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FULL PAPER

The viability of probiotic *Lactobacillus acidophilus* IFO 13951 and *Bifidobacterium longum* ATCC 15707 in gummy candies decreased during 4 weeks of storage

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Abstract

In addition to fermented milk, incorporation of probiotic bacteria into other food products has been increasing in recent years. Incorporation of probiotic bacteria into gummy candies, a popular chewy gelatin-based candy, is aimed to increase the consumption of probiotics since they have many health benefits. The purpose of this study was to evaluate the viability of probiotic bacteria *Lactobacillus acidophilus* IFO 13951 and *Bifidobacterium longum* ATCC 15707 in gummy candies during storage. This research used a true experimental design to evaluate the viability of probiotic bacteria during storage at room temperature (25-30^{o}C) and cold temperature (4-10^{o}C). The viable cells of *L. acidophilus* IFO 13951 were counted with Rogosa agar medium, while *B. longum* ATCC 15707 was counted with Bifidobacterium Selective Medium. The results showed that the reduction of viable cells during 4 weeks of storage ranged between 1.15 to 1.95 log CFU/g. The highest reduction of probiotic bacteria was found in *B. longum* ATCC 15707 that has been stored in room temperature. Meanwhile, the viability of *L. acidophilus* IFO 13951 in cold storage temperature was higher than the other probiotic bacteria. Even though there were reductions in cell viability after 4 weeks of storage, the number of probiotic cells ranged between 6.27 to 7.03 log CFU/g. In conclusion, the number of probiotics in the gummy candy met the criteria of probiotic products based on the cell number. However, further study is needed to extend the self-life of this probiotic gummy candies.

1. Introduction

Dairy products are the main carriers of probiotic bacteria to humans because milk provides an appropriate environment for probiotic bacteria to support their growth and viability. Nevertheless, with the low milk consumption in ASEAN countries such as Indonesia which is only 14.3 liters per capita, it is particularly challenging to integrate probiotic bacteria into non-dairy probiotic food products, as it must fulfill consumers' standards and expectancy for their health benefits (Stanton *et al*., 2003).

According to the United Nations World Health Organization for Food and Agriculture, probiotics bacteria are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2002). Hence, the probiotic bacteria have to be alive, while heat-killed or tyndalized probiotics do not meet with these criteria. Monitoring the viability of probiotic bacteria is essential to make sure that the probiotic cells are still alive.

Among the probiotics, Lactobacillus spp. and Bifidobacterium spp. are well known because they have many beneficial characteristics such as pathogenic microbes control (Garcia *et al*., 2016). We used two strains of probiotic bacteria namely *Lactobacillus acidophilus* IFO 13951 and *Bifidobacterium longum* ATCC 15707. *L. acidophilus* is gram-positive rod-shaped microbes with rounded ends. Usually, the width is 0.6±0.9 µm and the length is 1.5±6.0 µm. These are

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non-flagellated, non-motile and non-spore forming, and salt-intolerant. *L. acidophilus* is widely used as probiotic bacteria and has been studied intensively for its health benefits (Gomes and Malcata, 1999). *B. longum* is anaerobic, non-sporulating Gram-positive rod-shaped microbes representing ubiquitous inhabitants of the human gastrointestinal tract and vagina (Esaiassen *et al*., 2017). *B. longum* may be considered the most common species of Bifidobacterium, being found both in infant and adult feces (Bivati *et al*., 1984). The health benefits of *B. longum* are action toward intestinal pathogens, improved lactose utilization, anticarcinogenic action and control of serum cholesterol levels (Ibraheem *et al*., 2015).

It is important to assess the viability of strain in the carrier food at the time of consumption. Survival of probiotic strain depends on the pH, processing and storage temperature, and presence of microbial inhibitors such as NaCl and hydrogen peroxide in the food matrix (Kurmann and Rasic, 1991). In addition, it is important that probiotic products at the moment of consumption contain a sufficient number of active cells, i.e. at least 10^6 CFU/g (Rasic and Kurmann, 1983). A probiotic strain must, therefore, have good longevity, both in the product and in the gastrointestinal tract after digestion. During the gummy candies processing, there are several heating processes that might kill the probiotic bacteria. To monitor the survivability of probiotic bacteria in gummy candies, we evaluated the viability of probiotic cells after processing of gummy candies and during storage at room and cold temperature.

2. Material and methods

2.1 Gummy candies formula and preparation

L acidophilus IFO 13951 and *B. longum* ATCC 15707 were obtained from the Food and Nutrition Culture Collection (FNCC), Center for Food and Nutrition Studies, Universitas Gadjah Mada, Indonesia. The cultures were kept at -20°C in 10% skim milk (w/v) and 10% glycerol (w/v). For the preparation of gummy candies, frozen cultures were streaked onto Rogosa agar medium (Oxoid) for *L. acidophilus* IFO 13951 and Bifidobacterium Selective Media (BSM, Himedia) for *B. longum* ATCC 15707, followed by incubation at 37°C for 24-48 hrs. Then, one colony from each plate was transferred into 10 mL of MRS broth (Oxoid) and incubated overnight. The cell suspension was centrifuged at 3000 rpm for 10 min, and the pellet was washed with phosphate buffered saline twice. The number of *L. acidophilus* IFO 13951 and *B. longum* ATCC 15707 in the cell suspensions were 1.12 x 10^9 and 3.40 x 10^9 CFU/ mL, respectively.

The formula of gummy candies consisted of fructooligosaccharide (FOS, Orafti P95), glucose syrup, water, bovine gelatine 40% (Gelita), citric acid, and food additives. First, gelatin powder was soaked for 30 mins in water, then gelatin was melted at 60°C for 15 mins by heating. In the mixture under heating, FOS after glucose syrup was added and dissolved. Citric acid, food additive (fruit flavoring, food coloring, and sucralose), and probiotics were incorporated into the gummy candy mixture at the end of the process (temperature 40°C), then the mixture was poured into casts (Kusuma *et al*., 2018; Nurhayati *et al*., 2019).

2.2 The evaluation of cell viability

The viable cells of *L. acidophilus* IFO 13951 and *B. longum* ATCC 15707 were counted using different agar mediums. Rogosa agar medium was used to count the viable cells of *L. acidophilus* IFO 13951, while Bifidobacterium Selective Count Agar Base (BSC, Himedia) supplemented with Bifidobacterium Selective Supplement A (FD250, Himedia) and Bifidobacterium Selective Supplement B (FD251, Himedia) were used to count the viable cells of *B. longum* ATCC 15707.

As much as 90 mL of phosphate buffered saline (PBS) homogenized with 10 g of sample. Serial dilution of 10^{-5} to 10^{-7} with PBS was used for cell counting. As much as 1 mL of diluted sample was put into a sterile Petri dish, poured with Rogosa agar medium or BSC agar, and then mixed thoroughly by shaking the Petri dish. The Petri dish/plates were incubated at 37°C for 48 hours, under microaerobic condition for *L. acidophilus* or anaerobic for *B. longum.* The number of bacterial colonies per gram of samples was measured and expressed as a decimal log of colony forming units (log CFU/g).

2.3 Statistical analysis

The data were presented in mean $\pm$ standard deviation and analyzed with SPSS 16.0 statistical software (SPSS, Inc., Chicago, IL, USA). One-way ANOVA test was used to compare the number of viable cells during storage. The two-way ANOVA was performed to evaluate which variable that affects the cell viability and the interaction between variables/factors. The value $p < 0.05$ was considered statistically significant.

3. Results and discussion

There are several factors that must be addressed in evaluating the effectiveness of the incorporation of the probiotic strains into food products, namely safety, the compatibility of microorganism in the product, and the

viability of the cells during processing, packaging, and storage (Kechagia *et al*., 2013). Most probiotic products contain *Lactobacillus plantarum, Lactobacillus acidophilus, Lactobacillus fermentum, Lactobacillus rhamnosus, Bifidobacterium bifidum*, or *Bifidobacterium longum*. However, in commercial probiotic gummy candies there are only spore forming bacteria, namely *Bacillus coagulans* and *Bacillus subtilis* since they form heat resistant spores that could survive during processing, packaging, and storage (Posnick, 2018). Although *Bacillus coagulans* and *Bacillus subtilis are* already approved by Food and Drug Administration (FDA) as Generally Recognized as Safe (GRAS), however since they are in the form of spores instead of vegetative cells hence there are contradictory points of view between scientists regarding these spores forming probiotics.

The viable probiotic cells count in gummy candies during storage is given in Figure 1. After processing and in day 0 of storage, the number of viable cells in all gummy candies were higher than 8.0 $\log_{10}$ CFU/g. Hence, the number of probiotic cells in the food product has met the recommended number of probiotic cells which is minimal 6.0 $\log_{10}$ CFU/g or mL of food product (Boylston *et al*., 2004). However, the food industry suggested daily intake of at least 10^8–10^9 viable cells in order to provide a probiotic effect since we have to consider survival rates of probiotic cells in the gastrointestinal tract. Health benefits of probiotics include they are anti-pathogenic bacteria, lower serum cholesterol level, increase nutrient absorption, and decrease the use of antibiotics (Guo *et al*., 2010).

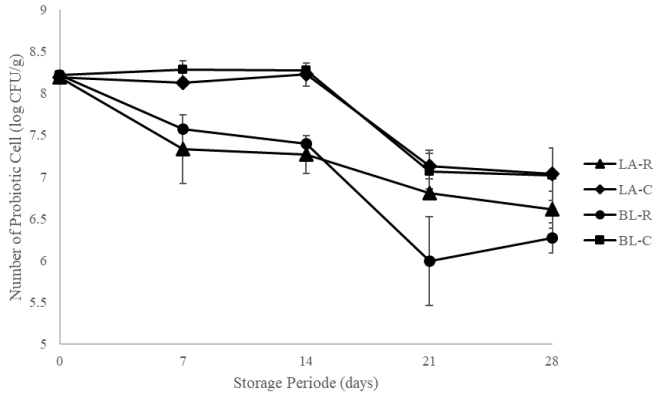

Figure 1. Viable probiotic cells count in gummy candies during storage. LA-R: *L. acidophilus* IFO 1395, stored in room temperature, LA-C: *L. acidophilus* IFO 1395, stored in cold temperature, BL-R: *B. longum* ATCC 15707, stored in room temperature, and BL-C: *B. longum* ATCC 15707, stored in cold temperature.

Storage at cold temperature resulted in the higher survival rate compared to at the room temperature. *L. acidophilus* IFO 13951 had good viability compared to *B. longum* ATCC 15707. The results showed that the reduction of viable cells during 4 weeks of storage ranged between 1.15 to 1.95 log CFU/g (Figure 2). The highest reduction of probiotic bacteria was found in *B. longum* ATCC 15707 that has been stored in room temperature. Meanwhile, the viability of *L. acidophilus* IFO 13951 in cold storage temperature was higher than other probiotic bacteria. The number of *L. acidophilus* IFO 13951 and *B. longum* ATCC 15707 stored at room temperature significantly decreased after 7 days of storage. However, when they were stored at cold temperature, the number of *L. acidophilus* IFO 13951 was significantly decreased after 21 days of storage, while the number of *B. longum* ATCC 15707 did not change (Table 1). Even though there were reductions of cell viability after 4 weeks of storage, the number of probiotic cells ranged between 6.27 to 7.03 log CFU/g, hence the probiotic gummy candy met the criteria of a probiotic product based on the cell number.

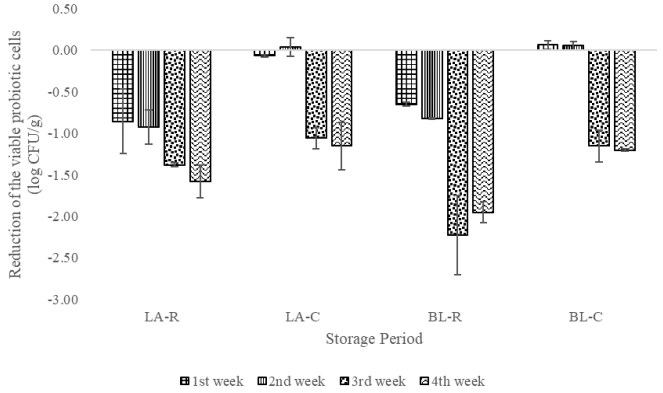

Figure 2. Reduction of the viable probiotic cells during storage. LA-R: *L. acidophilus* IFO 1395, stored in room temperature, LA-C: *L. acidophilus* IFO 1395, stored in cold temperature, BL-R: *B. longum* ATCC 15707, stored in room temperature, and BL-C: *Bifidobacterium longum* ATCC 15707, stored in cold temperature.

The reduction of probiotic cells might be due to the use of free cells instead of immobilized cells. Immobilized cells have many advantages such as protecting cells against damage and contamination during processing. On the other hand, immobilization of probiotic cells would increase its cost (Burgain *et al*., 2011).

The viability of *B. longum* ATCC 15707 in gummy candies stored in room temperature was lower than *L. acidophilus* IFO 13951 at the same storage condition. This result might be due to the requirement of specific amino acids and small peptides to stimulate the growth of Bifidobacteria. Bifidobacteria are also very sensitive to environmental parameters and require expensive media for propagation and the addition of growth-promoting factors, due to their stringent growth requirements (Ibrahim and Bezkorovainy, 1994). Gummy candies contain gelatin that is rich in amino

Table 1. The number of probiotic cells, expressed as log CFU/g, in gummy candies during storage at room and cold temperature

Storage Time (days)	*Lactobacillus acidophilus* IFO 13951		*Bifidobacterium longum* ATCC 15707	
	Room Temperature (28°C)	Cold Temperature (4°C)	Room Temperature (28°C)	Cold Temperature (4°C)
0	8.19±0.02[a]	8.19±0.02[a]	8.22±0.06[a]	8.22±0.06
7	7.33±0.41[b]	8.12±0.01[a]	7.57±0.04[b]	8.29±0.04
14	7.26±0.23[b]	8.22±0.14[a]	7.39±0.05[b]	8.28±0.01
21	6.81±0.05[b, c]	7.13±0.15[b]	5.99±0.53[c]	7.07±0.25
28	6.61±0.22[c]	7.03±0.31[b]	6.27±0.18[c]	7.02±0.04
P value	0.007	0.002	0.001	0.12

acids which are varied among different sources, i.e. types of animal (pork, bovine, fish, etc.) and also different parts of animal bodies (skins or bones) (GMIA, 2012; Arsyanti *et al*., 2018). However, due to low water activity, *Bifidobacteria* could not utilize these amino acids.

Based on the two-way ANOVA test, it was found that storage time and temperature significantly affect the viability of *L. acidophilus* IFO 13951 and *B. longum* ATCC 15707 in gummy candies since both factors have p-value < 0.001. However, there was no significant interaction effect of storage time and temperature (p-value = 0.793).

4. Conclusion

L. acidophilus IFO 13951 and *B. longum* ATCC 15707 could survive after processing of gummy candies. Although the viable cells of *L. acidophilus* IFO 13951 and *B. longum* ATCC 15707 decreased, the number of these probiotic bacteria in gummy candies ranged between 6.27 to 7.03 log CFU/g so that they had met the criteria of probiotic products based on the cell number.

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Conflict of interest

The authors declare no conflict of interest.

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