Vol 11(1) (2022): 33-40
DOI: http://dx.doi.org/10.24235/sc.educatia.v11i1.10159

SCIENTIAE EDUCATIA: JURNAL PENDIDIKAN SAINS

http://www.syekhnurjati.ac.id/jurnal/index.php/sceducatia

Viability of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* in Encapsulated Probiotic Candy With Freeze-Dry Method

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article info

Article history:
Received: 29 March 2022
Received in revised form: 01 April 2022
Accepted: 19 June 2022
Available online: 30 June 2022

Keywords:
Candy
Encapsulant
Freeze dry
Probiotic
Viability

abstract

The level of viable cell count in probiotic candy, based on WHO standard is $>10^6$ CFU/ml or $>10^6$ CFU/g. Meanwhile, information on the bacterial viability of probiotic candy according to WHO standards is still limited. The experimental study was conducted to discover the viability of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* in encapsulated probiotic candy. Encapsulants consisted of maltodextrin, gum, corn starch, and skim milk. Encapsulation of probiotic bacteria used freeze-dry method. Probiotic candies were stored at room temperature for 14 days in an aerobic condition. The viability of probiotics candy was tested and the results showed significance for each type of encapsulation ($p<0.05$). The gum and corn starch encapsulations showed bacteria viability that met WHO standards for functional foods, which ranged from 20,333,333±7,637,626 to 31,553,333±2,741,894 CFU/g ($>10^6$ CFU/g). The most preferred encapsulant in terms of taste, texture, and aroma was identified as skim milk encapsulation. Further research on the long-term storage of probiotic candy and the viability of probiotic candy bacteria in the gastrointestinal tract (*in vivo*) is needed.

1. Introduction

Probiotics are defined as live microorganisms that when administered in sufficient quantities have a positive impact on the health of the individual who consumes them or their host (Khalighi et al., 2016). Probiotic bacteria generally come from lactic acid bacteria (Widyadnyana et al., 2015; Rahmiati & Mumpuni, 2017; Sunaryanto, 2017; Suryani et al., 2017). In this study, two bacterial starters were used, namely *Lactobacillus bulgaricus* FNCC 0041 and *Streptococcus thermophilus* FNCC 0040. The use of more than one starter species will cause protocooperation (Yansyah et al., 2016). Protocooperation is an interaction between two or more bacteria in a mixed culture which will produce a higher acid level than the respective cultures. According to Hadi and Fardiaz (Yansyah et al., 2016), *L. bulgaricus* will play a greater role in the formation of aroma, while *S. thermophilus* will play a greater role in the formation of taste.

Probiotics is often considered to be a prerequisite for the health benefits. Probiotics are reported to be useful as agents to increase the immune system, reduce the risk of colitis, improve protein and fat digestion, prevent colon cancer, lower cholesterol, lower blood pressure, improve

immune function and prevent infection, reduce inflammation, irritable bowel syndrome, manage urogenital health. Consuming probiotics regularly also serves to inhibit the growth of pathogenic organisms, prevent diarrhea from various causes, prevent cancer, synthesize vitamin, detoxify toxins, manage lactose intolerance, increase mineral absorption, and prevent the growth of pathogenic bacteria and anti-microbial activity (Himawan et al., 2018; Yulimatussa et al., 2016; Amin et al., 2013; Firmansyah, 2016; Hossain et al., 2021).

In terms of viability of probiotic micro-organisms, probiotic bacteria must be able to grow in milk and survive insufficient numbers (Karimi et al., 2011). Probiotic products must contain bacterial cells that can survive and in sufficient quantities when they are consumed, considering some organisms may not be able to grow in milk (Adib et al., 2013). Viability and activity of the bacteria are important considerations. The standard of bacterial cell viability in probiotic products should be 10^6-10^7 CFU/mL or CFU/g (Khalighi et al., 2016). Maintenance of microorganisms (viability of probiotic bacteria) in functional food is a technological challenge because microorganisms cannot survive in suboptimal conditions (Ghasemnezhad et al., 2017). One way to improve the resistance and viability of probiotic bacteria is by encapsulation (Sumanti et al., 2016; Pavli et al., 2018; Sarkar, 2020). Microorganisms can be significantly protected by microencapsulation and immobilization on various substrates, including milk proteins and polysaccharides (Etchepare et al., 2016). Encapsulation is a technique of coating bacteria in an encapsulation matrix aimed at maintaining viability, protecting probiotic bacteria from damage caused by unfavorable environmental conditions, such as heat and, chemicals, and supporting the survival of bacteria during processing, storage, and travel through the digestive tract (Etchepare et al., 2016; Ghasemnezhad et al., 2017; Calinoiu et al., 2019).The advantage of encapsulated probiotic bacteria is that they can last longer because they are in powder form and are easier to use. The method used in the encapsulation process is freeze drying (Sumanti et al., 2016). The principle of freeze-drying technology begins with the freezing process of food and continues with drying by removing/separating most of the water in the material that occurs through the sublimation mechanism (Hariyadi, 2013). In this study, maltodextrin, gum, corn starch, and skim milk were used as encapsulants. In 1965, probiotics were discovered, and in 2002, candy products containing probiotics began to be produced. Currently, research on probiotic candy is still being conducted using various methods. The purpose of this study was to determine the effect of encapsulation by freeze drying method on candy performance and viability of probiotic bacteria with starter *Lactobacillus bulgaricus* FNCC 0041 and *Streptococcus thermophilus* FNCC 0040. This study is important to provide information to the industry in developing probiotic candy products. The viability of probiotic bacteria in candy still meets WHO standards and the candy has an organoleptic character that consumers like. Candies with these criteria have a health effect, so they are called functional foods.

2. Method

Place and time of study

This study was conducted at the Biotechnology Laboratory, Faculty of Science and Technology, Muhammadiyah University of Bandung from June-November 2021. The freeze-dry process was conducted at the Food Technology Laboratory of Padjadjaran University and candy molding was conducted at the Pharmacy Laboratory of Padjadjaran University. The organoleptic test was done in the city of Bandung with the number of respondents aged 22-55 years consisting of 21 male respondents and 19 female respondents.

Materials

Equipment for candy making included disc mill, analytical balance 0.01g, bowl, spoon, stirrer, aluminum foil, candy dough tube, freeze-dryer CHRIST ALPHA 1-4 plus, rotary tablet machine,

plastic, tissue, and gloves. Equipment for testing the viability of probiotic bacteria included a 250mL Erlenmeyer flask, petri dish, test tube and rack, analytical balance 0.01g, spatula, plastic wrap, aluminum foil, tissue, heat-resistant plastic, heat-resistant rubber autoclave, laminar flow cabinet, micropipette and micropipette tips, Bunsen burner, measuring cup, incubator, and magnetic stirrer. Equipment for organoleptic tests was in the form of stationery and questionnaire forms.

The composition of the probiotic candy formula was 70% powdered granulated sugar, 15% probiotic bacteria, 4% gelatin, 3% magnesium stearate, 3.6% Cilembu sweet potato flour, 5% probiotic bacteria encapsulation, 0.1% food coloring, and vanilla flavoring. 0.3%. Materials for viability testing included sterile physiological 0.85% NaCl, MRSA, Bacto Agar, distilled water, and 70% alcohol.

Method

This study was conducted experimentally with a completely randomized design. The treatments tested were the types of encapsulation, namely: control (P1), maltodextrin (P2), guar gum (P3), corn starch (P4), and skim milk (P5). Each sample was repeated 3 times for the calculation of bacterial viability. The number of respondents for the organoleptic test was 40.

Probiotic candy making

Probiotic candy was made by dissolving gelatin in boiling water until it was homogeneous as a candy binder solution. Encapsulation was done by dissolving the encapsulant in mineral water, then probiotic bacteria were inserted into the encapsulation. Candy dough contained Cilembu sweet potato flour, candy binder solution, encapsulant solution, sugar flour, natural coloring, and was continuously mixed until homogeneous dough was obtained. The candy dough was put in the freezer before the freeze dry process. Freeze drying was carried out in a freeze-dryer CHRIST ALPHA 1-4 plus and, the freeze dryer was set at -50°C for 48 hours until the dough was dry. Dried candy dough was made into powder form using a disc mill and then sieved using an 80 mesh sieve to produce fine candy granules. The candy granules were molded into candy tablets with a candy diameter of 1cm and a candy weight of 700mg in a rotary tablet machine.

Probiotic bacteria viability test

Bacterial viability test of probiotic candy was conducted on probiotic candy after freeze-drying, provided that the candy was stored at room temperature for 14 days. Bacterial viability of probiotic candy was tested on Man Rogosa Sharpe (MRS) agar media containing 70g MRS broth and 15g Bacto Agar in 1L sterile water. One gram of probiotic candy was diluted in 9mL of sterile physiological 0.85% NaCl (10x dilution of suspension), the suspension was diluted up to 107.1mL, sample suspension was poured into a petri dish, and 20ml of MRSA medium was poured over the sample suspension. The petri dish was closed and sealed with plastic wrap, then incubated in an incubator set at 37°C for 48 hours. Bacterial colonies appeared on the surface of the media in round shape and milky white in color. The amount of bacterial viability is expressed in CFU/g.

Probiotic candy organoleptic test

Organoleptic test was conducted on 40 respondents consisting of 19 female respondents and 21 male respondents. The age of the respondents ranged from 20-55 years. Candies were given to respondents to test organoleptic properties of the candy, namely, taste, aroma, texture, color, and shape. In the questionnaire, respondents were provided with choices of preference level including dislike, moderately like, like, and extremely like.

Data analysis

Before ANOVA analysis was conducted, bacterial viability data were analyzed by root transformation. The organoleptic test data were in the form of preference level, therefore a scoring system was made, with a score of 1 for dislike, a score of 2 for moderately like, a score of

3 for like, a score of 4 for extremely like. The results of bacterial viability and organoleptic tests have shown a significance. Therefore, it was continued with the honest significant difference (Tukey HSD) test with a 95% confidence level. ANOVA analysis was performed using SPSS version 16.

3. Result and Discussion

Viability of probiotic candy bacteria

The level of viable cell counts of probiotic candy bacteria with various encapsulations using the freeze-dry method stored at room temperature for 14 days ranged from 0-23,666,667 CFU/g. In this study, a comparative control, namely, probiotic candy that was encapsulated differently without the freeze-dry method, was used. The results showed that the level of viable cell counts of probiotic candy bacteria with various encapsulations without the freeze-dry method stored at room temperature for 14 days ranged from 0-63,000 CFU/g (7,867±1,659).

Based on ANOVA analysis, the type of encapsulation had a significant effect on the viability of probiotic bacteria with a shelf life of 14 days after freeze-drying ($p<0.05$). Based on further test using the Tukey HSD test, the shelf life of 14 days after freeze-drying showed that the gum encapsulation was relatively the same as corn starch but significantly different from the skim milk and maltodextrin encapsulants. The highest bacterial viability of candy with a shelf life of 14 days after freeze-drying was found in the gum and corn starch encapsulations (Table 1).

Table 1. The effect of various encapsules on viability of probiotic candy bacteria

No	Types of Encapsulant	14 days (CFU/g) with freeze-dry	14 days (CFU/g) without freeze-dry
1	Control (P1)	0.00±0.00[a]	0.00±0.00
2	Maltodextrin (P2)	280,000±91,651[a]	0.00±0.00
3	Gum (P3)	20,333,333±7,637,626[b]	350,± 49.49
4	Corn Starch (P4)	23,666,667±2,886,751[b]	15±7.07
5	Skim milk (P5)	366,667±288,675[a]	5± 7.07

Annotation description: different superscrift letter showed significant results with the tukey HSD test at the 95% confidence level.

The level of viable cell counts of the probiotic candy in the treatment without encapsulation showed 0 CFU/g in both encapsulated candy with and without freeze-drying. Bacterial viability of probiotic candy that was encapsulated without freeze-drying showed that it did not maintain the viability of probiotic bacteria, which resulted in units of up to hundreds of viable bacteria. The viability of the freeze-dried candy on day 14 showed tens of thousands to tens of millions of viable bacteria, and only gum and corn starch encapsulants were effective in maintaining bacterial viability, so it could be analyzed that the encapsulation would be effective with the freeze-dry method. The effective value that becomes the standard is the standard of viable bacteria in functional food according to WHO, which contains a minimum of 10^6 CFU/g (Table 1).

In this study, the percentage of encapsulant used was 5%. Drying with freeze-dry technique aims to obtain encapsulated cells in dry form, in order to facilitate use and packaging as well as increase the shelf life of the starter (Krasaekoopt et al., 2003). The drying process of the encapsulated bacteria can also be done using a spray drying technique (Triana et al., 2006). Lactic acid bacteria (LAB) were able to survive during frozen storage by using encapsulation (Pratama et al., 2019; Silaban et al., 2020). Encapsulation is a way to protect probiotic bacteria from harmful/extreme environmental factors such as heating, freezing, and low pH. Bacteria undergo a coating process by a polymer wall layer. The advantage of encapsulation is that it has a

semipermeable and strong membrane so that bacterial cells can withstand extreme conditions (Samedi & Charles, 2019; Haffner et al., 2016; Bilang et al., 2018; Kamil et al., 2020; Trimudita, 2021).

Drying with freeze-dry method causes a decrease in the number of bacterial cells from starter *Lactobacillus paracasei*. Although there was a decrease in bacterial viability after freeze drying, the dry bacterial cell population of *Lactobacillus paracasei* still met WHO standards because sodium alginate and skim milk were able to provide protection against bacterial cells, thereby reducing direct contact with the environment (Diza et al., 2020). The decrease in cell viability during freeze-drying may be caused by the freezing and drying processes. The freezing process causes cells to lose their stability, so they become easily damaged during drying. The main factors causing damage due to drying of bacterial cells are probably membrane damage from osmotic shock, and hydrogen bond displacement that affects the properties of hydrophilic macromolecules in cells (Puspawati et al., 2010).

The matodextrin coating material used in the microencapsulation of the probiotics *Streptococcus thermophilus*, *Lactobacillus murinus*, and *Pediococcus acidilactici* was able to maintain 60-80% viability after spray drying (Pradipta, 2017). The viability results revealed that *Lactobacillus sp* encapsulated with 2% chitosan coating could maintain its viability with the number of colonies log 7.41CFU/g in simulated gastric acid pH 3 for 120 minutes and log 4.78CFU/g in simulated gastric acid fluid pH 1.2 for 120 minutes (Fransiska & Djaenudin, 2021). Based on study conducted by (Lestari et al., 2020), probiotic chewing gum with starter bacteria *Lactobacillus acidophilus* IFO 13951, and *Bifidobacterium longum* ATCC 15707, and with encapsulated type at room temperature storage for 4 weeks experienced a decrease in the level of viable cell counts from 6.27 to 7.03 log CFU/g.

Level of preferences for probiotic candy

Organoleptic test is a method used to test the quality of a material or product using the five human senses. The variables tested were the flavor, taste, texture, color, and texture of the candy. Based on the results of the questionnaire, the respondents' preferences for the taste, flavor, texture, and color of probiotic candy, sorted by the most popular, were P5, P4, P1, P2, and P3. The respondents' preferences for the most popular form of probiotic candy were P5, P2, P1, P4, and P3 (Table 2).

Table 2. The effect of various encapsulants on respondents' level of preference for probiotic candy

No	Types of encapsulant	Taste	Flavor	Texture	Color	Form
1	Control (P1)	2.40 ± 0.63^{b}	2.43 ± 0.55^{b}	2.43 ± 0.87^{b}	2.53 ± 0.78^{a}	2.30 ± 0.85^{a}
2	Maltodextrin (P2)	2.23 ± 1.07^{b}	2.28 ± 0.78^{b}	2.25 ± 0.92^{b}	2.38 ± 0.90^{a}	2.33 ± 0.86^{a}
3	Gum (P3)	1.55 ± 0.93^{a}	1.83 ± 0.87^{a}	1.70 ± 0.79^{a}	2.30 ± 0.91^{a}	2.13 ± 0.88^{a}
4	Corn Starch (P4)	2.63 ± 0.93^{b}	2.60 ± 0.81^{b}	2.45 ± 0.85^{b}	2.47 ± 0.93^{a}	2.25 ± 0.90^{a}
5	Skim milk(P5)	3.13 ± 0.82^{c}	3.03 ± 0.89^{c}	2.95 ± 0.78^{c}	2.60 ± 0.84^{a}	2.35 ± 0.90^{a}

Annotation description: different superscrift letter shows significant results with the Tukey HSD test at the 95% confidence level.

Based on the results of the one-way ANOVA analysis, the type of encapsulation had a significant effect on the taste, flavor, and texture parameters of probiotic candy ($p<0.05$), but did not significantly affect the shape and color parameters of the candy ($p>0.05$). The results of further test using the Tukey HSD test showed that skimmed milk encapsulation was the most popular, while gum encapsulation was the least favored by the respondents in terms of the parameters of taste, aroma, and texture of the candy. Maltodextrin and corn starch

encapsulations had relatively the same level of preference as probiotic candy without encapsulation in terms of the parameters of taste, flavor, and texture of the candy. This study, is relatively the same as the study conducted by (Osmand et al., 2012), which reveated that the flavor of candy with skim milk treatment was the most preferred flavor by panelists with a score of 3.1, which is in the range of "favorable" and "highly favorable". Milk has little sweet taste caused by lactose. Besides sweet taste, salty taste sometimes is also present in milk because of the content of chloride, citrate, and other mineral salts (Okarini & Suartiningsih, 2017). The savory taste of milk is caused by the component fat and protein in milk.

4. Conclusion

Based on the results and discussion, it can be concluded that the probiotic candy with various types of encapsulation using the freeze-dry method significant results. Gum, and corn starch encapsulants showed the level of viable cell counts of bacteria that met WHO standards for functional foods, which ranged from 20,333,333±7,637,626 to 31,553,333±2,741,894 (>10^6 CFU/g). The most favored taste, texture, and aroma belonged to skim milk encapsulation. Further research on the long term storage of probiotic candy and test of probiotic candy bacteria viability in the gastrointestinal tract (*in vivo*) are needed.

Acknowledgments

We would like to express our gratitude for the funding provided from "Penelitian Dosen Pemula (PDP) Kementerian Pendidikan, Kebudayaan, Riset, dan Teknologi" for the 2021 fiscal year.

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