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FULL PAPER

Development of probiotic gummy candy using the indigenous *Lactobacillus plantarum* Dad-13 strain; evaluation of its gastrointestinal resistance and shelf-life prediction

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Abstract

Nowadays, functional food especially probiotic products have gained more interest, and gummy candy *L. plantarum* Dad-13 is an innovation. However, the efficacy of probiotics relies on their viable cells and resistance in the gastrointestinal tract. Therefore, this research aimed to evaluate the gastrointestinal resistance and probiotic cells' shelf life and investigate gummy probiotics' characteristics. A market survey was carried to understand the consumer's knowledge on the health benefit of probiotics and their product, also intend to buy the product. Gastrointestinal simulation with pepsin and the pancreatic enzyme was conducted to evaluate the gastrointestinal tract's probiotic resistance, probiotic shelf life was evaluated with the Accelerated Shelf-life Test (ASLT). Proximate analysis was carried out according to the Association of Official Analytical Chemists (AOAC), and physical characteristics were analysed before and after 90 days of storage at 4°C. The result showed that the supplementation of *L. plantarum* Dad-13 in the gummy probiotic had an excellent survival during gastrointestinal system simulation with the predicted shelf life was 75.17 days or 2.5 months at 4°C of storage. However, the moisture content of gummy probiotics was more than 20%. Considering the survival of *L. plantarum* Dad-13 during gastrointestinal simulation, it can be concluded that gummy probiotics can be used as a carrier for *L. plantarum* Dad-13.

1. Introduction

In recent years, functional foods have gained increased interest. Functional food is a type of dietary item that is pleasant in the sensory attributes and provides essential nutrition and brings health benefits. Several functional ingredients are typically added to make functional foods, ranging from the phenolic compound, resistant starch, insoluble dietary fibre, prebiotic, to even a live microorganism, which was probiotic (Abuajah *et al*., 2015; Gul *et al*., 2016). Probiotic is a live microorganism that, when consumed in an adequate amount, will give health benefits to the host (Food and Agriculture Organization/World and Organization, 2002). Several health benefits of probiotics are known to maintain gastrointestinal health, improve immune response, inhibit pathogen colonisation in the colon, and produce short-chain fatty acid (Kechagia *et al*., 2013).

Of many probiotics, *L. plantarum* Dad-13 is worth mentioning. *L. plantarum* Dad-13 is an indigenous probiotic isolated from a spontaneously fermented buffalo milk called *dadih*. The strain has been characterised for its probiotic potentials such as antimicrobial activity and its resistance in gastrointestinal *in vitro* (Rahayu *et al*., 2015). Molecular analysis showed that the strain belonged to *L. plantarum* species (Rahayu *et al*., 2015), and a safety assessment of *L. plantarum* Dad-13 using Sprague Dawley rats demonstrated that no translocation was observed in the rats' organ and blood (Rahayu *et al*., 2019). Indeed, several clinical trials showed that *L. plantarum* Dad-13 could survive in the gastrointestinal tract (Rahayu *et al*.,

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2016; Banin *et al*., 2019; Rahayu *et al*., 2019). *Lactobacillus plantarum* Dad-13 has anti-diarrhoea and immune-modulator properties (Nurliyani *et al*., 2015; Tari and Handayani, 2015), and it is considered an intracellular uricase producer (Handayani *et al*., 2017).

For as much as the background mentioned above, *L. plantarum* Dad-13 has a considerable potential to be applied in food products other than yoghurt and other fermented foods. Supplementation of *L. plantarum* Dad-13 into food products has been conducted in several studies, such as in yoghurt, yoghurt with purple sweet potato extract, fermented milk, and *tape ketan* (fermented sticky rice) (Tari *et al*., 2016; Utami *et al*., 2016; Rahayu *et al*., 2016; Pamungkaningtyas *et al*., 2018). However, all of those probiotic products are limited to fermented food. Consumers prefer food that is not only nutrient-enriched but also convenient (easy to handle).

The number of viable probiotics is essential to give health benefits, and a probiotic product should have viable cells count of 10^6-10^7 CFU/g or mL at the end of the expired date for their efficacy (Barbosa *et al*., 2015). These factors make predicting their shelf-life crucial. The accelerated test is one of the methods to predict the shelf-life in a short-term test under harsh conditions by extrapolating the kinetic parameter and the storage environment. *L. plantarum* Dad-13 supplemented with gummy probiotics should retain its viable cells against gastrointestinal conditions to colonise in the colon. Therefore, this research aimed to develop a new indigenous gummy probiotic *L. plantarum* Dad-13, as a carrier of the probiotic cell. With that in mind, resistance in the gastrointestinal tract, probiotic cells' shelf-life prediction, and gummy probiotics' physicochemical characteristics were evaluated.

2. Materials and methods

2.1 Survey of consumer's behaviour and acceptance

The descriptive questionnaire was designed to understand consumers' behaviour, mainly knowledge on the health benefit of probiotics and their products. A consumer survey was conducted between teenagers and adults, which represents the target market for probiotic products. Each right answer was scored as 1, while each wrong answer was scored 0, which then divided into three categories: good (>75% of the right answer), fair (50-75% of the right answer), and low (<50% of the right answer). Consumer's interest in the indigenous gummy probiotic product was asked as "yes" and "no" answers.

2.2 Bacterial used in this study

Skim milk powder containing *L. plantarum* Dad-13 was obtained from the Centre for Food and Nutrition Studies, Universitas Gadjah Mada.

2.3 Gummy probiotic production

The production of gummy probiotics was modified from Lele *et al*. (2018). The formula consisted of bovine gelatine (11 g), sucrose (20 g), glucose syrup (10 g), water (20 g), and skim milk powder containing *L. plantarum* Dad-13 (5 g), with the initial cell counts 7.58×10^9 CFU/g. In brief, the gelatine was soaked with water for 15 mins to bloom. The sugar solution was prepared by diluting glucose syrup, sucrose, and water by heating at 115-120^{o}C for 15 mins. After that, the sugar solution and bloomed gelatine were mixed until there was no lump, and then the mixture was cooled until it reached 40^{o}C. Before the addition of probiotics, skim milk powder containing *L. plantarum* Dad-13 was dissolved with water and mixed into a candy mixture at 40^{o}C. The citric acid (0.1 g) was incorporated into the candy mixture at the end of the process. Flavour agents could be added at the desired amount. The candy mixture was then poured into a mould and set at room temperature for 30 mins and continued in a chiller. Once it had finished, the gummy probiotic was packed and sealed into an aluminium bag and was kept dry until the analysis.

2.4 Microbial count

The viable cells of *L. plantarum* Dad-13 were count by pour plate method. The samples (1 g) were diluted into 9 mL saline water (0.85% NaCl, w/v) and homogenised with a stomacher followed by serial dilution. At appropriate dilution series, 1 mL of suspension was plated on De Man, Rogosa, and Sharpe (MRS) agar medium (Oxoid) and incubated at 37^{o}C for two days. The counted *L. plantarum* Dad-13 was then expressed as CFU/g samples.

2.5 Gastrointestinal simulations

The survival of probiotic *L. plantarum* Dad-13 in the simulated gastrointestinal tract was analysed *in vitro* according to Tokatl *et al*. (2015) with modification. Briefly, for the gastric simulation, the samples (1 g) were incubated in a gastric solution at 37^{o}C for 2 hrs, which was prepared by suspending 3 mg/mL pepsin (Sigma-Aldrich; P 7000) into sterile saline water (0.85% NaCl, w/v) and adjusting the pH to 2.5. Meanwhile, for the intestinal simulation, the samples were incubated in an intestinal solution at 37^{o}C for 4 hrs, which was prepared by suspending 1 mg/mL pancreatic (Sigma-Aldrich; P7545) and 0.3% (w/v) bile salt into sterile saline water (0.85% NaCl, w/v) and adjusting the pH to 8. The viable count of *L. plantarum* Dad-13 was determined before and at each hour interval of incubation.

2.6 Probiotic shelf-life prediction by accelerated test

Packed gummy probiotic samples were stored at four different temperatures (4, 11, 30, and 37°C). The cell viability of *L. plantarum* Dad-13 was counted at intervals 0, 2, 4, 6, 8, 12, 16, and 22 days for samples that were incubated at 30 and 37°C. Meanwhile, at intervals 0, 2, 4, 6, 8, 12, 16, 22, 28, and 34 days was applied for samples were incubated at 4 and 11°C. The viable count was fitted to zero as well as to the first-order reaction equation model, as shown in equations 1 and 2, respectively.

$$N = N_o - k_t \quad (1)$$

$$\ln N = \ln N_o - k_t \quad (2)$$

N_o is the initial cells count (CFU/g), while N is viable cells count in everyday storages (CFU/g). On the other hand, $_t$ is a time of storage, and k is the inactivation rate constant (day^{-1}). For estimating the probiotics shelf-life, the inactivation rate at each incubation temperature was plotted to obtain the Arrhenius equation, as shown in equation 3.

$$\ln k = \ln k_o - Ea/RT \quad (3)$$

Ea is the activation energy (kcal/mol), while R and T is the universal gas constant (R=1.987 $cal \cdot mol^{-1} \cdot k^{-1}$), and the absolute temperature (°K), in turn.

Packed gummy probiotics, which were incubated at 4°C, were collected and analysed for the viable cells at a regular interval of 10 days until the viable cells reached the limit dose of probiotic 1×10^7 CFU/g.

2.7 Physicochemical analysis

Proximate analysis was done by using the standard procedure of AOAC (2005). The gummy probiotic energy content was calculated using Atwater's conversion factors (Araro *et al.*, 2020). Physical characteristics of gummy probiotics were analysed before and after 90 days of storage at 4°C. The colour parameters (L*, a*, and b*) were measured using chromameter (Minolta CR-310), and a_w was measured using a_w meter (pa_wkit). Meanwhile, the texture of gummy probiotics was measured using a texture analyser (NEXYGEN plus).

2.8 Statistical analysis

Any significant difference in all samples was evaluated with one-way ANOVA followed by the Duncan Multiple Range Test. A paired t-test was performed to assess any significant difference in physical characteristics after storage. All statistical analysis was performed by IBM SPSS Statistic 24 confidentially (p value<0.05).

3. Results and discussion

3.1 Consumer's behaviour and acceptance

A total of 200 respondents (age:18-24 years old) filled out the questionnaire. As shown in Figure 1, aside from the sensory aspect, the nutritional value was one of the considerable aspects of selecting food. More than half of respondents (62%) had good knowledge regarding probiotics, with acceptance and intention to buy gummy probiotics reach for 92% of respondents. The probiotic consumption trend is inspired by the Balkan region, which has a longevity life span due to their habit of consuming fermented milk. The trend is widespread and has become a new lifestyle, particularly with the role of aggressive advertising in the media. Besides that, awareness of healthy lifestyles among the community has dramatically increased. According to Lerner *et al.* (2019), citing from global market analysts, probiotics' global market size is predicted to exceed 3 billion US dollars by 2024. Therefore, with the growth of the probiotics market, and consumer knowledge regarding probiotics, the development of indigenous gummy probiotics is possible to fulfil the demand in the probiotic market.

3.2 Resistance to the gastrointestinal simulations

An adequate amount of probiotic cells in a probiotic product does not guarantee its resistance throughout the gastrointestinal system. The initial cell count of the gummy probiotic was 5.88×10^8 CFU/g. As seen in Figure 2 (a), after 2 hrs of incubation at the simulated

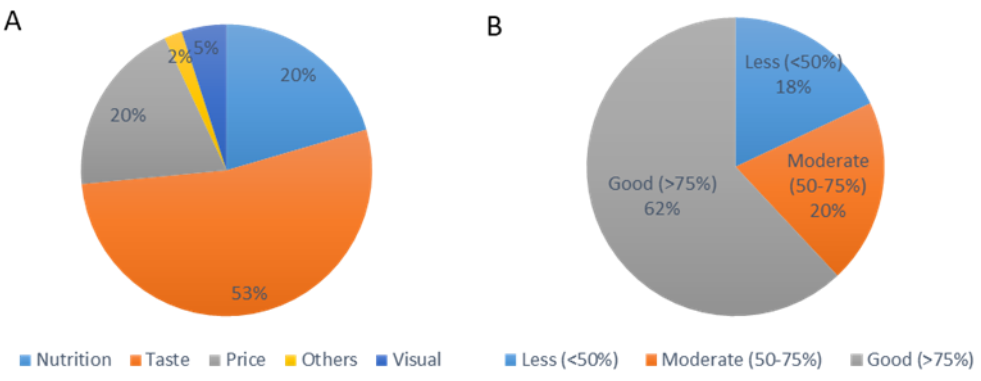

Figure 1. Consumers behaviour. (A) Consideration for selecting food. (B) Consumer's knowledge regarding probiotic

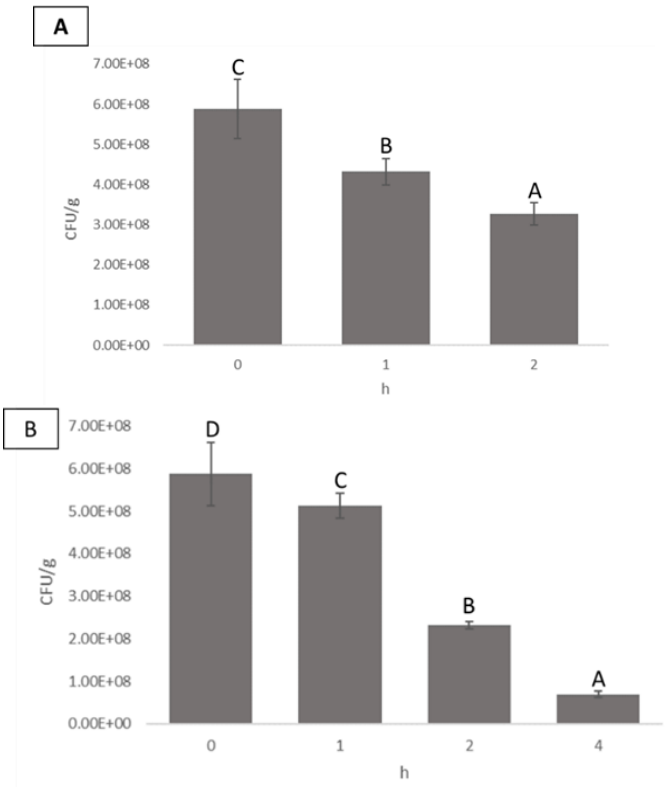

Figure 2. Gastrointestinal simulations of *L. plantarum* Dad-13. (A) Resistance in simulated gastric solution. (B) Resistance in simulated intestinal solution. Values are presented as mean±SD, n = 2. Different letters are significantly different ($p<0.05$) by one-way ANOVA followed by Duncan Multiple Range Test.

gastric juice, the final viable cell count was 3.28×10^8 CFU/g (44.26% reduction from the initial cells). A significant difference was observed at the simulated intestinal after 4-hour incubation, which was 6.98×10^7 CFU/g (88.13% reduction) from initial cells (Figure 2B).

Gummy probiotic can be classified as a hydrogel due to its main ingredient (i.e., gelatine, agar, and pectin). Protein-based hydrogels are applicable for delivery systems, especially nutraceuticals, due to their excellent functional properties, low toxicity, and high nutrition value (Abaee *et al*., 2017). According to Young *et al*. (2005), gelatine-based controlled-release systems have been developed because their tailored electrical and physical properties depend on the operation process. Several studies have reported that gummy candy can be used as a carrier for bovine colostrum, essential oils, and also probiotics (Bartkiene *et al*., 2018; Lele *et al*., 2018). In addition, gelatine also acts as a protective agent in delivery systems (Santoro *et al*., 2014). Interaction between gelatine and milk protein within the candy mixture is expected to occur since those polymers carry an opposite charge (Pang *et al*., 2013). According to Pang *et al*. (2015), gelatin and skim milk protein will increase the gel's firmness. Therefore, the diffusion of a gastric and intestinal solution to contact with probiotic cells will be delayed.

Powder *L. plantarum* Dad-13 was obtained with microencapsulation by a freeze-dried process using skim milk as a protectant. Microencapsulated probiotics had more stable viability against the harsh condition than free cell form, as reported in other studies (Liao *et al*., 2017; Moayyedi *et al*., 2018; Kamil *et al*., 2020). The microencapsulation process's role is to immobilise probiotic cells within a matrix and protect against harsh conditions (Anal and Singh, 2007; Abd El-Salam and El-Shibiny, 2015; Eckert *et al*., 2017). The probiotic gummy candy can retain its minimum viable cells at 10^7 CFU/g in gastrointestinal simulation. Therefore, it can be estimated that the viable cells of *L. plantarum* Dad-13 are high enough to colonise in the colon.

3.3 Viable cells count of probiotic at different temperature

As seen in Figure 3, the initial cell count of *L. plantarum* Dad-13 in gummy probiotics was 7.80×10^8 CFU/g. A loss of viable cell count had been observed at the time of storage. After 22 days of storage, gummy probiotics stored at 30 and 37°C had a higher reduction of the counted viable cell, from the initial cells to 6.50×10^3 and 1.52×10^3 CFU/g, respectively. In addition, gummy probiotic, which was stored at 11°C, had a viable cell count of 2.50×10^7 CFU/g after 34 days. Meanwhile, storing at 4°C, the gummy probiotic had more stable viable cells, which count 1.16×10^8 CFU/g.

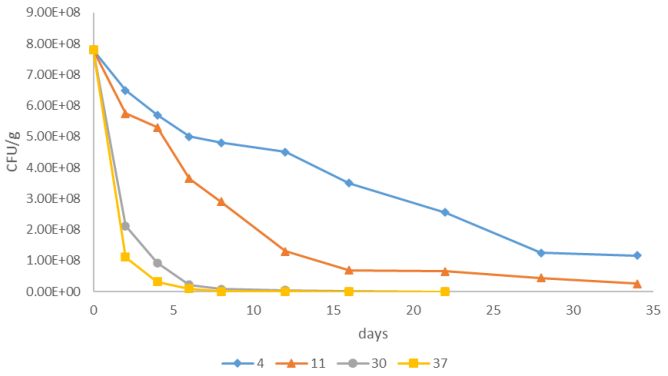

Figure 3. Changes in probiotic viability at different temperatures. Values are presented as mean±SD, n = 2.

Lestari *et al*. (2020) reported a similar observation, where good stability of *L. acidophilus* IFO 13951 and *Bifidobacterium longum* ATCC 15707 in gummy probiotic is achieved at 4°C of storage. Conversely, an expressive decrease of viable cells was observed at storage over 25°C, and it is reported in other research (Klayraung *et al*., 2009; Nagashima *et al*., 2013; Lestari *et al*., 2020). Another observation by Lele *et al*. (2018) showed that the use of gelatine as an ingredient of gummy probiotics gives good stability during storage.

High temperature is one of the considered factors affecting probiotic stability, besides pH, water activity,

chemical compound, and oxygen content (Gueimonde and Sánchez, 2012). Also, gelatine as hydrogel could be affected by temperature. According to Ullah *et al*. (2015), at high temperatures, hydrogel's structure will dissociate and swell due to the breaking of the hydrogen bonds. It causes the inability to protect the probiotic cells. On the contrary, at low temperatures, a complex structure of gelatine hydrogel is formed by hydrogen bonds and entraps the probiotic cells to become more settled.

3.4 Prediction of probiotic shelf-life

The change of food characteristics follows either zero or first-order reaction (Calligaris *et al*., 2019). Therefore, the changes in the viable cells are plotted into zero and first-order reactions. As seen in Table 1, the first-order reaction has excellent linearity compared to the zero-order. Therefore, it will be used further in the Arrhenius equation.

The slope of the equation refers to the inactivation rate of probiotic cells stored at different temperatures. Increasing the temperature of incubation aligns with a high inactivation rate. It shows that the accelerated test's kinetic model is temperature-dependent, and the same result was observed in other research (Achour *et al*., 2001; Park *et al*., 2018; Zhi *et al*., 2018; Li *et al*., 2019). The ln value of the inactivation rate was plotted with reciprocal absolute temperature, which the Arrhenius equation will obtain. The obtained equation was further used to estimate the shelf-life according to the first-order reaction in equation 4.

$$t_s = \ln (No-Nt)/k \quad (4)$$

t_s is estimated shelf-life, N_o is the initial cells count (CFU/g), N_t is the viable limit cells at the end of storage (10^7 CFU/g), and k is the inactivation rate constant (day^{-1}).

As seen in Table 2, the estimated shelf-life of viable *L. plantarum* Dad-13 at 4, 11, 30, and 37°C was as follows: 75.17, 43.49, 11.19, and 7.07 days, respectively. According to the estimated shelf-life, cold storage (4°C) was recommended to maintain the viable cells of probiotic *L. plantarum* Dad-13. However, the observed shelf-life to reach the minimal count of viable cells at 1.9×10^7 CFU/g was 90 days at 4°C of storage or with a relative error of 16.48%. Even though the incubation condition was maintained stable during storage, food deterioration is more complicated. Other factors, such as humidity, water activity, and packaging materials, may affect the rate of food deterioration. Otherwise, the accelerated test can be applied as a model to predict the shelf-life of gummy probiotic *L. plantarum* Dad-13.

3.5 Physicochemical characteristics of gummy probiotic L. plantarum Dad-13

Physicochemical characteristics of gummy probiotics can be seen in Table 3. According to the Indonesian standard about gummy probiotics (SNI 3547.2-2008) (Badan Standardisasi Nasional, 2008), the maximum moisture content is 20%. In this research, gummy probiotics' moisture content was higher than 20%. Moisture content in gummy probiotics has a relation with the amount of the used gelatine (Susanty and Pujilestari, 2016). The more gelatine used, the more free water molecule will interact. Pang *et al*. (2015) reported that gelatine enhances water holding capacity in combination with skim milk protein, which may be the cause of high moisture content in gummy probiotics. The final moisture content of food determines its physical characteristics as well as its shelf-life. The lower the moisture content, the more rigid texture of gummy probiotics with a longer shelf-life (Ergun *et al*., 2010).

In line with the high moisture content, high water activity was also observed in gummy probiotics. In confectionery, water activity is an important parameter to describe microbiological and physical characteristics. According to Ergun *et al*. (2010), as a confectionery product, gummy candy has water activity in the range of 0.5-0.75. Besides, in the modern confectionery factory, convection drying in tunnels or chambers is done after the moulding process (Delgado and Bañón, 2015). In gummy probiotic production, on the other hand, the drying process is not carried out. However, the ash content of gummy probiotic is within the SNI 3547.2-2008 standard.

Table 1. Linear regression equations for the estimated shelf-life

Temperatures (°C)	Zero-order reaction		First-order reaction	
	Linear Equations	R^2	Linear Equations	R^2
4	$y = -1.82\times10^7x + 6.69\times10^8$	0.9484	$y = -0.0562x + 20.453$	0.9674
11	$y = -1.99\times10^7x + 5.51\times10^8$	0.7466	$y = -0.1022x + 20.28$	0.9487
30	$y = -2.23\times10^7x + 3.35\times10^8$	0.3865	$y = -0.4236x + 19.942$	0.9802
37	$y = -1.98\times10^8x + 2.90\times10^8$	0.2995	$y = -0.5734x + 19.613$	0.9785

Linear equations of zero-order reaction are obtained from reciprocal incubation time in days (X) and viable cells count in CFU/g (Y). While linear equations of the first-order reaction are obtained from reciprocal incubation time in days (X) and viable cells count in ln CFU/g (Y)

Table 2. Shelf-life predicted by ASLT

T (°C)	ln k (day^{-1})	Arrhenius equation	R^2	N_o (CFU/g)	N_t (CFU/g)	ESL (days)	OSL (days)	RE (%)
4	-2.879	y = -6149.7x + 19.353	0.9964	7.80×10^8	1×10^7	75.17	90	16.48
11	-2.281					43.49		
30	-0.859					11.19		
37	-0.556					7.07		

Arrhenius equation is obtained from the first order's inactivation rate reaction at different temperatures. ESL: Estimated shelf-life; OSL: Observed shelf-life; RE: Relative error, between observed shelf-life and estimated shelf-life at 4°C

Moreover, gummy probiotic has a milky white colour, as indicated in the value of L*, a*, b*. The yellowish-white colour of gummy probiotics is due to the existence of skim milk as an ingredient in the making of candy. Also, for gummy probiotics, the texture is one of the primary determinants for its quality. In this research, after 90 days of storage at 4°C, a significant difference was observed in the gummy probiotic's hardness and gumminess. An increment of hardness gum candy was also observed by Csima *et al*. (2014) during storage at several temperatures. The increment of hardness and gumminess of gummy probiotics may be due to a complex inner structure of gelatine that formed during cold storage (Ullah *et al*., 2015).

Table 3. Physicochemical characteristics of gummy probiotic

Proximate Content		
Moisture (%)	27.83±0.21	
Ash (%)	0.61±0.01	
Crude fat (%)	0.08±0.01	
Crude protein[#] (%)	16.38±0.08	
Available carbohydrate (%)	55.12±0.12	
Energy (Kcal/100 g)	286.66±0.88	
Physical characteristics		
	Before	After
Colour		
L*	56.10±0.90	56.07±1.23
a*	-1.45±0.16	-1.38±0.22
b*	11.93±0.34	12.32±0.42
Texture		
Hardness (N)	4.37±0.37	5.38±0.70[ɤ]
Gumminess (N)	4.07±0.36	5.00±0.61[ɤ]
Chewiness (N)	3.84±0.35	4.20±0.56
aw	0.81±0.01	0.82±0.01

Values are presented as means±SD, n = 2.
[#]conversion factor: 6.25; [ɤ]significantly different ($p < 0.05$) by paired t-test.

4. Conclusion

A non-fermented food product of indigenous probiotic *L. plantarum* Dad-13 in the form of gummy probiotic was developed and it has a good acceptance according to consumer's survey. In vitro analysis showed that *L. plantarum* Dad-13 supplemented into gummy probiotics has a good survival during gastrointestinal tract simulation, which means that gummy probiotics can be used as a carrier to deliver probiotics. According to the result of shelf-life prediction, cold storage (4°C) is preferable to maintain the probiotic cells with no major changes in the physical characteristics of gummy probiotics.

Conflicts of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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