MedPharmRes

Journal of University of Medicine and Pharmacy at Ho Chi Minh City
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Original article

Formulation of chewing gum containing *Lactobacillus brevis* NB10

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Received April 25, 2022: Revised June 15, 2022: Accepted June 30, 2022

Abstract: ***Introduction:*** *Lactobacillus brevis* NB10 isolated from human saliva, showed *in vitro* effect on inhibiting the growth and the biofilm formation of *Streptococcus mutans* - a major pathogen that initiates dental plaque and caries. Formulation of chewing gum containing *L. brevis* NB10 is the next step in using this probiotic for the prevention of dental diseases related to *S. mutans.* ***Methods:*** Cryoprotectants such as sorbitol, mannitol, xylitol, lactose, maltodextrin, skim milk and potassium ascorbate were selected to formulate the freeze-dried powder containing *L. brevis* NB10. Next, the freeze-dried powder of *L. brevis* NB10 was used to make chewing gum by direct compression method using HIG-04 gum base (Heath in Gum ®). Other ingredients such as xylitol, strawberry flavor powder, glycerin, aerosil, talc, and magnesium stearate were selected to formulate chewing gum with suitable chewing feel, taste, and flavor. ***Results:*** The freeze-dried powder showing the highest bacterial survival rate (75.5 ± 7.6%) consists of 6% maltodextrin, 6% skim milk, and 0.25% potassium ascorbate. The optimal formula of chewing gum tablet consists of 10% *L. brevis* NB10 freeze-dried powder, 10% xylitol, 2% strawberry powder, 1.5% glycerol, 1.5% magnesium stearate, 1.5% talc and 2.5% aerosil. The bacterial survival rate after tablet compression is 34.34 ± 0.55 %. The product is stable for at least 3 months at 5 ºC ± 3 ºC. ***Conclusion:*** This study was successful in formulating chewing gum containing at least 10^7 CFU/tablet of *L. brevis* NB10.

Keywords: Probiotic; Lactobacillus; dental caries; freeze-drying; chewing gum.

1. INTRODUCTION

Oral diseases are among the most common noncommunicable diseases in the world which relate to serious health issues and economic burdens. According to The Global Burden of Disease Study (2019), it was estimated that 3.5 billion people suffering from oral diseases around the world. Among these, 2.3 billion people had permanently damaged their teeth in form of caries and tooth decay, and no less than 530 million children suffer from caries of their primary teeth [1]. Oral diseases are closely related to sugar consumption, tobacco, and alcohol abuse as well as poor oral hygiene habits. Cariogenic bacteria, especially the mutans streptococci group (*S. mutans*, *S. sobrinus*…) can attach onto tooth surfaces and produce biofilm in the presence of sugars to initiate dental caries. As the caries progress, the metabolisms of the microbial community in biofilms reduce the pH and induce the demineralization process that causes unreversible damage to the enamel layer and finally lead to tooth decay. The levels of mutans streptococci in plaque and saliva are also the reliable risk indicator for early childhood caries [2, 3].

Dental caries and tooth decay can be prevented by maintaining oral hygiene. Nowadays, besides fluoride therapies, the use of probiotics for the replacement or displacement of cariogenic bacteria in the oral cavity is a promising approach in prevention of caries. Probiotics are defined by FAO/WHO (2001) as living microorganisms that, when administered in adequate amounts, confer health

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DOI: 10.32895/UMP.MPR.7.2.2

benefits to the host. Most of probiotics bacteria used today are Lactic acid bacteria (LAB) and are belong to the genera *Lactobacillus* or *Bifidobacterium* [4]. Recent studies found that *Lactobacillus* spp. exhibiting potential properties that could make them become the promising tool to fight against *S. mutans* and their relating diseases. Many of Lactobacillus spp. such as *L. paracasei, L. rhamnosus, L. lactis, L. fermentum, L. reuteri, L. plantarum*… were found to show antagonistic activity angainst *S. mutans* by competing for adherence sites and nutrients; producing sort of antimicrobial secondary metabolites to inhibit *S. mutans* growth; and down regulating responsible genes in biofilm formation [5 -14]. Chewing gum containing *L. reuteri* ATCC 55730 and *L. reuteri* ATCC PTA 5289 showed a statistically significant reduction ($p < 0.05$) of salivary mutans streptococci after 3 weeks of administration [15]. Also, the use of chewing gum containing these two strains reduced gingival inflammation and the levels of selected inflammatory mediators in the gingival crevicular fluid after 2 weeks [16]. Today, many chewing gum products containing probiotics can be found in the marketplace for the prevention of dental caries, improving oral health, and fighting bad breath. However, scientific papers about the preparation of probiotic chewing gum are hard to find throughout the literature, and to our knowledge, there is no paper related to this field of research was published yet in Vietnam.

From the previous study on screening of probiotics that antagonized *S. mutans*, *L. brevis* NB10 was among the most promising strains. Besides showing good *in vitro* effect on inhibiting the growth and biofilm formation of *S. mutans*, this strain meets the requirements of FAO/WHO on probiotic characteristics such as high tolerance to artificial gastrointestinal conditions and showing no risk of transferring antibiotic resistance genes [17]. In addition, being isolated from human saliva and showing high tolerance to salivary amylase, *L. brevis* NB10 is predicted to be able to last long in the oral cavity. *L. brevis* N10 is also a non-sucrose fermenter so the use of this strain would not increase the acid load in the oral cavity compared to typical Lactobacilli. For all of these reasons, it is considered that *L. brevis* NB10 is a good candidate to be used in chewing gum products for the prevention of *S. mutans*-related diseases. The current study aims to formulate the chewing gum tablet containing at least 10^7 CFU/tablet of *L. brevis* NB10. To achieve this, firstly, *L. brevis* NB10 freeze-drying powder was formulated. Low cariogenic cryoprotective agents were selected and screened to form a suitable protective medium that excerts a high bacterial survival rate after freeze-drying and during long-term storage. Next, chewing gum tablets were prepared from *L. brevis* NB10 freeze-dried powder using the direct compression method. The gum formulation was optimized for a high bacterial survival rate and good organoleptic properties. The result of this study would provide fundamentals in applying this beneficial bacteria for the prevention of dental diseases relating to *S. mutans*.

2. MATERIALS AND METHOD

2.1. Materials

HIG04 gum base (Health in Gum ®) was purchased from Cafosa (Barcelona, Spain). De Man, Rogosa and Sharpe (MRS) agar and MRS broth were purchased from Merck Millipore (USA). Skim milk powder was purchased from The A2 Milk Company (Australia) Pty Ltd.. All other excipients used in formulation of freeze-dried powder and chewing gum were of pharmaceutical grade. All reagents used in the analyses were of analytical grade.

2.2. *L. brevis* NB10 growth and harvesting condition

L. brevis NB10 stored in -80 ºC glycerol stock was thawed and streaked onto MRS agar, incubated in 24 h at 37 ºC, 5% CO_2. Transfer single colony from this plate into 25 ml of MRS broth and incubate in 24 h at 37 ºC, 5% CO_2. Finally, transfer bacteria from this broth into 250 ml of MRS broth and incubate in 24 h at 37 ºC, 5% CO_2. Collect bacterial cell by centrifuge at 10.000g, 4 ºC for 5 min, wash the cell twice by 20 ml of cold 0.9% NaCl. Resuspend bacterial pellet into 0.9% NaCl solution to make bacterial suspension at 2 x10^9 CFU/ml.

2.3. Viable bacteria enumeration

The number of viable *L. brevis* NB10 in samples (bacterial suspension, freeze-dried powder, or chewing gum) was determined by the spread plate method. Briefly, ten-fold serial dilutions were made from 1 ml of bacterial suspension or 1 gram of examined powder, or 1 tablet of chewing gum. Then, transfer and spread 0.1 ml of each dilution onto three dishes of MRS agar, incubate these dishes in 24 h at 37 ºC, 5% CO_2. Select and calculate the mean colony-forming unit (CFU) from the plates corresponding to a given dilution that shows the number of colonies between 30 – 300. The quantity of viable *L. brevis* NB10 in samples was calculated as:

$$\boldsymbol{Q} = \frac{\overline{N} \times 10^{d}}{0.1} \quad \text{(CFU/ml or CFU/g or CFU/tablet)}$$

Wherein, $\overline{N}$ is the mean CFU from the plates of given dilution; d is the dilution factor

For freeze-dried powders, three samples will be taken to enumerate viable bacteria and calculate the average value.

To quickly estimate the viable amount of *L. brevis* NB10 by OD600, a series of bacterial suspensions with OD600 value from 0.1 to 2.5 was prepared using colonies from overnight MRS agar plate. Then, the logarithm of viable cell concentration (log CFU/ml) from these suspensions was determined by the spread plate method and plotted versus their OD600 values. As a result, the linear regression equation between OD600 and Log CFU/ml was made as: $\hat{y} = 2{,}579x + 5{,}014$ ($R^2 > 0{,}9994$, data not shown). Therefore, the cell density of bacterial suspensions will be adjusted appropriately based on OD600 using this equation before mixing with cryoprotectants to make freeze-dried powder.

2.4. Freeze drying of *L. brevis* NB10

To choose the most suitable protective agents that providing the best survival rate of *S. brevis* NB10 after freeze-drying as well as during storage, protectants such as sorbitol, mannitol, xylitol, lactose, maltodextrin, skim milk, and

potassium ascorbate were used to formulate the *L. brevis* NB10 freeze-dried powders. Each agent was prepared as 20% solution and autoclaved at 115 ºC for 15 minutes before use, except potassium ascorbate was sterilized through 0.22 µm filter. These protective agents were used as single agent or in mixture with other agents to form different protective mediums. Then, *L. brevis* NB10 suspension at 2×10^9 CFU/ml was mixed with each protective medium at a ratio 1:1 to make the pre-freeze-dried samples. Determine the amount of *L. brevis* NB10 (CFU/ml) in these samples before drying ($\mathbf{Q_1}$).

Pre-freeze-dried samples were kept at -20 ºC for 4 hours then at -80 ºC for 4 hours. Next, transfer these samples to the freeze-dryer (FreeZone® 4.5L, Labconco, USA). The condenser temperature was set at -50 ºC, the drying chamber was set at -20 ºC, 0.15 mbar for 16 hrs and then -10 ºC for 6 hrs. The freeze-dried powder was collected and stored in PE bag with moisture absorber. Determine the amount of *L. brevis* NB10 (CFU/g) in these powders after drying ($\mathbf{Q_2}$). The survival rate ($\mathbf{S_1}$) of *L. brevis* NB10 after freeze-drying corresponding to a given protective medium was calculated as:

$$\mathbf{S_1 = 100 \times (Q_1 \times V_1)/ (Q_2 \times M_2)\ (\%)}$$

Wherein, $\mathbf{V_1}$ is the total volume of the sample before freeze-drying; $\mathbf{M_2}$ is the total weight of the sample after freeze-drying.

To choose the most suitable protective agents that providing the best stability of *S. brevis* during storage, the freeze-dried powders were stored at 5 ºC ± 3 ºC or at 25 ºC ± 2 ºC, RH 60%. After certain periods, examine for the change in appearance of these powder. Also, the amount of *L. brevis* NB10 (CFU/g) in these powders was determined ($\mathbf{Q_3}$), and the survival rate ($\mathbf{S_2}$) of *L. brevis* NB10 during storage was calculated as:

$$\mathbf{S_2 = 100 \times Q_3 / Q_2\ (\%)}$$

2.5. Formulation of chewing gum containing *L. brevis* NB10

The basic formulation of the chewing gum containing *L. brevis* NB10 was used as showing in Table 1. The chewing gum was prepared by direct compression method to prevent the exposure of bacteria to water, solvents or moisture during granulation as well as to reduce the preparation time thus increasing the survival rate of *L. brevis NB10* after formulation. Briefly, the freeze-dried powder of *L. brevis* NB10 was mixed with xylitol and strawberry flavor powder, while HIG04® was mixed with aerosil, and glycerol. Next, these mixtures were blended, then mixed with lubricants. The gum tablet was made using rotary tablet machine with 15 mm dice equipped (CIP - 8. STN. O. Lab Press, India). The compression was operated manually at the speed 7 rpm. Also, all the preparation process was taken place in 20 ºC and 50% RH room.

Table 1. Compositions of gum basic formulation

No.	Ingredients	Amount (%)	Aims
1	*L. brevis* NB10 freeze-dried powder (> 10^9 CFU/g)	10%	Provide alive *L. brevis* NB10 for minimum of 10^7 CFU/tablet
2	Aerosil	1 - 5%	Increase flowability, prevent of gum base aggregation during preparation
3	Xylitol	5 - 20%	Diluent, sweetener, help to reduce tooth decay
4	Strawberry flavor powder	0.5 - 3%	Flavor
5	Glycerol	0.5 - 2%	Softener, adjust the texture feel when chewing
6	Talc	0 - 1.5%	Lubricant, increase flowability, prevent the adhesion of gums into tablet press die and punches
7	Magnesium stearate	0 - 1.5%	Lubricant, increase flowability, prevent the adhesion of gums into tablet press die and punches
8	HIG04® powder	Adjust to total of 100%	Binder, gum base, adjust total mass of gum tablet to make chewable mass

To determine the appropriate amount of each composition, series of formulations based on the basic formulation were made in which individual excipient was changed at a time to examine the effect of its on the product properties. The gum tablet properties were evaluated by the following tests.

Tablet appearance and weight uniformity

Choose 20 random gum tablets from each batch and observe each one by the naked eye for shape, color, surface roughness, and appearance uniformity between tablets. Evaluate of weight variant according to the Vietnamese Pharmacopoeia V. No more than 2 units have the mass that are outside the limits of 95–105% of the average mass, and no tablet has the weight that is outside the limits of 90–110% of the average mass.

Viable bacterial count

The amount of *L. brevis* NB10 (CFU/g) before and after tablet compression was determined, and the survival rate (S3) of *L. brevis* NB10 was calculated as:

$$\mathbf{S_3 = 100 \times \frac{Q_5}{0{,}1 \times m_t \times Q_4}\ (\%)}$$

Wherein, $\mathbf{Q_4}$ is the amount of *L. brevis* NB10 (CFU/g) in the semi-finished powder before compression; $\mathbf{Q_5}$ is the amount of *L. brevis* NB10 (CFU/tablet) in the gum tablet right after compression; $\mathbf{m_t}$ is the weight of gum tablet.

For the content uniformity test, count the number of viable *L. brevis* NB10 of 10 random gum tablets from each batch. No more than one unit has the viable bacterial number that is outside the limit of 80 – 120% of average viable bacterial

number, and no unit has the viable bacterial number that is outside the limit of 60 – 140% of average viable bacterial number.

Evaluation of chewing gum organoleptic properties

Organoleptic properties of the gum tablet include chewable mass, sweetness, flavor (intense and lasting time), and softness were evaluated by 6 healthy volunteers. After signing informed consent form, each volunteer was asked to chew a gum tablet for 10 seconds, then spat out and give a score according to the product properties assessment scorecard (Table 2). The chewing gums used in these assessments were made from freeze-dried powder without *L. brevis* NB10.

Table 2. Score scale for organoleptic properties of chewing gum tablet

Properties	Score					Chewing time
	1	2	3	4	5	
Chewable mass	Too small	Small but chewable	Optimum to chew	Big, but chewable	Too big, hard to chew	10 seconds
Sweetness	No sweet or very little sweet	Little sweet	Good sweetness	Strong sweet	Very strong sweet	10 seconds
Flavor intense	No or very little odor	Weak odor	Good odor	Strong odor	-	10 seconds
Flavor lasting time	Keep chewing until no feeling of odor, record the time					
Softness	Too hard	Hard	Acceptable	Soft	Too soft	10 seconds

Bacterial stability in gum tablet

Gum tablets were kept in PE bags with moisture absorber and stored at 5 ºC ± 3 ºC. After periods of time, choose 10 random tablets to detect the number of viable *L. brevis* NB10 in each tablet.

3. RESULTS

3.1. Freeze drying of *L. brevis* NB10

Firstly, cryoprotectant agents were used individually to make pre-freeze-dried samples. The concentration of each agent used in pre-freeze-dried sample was 10%. The total volume of each pre-freeze-dried sample was 100 ml and the initial amount of bacteria was adjusted to approx. 10^9 CFU/ml. The appearance, moisture and the bacterial survival rate of each corresponding freeze-dried powder was shown (Table 3).

From this result, lactose, sorbitol and xylitol were not able to protect *L. brevis* NB10 during freeze-drying process. The bacterial survival rates in freeze-dried powder using these agents were lower than 10%. Also, these powders were not suitable to use in gum preparation due to rapid moisture absorbing as well as hard to grind into fine particle.

Table 3. Properties of *L. brevis* NB10 powders using single protectant

Cryo-protectant agent (10%)	Properties of Freeze-dried powder		
	Appearance	Moisture (%)	Survival rate (%)
Lactose (Lac)	Whitish powder with visible clumps, rapid moisture absorbing	3.97	0.05
Sorbitol (Sor)	Whitish powder with visible clumps, rapid moisture absorbing	4.85	3.3
Mannitol (Man)	Whitish powder, easy to grind into fine particles, low moisture absorbing	1.75	**24.98**
Xylitol (Xyl)	Whitish powder with visible clumps, rapid moisture absorbing	3.01	0.75
Maltodextrin (Mal)	Whitish powder, easy to grind into fine particles, low moisture absorbing	1.89	**22.87**
Skim milk (SM)	Yellowish powder with visible clumps, hard to grind into fine powder	2.40	**33.19**

Table 4. Properties of *L. brevis* NB10 powders using mixture of protectants at different mixing ratio

Cryo-protectant agent at 10% (w/v)	Mixing ratio (w:w)	Properties of Freeze-dried powder		
		Appearance	Moisture (%)	Survival rate (%)
Man: SM	**1:9**	Yellowish powder, visible clumps, rapid moisture absorbing	2.95	31.81
	3:7	Ivory powder, easy to grind into fine particles, low moisture absorbing	3.06	**53.49**
	5:5	Ivory powder, easy to grind into fine particles, rapid moisture absorbing	2.13	37.54
	7:3	Ivory powder, easy to grind into fine particles, rapid moisture absorbing	2.47	25.24
Mal: SM	**1:9**	Ivory powder, easy to grind into fine particles, rapid moisture absorbing	3.67	33.76
	3:7	White powder, easy to grind into fine particles, low moisture absorbing	2.46	**48.43**
	5:5	White powder, easy to grind into fine particles, low moisture absorbing	3.02	**61.21**
	7:3	White powder, easy to grind into fine particles, low moisture absorbing	2.85	28.15

Table 5. Properties of *L. brevis* NB10 powders using mixture of protectants at different use amount

Cryo-protectant agent (w:w)	Use amount (% w/v)	Properties of Freeze-dried powder		
		Appearance	Moisture (%)	Survival rate (%)
Man: SM (3:7)	**8**	Ivory powder, easy to grind into fine particles, low moisture absorbing	2.23	41.28
	10	Ivory powder, easy to grind into fine particles, low moisture absorbing	2.76	**55.15**
	12	Ivory powder, easy to grind into fine particles, low moisture absorbing	2.51	47.92
Mal: SM (3:7)	**8**	Ivory powder, easy to grind into fine particles, low moisture absorbing	3.03	39.41
	10	Ivory powder, easy to grind into fine particles, low moisture absorbing	2.70	**51.34**
	12	Ivory powder, easy to grind into fine particles, low moisture absorbing	2.79	42.06
Mal: SM (5:5)	**8**	White powder, easy to grind into fine particles, low moisture absorbing	3.12	53.88
	10	White powder, easy to grind into fine particles, low moisture absorbing	2.86	57.66
	12	White powder, easy to grind into fine particles, low moisture absorbing	2.37	**65.69**

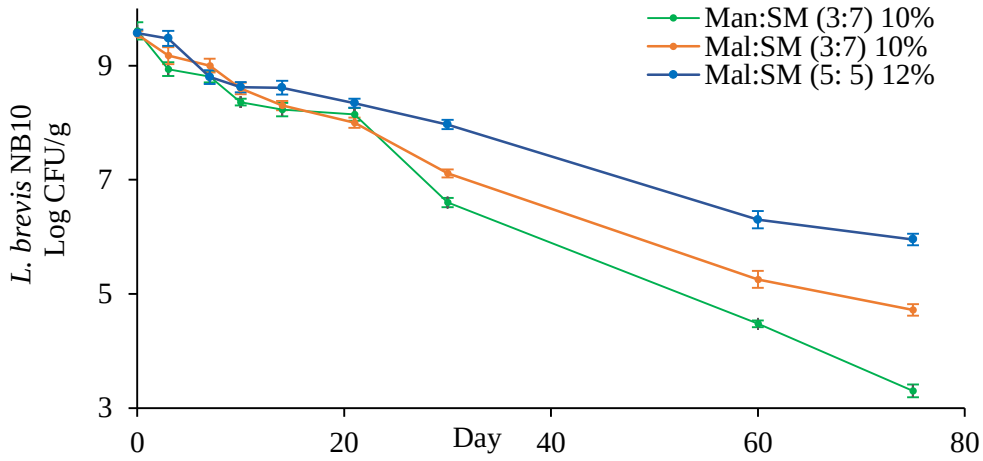

Figure 1. Viable number of *L. brevis* NB10 in freeze-dried powders at room temperature over 75 days

Table 6. Properties of *L. brevis* NB10 powders using 12% of Mal:SM (5:5) supplied with different amount of potassium ascorbate

Cryo-protectant agent (w:w)	Potassium Ascorbate % (w/v)	Properties of Freeze-dried powder			
		Appearance	Moisture (%)	Survival rate (%)	RSD (%) n = 3
Mal:SM (5:5) 12% (w/v)	0	Whitish powder, easy to grind into fine particles, low moisture absorbing	2.08	62.51	3.29%
	0.25	Whitish powder, easy to grind into fine particles, low moisture absorbing	2.16	68.15	2.61%
	0.5	Yellowish powder, easy to grind into fine particles, low moisture absorbing	2.87	70.92	4.48%
	1.0	Brownish powder with visible clumps, hard to grind into fine powder,	2.71	63.39	5.93%

Mannitol, maltodextrin, and skim milk showed promising ability in preserving *L. brevis* NB10 during freeze-drying process with bacterial survival rates higher than 20%. Hence, these agents were chosen and use in the mixture to formulate the protective medium for freeze-drying of *L. brevis* NB10 (Table 4).

According to the results, the combination of mannitol or maltodextrin with skim milk improved the survival rate of *L. brevis NB10* after the freeze-drying process, wherein three combinations showing the highest bacteria protection were Man: SM (3:7); Mal: SM (3:7) and Mal: SM (5:5) with the bacterial survival rate of 53.49%; 48.43% and 62.21%, respectively. Hence, these three combinations were selected to adjust the use amount to further improve bacterial survival (Table 5).

Of all the combinations, the three showing the best *L. brevis* NB10 protection ability were Man:SM (3:7) at 10%, Mal:SM (3:7) at 10%, and Mal:SM (5:5) at 12%. Next, the stability of *L. brevis* NB10 in these three freeze-dried when stored at room temperature was examined. The amount of live *L. brevis* NB10 in these powders during 75 days of storage were shown (Figure 1)

The amount of live *L. brevis* is gradually decreased in all tested powders during storage time, wherein, the combination of Mal:SM (5:5) at 12% (w/v) provided the highest bacterial stability. Hence, the combination of Mal:SM (5:5) at 12%

(w/v) was chosen to prepare *L. brevis* NB10 freeze-dried powder. Next, potassium ascorbate was added to the freeze-dried formulation to increase *L. brevis* NB10 stability over time. The properties of these freeze-dried powders were shown in Table 6.

The addition of 0.25% - 0.5% of potassium ascorbate slightly increased the survival rate of *L. brevis* NB10 during the freeze-drying process ($p < 0.05$). However, the higher the amount of potassium ascorbate was, the darker the color of freeze-dried powders was. Also, when adding 1% of potassium ascorbate to the formulation, the freeze-dried product could not be ground into a fine powder which is not suitable to use as material in gum formulation. Hence, the freeze-dried formulations containing 0.25% and 0.5% of potassium ascorbate were selected to study the bacterial stability at room temperature (Figure 2).

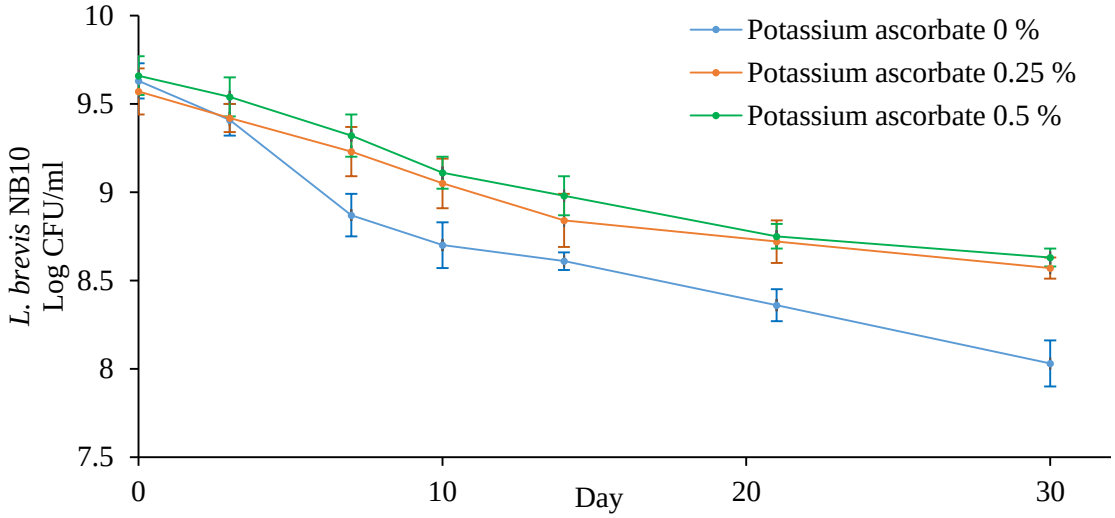

Figure 2. Viable number of *L. brevis* NB10 in freeze-dried powders supplied with potassium ascorbate at room temperature over 30 days

Potassium ascorbate showed a positive effect on the *L. brevis* NB10 survival in 30 days. However, there was no significant difference in the protective effect of potassium ascorbate at the concentrations of 0.25% or 0.5%. Thus, the optimal formulation for preparing freeze-dried *L. brevis* NB10 consisted of 6% maltodextrin, 6% skim milk, and 0.25% potassium ascorbate (Table 7).

Table 7. Optimum formulation of *L. brevis* NB10 freeze-dried powder

No.	Composition	Amount
1	*L. brevis* NB10	1 x 10^{12} CFU
2	Sodium chloride	3.15 g
3	Potassium ascorbate	2.5 g
4	Maltodextrin	60.0 g
5	Skim milk	60.0 g
6	Water	To 1000 ml

To evaluate the consistence of freeze-drying formulation and preparation process, 3 lots of *L. brevis* NB10 freeze-dried powders were made. The total volume of each pre-freeze-dried sample from each lot was 500 ml. The properties of freeze-dried powders from these three lots were shown in Table 8.

There was no significant difference in the appearance, moisture content, and bacterial survival rate between the three studied lots. Therefore, the formulation and preparation process of *L. brevis* NB10 powder was consistent.

The number of viable *L. brevis* NB10 decreased over time when stored at 5 ºC ± 3 ºC (Figure 3). However, after 6 months of storage, the number of live bacteria in all 3 lots remained higher than 10^9 CFU/g. Also, there were no significant differences in the bacterial stability between the three studied lots ($p < 0.05$). Therefore, *L. brevis* NB10 freeze-dried powders were stable at 5 ºC ± 3 ºC for at least 6 months.

3.2. Formulation of chewing gum containing *L. brevis* NB10

From the basic formulation, a series of formulations (F1 – F20) were made in which individual excipient was changed at a time to examine the effect of its on the product properties (Table 9). The formulations F1 – F4 were used to optimize chewable gum mass; F5 – F9 for selection of suitable sweetness; F10 – F13 for adjusting gum flavor intense and lasting time; F14 – F17 for modifying gum softness; and F18 – F20 for prevention of the adhesion of the gum tablet into die and punches. The number of gum tablets prepared from each formulation was 30.

3.2.1. Optimization of chewable gum mass

To obtain optimal gum mass for chewing, four formulations (F1-F4) were made and evaluated by healthy human volunteers (n = 6). The number of viable *L. brevis* NB10 and chewing feel scores from response of human testers were shown in Table 10.

The average number of *L. brevis* per tablet in all formulations was higher than 10^7 CFU. The formulation F3 (1800 mg) was rated as the most suitable size for chewing. Hence, the total mass of gum tablets prepared for subsequent experiments was fixed as 1800 mg.

3.2.2. Adjusting gum sweetness

The freeze-dried *L. brevis* NB10 powder has unpleasant taste, thus addition of sweetener is necessary to achieve better taste and enhance attraction to consumers. Xylitol was selected as sweetener in this study because of its anticariogenic ability and safeness [18], [19], [20].

The increase of xylitol concentration in gum formulations (F5 – F9) enhanced the sweetness of the gum tablets while not affected the viability of *L. brevis* NB10 (Table 11). The formulation F7 showed the most appropiate sweetness (according to 5/6 testers). Hence, the concetration of xylitol used in gum formulation was selected as 10% (w/w) (180 mg/1800 mg of gum tablet).

Table 8. Properties of 3 Lots of *L. brevis* NB10 freeze-dried powders

Lot	Properties of Freeze-dried powder		
	Appearance	**Moisture (%)**	**Survival rate (%)**
Lot P1.11.5.21	Whitish powder, easy to grind into fine particles, low moisture absorbing	1.38	75.3
Lot P2.14.5.21	Whitish powder, easy to grind into fine particles, low moisture absorbing	1.37	74.9
Lot P3.17.5.21	Whitish powder, easy to grind into fine particles, low moisture absorbing	1.45	76.3
Average survival rate			**75.5 ± 0.9**

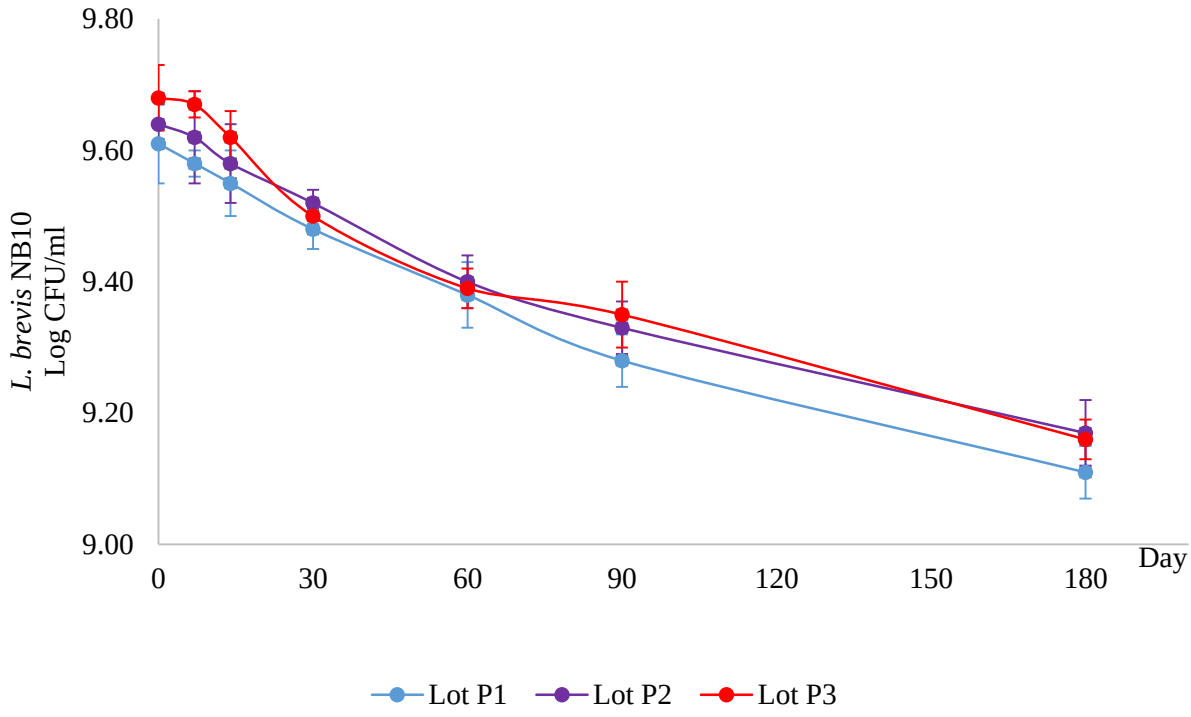

Figure 3. Viable number of *L. brevis* NB10 in 3 lots of freeze-dried powder at 5 ºC ± 3 ºC over 180 days

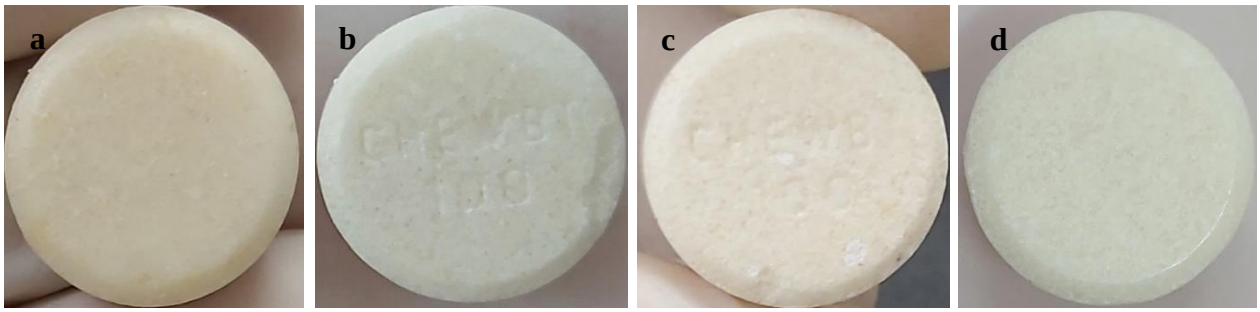

Figure 4. Appearance of gum tablets from the formulation F17 – F20. (a) Gum tablet from F17 tend to stick into punches and die; (b) Gum tablet from F18 showed flaw and crack in the edge; (c) Gum tablet from F19 show ununiform surface with white spots; (d) Gum tablet from F20 showed the best appearance

Table 9. Formulations of chewing gum prepared to select the content of excipients

Ingredients	Amount (mg)								
	F1	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**	**F9**
Freeze-dried *L. brevis* NB10 (*)	100	150	180	200	180	180	180	180	180
Xylitol	100	150	180	200	45	90	180	270	360
Strawberry powder	-	-	-	-	-	-	-	-	-
Glycerol	-	-	-	-	-	-	-	-	-
Talc	-	-	-	-	-	-	-	-	-
Magnesium stearate	-	-	-	-	-	-	-	-	-
Aerosil	24	36	45	48	45	45	45	45	45
HIG04® powder	776	1164	1395	1552	1530	1485	1395	1305	1215
Total weight	1000	1500	1800	2000	1800	1800	1800	1800	1800
Aims	Optimize chewable mass				Adjust gum sweetness				

Ingredients	Amount (mg)										
	F10	**F11**	**F12**	**F13**	**F14**	**F15**	**F16**	**F17**	**F18**	**F19**	**F20**
Freeze-dried *L. brevis* NB10 (*)	180	180	180	180	180	180	180	180	180	180	180
Xylitol	180	180	180	180	180	180	180	180	180	180	180
Strawberry powder	9	18	36	54	36	36	36	36	36	36	36
Glycerol	-	-	-	-	9	18	27	36	27	27	27
Talc	-	-	-	-	-	-	-	-	54	0	27
Magnesium stearate	-	-	-	-	-	-	-	-	0	54	27
Aerosil	45	45	45	45	45	45	45	45	45	45	45
HIG04® powder	1386	1377	1359	1341	1350	1341	1332	1323	1278	1278	1278
Total weight	1800	1800	1800	1800	1800	1800	1800	1800	1800	1800	1800
Aims	Adjust gum flavor				Adjust gum softness				Reduce stickiness		

(*) Chewing gums used in the human evaluation test were made from placebo freeze-dried powder which did not contain L. brevis NB10

Table 10. Chewing gum tablet properties and chewing mass feedback from volunteer

Formulation	Average gum mass (g)	Average viable number of *L. brevis* NB10 (Log CFU/tablet)	Volunteer score feedback					
			A	**B**	**C**	**D**	**E**	**F**
F1	1,00 ± 0,02	7,01 ± 0,04	1	2	1	1	1	2
F2	1,51 ± 0,12	7,84 ± 0,07	2	3	2	2	2	3
F3	**1,81 ± 0,07**	8,11 ± 0,15	**3**	**3**	**3**	**3**	**3**	**3**
F4	1,98 ± 0,06	8,18 ± 0,06	4	4	3	4	4	4

3.2.3. Adjusting gum flavor intense and lasting time

Like sweeteners, flavors are important ingredients in gum formulation which provide product attractive scent. Spearmint and peppermint are among the most used flavor in chewing gum [21]. However, these mints contain essential oil components which can exert antibacterial effect, and hence, can affect the viability of *L. brevis* NB10 in the formulation. Thus, flavor from fruits, i.e., strawberry flavor powder in this study was chosen to adjust gum flavor.

From the results shown in table 10, strawberry flavor did not cause negative effects on *L. brevis* NB10 during formulation. The gum flavor become more intense and longer-lasting when increasing the concentration of strawberry flavor, wherein, the formulations F12 and F13 showed good lasting flavor (> 5 mins) (Table 12). However, the formulation F12 had the most acceptable flavor as voted by 6/6 tester. Hence, the concentration of strawberry flavor used in gum formulation was set as 2% (w/w) (36 mg/ 180 mg of gum tablet)

3.2.4. Modifying gum softness

Chewing gums should have the proper softness to confer a comfortable chewing feel. If the gum texture physically becomes too rigid or too soft, it will make the chewing feel less joy and reduce the consumer interest in the product. Glycerol is used widely in chewing gum formulation as moisture-retaining and flexibility enhancing [22]. Also, glycerol has no data for its cariogenic effect.

The gum tablet texture from the formulation F14 - F17 became softer when the glycerol concentration increased, wherein the gum tablet made from F16 showed the most appropriate softness for chewing (rated by 5/6 testers) (Table 11). Therefore, the concentration of glycerol used in the formulation was fixed as 1.5 % (w/w) (27 mg/ 180 mg of gum tablet).

Table 11. Chewing gum tablet properties and organoleptic properties feedback from volunteers

Formulation	Average viable number of *L. brevis* NB10 (Log CFU/tablet)	Volumteer score feedback						Aims
		A	B	C	D	E	F	
F5	8,12 ± 0,02	1	1	1	2	1	1	Adjusting sweetness
F6	8,11 ± 0,03	2	2	2	3	2	2	
F7	8,13 ± 0,06	**3**	**3**	**3**	**4**	**3**	**3**	
F8	8,09 ± 0,07	4	4	4	5	4	4	
F9	8,15 ± 0,06	4	5	4	5	5	5	
F10	8,15 ± 0,06	1	1	1	1	1	1	Adjusting gum flavor intense and lasting time
F11	8,10 ± 0,03	2	2	2	2	2	2	
F12	8,08 ± 0,04	**3**	**3**	**3**	**3**	**3**	**3**	
F13	8,13 ± 0,04	4	4	4	4	4	4	
F14	8,13 ± 0,06	1	1	1	1	1	2	Adjusting gum softness
F15	8,10 ± 0,05	2	2	2	2	3	3	
F16	8,09 ± 0,06	**3**	**3**	**3**	**3**	**3**	**4**	
F17	8,12 ± 0,05	4	4	4	4	4	5	

Table 12. Flavor lasting time recorded from F10 – F13

	Formulation			
	F10	**F11**	**F12**	**F13**
Flavor lasting time (second)	61.5 ± 10.5	178.8 ± 10.3	329.5 ± 31.8	389 ± 16.9

Table 13. Flowability and properties of gum powder and gum tablets from F18-F20

Properties	Formulation		
	F18	**F19**	**F20**
Angle of repose	32.14 ± 0.22	33.22 ± 0.41	31.26 ± 0.23
Hausner ratio	1.19 ± 0.00	1.13 ± 0.01	1.13 ± 0.00
Compressibility index (%)	18.85 ± 0.19	11.63 ± 0.76	11.29 ± 0.18
Moisture (%)	2.31	2.36	2.44
Gum tablet's appearance	Gum tablets didn't stick to die and punches. The gum tablet surface was lightly cracked	Gum tablets didn't stick to die and punches. The gum tablet surface had some white spots	Gum tablets didn't stick to die and punches. The gum surface was uniform and flawless.
Average viable *L. brevis* NB10 (CFU/tablet)	8.10 ± 0.05	8.15 ± 0.06	8.11 ± 0.04

3.2.5. Optimization of anti-adhesion agents

Through a series of screening experiments for sweetener, flavor, and softener, the *L. brevis* NB10 chewing gum formulation consisted of 10% *L. brevis* NB10 freeze-dried powder, 10% xylitol, 2% strawberry flavor powder, 1.5% glycerol. Flow properties of the gum powder (before compression) from this formulation showed good flowability. The angle of repose was 33.66 ± 0.38 (≤ 35 for good flowability) and the Hausner ratio was 1.14 ± 0,00 (≤ 1.18 for good flowability). Also, the compressibility index of the gum powder was 12.57 ± 0.13 which is considered good compressibility and suitable for the direct compression method. However, gum tablets obtained from this formulation were shown to adhere to die and punches of tablet compression machine. Hence, lubricants such as talc, magnesium stearate were added to overcome this issue (F18 – F20). The properties of gum powders and gum tablets from these formulations were shown (Table 13).

The addition of talc and magnesium stearate into gum formulations did not cause harmful effects on *L. brevis* NB10 viability during gum preparation. Also, these lubricants did reduce the sticking of tablets to die and punches and made them easier to eject from the die. However, the use of 3% talc (w/w) in the formulation F18 may reduce the adhesion of the gum powder particles, which in turn, make the gum tablet surface become cracking after compression. Meanwhile, in the formulation F19, magnesium stearate at 3% (w/w) was not completely dispersed throughout the gum powder mixture,

thus, the gum tablet surface from F19 showed some white spots and lack of uniformity. In the formulation F20, when adding 3% (w/w) of talc: magnesium stearate mixture (1:1), the gum tablets were perfectly made without cracking and flaw on the surface. Therefore, formulation F20 was chosen as the final formulation of *L. brevis* NB10 chewing gum.

To evaluate the consistence of gum tablet formulation and compression process, 3 lots of *L. brevis* NB10 chewing gum were made. The number of tablets made for each lot was 100 tablets. The number of viable *L. brevis* NB10 in gum powders and tablets from each lot showed no significant difference (Table 14).

Next, the bacterial stability in gum tablets from these three lots at 5 ºC ± 3 ºC were studied. The average numbers of live *L. brevis* NB10 in the gum tablets in 90 days of storage were shown below (Figure 5).

Table 14. Chewing gum tablet properties from three lots of optimized formulation

Properties		Lot		
		Lot G1.21.10.21	Lot G2.22.10.21	Lot G3.23.10.21
Viable *L. brevis* NB10	*L. brevis* NB10 freeze-dried powder (Log CFU/g)	9.43	9.39	9.33
	Gum powder (Log CFU/g)	8.37	8.33	8.31
	Gum tablet (Log CFU/tablet)	8.16 ± 0,06	8.13 ± 0,05	8.10 ± 0,05
	Survival rate after tablet compression (%)	34.10	34.89	34.02
Weight uniformity	Average gum weight (g)	1.82 ± 0.01	1.82 ± 0.01	1.81 ± 0.01
	Range of acceptance (g)	1.73 – 1.91	1.73 – 1.91	1.73 – 1.91

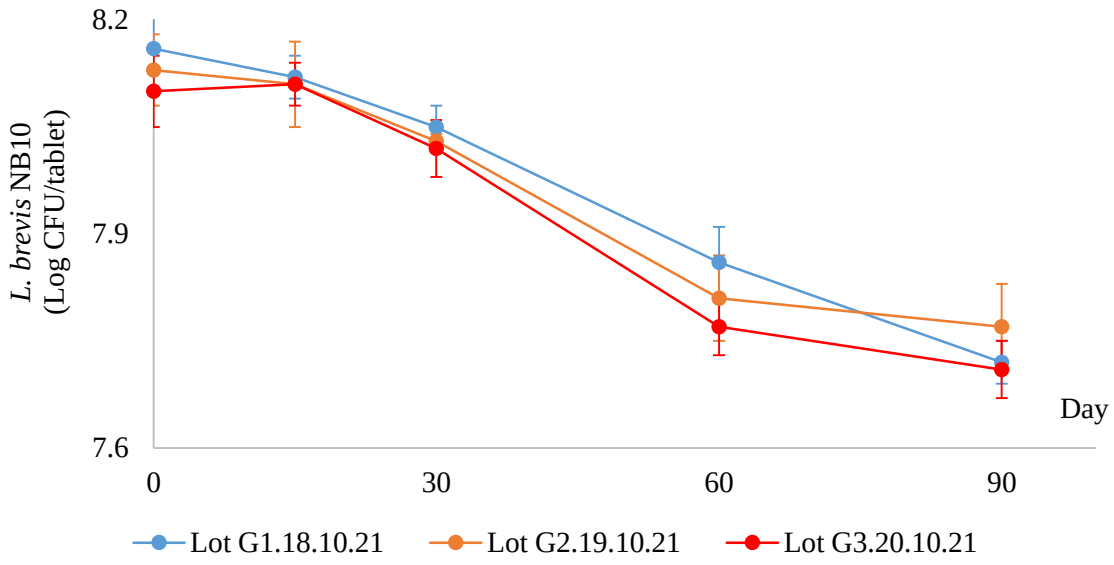

Figure 5. The average viable number of *L. brevis* NB10 in tablets from 3 lots of chewing gum over 90 days

In all chewing gum lots, the number of live *L. brevis* NB10 in gum tablets was decreased over time but remained higher than 10^7 CFU/tablet in 3 months of storage. Therefore, the chewing gum product was shown to be stable at 5 ºC ± 3 ºC for at least 3 months.

4. DISCUSSION

4.1. Freeze-drying of *L. brevis* NB10

Freeze-drying (as known as lyophilization) is used widely as a preservative method for various kinds of pharmaceutical and biological products such as antibiotics, vaccines, recombinant proteins, biologics, and probiotics. Freeze-drying takes place in 3 stages: (1) freezing – the product is frozen at ultra-low temperate so as all the water (or solvent) in the formulation becomes ice (crystallization); (2) primary drying – most of the water content in the product as ice crystals is removed through sublimation process at low pressure; (3) secondary drying – the remaining water is further removed from the product by desorption [23]. During the freeze-drying process, microorganisms are suffered from different stresses such as cold shock, osmotic stress (due to the concentration of solutes), mechanical forces such as lethal damage from ice crystals. Also, the storage and the rehydration conditions of the products also strongly affect the viability of bacteria [23 - 25].

Methods used to improve bacterial survival rate in the freeze-dried product include (1) adjustment of culture condition and pre-stressing strategies to alter the composition of bacterial cell wall and membrane which help to increase the

tolerance of bacteria to cold and water loss effect; (2) formulation of suitable protective media which help to reduce the negative effect of mechanical and osmotic stresses during freezing stage (cryoprotectants) or during drying stages (lyoprotectants); (3) optimization of freeze-drying process parameters such as freezing temperature and cooling rate (which results in different size and amount of ice crystal) or the temperate and pressure at which the drying stages take place (which affect the time needed to complete the process). While the screening of culture conditions is time-consuming and cannot replace the need for the two latter strategies, the optimization of lyophilization parameters requires sophisticated equipment and depends on the protective formulation used. Optimization of the freeze-drying formulation is often the method of choice [23 - 25].

Various types of substances were studied as protective agents for preserving probiotics during the freeze-drying process. However, their mode of action was not yet fully understood. Low molecular sugars (glucose, lactose, trehalose…), polyols (sorbitol, mannitol, xylitol…), or amino acid (lysine, glycine, glutamine…) can act as water substitutions to bind to bacterial phospholipids and proteins, thus, reducing the damage to these structures during freezing, dehydration, or rehydration process. Large molecular protectants such as polysaccharides (maltodextrin, inulin, FOS, GOS…) or skim milk tend to accumulate outside of the cells and enlarge the unfrozen fractions during the freezing stage, thus, reducing the harmful effects of mechanical and osmotic stresses. Also, most protective agents can lower the nucleation temperature. Therefore, they can reduce the size of ice crystals and their damage to the cells. However, there is no such universal formulation of protectants that is suitable for all bacteria. The effects of protective agents are different for each strain [24, 25].

To prepare a freeze-dried powder of *L. brevis* NB10 for use in chewing gum, protective agents that exhibit low or no cariogenic activities were selected [25]. As such, mannitol, xylitol, sorbitol, lactose, maltodextrin, and skim milk were screened to design the freeze-dried formulation that provided the highest *L. brevis* NB10 viability and stability. The results showed that the combination of protectants showed a better protective effect on *L. brevis* NB10 than each protectant when used alone, wherein, the mixture of maltodextrin 6% and skim milk 6% showing the best performance. The augmented effect when using mixture of protectants was also observed from different studies (Table 15). Also, the suitable protectants and their use amount are varied for each strain.

Table 15. Optimum freeze-drying media for preservation of Lactobacillus strains

Lactobacilli	Optimal freeze-drying formulation	% survival	Ref
L. acidophilus (NCDC 291)		69.86	[27]
L. acidophilus (NCDC 015)	1.2% Sucrose + 6% skim milk	68.84	
L. acidophilus (MTCC 10307)		67.92	
L. lactis Sr. 3.54	10% (Sucrose + skim milk)	62.0	[28]
L. paracasei subsp. *tolerance* (DSM 20258)	6% Skim milk + 8% Trehalose + 4% sodium ascorbate	82.0	[29]
L. delbrueckii subsp. *bulgaricus* (DSM 20081)		74.0	
L. plantarum JH287	10% Skim milk + 10% Sucrose	77.15	[30]
L. brevis NB10	6% maltodextrin + 6% skim milk + 0.25% potassium ascorbate	75.5	This study

4.2. Formulation of *L. brevis* NB10 chewing gum

As a probiotic containing chewing gum, the exposure of freeze-fried bacteria to water, solvent, high temperature, oxygen… during the preparation process affect greatly on its survival in the product tablets. Thus, the direct compression method using gum base was chosen to reduce the preparation time and get rid of harmful events. Other excipients were examined one by one for the selection of suitable amounts and suspected of any negative effect on *L. brevis* NB10 viability.

Though not showing the protective effect on *L. brevis* NB10 during the freeze-drying process in this study, xylitol is a common sweetener in chewing gum formulation. Due to its anti-cariogenic effect, the combination of xylitol and *L. brevis* NB10 may show the synergic effect on the prevention of tooth decay caused by *Streptococcus mutans*.

In this study, healthy human volunteers (n = 6) were recruited to evaluate some organoleptic properties of chewing gum tablets such as chewability mass, sweetness, flavor lasting time, and softness to optimize the amount of each excipient. All chewing gum tablets used in human evaluation tests were prepared using placebo freeze-dried powder which was not contained live *L. brevis* NB10. The limitation of the current study was the low number of human participants. Therefore, the organoleptic evaluation recorded by this study might not represent the feedback of larger group of people. However, all the excipients used in the chewing gum formulation did not affect the viability of *L. brevis* NB10 during the preparation process. Thus, if using a larger number of human testers to further optimize chewing gum formulation, one can easily re-establish the amount of each excipient without affecting the survival rate of *L. brevis* NB10 as achieved in this study.

Conclusion

This study was successful in formulating chewing gum containing at least 10^7 CFU/tablet of *L. brevis* NB10. The prepared chewing gums were stable for at least 3 months at 5 ^oC ± 3 ^oC. Further studies could be conducted to improve bacterial stability such as anti-moisture film coating or optimization of the preparation process.

FUNDING

The authors received no financial support for the research, authorship, and/or publication of this article.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ACKNOWLEDGEMENTS

We acknowledge the Pham Ngoc Thach University of Medicine for supporting us with some critical equipment to complete this study.

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