Shelf life of Microencapsulated and Free Cells of *Lactobacillus plantarum* IS-10506 at Different Temperatures

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Abstract. Survival of microencapsulated probiotic *Lactobacillus plantarum* IS-10506 of dadih origin at different storage conditions has been investigated. *L. plantarum* cells suspension was dispersed in Flocel pH 101 mixed with skim milk powder and dried by Fluid Bed Dryer (FBD) at 37°C for 2 hours, then microencapsulated by 4.75% (w/v) sodium alginate and 5.5% (w/v) calcium chloride. The microencapsulated and free cells probiotics were packed in standing aluminium pouch and stored at three different temperatures, 24°C (room temperature), 4°C (chiller), and -20°C (freezer) for 24 weeks, and the viable counts of probiotic were enumerated using MRS Agar medium every two weeks. Microencapsulated and free cells of probiotic *Lactobacillus plantarum* IS-10506 showed stable viability at -20°C for 24 weeks. At 4°C free cells showed significant decreased of viability while microencapsulated cells showed stable viability at week 24. Storage at 24°C led to significant loss of viability (p< 0.05) of both free cells and microencapsulated cells after 8 and 10 weeks, respectively, to undetectable level. Moisture content in both of free cells and microencapsulated cells at 24°C significantly increased at week 2. While at 4°C, there was significant increase of moisture content observed in free cells at week 2, and microencapsulated cells showed significant increase of moisture content at week 10. Likewise, at -20°C, moisture content of both free and microencapsulated cells showed significant increase at week 2 and 10, respectively.

1. Introduction

*Lactobacillus plantarum* IS-10506 is a novel indigenous probiotic isolated from dadih, a spontaneous fermented buffalo milk from West Sumatera, Indonesia [1]. The probiotic properties such as acid and bile tolerance [2], adhesion properties [3], and competitiveness against pathogens has been proven *in vitro*[4]. *L. plantarum* IS-10506 showed antimutagenic property [5], as well as significant increase offaecalsIgAand improving zinc status of young children [6]. Moreover, the probiotic strain had the potential in treating Atopic Dermatitis’ children [7].

Viability plays important role, and the efficacy of probiotic function is strain dependent. All industrial applications require to maintain viable counts of probiotic population during handling, processing and storage. Effort to protect the viable cells is very important to anticipate high temperature, oxygen and humidity in the environment. Microencapsulation technique can help in
maintaining stable viability of probiotic. Several studies had been carried out to investigate stable viability at different storage conditions[8].

Microencapsulation is an innovative and effective technique applied in food industries for protecting probiotic cells against unfavorable conditions and releasing the cells as living, and metabolically active in the gut [9]. Fluidized bed drying is an efficient and cost-effective alternative in drying starter cultures as well as probiotic compared to freeze drying. Hence, it is interesting to investigate the effect of process variables and conditions of fluidized bed drying on viability of probiotic bacteria [10].

Packaging materials and storage condition also play a role in maintaining stable viability of probiotics. Different packaging materials such as glass, metal pouch and others have been suggested. The purpose of this study was to investigate the survival of microencapsulated probiotic Lactobacillus plantarum IS-10506 isolated from dadih at different storage temperatures.

2. Materials and methods
2.1. Bacterial strain
Probiotic Lactobacillus plantarum strain IS-10506 (GenBank accession no. DQ860148) of dadih origin[1] was used in this study. The lactic acid bacteria were cultured in de Man-Rogosa-Sharpe Broth (Oxoid, UK), at 37°C overnight and then cultivated in fermentor SP30 L (BIOTRON, Korea) for 20 hours at 37°C. The cells of Lactobacillus plantarum IS-10506 were concentrated using cold centrifuge (THERMO, USA), at 3,000 × g for 20 min at -4°C and the cell pellet was washed once with sterile aquadest. The initial population of bacteria in the suspension was 2.58 × 10^{12} CFU/mL.

2.2. Fluidized bed dryer
Filler materials were consist of 1 kg of microcrystalline cellulose (Gujarat, India) and 1 kg of Skim Milk Powder (Dairy America, USA) which were previously loaded and mixed into fluid bed dryer (Hong Dau, Taiwan). The fluid bed drying and filler materials were sterilized at 120°C before drying process. One hundred ml of L.plantarum cells were added with 100 ml of UHT milk, atomized by fluid nozzle in side-spray position using a peristaltic pump applying a spraying air pressure. The pressure used was 1.3kPa with a maximal 37°C bed temperature, with spraying rate in a range of 10 ml/ min. Sodium alginate 4.75% (w/v) and calcium chloride 5.5% (w/v) were used to encapsulated cells.

2.3. Experimental design
After drying, ten gram of microencapsulated and free cells of probiotics each was kept in 70 g shiny gold standing aluminium pouch, 125 micron thickness, with one way valve and zipper, and stored at 24°C (room temperature), 4°C (chiller), -20°C (freezer) for six months. Storage temperature was monitored by laboratory thermometer. Viable counts of probiotic were enumerated by pour plate on MRS Agar (SNI 2981: 2009), in every two weeks. Viable counts were assessed in triplicate.

2.4. Enumeration of Viability
Microencapsulated and free cells of probiotic L. plantarum IS-10506 were enumerated on MRS Agar (Oxoid, Basingstoke, UK). One gram of sample was mixed with 9 ml sterile Ringer (NaCl) solution. Followed by serial dilution and then pour on the MRS Agar plate and incubated at 37°C for 48 hours. The viability of the sample was reported as CFU/g.

2.5. Moisture content determination
Moisture content of the samples were analyzed using Moisture Analyzer (KERNandSOHN GmbH, Germany). Three grams of sample was poured into aluminium plate, then heated by halogen lamp for 3 minutes at 100 °C and confirmed by oven drying method at 102 °C for 3 hours.
2.6. Statistical Analysis
SAS Software version 9.4 was used to analyze the data followed by Duncan’s multiple range tests for significant difference among mean values at the 95% confidence interval.

3. Results and Discussion
3.1. Viable counts
Temperature (p = 0.0001), combination of microencapsulation and temperature (p=0.019), keeping time (p = 0.0001), combination of temperature and keeping time (p = 0.0001), combination of microencapsulation, temperature and keeping time (p = 0.044) showed significant difference on viability, but microencapsulation did not show significant effect on viable counts (p= 0.349) (Table 1). Storage at 24°C led to significant loss of viability (p< 0.05) in free cells as well as microencapsulated cells after 8 and 10 weeks, respectively, to undetectable level (Fig 1.A). At 4°C, viability of free cells of probiotic was significantly decreased by 2.34 Log CFU/g, and no significant decreased of viable counts of microencapsulated probiotic, relatively stable after 24 weeks storage (Fig 1.B). On the other hand, at -20°C, there was no significant loss of viability of free cells of probiotic L. plantarum during 24 weeks period of storage (p> 0.05), decreased by 1.03 Log CFU/g. Viability of microencapsulated L. plantarum IS-10506 at week 24, showed significant decreased by 1.41 Log CFU/g(p< 0.05) (Fig 1.C).

A. Room temperature (24°C)
The results also showed that viability of free cells of *L. plantarum* –IS 10506 was relatively stable at 4°C for 22 weeks, while microencapsulated probiotic was stable during 24 weeks. At week 24, the viable counts of microencapsulated probiotic powder was not significant as compared to week 22, however the declined viable counts was significant compared with week 0. Sulaiman [11] reported
that at 4°C, microencapsulation showed significant higher of viable counts of *L. plantarum* –IS 10506 as compared to free cells during 5 weeks, which were decreased by 0.8 Log CFU/g and 1.57 Log CFU/g, respectively.

At -20°C, both free cells and microencapsulated cells showed stable viability at week 24. The significant reduction of viability of microencapsulated probiotic stored at -20°C at week 24 could indicate non homogeneity of the powder. The free cells of probiotic *L. plantarum* IS-10506 showed stable viability stored at -20°C for 24 weeks. Sulaiman [11] reported that the estimated shelf life for maintaining viability of $10^{12}$ CFU/g, at -20°C was 11 weeks and 45 weeks, for free cells and microencapsulated probiotic powder, respectively, with 2 log cycles decreased to $10^{10}$ CFU/g.

Horison and Suro [12] reported that microencapsulation showed no significant effect on the half-life period of *Enterococcus faecium* IS-27526. Further study needs to confirm one year shelf life of microencapsulated *L. plantarum* –IS-10506, to confirm the results reported by Sulaiman [11]. In a validated in vitro model of upper GI tract, Suro et al [13] reported that microencapsulation significantly increased survival of *L. plantarum* IS-10506, which is an important factor for probiotic to reach intestinal alive.

### Table 1. Analysis of variance on microencapsulation and storage condition to viability of *L. plantarum* IS-10506 (log$^{10}$CFU/g)

| Source                        | DF | Type III SS | Mean Square | F Value | P-value |
|-------------------------------|----|-------------|-------------|---------|---------|
| Microencapsulation            | 1  | 0.390       | 0.390       | 0.960   | 0.349   |
| Temperature                   | 2  | 1925.114    | 962.557     | 2428.140| 0.0001  |
| Microencapsulation *temperature| 2  | 3.250       | 1.625       | 4.100   | 0.019   |
| r(Microencapsulation *Temperature) | 10 | 4.048       | 0.405       | 1.020   | 0.430   |
| Week                          | 12 | 805.514     | 67.126      | 179.850 | 0.0001  |
| r(Week)                      | 24 | 8.958       | 0.373       | 0.940   | 0.547   |
| Microencapsulation *Week      | 12 | 5.908       | 0.492       | 1.220   | 0.384   |
| Temperature*Week              | 24 | 1111.695    | 46.321      | 116.850 | 0.0001  |
| Microencapsulation *Temperature*Week | 24 | 15.573     | 0.649       | 1.640   | 0.044   |
| Error                         | 120| 47.570      | 0.396       |         |         |
| Corrected Total              | 233| 3929.296    |             |         |         |

### 3.2. Moisture content

Table 2 shows that temperature and microencapsulation showed significant effect on moisture content of probiotic powder (p=0.0001); interaction between microencapsulation and temperature showed significant effect on moisture content (p =0.0003). Interaction of microencapsulation, temperature and keeping time also showed significant effect on moisture content of probiotic powder (p= 0.0001). Moisture content of microencapsulated cells was significantly lower than free cells at 24˚C (p <0.05) in a range of 3.2 – 6.7%. Free cells showed wider range of moisture content, 3.2–7.7%. At 24˚C, there was significant increased (p<0.05) of moisture content observed in both of free cells and microencapsulated cells at week 2 (Fig 2). While at 4˚C, there was significant increased (p<0.05) of moisture content observed in free cells at week 2, while microencapsulated cells showed significant increase of moisture content at week 10 (p<0.05) (Fig 2). Likewise, at -20°C, moisture of both free cells and microencapsulated cells were significant increase (p<0.05) at week 2 and 10, respectively. The loss of viable counts was inline with increasing moisture content, which facilitated oxidation and enzymatic hydrolysis during storage[14]. Overall, microencapsulation resulted in stable viability and moisture content at lower storage temperature.
A. Room temperature (24°C)

B. Chiller (4°C)
C. Freezer (-20 °C)

**Figure 2.** Moisture content at Room temp (A) Chiller (B) and Freezer (C) for 24 weeks. A1: Free cells (24°C); A2: Free cells (4°C); A3: Free cells (-20°C); B1: Microencapsulated (24°C); B2: Microencapsulated (4°C); B3: Microencapsulated (-20 °C). (*): significant difference

**Table 2.** Analysis of variance of microencapsulation and storage condition to viability of *L. plantarum* IS-10506 (log$_{10}$CFU/g) Interaction between microencapsulation and storage temperature towards moisture content

| Source                  | DF | Type III SS | Mean Square | F Value | P-value |
|-------------------------|----|-------------|-------------|---------|---------|
| Microencapsulation      | 1  | 98.385      | 98.385      | 6609.480| 0.0001  |
| Temperature             | 2  | 134.151     | 67.076      | 2690.560| 0.0001  |
| Microencapsulation *Temperature | 2  | 0.431       | 0.216       | 8.650   | 0.0003  |
| r(Microencapsulation *Temperature) | 10 | 0.149       | 0.015       | 0.600   | 0.8137  |
| Week                    | 12 | 118.652     | 9.888       | 671.070 | 0.0001  |
| r(Week)                 | 24 | 0.354       | 0.015       | 0.590   | 0.9318  |
| Microencapsulation *Week | 12 | 23.365      | 1.947       | 130.800 | 0.0001  |
| Temperature*Week        | 24 | 33.700      | 1.404       | 56.320  | 0.0001  |
| Microencapsulation *Temperature*Week | 24 | 4.531       | 0.189       | 7.570   | 0.0001  |
| Error                   | 120| 2.992       | 0.025       |         |         |
| Corrected Total         | 233| 416.867     |             |         |         |

4. Conclusion
Microencapsulated and free cells of probiotic *Lactobacillus plantarum* IS-10506 showed stable viability at -20°C for 24 weeks, as shown by no significant reduction of viability (p>0.05). At 4°C free cells showed significant decreased of viability, by 2.34 Log CFU/g while microencapsulated showed
stable viability, as shown by insignificant decreased, by 0.84 Log CFU/g after 24 weeks. Moisture content in both of free cells and microencapsulated cells at 24°C were significantly increased at week 2. While at 4°C, there was a significant increase of moisture content observed in free cells at week 2, and microencapsulated cells showed significant increase of moisture content at week 10. Likewise, at -20°C, moisture of both free cells and microencapsulated cells were significant increase at week 2 and 10 respectively. Higher temperature increased the moisture content of probiotics powder and decreased viability of L. plantarum IS-10506. Longer storage time more than 24 weeks at -20°C and 4°C need to be explored to find out maximum storage time of microencapsulated probiotic and free cells by assessing viable counts, moisture content and water activity, to explore the role of encapsulant for L. plantarum IS-10506, which is not only protect the probiotic strain in gastric conditions, but also for maintaining stable viability during storage.

References
[1] Surono IS 2015Traditional Indonesian dairy foods Asia Pac. J. Clin.Nutr. 2426-30
[2] Surono I S2003 In vitro probiotic properties of indigenous dadih lactic bacteria. Asian-Aust J. Anim.Sci.16576–731
[3] Dharmawan J and Surono I S 2006 Adhesion Properties of Indigenous Dadih Lactic Acid Bacteria on Human Intestinal Mucosal Surface Asian Australasian J. Anim. Sci. 19 751 - 755
[4] Collado MC, Surono IS, Meriluoto J and Salminen S 2007 Potential probiotic charac-teristics of Lactobacillus and Enterococcus strains isolated from traditional dadih fermented milk against pathogen intestinal colonization J. Food Prot.70700–705
[5] Surono IS,Pato U,Koesnandar,Hosono A 2009 In vivo Antimutagenicity of Dadih Probiotic Bacteria towards Trp-P1. Asian-Aust.J. Anim. Sci. 22 119 – 123
[6] Surono I S, Martono PD, Kameo S,Suradjji EW and Koyama H2014 Effect of probiotic L. plantarum IS-10506 and zinc supplementation on humoral immune response and zinctatus of Indonesian pre-school children J. Trace Elem. Med. Biol. 28465-469
[7] Prakoewsac R S,Herwanto N,Prameswari R,Astari L,Sawitri S,Hidayati AN,Indramaya DM,Kusumowidagdo ER and Surono IS 2017 Lactobacillus plantarum IS-10506 supplementation reduced SCORAD in children with atopic dermatitis Benef. Microbes 8833-840
[8] Anal AK and Singh H 2007 Recent advances in microencapsulated of probiotics for industrial application and target delivery Trends Food Sci.Tecnol. 18240-251
[9] Mansouripour S, Esfandiari Z and Nateghi L 2013 The effect of heat process on the survival and increased viability of probiotic by microencapsulated: A review Annals Biol.Res. 4 83-87
[10] Bensch G, Ruger M, Wassermann M,Weinholz S 2014 Flow cytometric viability assessment of lactic acid bacteria starter cultures produced by fluidized bed dryingApp. Microbiol. Biotechnol. 98 4897-4909
[11] Sulaiman F 2019 Accelerated Shelf Life Test (ASLT) untuk Pendugaan Umur SimpanSerbuk Probiotik Mikroenkapulasi dan Non-Enkapsulasi dengan dan Tanpa Absorben. B.A. Thesis. Binus nivesity
[12] Horison R, and Surono I S 2020Half-life Estimation of Encapsulated Enterococcus faecium IS-27526 by Accelerated Shelf Life Testing (ASLT) IOP Conf. Series Earth and Environmental Science 426
[13] Surono I S, Verhoeven J,Verbruggen S and Venema K 2018 Microencapsulation increases survival of the probiotic Lactobacillus plantarum IS-10506 but not Enterococcusfaecium IS-27526 in a dynamic, computer-controlled in vitro model of the uppergastrointestinal tractJ. Appl. Microbiol. 124 1604-1609
[14] Guergoletto K B 2012 Dried Probiotics for Use in Functional Food Food Industrial Processes–Methods and Equipment(Brazil: IntechOpen)