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FULL PAPER

Microencapsulation of indigenous probiotic *Lactobacillus plantarum* Dad-13 by spray and freeze-drying: strain-dependent effect and its antibacterial property

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Abstract

Lactobacillus plantarum Dad-13 is a potential probiotic candidate, and microencapsulation is the method for extending its shelf-life. Furthermore, spray or freeze -drying is a common method for microencapsulation, and its effect on the stability of probiotic is strain-dependent. This research was aimed to evaluate the strain-dependent effect of probiotic microencapsulation and to choose a suitable method for further research. In this research, for strain-dependent analysis, *L. plantarum* strain Mut-7 was used as a comparison. Probiotics were encapsulated with 10% (w/v) skim milk and 1 % (w/v) sucrose as wall material by spray and freeze-drying method. The obtained probiotics powder was then analyzed for physicochemical, sublethal injury and stability during storage. Freeze-dried *L. plantarum* Dad-13 was than analyze the antibacterial activity against pathogenic bacteria. The results showed that a_w, color and particle diameter were significantly different between the spray and freeze-dried probiotics. Hence, the microstructure of spray-dried microcapsule was spherical and wrinkle, while the freeze-dried microcapsule was porous. However, the viable cell of freeze-dried probiotic was significantly higher and more stable during eight weeks of storages. From both strains, we found that there was no strain-dependent effect on viable cells after the drying method. Thus, we deduced that freeze-drying was the suitable method for microencapsulation *L. plantarum* strain Dad-13, considering the freeze-dried *L. plantarum* Dad-13 still had antibacterial activity.

1. Introduction

The definition of probiotics, which has been clearly described by FAO/WHO (2002), is a live microorganism that when administered in an adequate amount, could have a beneficial effect on the host. The beneficial effect of probiotic is improving gastrointestinal function, modulation immune response, pathogen inhibitor, reduce irritable bowel syndrome/inflammatory bowel disease's symptoms, and prevention of colon cancer (Parvez *et al*., 2006; Anal and Singh, 2007; Kechagia *et al*., 2013). Probiotic has an enormous potential to be extended to another form, and it is not only limited to dairy products. Furthermore, probiotic-supplemented food can be considered as a functional food due to its health benefits (Floch, 2014). Nowadays, people have developed an interest in foods having functional properties.

Most of the probiotic candidates are lactic acid bacteria from *Lactobacillus* and Bifidobacterium genus (Holzapfel *et al*., 2001). Also, yeast such as *Saccharomyces cerevisiae* has also been identified to exhibit probiotic properties (Floch, 2014). A new strain of indigenous probiotic has been successfully isolated from Indonesian traditional fermented food, it is *L. plantarum* Dad-13 which was isolated from Dadih, a spontaneously fermented buffalo milk. Characterization of probiotic properties of *L. plantarum* Dad-13 had been done by Rahayu *et al*. (2015) which showed that *L. plantarum* Dad-13 has antimicrobial activity and tolerance to the gastrointestinal tract.

Several clinical research of *L. plantarum* Dad-13 is promising for its development, and one of them is probiotic-supplemented food. According to Pamungkaningtyas *et al*. (2018), yoghurt fermented by *L.*

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plantarum Dad-13 in combination with indigenous *Streptococcus thermophilus* Dad-11 has better sensory acceptance compared to the combination of *Lactobacillus bulgaricus* and *S. thermophilus*. As the definition of probiotic by FAO/WHO that probiotic should be consumed in live cell and adequate amount, maintaining the stability and viability of probiotic cells incorporated in food is very important yet challenging. Factors such as temperature, pH, oxygen availability, water activity, and moisture content are the most crucial factors affecting the stability of probiotic. Meanwhile, according to (Barbosa *et al*., 2015), probiotic-supplemented food should retain the viability of 10^6-10^7 CFU/g or /mL at the end of the expired date. Probiotics supplemented into cornelian cherry juice could not withstand the natural pH of the juice during seven days of storages (Nematollahi *et al*., 2016). The sufficient probiotic viability is targeted to survive under the gastrointestinal condition and colonized on the colon. Therefore, the development of new strain probiotic-supplemented food is concerned with maintaining the viability during process and storage.

Microencapsulation of probiotic is one of the methods that can be applied to extend the probiotic. It is a technology to protect the unstable compound (gas, liquid or solid) using protective wall material (polysaccharide, lipid, and protein) and control release of its compound to reach the target (Anal and Singh, 2007; Quintanilla-Carvajal *et al*., 2010; Eckert *et al*., 2017). The product of microencapsulation is then called as a microcapsule. Spray-drying and freeze-drying are the two most common methods of probiotic microencapsulation (Barbosa *et al*., 2015; Dianawati *et al*., 2016; Moayyedi *et al*., 2018). In addition, a different method may give different characteristics of a microcapsule. However, although the microencapsulation of probiotic has been conducted in several research projects, its effect on probiotic is strain-dependent (Del Piano *et al*., 2006; Huang *et al*., 2017).

Therefore, in this research, microencapsulation of indigenous probiotic strain *L. plantarum* Dad-13 using spray-drying and freeze-drying method was studied in which 10% (w/v) skim milk and 1% (w/v) sucrose was used as the wall material. This research aimed as a preliminary study to choose the suitable method for microencapsulation of indigenous probiotic strain for further research. The strain-dependent effect of microencapsulation method was evaluated from injured cells and stability of probiotic after the process, in which *L. plantarum* Mut-7 was used for comparison.

2. Materials and methods

2.1 Bacterial strains

To analyze strain-dependent effect, *L. plantarum* Mut-7, which was isolated from fermented cassava, was used as a comparison. Four pathogenic bacteria were used for antibacterial activity analysis, which was: *Listeria monocytogenes* ATCC 7644, *Staphylococcus aureus* ATCC 6538, *Escherichia coli* IFO 3301 and *Salmonella enterica ser.* Typhimurium IFO 13245. All the bacterial strains were obtained from the Center for Food and Nutrition Study, Universitas Gadjah Mada. For culture stock, the cell was kept at -40^{o}C within vial containing 1:1 20% glycerol/10% skim milk.

2.2 Growth Condition

Before the microencapsulation, probiotic strains were inoculated in MRS (de Man, Rogosa and Sharpe) broth. Meanwhile, the pathogenic bacteria were inoculated in nutrient broth. All the bacteria were incubated at 37^{o}C for 24 hrs and sub-cultured twice into a new broth medium.

2.3 Microencapsulation of L. plantarum strain Dad-13 and Mut-7

Microencapsulation was adapted from (Harmayani *et al*., 2001) with modification. After the propagation step, the probiotic cell was harvested by centrifuging at 4^{o}C 4000 rpm for 15 mins (Thermo scientific). Wall material solution was prepared by diluting 10% (w/v) skim milk and 1% (w/v) sucrose into aquadest followed by pasteurizing the solution at 100^{o}C for 15 mins. The bacterial pellet was then suspended into the solution and homogenized afterwards. Spray-drying was carried out with a lab-scale co-current spray-dryer (LabPlant spray-dryer SD-05) with process condition was as followed: inlet-outlet temperature (100-60^{o}C) and feed velocity (400-450 mL/h). For freeze-drying, the bacterial suspension was frozen at -40^{o}C for 24 hrs and then freeze-dried for 72 hrs. The freeze-dried microcapsule was then crushed with pestle and mortar. Both spray and freeze-dried microcapsules were seal-packed within an aluminum foil and stored at two temperatures (25^{o}C and 4^{o}C) for 8 weeks.

2.4 Characteristics analysis

2.4.1 Yield process

After each process, microencapsulation yield (%) was determined with the formulas as follow:

$$\%\,Yield = \frac{M1}{M0} \times 100\%$$

Where M1 is the weight of microcapsule after the process and M0: the weight of initial total solid in liquid

feed

2.4.2 Moisture content and water activity

Moisture content was measured by gravimetric method while water activity using a_w meter (pa_wkit).

2.4.3 Color measurement

The color of microcapsule was measured using chromameter (Minolta CR-310) with three parameters (L*, a* and b*).

2.4.4 Dissolution test

This test is according to (Quek *et al.,* 2007) where 50 mg of each microcapsule was mixed with 1 mL of distilled water using a vortex. Dissolution was measured as the time (s) taken to dissolve the powders completely.

2.4.5Microstructure analysis

The analysis of microstructure of probiotic was performed by Scanning Electron Microscopy (SEM JEOL JSM-6510 LA). The samples were mounted on a stub of metal with adhesive, coated with 40 - 60 nm of metal such as Gold/Palladium and then observed in the microscope.

2.4.6 Size particle analysis

The particle size was measured by a laser particle size analyzer (Malvern Zetasizer Nanoseries Nano ZS ver 7.01). Prior to the analysis, the probiotic microcapsule was dispersed into aquadest (1:10 w/v).

2.5 Survival and stability of probiotic microcapsule during process and storage

2.5.1 Enumeration of a bacterial cell, survival and sublethal injury analysis

The viable cell was enumerated before and after the drying process and the weekly interval during eight weeks of storage. After rehydration of 1 g microcapsule into 9 mL of 0.85% NaCl and homogenization for 1 min, at proper dilution, the suspension was pour plated on MRS medium and incubated at 37°C for 48 hrs. Meanwhile, for sublethal injury analysis, it was pour-plated on MRS medium added with 0.15% bile salt (BS) and incubated at 37°C for 5 days. The viable cell was express as log CFU/g.

2.5.2 Inactivation rate during storage

During eight weeks of storage, the viable cell was analyzed weekly, and the inactivation rate was expressed as logarithmic following the first-order kinetics as described below:

$$\text{Log } Nt = \log No + k_T t$$

where No is initial viable cell (CFU/g), Nt is viable cell counts in every week storages (CFU/g), t is a time of storage, and k_T is the inactivation rate constant at temperature T ($week^{-1}$)

2.6 Antibacterial activity

Antibacterial activity analysis was conducted according to Papamanoli *et al*. (2003) with modification. In brief, 1 mL of pathogen bacteria (viability 10^6 CFU/ mL) were pour platted into NA medium and waited until the medium solidified. The solid medium was then plugged for making well (4 wells in each Petri). Cell-free supernatant of a fresh overnight culture of *L. plantarum* Dad-13 and powder *L. plantarum* Dad-13 grown in MRS broth was used for antibacterial analysis. Approximately 50µL of supernatant were dropped into the wells. Sterile water and amoxicillin (10µg/100µL) were used respectively at the same volume for negative and positive control. Petri was incubated 24 hrs, 37°C. Antimicrobial activity was determined by clear zone measurement around the wells (mm).

2.7 Statistical analysis

A one-way ANOVA followed by the Duncan Multiple Range Test (DMRT) was performed to evaluating any significant differences in all samples and independent t-test for each treatment. All statistical analysis was performed using IBM SPSS Statistic 24 with confidential (p value<0.05).

3. Results and discussion

3.1 Probiotic's microcapsule characteristics

The yield process shows the efficiency of both microencapsulation methods. Spray and freeze-dry had different yields, which were 45.78% and 44.88% for spray-dry of Dad-13 and Mut-7 strains respectively, while 69.80% and 67.53% for freeze-drying of Dad-13 and Mut-7 strains respectively. The same result was obtained by Wilkowska *et al*. (2016), who explained the lower yield in spray-drying process is due to some of the powder stuck on the cyclone or lost blown during the process. The overall characteristic of probiotic's microcapsules can be seen in table 1. As can be seen, there was no significant difference in dissolution and moisture content from both processes and strains. However, freeze-dried microcapsule had faster dissolution time and higher moisture content. Dissolution time expresses the required time for the microcapsule to well rehydrated in the water. It is an essential aspect for the homogeneity of probiotic supplemented into food. The high moisture content of microcapsule has a tendency to agglomerate and quickly reconstitute in the water (Quek *et al*., 2007). In addition, freeze-dried

Table 1. Characteristic of spray and freeze-dried *L. plantarum* strain Dad-13 and Mut-7

Characteristics	Spray drying		Freeze drying	
	Dad-13	Mut-7	Dad-13	Mut-7
dissolution (s)	14.33±0.57	13.33±3.51	12±3.6	11±1.0
moisture content (%)	6.28±0.26	6.47±0.25	6.19±0.28	6.28±0.96
a_w	0.43±0.01^b	0.47±0.02^c	0.44±0.01^b	0.40±0.02^a
Color				
L*	83.26±0.04^a	83.31±0.07^a	87.02±0.01^b	87.12±0.09^b
a*	5.17±0.02^b	4.65±0.19^a	4.69±0.01^a	4.69±0.02^a
b*	12.59±0.07^b	12.68±0.40^b	9.65±0.06^a	9.69±0.04^a
particle diameter (µm)	0.28±0.003^a	0.30±0.001^b	0.47±0.008^c	0.51±0.007^d

Values with different letters within the same column differ significantly ($p<0.05$)

microcapsule has high rehydration capacity (Barbosa *et al*., 2015), which due to the porous structure as can be seen in Figure 1.

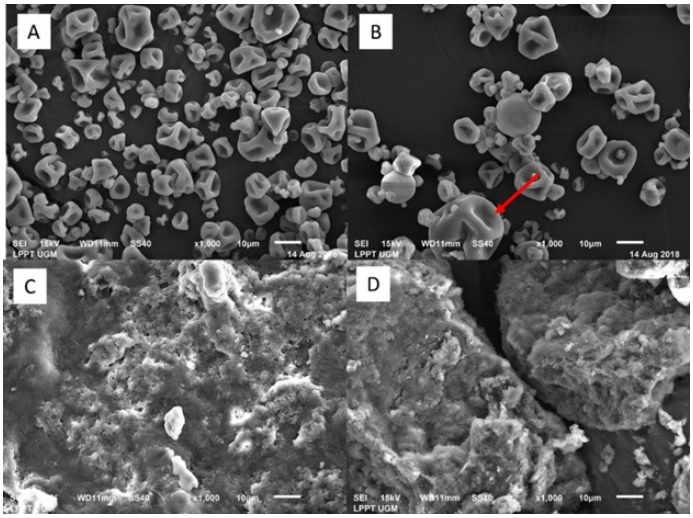

Figure 1. Morphology of spray (A: *L. plantarum* Dad-13; B: *L. plantarum* Mut-7) and Freeze (C: *L. plantarum* Dad-13; D: *L. plantarum* Mut-7) Dried Probiotic. The arrow showed the wrinkle on the surface of spray-dried microcapsule.

Significant differences were observed in water activity, color and particle diameter. Freeze-dried microcapsule had higher water activity compared to spray-dried microcapsule. Besides that, freeze-dried microcapsule also had a higher lightness and less yellowish intensity. The use of high temperatures in spray-drying leads to a lot of water removal, yet induce browning reactions (Koc *et al*., 2010). However, according to Kumar and Mishra (2004), to maintain the stability of probiotic's microcapsules, it should have water activity below 0.20-0.25 and moisture content below 4-5%.

Freeze-dried microcapsule had bigger particle diameter compared to that of the spray-dried microcapsule. Since the feed solution was the same for both methods, the difference in particle size may be affected by the process parameter and post-treatment. The same result is also reported by Moayyedi *et al*. (2018) and Ying *et al*. (2010). The smaller size of spray-dried microcapsule due to the feed solution is sprayed through the nozzle. Meanwhile, freeze-dried microcapsule must be manually grinding after the process.

Figure 1 shows the morphology of microcapsule from both methods. Spray-dried-microcapsule had spherical and wrinkle appearance on the surface, while freeze-dried microcapsule had a porous and irregular shape. The wrinkle appearance of spray-dried microcapsule was probably a result of a rapid moisture loss during the process (Wilkowska *et al*., 2016).

3.2 Effect of different methods on cell viability

Several research projects have been reported that skim milk is one of the suitable wall materials for probiotic microencapsulation (Ananta *et al*., 2005; Liao *et al*., 2017). Skim milk has the buffering capacity and forms hydrogel on the surface of the probiotic cell, that able to inhibit diffusion of acid when it passes the gastrointestinal tract (Livney, 2010). Therefore, in this research, skim milk was used as a wall material. The effect of spray and freeze-dry on probiotic cell viability is shown in Figure 2.

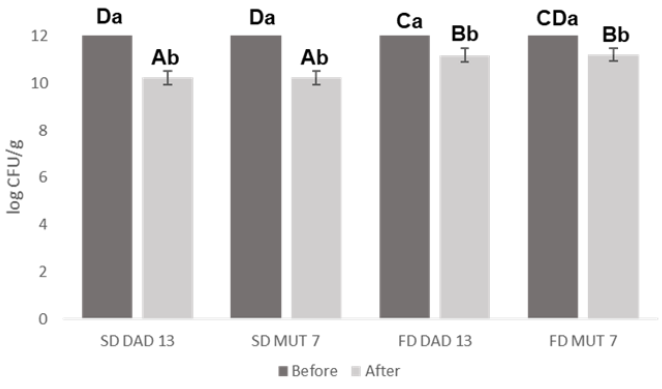

Figure 2. Viable cell of probiotics cell before and after process. Bars with different capital letters above are significantly different ($p<0.05$) between samples while bars with different lowercase letters are significantly different ($p<0.05$) between treatments. SD: Spray dry, FD: Freeze dry.

The use of skim milk as a wall material during spray-drying seemed to be less able to protect a probiotic cell from the high temperature of spray-drying. The spray-drying method resulted in the loss of approximately 2 log cycles of viable cells in both strains. Meanwhile, the loss due to freeze-drying was only 0.9 log cycle in both strains. There was no significant difference in viable cells between strains Dad-13 and Mut-7 in both methods. Stress such as heat, osmotic, oxidative and desiccation may have occurred and caused cells inactivation during the drying process (Huang *et al*., 2017). The use of high temperature during spray-drying lead to rapid water removal, loss of membrane permeability and protein denaturation (Iaconelli *et al*., 2015). According to Teixeira *et al*. (1997), not only heat stress occurred during spray-drying but also osmotic stress and oxidative stress. The freeze-drying method is much mild process compare to spray-drying, and water removal is based on the sublimation process from the ice crystals (Broeckx *et al*., 2016). However, during slow freezing, coarse ice crystal formed and can be a damage for the cell (Fowler and Toner, 2005). The use of skim milk is preventing cell leakage during slow freezing. Skim milk is a non-colligative cryoprotectant and inhibits the formation of coarse crystal ice, which prevents the cell run into mechanical or osmotic stress (Broeckx *et al*., 2016).

In order to investigate the injured cell after the drying process, the sublethal injury was performed by cultivated on MRS media with BS addition. According to Espina *et al*. (2016), in the state of sublethal injury, the bacterial cell is metabolically active but unable to grow in laboratory culture media. As can be seen from Figure 3, there was no significant difference in a viable cell on MRS and MRS+BS from both strains with freeze-drying. Meanwhile, a significant difference was observed in both strains with spray-dry. The grown cell on MRS+BS indicates a healthy cell, in which the difference of viable cell between MRS and MRS+BS is the injured cell. Base on that, spray-dried microcapsules had more injured cell compared to freeze-dried microcapsules. In addition, there was no observed significant difference of injured cell between strains Dad-13 and Mut-7 in both methods. Therefore, the strain-dependent effect of the drying process did not observe in strain Dad-13 and Mut-7. According to Laconelli *et al*. (2015), *L. plantarum* is least affected by stress during spray and freeze-drying without a protective agent.

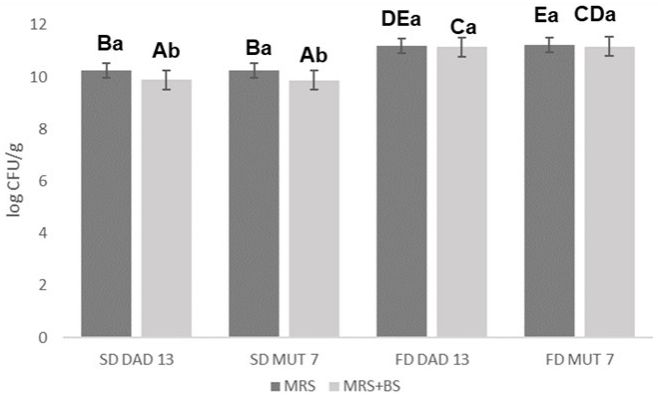

Figure 3. Sublethal Injury of spray and freeze-dried probiotics. Bars with different capital letters above are significantly different ($p<0.05$) between samples while bars with different lowercase letters are significantly different($p<0.05$) between treatments. SD: Spray dry, FD: Freeze dry.

On the contrary, in this research, the spray-drying process caused more injury cells than the freeze-drying process, and it was observed in both strains. Some protein and genes of *L. plantarum* are known to play a role in heat, oxidative and heat osmotic adaptation mechanisms (Bove *et al*., 2012; Zotta *et al*., 2013; Wu *et al*., 2016). Both strains *L. plantarum* Dad-13 and Mut-7 are isolated from spontaneously fermented food, which there is no heat treatment (Rahayu *et al*., 2015). That is maybe because both strains did not have an active adaptation mechanism during high-temperature treatment and have the same viable pattern against the drying process.

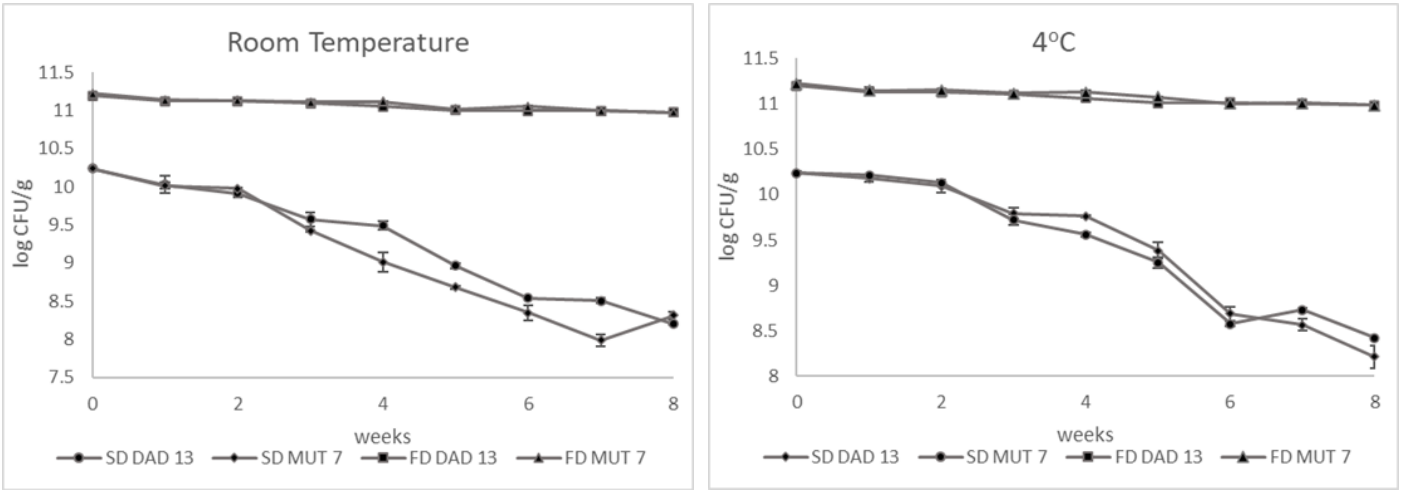

Figure 4. Stability of spray and freeze-dried probiotics during 8 weeks of storage. SD: Spray dry, FD: Freeze dry

3.3 Stability of Microcapsule during eight weeks storage

Stability during eight weeks of storage at two different temperatures of spray and freeze-dried microcapsule can be seen in Figure 4. The viable cell reduction of spray-dried microcapsule during storage was really visible compared to freeze-dried probiotic microcapsule. Furthermore, the effect of storage temperature did not affect the stability of spray and freeze-dried microcapsule. The stability of microcapsules during storage is an important parameter and relates to its shelf-life. Stability at room temperature (25°C) is desired from probiotic microcapsule due to its low utility cost.

The inactivation rate shows the effect of the environment on the stability of probiotic microcapsule. The less of kT means that the probiotic microcapsule is more stable. All of the microcapsules follow the ordo 1 kinetic reaction, which kT of freeze-dry probiotic was smaller compare to spray-dried probiotic (Table 2). As those mentioned above, it is clear that freeze-dried probiotic microcapsule was the most stable compared to spray-dried probiotic at the analyzed temperature.

Table 2. Inactivation rate of spray and freeze-dried probiotic during 8 weeks of storage at 25°C and 4°C

Method	Strain	25°C		4°C	
		k (week^{-1})	R^2	k (week^{-1})	R^2
Spray drying	Dad-13	0.2682	0.9735	0.2684	0.9303
	Mut-7	0.2833	0.9481	0.2544	0.9443
Freeze drying	Dad-13	0.027	0.9359	0.0262	0.9408
	Mut-7	0.0282	0.9051	0.0284	0.9276

High residual moisture content due to inadequate drying process affects the bacteria survival during storage since water is required for physic-chemical reaction (Dianawati *et al*., 2013). Even though in this research freeze-dried probiotic had slightly higher moisture content compared to spray-dry probiotic, freeze -dried probiotic was more stable during storage. The low survival of spray-dried probiotic may be due to a high number of injured cells during the process. Therefore, the freeze-drying process is more suitable for *L. plantarum* Dad-13 microencapsulation and will be used for further analysis.

3.4 Antibacterial activity

Antibacterial activity is one of the probiotic characteristics, which related to the anti-pathogen ability of probiotic. The antibacterial activity comes from bacteriocin, which a protein or polypeptide metabolite produced by the cell (Zacharof and Lovitt, 2012). The result of the antibacterial activity of freeze-dried microcapsule *L. plantarum* Dad-13 can be seen in Table 3. Although there is an injured cell after the freeze-drying process, freeze-dried *L. plantarum* Dad-13 still showed inhibition against the pathogens, which no significant different with free cell for *L. monocytogenes*, *E. coli* and *S. aureus* pathogens. However, the inhibition's spectrum was varied in each pathogen. According to Bagad *et al*. (2017), each antibacterial activity of lactic acid bacteria has its intrinsic biological properties against pathogens. Lacticin, lactocin, pediococin, pisciolin, enterocin, reuterin, plantaricin, eneterolysin and nisin are the common bacteriocin from *L. plantarum* (Arqués *et al*., 2015). Therefore, the freeze-drying process of *L. plantarum* Dad-13 did not eliminate its probiotic characteristics.

4. Conclusion

A significant difference was observed in water activity, color, and particle diameter of spray and freeze-dried probiotics. Moreover, freeze-dried probiotic had porous morphology, causing it to dissolve easily. Strain dependent effect of spray and freeze-dry probiotic microencapsulation was not observed between *L. plantarum* Dad-13 and Mut-7. Hence, freeze-dried probiotic had higher viable cell and was more stable during storage. Therefore, freeze-dry was considered as a suitable method for *L. plantarum* DAD- 13 `microencapsulations, which freeze dried *L. plantarum* Dad-13 still had inhibition against pathogens. The use of suitable wall material will be our focus for further research.

Conflict of Interest

The authors declare that no conflict of interest

Table 3. antibacterial activity of freeze-dried *L. plantarum* Dad-13

	Clear Zone (cm)			
	L. monocytogenes	*E. coli*	*S. aureus*	*S. enterica* ser. Typhimurium
Water	0	0	0	0
Amoxicillin	1.88±0.05^{b}	1.83±0.13^{b}	2.20±0.22^{b}	1.55±0.66^{a}
Free cell	1.78±0.10^{ab}	1.58±0.10^{a}	1.55±0.10^{ab}	1.95±0.06^{c}
Powder	1.73±0.10^{a}	1.60±0.08^{a}	1.30±0.10^{a}	1.73±0.05^{b}

Values with different letters within the same column differ significantly ($p<0.05$)

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