcapsule powder dissolved in 9 ml of NaCl 0.85% sterile, the result of viability was calculation by means of *Total Plate Count* (TPC), dilution solvent media isolated at 10-4, 10-5, and 10-6 using NaCl 0.85%. Observation was incubated 48 hours at 37 °C [15].

2.8. **The preparation probiotic chocolate**

The preparation chocolate candy with composition 50% chocolate paste, 20% chocolate fat, 11.5% skim milk and 18.5% stevia sugar. The process of chocolate candy by tempering is heating different with variation temperature. Addition in the chocolate 20 g microcapsule *L. plantarum* $10^{10}$ cfu/g, then mixed evenly, inserted into the mold, and cooling. Chocolate is removed from the mold after reaching 10 °C [16].

2.9. **Viability test L. plantarum in probiotic chocolate candy**

Probiotic chocolate candy is divided into 3 different places and stored in 3 different temperatures is 4 °C, 27-30 °C and 37 °C. Viability testing is carried out in 3 stages, after the encapsulation process, after storage for 7 days and after storage for 14 days. It was taken 1 g of microcapsule powder dissolved in 9 ml of NaCl 0.85% sterile, the result of viability was calculation by means of *Total Plate Count* (TPC), dilution solvent media isolated at $10^{-3}$, $10^{-4}$, and $10^{-5}$ using NaCl 0.85%. Observation was incubated 48 hours at 37 °C [15].

2.10. **Viability of L. plantarum per gram (mL) is calculated using the following formula [15]**

$$
\text{cfu} = \Sigma \text{colonies} \times \frac{1}{\text{dilution}} \times \frac{1}{\text{vol plating}}
$$

Information :

- $\Sigma$ bacteria (cfu)/g : the amount of cells bacteria
- $\Sigma$ colonies : the amount of colonies after plating process
3. Results and Discussion

3.1 Clear zone produce on the essay degradation uric acid activity of L. plantarum Mar 8 and KMar C2

The essay result of clear zones on bacteria that grown in GYP uric acid medium can be seen in Figure 1. The clear zones formed on activity testing L. Plantarum Mar 8 and K Mar C2 in degrade uric acid, diameter clear zones 3 cm in petri dish. Clear zone around the colony proved bacteria produce acid and uricase enzymes, so can neutralize CaCO$_3$ than degradation uric acid. The catalase reaction is negative (not bubbly) after H$_2$O$_2$ drops [17]. Both bacteria to use for research the viability of bacteria after microcapsule process, to decrease uric acid in the blood.

![Figure 1](image)

**Figure 1.** The clear zona L. plantarum Mar 8 and KMar C2

3.2 The total cells L. plantarum Mar 8 and K Mar C2 before and after encapsulation

The total cells L. plantarum colonies after encapsulation process decreased compared to before encapsulation process and on chocolate candy decreased compared to microcapsule powder.

| Treatment L. plantarum | Before encapsulation | Encapsulation | After mixing chocolate |
|------------------------|----------------------|---------------|------------------------|
|                        | A        | B        | Average               |
| Before encapsulation   | 1.03 x 10$^{11}$ | 1.05 x 10$^{11}$ | 1.04 x 10$^{11}$      |
| Encapsulation          | 1.88 x 10$^{10}$  | 1.977 x 10$^{10}$ | 1.925 x 10$^{10}$    |
| After mixing chocolate | 1.49 x 10$^{9}$   | 1.91 x 10$^{9}$   | 1.70 x 10$^{9}$     |

The of the research viability before and after encapsulation process decreased one logarithmic from 1.04 x 10$^{11}$ cfu/g to 1.925 x 10$^{10}$ cfu/g, decreased viability because heating during the spray drying process [18]. The amount of bacteria cell mixed chocolate was one logarithmic decreased become 1.70 x 10$^9$ cfu/g, because change in pH for microcapsules has 6.0 and after process has 4.0. Decreased pH can cause changes enzyme activity in bacteria [18]. The result amount probiotic doses in chocolate candy 3.4 x 10$^7$ cfu/g are calculated from 20 g microcapsule for 1000 g chocolate candy, still qualifying for use as probiotics [6]. The purposespf protection agent to caot bacteria during high temperature when spray drying process cease protection bacteria death [19].
Table 2. The amount of *L. plantarum* cell (cfu/g) is based on storage temperature in microcapsule powder after 14 days

| Initial concentration | Storage temperature |
|-----------------------|---------------------|
| 1.925 x 10^10         | 4 °C                |
| 1.745 x 10^10         | 27 – 30 °C          |
| 1.49 x 10^10          | 37 °C               |
| 1.385 x 10^10         |                     |

The result viability of cells during storage at 4 °C dropped 9% to 1.745 x 10^10 cfu/g, storage at 27 – 30 °C dropped 23% to 1.49 x 10^10 cfu/g and storage 37 °C dropped 28% to 1.385 x 10^10 cfu/g.

Bacterial cells in the form of microcapsules have better resistance, possibly to the use skim milk and chocolate fat is a good substrate for *L. plantarum* growth at storage temperature 37 °C because the temperature of 37 °C is the optimal temperature for the growth of *L. plantarum* bacteria [17]. The higher outlet temperature and rapid drying influences the cell survival after drying as well as during storage, so in order to retain the maximum cell viability, lower outlet temperature. The viability loss during storage the big impact on survival during storage, cause membrane lipid oxidation, storage temperature and moisture content the key factor that effect cell viability [9].

Table 3. The total cells of *L. plantarum* (cfu/g) are based on storage temperature in chocolate after 7 and 14 days

| Treatment       | Before storage | Temperature storage |
|-----------------|----------------|---------------------|
|                 |                | 4 °C                |
| 0 Day           | 1.7 x 10^9     |                     |
| 7 days          | 1.53 x 10^9    | 1.49 x 10^9         | 1.42 x 10^9 |
| 14 days         | 1.465 x 10^9   | 1.22 x 10^9         | 1.07 x 10^9 |

Decreasing bacterial cells probiotic chocolate candy after the storage 7 and 14 days but do not meaningful. Viability microorganism in chocolate must be able to have positive effect on health. The encapsulation process can stable viability under extreme condition, so during storage amount of encapsulated probiotic is more stable than probiotic without encapsulation [20]. Use chocolate fat has the advantage to contains lipids and proteins which give an additional protection to bacteria, so improve the microcapsules increasing the elasticity of the protectant film formed during spray drying, helping to avoid cracked particles and protecting the cells inside them [19].

References

[1] Tari A I N, Handayani C B, and Sudami 2016 *Agritech* **36** 7
[2] Arief M, Fitriani N, and Subekti S 2014 *J. Ilm. Perikan. dan Kelaut.* **6** 49
[3] Yulinery T & Nurhidayat N 2015 Proc. Seminar Nasional Masyarakat Biodiversitas Indonesia **1** 270.
[4] Yuniastuti A 2014 *Monograf probiotik (dalam persepektif kesehatan)* (Semarang: UNNES Press) p 99
[5] Firdaus M, Setijawati D and Kartikaningsih 2014 *Res. J. Life Sci.* **01** 27
[6] Coghetto C C, Flores S H, Brinques G B and Ayub M A Z 2016 *LWT - Food Sci. Technol.* **71** 54
[7] Özkan G and Bilek S E 2014 *Int. J. Nutr. Food Sci.* **3** 145
[8] Sumanti D M, Lanti I, Hanidah I, Sukarminah E and Giovanni A 2016 *J. Penelit. Pangan* **1** 7
[9] Rajam R & Anandharamakrishnan C 2015 *LWT - Food Sci. Technol.* **60** 773
[10] Natasya and Budiman I 2013 *Efek dark chocolate terhadap penurunan tekanan darah* Universitas Kristen Maranatha **75** pp 0 – 3
[11] Ramlah S and Yumas M 2017 *J. Ind. Has. Perkeb.* **12** 58
[12] Carolia N and Ayuning L G I 2016 *Majority* **5** 59
[13] Esser D M, Mars E, Oosterink A, Stalmach M, Mülle, and Afman L A 2014 *FASEB J.* **28** 1464
[14] Maciel G M, Chaves KS, Grosso CRF, and Gigante M L 2014 *J. Dairy Sci.*, **97** 1991
[15] Triana E and Yulinery T 2015 *Proc. Seminar Nasional Masyarakat Biodiversitas Indonesia* **1** 278
[16] Indarti E N, Arpi N and Budijanto S 2013 *J. Teknol. dan Ind. Pertan. Indones.* **5** 1
[17] Yulinery T and Nurhidayat N 2013 *J. Ilmu Kefarmasian Indones.* **11** 147
[18] Lapsiri W, Bhandari B, and Wanchaitanawong P 2012 *Dry. Technol.* **30** 1407
[19] Bustos P and Bórquez R 2013 *Dry. Technol.* **31** 5
[20] Jati A U P, Jenie B S L, and Suluantari 2015 *J. Teknol. dan Ind. Pangan* **26** 135