Vol. 12, No. 4 (2023) : 899 - 908
DOI : http://dx.doi.org/10.23960/jtep-l.v12i4.899-908

JURNAL TEKNIK PERTANIAN LAMPUNG

ISSN 2302-559X (print) / 2549-0818 (online)
Journal homepage : https://jurnal.fp.unila.ac.id/index.php/JTP

The Shelf-life, Microbiology Quality, and Characteristic Changes of Probiotic Lactobacillus plantarum Dad-13 Milk Jelly Candy during Storage

Rafli Zulfa Kamil[1]✉, Fida Hasna Fadhila[2], Angela Dea[2], Endang S. Rahayu[2], Subekti Hartiningsih[3]

[1]Department of Food Technology, Faculty of Animal and Agricultural Sciences, Universitas Diponegoro, Semarang, INDONESIA
[2]Department of Food and Agricultural Product Technology, Faculty of Agricultural Technology, Universitas Gadjah Mada, Yogyakarta, INDONESIA
[3]Department of Agricultural Technology, Nahdlatul Ulama University, Yogyakarta, Yogyakarta, INDONESIA

Article History :

Received : 7 July 2023
Received in revised form : 5 August 2023
Accepted : 7 August 2023

Keywords :

Jelly candy,
L. plantarum Dad-13,
Microbiological quality,
Probiotic,
Storage .

✉Corresponding Author:
raflizulfakamil@lecturer.undip.ac.id

ABSTRACT

Probiotics are live microorganisms that, when consumed in sufficient amounts, can provide health benefits to their host. L. plantarum Dad-13 milk jelly candies are non-fermented probiotic products. This study aims to evaluate the changes in the characteristics of probiotic milk jelly candies during storage and their microbiological quality. Characteristics such as pH, water activity (aw), hardness, chewiness, gumminess, brightness, and viability of lactic acid bacteria were analyzed over a period of 4 weeks at two temperatures, namely 4 and 30°C. Sub-lethal injury analysis was conducted to estimate the survival of probiotic cells during the jelly candy manufacturing process. Microbiological quality was assessed through Total Plate Count (TPC), Coliform, Escherichia coli, Salmonella, Mold, and Yeast analyses. The research results showed that the number of probiotic cells experiencing injury was less than 5%. Furthermore, changes in physical characteristics were observed during the 4-week storage period, but the temperature difference only affected pH and aw. The viability of lactic acid bacteria was more stable at a storage temperature of 4°C, and the microbiological quality met the jelly candy standards, except for TPC. Based on the research findings, milk jelly candies can be used as a carrier for probiotic cells as a non-fermented probiotic product innovation, with a recommended storage temperature of 4°C and best consumed within 47 days. Additionally, probiotic milk jelly candies are free from other microbial contamination.

1. INTRODUCTION

Probiotics are living microorganisms that, when adequately consumed, can confer host health, such as lowering cholesterol, improving immune response, maintaining gut health, and lowering the symptom of lactose intolerance (Wieërs *et al*., 2020). The awareness of a healthy lifestyle among society drives the development of new products which are not only pleasant in terms of sensory but also offer health benefits. In previous research, nutrition is the second consideration along with price and after taste of consumers' consideration for selecting food (Kamil *et al*., 2021). Probiotics, lactic acid bacteria, are often found in fermented products that nowadays can be easily found in the market, such as yogurt, kimchi, kefir, pickle, tape (fermented cassava), etc (Mukisa *et al*., 2019). However, along with the high demand for fermented products, consumers, especially children, still don't like the fermented taste and aroma. To conquer the problem, developing a new probiotic product in which no fermentation process is required is an innovation.

Jelly candy is one of the confectionary products children like due to its sweet taste and chewy texture. According to SNI 3547-2-2008 (BSN, 2008), jelly candy is a confectionary product with a soft texture, composed of hydrocolloid compounds such as agar, gum, pectin, starch, carrageenan, gelatin, and others which can improve the chewy texture. Other ingredients of jelly candy are glucose, sucrose, and flavoring agent. The most common ingredient for jelly candy is gelatin, a polymer with properties such as biodegradable, biocompatible, stable in broad pH ranges, and contains essential amino acids that are good for health. In addition, gelatin can act as a carrier in delivery systems for unstable compounds, not to mention probiotics (Santoro *et al*., 2014).

Several lactic acid bacteria have been known for their probiotic properties, such as Lactobacillus, Streptococcus, and Bifidobacterium. *L. plantarum* Dad-13 is an indigenous probiotic isolated from spontaneously fermented buffalo milk, which has the properties such as resistance in the gut simulation, antimicrobial activity against pathogens, and it does not translocate in the rat's blood and organ, which guarantee its safe use in food products (Rahayu *et al*., 2015; 2019). Several clinical trials also confirm its potentials, such as survival in the gut, anti-diarrhea and immune modulator, intracellular uricase producer, and folic acid producer, and also help to improve the growth of undernourished children (Handayani *et al*., 2017; Kamil *et al*., 2022; Nurliyani *et al*., 2015; Rahayu *et al*., 2016). With those promising potentials, *L. plantarum* Dad-13 has been incorporated into several food products such as yogurt, yogurt sweet potato extract, fermented sticky rice, and fermented milk (Nursiwi *et al*., 2018; Rahayu *et al*., 2016; Tari *et al*., 2016). However, again, those are fermented food products that may have been a limitation for accepting indigenous probiotic strains, especially *L. plantarum* Dad-13.

Concerning the efficacy of probiotics, they should be consumed as living cells with a minimal viable count ranging from 10^6-10^7 CFU/g or mL (Kamil *et al*., 2020). Several factors affect the viability of probiotics cells, such as temperature, pH, oxygen availability, moisture content, and water activity (Lizardi-mendoza *et al*., 2013). During storage, there are also changes in product quality that may affect the product quality. In addition, since jelly candy *L. plantarum* Dad-13 is an innovation of probiotic products, it is also important to evaluate the microbiology quality to ensure product safety. Therefore, this research aims to evaluate the change in jelly candy probiotics *L. plantarum* Dad-13 during storage and its microbiology quality.

2. MATERIALS AND METHODS

2.1. Jelly Candy Production

Bovine gelatine (Intisari, Yogyakarta), sucrose (Gulaku), glucose (inti sari, Yogyakarta), skim milk powder containing *L. plantarum* Dad-13 obtained from Center of Food and

Nutrition Study, Universitas Gadjah Mada which produce following previous research by Kamil *et al*. (2020), and water are the ingredients for jelly candy production. Briefly, sucrose (20 g) was added to water (10 mL) and brought to a boil until completely dissolved (±10 minutes). After that, the sugar solution was mixed with bloomed gelatin (10 g gelatin soaked in 10 ml water). The mixture was then cooled to reach 40 °C and mixed well with 5 g of skim milk containing *L. plantarum* Dad-13 (initial viable count log 9-10 CFU/g). The candy mixture was molded and waited until solidified at room temperature, then stored in a chiller (4 °C) for 24 hours. The jelly candy was then packed and sealed in aluminum foil packaging (20 jelly candies for each temperature) and stored at two different temperatures (4 °C, RH 80-90% and 30 °C, RH 60-70%) for four weeks.

2.2. Physical Characteristics Analysis

2.2.1. pH Analysis

The pH analysis was referring to Nurdjanah *et al*. (2023) with modification. One gram of jelly candy was dissolved in 9 mL aquadest and homogenized. Preceding pH analysis, the pH meter was calibrated using a buffer solution (pH 4 and 9). After calibration, the probe was directly dipped to the sample solution and measured the pH. pH analysis was done at two different storage temperatures before and after four weeks.

2.2.2. Texture Analysis

The texture analysis was performed using a texture analyzer (NEXYGEN Plus), according to Kamil *et al*. (2021). The sample to be tested was placed on the sample table. The probe speed is set with a test speed of 0.5 mm/sec and a wait time of 0.5 seconds. After the probe presses the sample, the results will measure hardness, chewiness, and gumminess. Texture analysis was done before and after four weeks at two different storage temperatures.

2.2.3. Lightness Analysis

The color was analyzed using a chromameter (Minolta CR-310) to measure the L value, according to Kamil *et al*. (2021). Lightness analysis was done at two different storage temperatures before and after four weeks.

2.2.4. Water Activity (a_w) Analysis

Water activity analysis was performed using a_w meter (Pa_w kit), according to Kamil *et al*. (2021). The sample is placed in a plastic container, then installed on the aw meter, and waited until the sound on the tool constantly marks the a_w value. Water activity analysis was done before and after four weeks at two different storage temperatures.

2.3. Probiotic Viability Analysis and Self-life Prediction

The pour plate method was used to count the viability of *L. plantarum* Dad-13 during storage. In brief, after several series of dilutions in 0.85% NaCl, 1 mL of suspension was plated on MRS (De Man, Rogosa, and Sharpe) (Oxoid) agar medium, followed by incubation for two days at 37 °C. Sublethal injury analysis was also performed following the method Kamil et al., (2020). Probiotic viability was done every week for four weeks at two different storage temperatures. The inactivation constant (k) was calculated using the first-order reaction equation as below:

$$\log N_t = \log N_o + k\, T_t \qquad (1)$$

After obtaining the k value, the shelf-life value can then be calculated with the formula:

$$t_s = [\ln(N_o - N_t)]/k \quad (2)$$

where N_o is the initial count of probiotics (CFU/g), N_t is the count of probiotics after several weeks of storage (CFU/g), and t is storage time (week).

2.4. Microbial Quality Analysis

Microbial quality analysis was done following the Indonesian Nasional Standard SNI 3547-2-2008 guideline regarding soft confectionary products (BSN, 2008). The analysis consists of total plate count, *Staphylococcus aureus*, Salmonella, Coliform, *Escherichia coli*, mold, and yeast. Microbial quality analysis was conducted before storage.

2.5. Statistical Analysis

A paired t-test was performed to analyze probiotic milk jelly candy changes during 4 weeks of storage. Meanwhile, an independent t-test was performed to compare each parameter on observed temperature. Significant differences were stated when $p < 0.05$. All statistical analysis was performed by using IBM SPSS Ver 26.0 for Windows. Shelf-life analysis of probiotics was performed by using Ms. Excel 365.

3. RESULTS AND DISCUSSION

3.1. Sublethal Injury

The heat was used to melt ingredients such as gelatin, glucose, and sucrose in making probiotic milk jelly candy. This heat induction can cause probiotic cells added at the end of the process to be disrupted and can sustain sublethal injury. Therefore, a sublethal injury test was carried out to determine the cells injured due to the processing of known healthy cells on the product. A sublethal injury state is defined as the condition where the bacterial cell is metabolically active but unable to be cultured in a growing medium (Espina *et al*., 2016).

Table 1. Sublethal injury of *L. plantarum* Dad-13 during processing

MRS agar (log CFU/g)	MRS agar + bile salt (log CFU/g)	Injured cell (%)
8.76	8.43	4

After making probiotic milk jelly candy, there were in Table 1, it can be seen that the number of cells of MRS agar was 8.76 log CFU/g. However, the number of cells is 8.76 log CFU/g are all living cells, cells that have experienced sublethal injury as well counted. The number of healthy cells after the process was then tested by adding bile salt to MRSA media, and the results obtained were 8,43 log CFU/g. Therefore, the injured cell was 0.33 log CFU/g or 4%, which means not significantly affected by heat. Heat treatment of *L. plantarum* at 42 °C for 1 hour, can improve its heat resistance by 3 -2 log cycles at temperatures up to 70 °C (De Angelis *et al*., 2004). This result indicates that the processing of probiotic milk jelly candy is a mild process and can potentially be used as a probiotic carrier.

3.2. The Physical Changes of Probiotic Milk Jelly Candy During Storage

According to the changes in probiotic candy's characteristics (Table 2), the notable change is pH, aw, and texture over storage time. The different storage temperature only affects the final pH and a_w. The pH of probiotic candy stored at 4 °C tends to be higher than at 30 °C. This is the same as research by Randazzo *et al*., (2013) on stored probiotic jam products at 5 °C and 25 °C, in which the pH decreased during storage. The decrease in pH at 25 °C was higher than at 5 °C. This decrease in pH can be caused by the use of skim milk as an ingredient of filler (*L. plantarum* Dad-13 filler). According to Widowati *et al*., (2007), lactic acid bacteria convert the sugar in milk (lactose) into energy and lactic acid. The formation of lactic acid is what causes a decrease in the pH. However, the final pH of candy stored at 4 °C is higher than the storage temperature of 30 °C. This is because 4 °C is not a suitable temperature for *L. plantarum* Dad-13 optimum growing, and the bacteria tend to slow its metabolism. In addition, we also did a sampling for the pH test of several commercial jelly candies. Based on that, the commercial jelly candies have a pH ranging from 3.53 – 4.03. It indicates that storing probiotic jelly candy in two different temperatures for 4 weeks is still acceptable in terms of acidity.

Table 2. The changes in Candy's characteristics during storage at different temperatures

Parameters	Temperature	Before	After
pH	4 °C 30 °C	5.65 ± 0.02^{a}	5.09 ± 0.08^{b} 4.83 ± 0.04^{c}
a_w	4 °C 30 °C	0.84 ± 0.01^{a}	0.84 ± 0.01^{a} 0.81 ± 0.01^{b}
Hardness (N)	4 °C 30 °C	18.45 ± 0.13^{a}	31.68 ± 5.10^{b} 29.44 ± 2.25^{b}
Chewiness (N)	4 °C 30 °C	15.93 ± 0.78^{a}	23.66 ± 2.73^{b} 21.53 ± 1.67^{b}
Gumminess (N)	4 °C 30 °C	16.88 ± 0.76^{a}	25.49 ± 2.78^{b} 23.42 ± 1.67^{b}
Lightness	4 °C 30 °C	61.72 ± 1.27^{a}	61.05 ± 1.69^{a} 59.17 ± 0.50^{a}

Data are presented as mean ± standard deviation. Different superscripts indicate significantly different in each observed parameter.

During four weeks of storage, a_w level was also tested, as shown in Table 2. At a storage temperature of 4 °C, the obtained a_w was 0.84 ± 0.01, whereas at 30 °C storage temperature was 0.81 ± 0.006. The a_w value at 4 °C storage is higher than at a storage temperature of 30 °C. In addition, a significant decrease of a_w was observed in candy stored at 30 °C, while no significant change at 4 °C. Water activity is closely related to water levels in the material and correlates to its self-life (Razak *et al*., 2020). According to Ergun *et al*., (2010), The a_w value of commercial gummies and jellies ranges from 0.5 -0.75 (based on a sampling test). The high used gelatine concentration influences the high aw of probiotic candy. Gelatine is a hydrocolloid which able to bind water in large numbers. This is in accordance with the research from Pang *et al*., (2015), the more gelatine added to the product, the more water can be bound.

Another parameter analyzed is texture. Texture plays an important role because it affects consumer preference for the product. After four weeks of storage, the texture of probiotic jelly candy was measured using a texture analyzer with parameters hardness, chewiness, and gumminess. After being stored for four weeks at two different temperatures, the hardness, chewiness, and gumminess increased in all treatments. At 4 °C average hardness increased to 31.683 ± 5.10 N, chewiness increased to 23.664 ± 2.73 N, and gumminess increased to 25.486 ± 2.78 N.

Meanwhile, at 30 °C hardness increased to 29.437 ± 2.25 N, chewiness increased to 21.525 ± 1.67 N, and gumminess increased to 23.415 ± 1.67 N. The increment of texture may be due to the formation of gelatine's inner structure, such as hydrogen bound and cross-linked with other substances, as well as sugar crystallization. However, there is no difference in the final texture (hardness, chewiness, and gumminess) of probiotic jelly candy after 4 weeks of storage at two temperatures.

In addition, the probiotic jelly candy brightness was also tested using a chromameter, the value of *L* represents the brightness level of the sample. After being stored for four weeks, the brightness values (*L*) in the two samples at 4 °C and 30 °C storage temperatures were not significantly different. Therefore, storage for 4 weeks at 4 °C and 30 °C did not significantly affect the levels of probiotic jelly candy product brightness.

3.3. Probiotic Viability during Storage and Its Shelf-life Prediction

The probiotic jelly candy was stored at two temperatures, namely 4 °C and 30 °C for four weeks. Every week pour plating is carried out to calculate the number of cells and the result can be seen in Table 3. At week 0, the number of *L. plantarum* Dad-13 cells present in probiotic jelly candy is as much as 4.7×10^8 CFU/g, where the initial cell number is added in jelly candy of 6.1×10^9 CFU/g. In the first week, cell viability at 4°C storage experienced a decrease to 2.4×10^8 CFU/g, and at room temperature 30°C decreased to 7.7×10^5 CFU/g, as in Table 3. The longer the storage time, the decreased the number of *L. plantarum* Dad-13 cells. At the end of the storage period (four weeks), the number of cells in storage temperature 4°C of 1.2×10^7 CFU/g, while at 30 °C, it is 4.1×10^4 CFU/g.

Table 3. The changes of *L. plantarum* Dad-13 during storage and its shelf-life

Week	CFU/g	
	4 °C	**30 °C**
0	4.7×10^8	4.7×10^8
1	2.4×10^8	2.2×10^5
2	4.8×10^7	3.1×10^5
3	4.6×10^7	6.5×10^4
4	1.2×10^7	4.1×10^4

Table 4. Linearity equation of probiotic at two temperatures

	Linearity equation	
	4 °C	**30 °C**
Ordo 0	$y = -1x10^8x + 5x10^8$ $R^2 = 0.822$	$y = -9x10^7x + 4x10^8$ $R^2 = 0.5004$
Ordo 1	$y = -0.1321x + 20.055$ $R^2 = 0.949$	$y = -0.2868x + 17.397$ $R^2 = 0.6567$

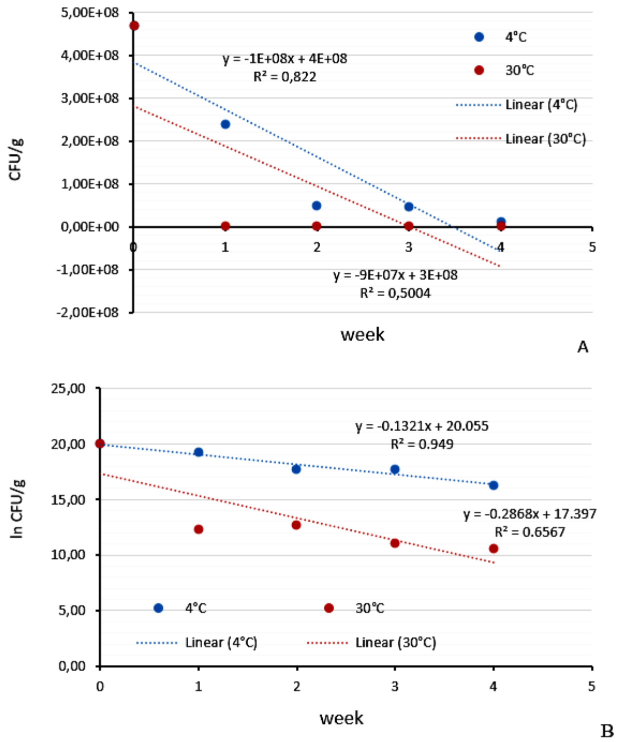

Figure 1. Linearity graph following Ordo 0 (A) and Ordo 1 (B)

Cell viability at 4 °C was higher than storage at 30 °C. According to Jannah *et al.* (2022), low-temperature storage causes probiotic viability to last longer and extends shelf life. Decreased number of LAB (lactic acid bacteria) colonies on probiotic products due to storage at high temperatures can result in the dehydration of cells, causing cells to burst (De Angelis *et al.*, 2004). Therefore, storage at room temperature is not recommended for probiotic products.

Decreased cell viability can also occur due to a decrease in pH product. This decrease in pH is due to the formation of organic acids by LAB. Rivera-Espinoza & Gallardo-Navarro (2010), reported that in some fermented products dairy, decreased viability of probiotic bacteria was caused by a decrease in pH to 4-5 and accumulation of organic acids as a result of growth and fermentation. In addition, sugar content with a concentration that is too high can cause LAB cells to undergo lysis due to pressure differences osmotic (Matouskova *et al.*, 2021). Hence, probiotic viability in food can be enhanced by the addition of a prebiotic, a specific substrate for probiotic bacteria (Nurdjanah *et al.*, 2023).

To determine the estimated shelf life, it is necessary to know the constant of inactivation (*k*), depicted from the linearity equation's slope—the rate of degradation reaction of probiotic jelly candy following Orde 1, due to its good linearity as can be seen in Figure 1 and Table 4. The number of cells (ln CFU/g) per week is plotted with the result R^2 value at a 4 °C storage temperature of 0.949 and 30 °C of 0.6567. It indicates that the shelf-life estimation best follows the 4 °C storage temperature due to its good linearity. At 4 °C storage temperature, the equation $y = -0.1321x + 20.055$ is obtained. It is known that the *k* value is -0.1321/day, meaning at storage temperature of 4 °C probiotic jelly candy decrease in quality by 0.1321 unit per day.

The shelf-life of probiotic jelly candy stored at 4 °C can be calculated by following equation (2). The efficacy of probiotics is when the minimal viable cell is 6 log CFU/g or mL. Therefore, probiotic candy's shelf-life (or so-called best before) is estimated from the time it reaches the minimum number of viable cells, which is 46.6 days. The calculation of shelf-life is as follows:

$$y = -0.1321x + 20.055$$

The shelf life t is calculated by using k = 0.1321 (day)$^{-1}$, N_o = 4.7 x 10^8 CFU/g, and N_t = 10^6 CFU/g as the following:

$$t\ (\text{orde 1}) = \frac{\ln(No - Nt)}{k} = \frac{\ln(4.7 \times 10^8 - 10^6)}{0.1321} = 46.6\ days$$

3.4. Microbial Quality of Probiotic Milk Jelly Candy

The microbiology quality of probiotic *L. plantarum* Dad-13 milk jelly candy can be seen in Table 5 with parameters Total Plate Count, Coliform*, Escherichia coli, Staphylococcus aureus,* Salmonella*,* mold/yeast, in accordance with Indonesian Nasional Standard (SNI 3547-2-2008) regarding soft confectionary products. The results showed that all the microbiology parameters of probiotic candy follow the standard of SNI, except the TPC. The TPC result exceeds the SNI standard for jelly candy products, which is a maximum of 5 x 10^4 for soft confectionery in the jelly category. This large result does not mean that the contamination of pathogenic microbes in the jelly candy is large, but because the *L. plantarum* Dad-13 colony is included in this jelly candy product. If the TPC results of 1.8 x 10^7 were compared with the initial number of probiotic *L. plantarum* Dad-13 jelly candy cell viability, the numbers were close, namely 4.7 x 10^8 CFU/gram. This is because the media used is Plate Count Agar (PCA) media which is a standard medium for the total plate count method for waste, water, and other food samples**.**

Table 5. Microbiology quality of probiotic *L. plantarum* Dad-13 milk jelly candy

	Unit	Probiotic candy	Standard
Total Plate Count (TPC)	colony/g	1.8 x 10^7	Max. 5 x 10^4
Coliform	MPN/g	4	Max. 20
Escherichia coli	MPN/g	< 3	< 3
Staphylococcus aureus	colony/g	< 25	Max. 1 x 10^2
Salmonella		Negative	Negative/25 g
Mold/yeast	colony/g	1.25x 10^1	Max 1x10^2

4. CONCLUSIONS

Milk jelly candy is possible as a non-fermentation carrier for probiotic *L. plantarum* Dad -13, with the best consumption for its health benefits before 47 days at 4 °C storage. Different storage temperatures affect the pH value although it is still acceptable. The microbial quality is also in accordance with SNI 3547-2-2008 except for TPC, which is due to the addition of probiotic cells. Further formula optimization is needed to control the a_w of probiotic jelly candy.

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