

Development of Milk Protein Edible Films Incorporated with *Lactobacillus rhamnosus* GG

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Bioactive edible films have the potential to be probiotic carriers. This innovative approach can replace plastic packaging and can benefit human health. This study demonstrated the incorporation of *Lactobacillus rhamnosus* GG (LGG) into whey protein isolate (WPI) and sodium caseinate (NaCas) edible films. Probiotic cells were directly incorporated into the film forming solutions, and the films were produced by the casting method. The physical, mechanical, and probiotic viability properties of the edible films were determined in the presence and absence of LGG. Furthermore, the viability of LGG was evaluated during the drying process and storage of 14 days at 4 °C and 25 °C, respectively. The results showed the incorporation of LGG increased the moisture content, puncture force, and lightness of both films. However, viability of LGG was lower in the WPI film regardless of storage temperature. At the end of storage days, both WPI and NaCas edible films maintained the LGG viability above the recommended levels when stored at 4 °C, which was 10⁶ CFU/g. The findings of this study suggested that edible films made of WPI and NaCas showed feasibility to immobilize LGG with chilled storage at 4 °C.

Keywords: Edible film; Whey protein isolate; Sodium caseinate; Probiotics; Bioactive packaging

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INTRODUCTION

In recent decades, probiotic research has gained considerable interest, as it exerts a broad spectrum of effects on human health. Probiotics are beneficial for the immune system modulation and intestinal related diseases, such as lactose intolerance, intestinal invasion by pathogenic bacterial species, and colon cancer (Kerry *et al.* 2018; La Fata *et al.* 2018). However, a preservation strategy is highly needed to retain the probiotic viability in final products. This is to ensure that the beneficial effects are successfully delivered to the human intestinal tract. Immobilization of probiotics in edible films is an amicable way to maintain the probiotic stability and efficiency (Altamirano-Fortoul *et al.* 2012).

An edible film is a thin layer of packaging material used for wrapping food, and it forms a protective barrier against the environment. It can maintain the quality and prolong the shelf life of food. The primary advantage of edible film is its biodegradability. Currently, a majority of food packaging is derived from plastic, which consequently results in deterioration of the environment (Salgado *et al.* 2015). Furthermore, tons of plastic trash also contribute to marine pollution, which disrupt marine life considerably (Webb *et al.* 2012). Currently, consumer demands are changing as they are more aware of the presences,

roles, and implications of food packaging. For this reason, bio-active packaging has been achieved and developed.

Antimicrobial agents including essential oils, herbs, spices, and probiotics are able to inhibit the growth of food spoilage microorganisms (Han 2014; Zaman *et al.* 2018; Choong *et al.* 2019; Rawdkuen 2019). The incorporation of an antimicrobial agent into film-forming formulations contributes to higher antimicrobial properties than direct addition of antimicrobial agent into the food (Kristo *et al.* 2008). The incorporation of *Lactobacillus* (L.) *plantarum* was found to improve the antibacterial properties of polysaccharide film against *Listeria innocua* (Sánchez-González *et al.* 2013). In addition, a protein film loaded with *L. acidophilus* and *L. paracasei* displayed higher antimicrobial activity than the control film (Abdollahzadeh *et al.* 2018).

Probiotic edible films are a type of practical food, in which bioactive agents are designed to be contained in the film. Bioactive packaging is different from the edible film itself, as it is a new packaging technology that has a direct impact on consumer health (Han 2014). The components of film-forming materials must be in accordance with the natural and bioactive agents acceptable in foods, thus maximizing the function of beneficial agents (Benbettaieb *et al.* 2017). For instance, protein-based films are suitable carriers for functional compounds, which can be applied in wide range of food products due to their variety of chain to chain interaction that contributes to good mechanical and barrier properties (Schmid and Muller 2019). Moreover, the edible films are made up of milk protein components that can hold and maintain the viable rate of probiotics for a long storage time (Pereira *et al.* 2016; Sánchez-González *et al.* 2013). Whey protein isolate (WPI) has extensive film-forming ability and good gas barrier property in low humidity conditions (Kurek *et al.* 2014). Sodium caseinate (NaCas) can be produced by adding sodium hydroxide into casein through neutralization. The NaCas is colourless and flavourless and it contributes to excellent nutritional value and shows excellent water-holding property (Sánchez-González *et al.* 2013).

Lactobacillus rhamnosus GG, ATCC 53103 (LGG) is highly studied due to its various beneficial effects such as alleviating rotavirus diarrhea and dental caries in children (Segers and Lebeer 2014). Besides, the anti-inflammatory effects possessed by LGG aids in maintaining regulation of immune response to infection which can slow down tumor growth and reduce the risk of colon cancer (Khailova *et al.* 2013). The LGG can extensively adhere to intestinal mucosa and produce antimicrobial substances because of its resistance to bile and low pH. In addition, it contains bacteriocin, which is active against anaerobic bacteria, such as *Clostridium*, *Bifidobacterium*, *Escherichia coli*, *Pseudomonas*, *Staphylococcus*, and *Salmonella* (Floch *et al.* 2017).

Although LGG is well known for its health beneficial properties, the information of LGG incorporated into edible film has not been extensively investigated. Hence, this study comprises the preparation of edible films based on WPI and NaCas with the addition of LGG. The relative physical, mechanical, and antimicrobial properties of the two edible films prepared were analyzed and compared. The viability of LGG in edible films was investigated through 14 days of storage. In addition, the effect of storage temperature and suitability of substrate on the viability of LGG in films was also examined.

EXPERIMENTAL

Materials

Lactobacillus rhamnosus GG (LGG) strain was purchased in capsule form from AA Pharmacy (Taman Connaught, Cheras, Malaysia). Other chemicals and materials used in this study were food grade whey protein isolate (Synertec, Kuala Lumpur, Malaysia), sodium caseinate (Synertec, Kuala Lumpur, Malaysia), glycerol (99.5%, Friendemann Schmidt, Parkwood, Australia), De Man, Rogosa and Sharpe (MRS) broth (Oxoid, Cheshire, UK), MRS agar (Oxoid, Cheshire, UK), disodium hydrogen phosphate (99%, Friendemann Schmidt, Parkwood, Australia), potassium dihydrogen phosphate (>99%, Bendosen, Hamburg, Germany), sodium chloride (99.5%, R&M Chemicals, Essex, England), Mueller-hinton agar (Oxoid, Cheshire, UK), and nutrient broth (Oxoid, Cheshire, UK).

Methods

Probiotic cells preparation

The LGG was cultured according to the procedure of Cheng (2015) with slight modifications. One capsule of commercial probiotic cells was inoculated in 100 mL of prepared MRS broth and cultured overnight at 37 °C in an incubator under anaerobic condition (INB 500, Memmert, Schwabach, Germany). Cell suspensions were then transferred into sterile centrifuge tubes and centrifuged (Centrifuge 5804 R, Eppendorf, Hamburg, Germany) at 4200 rpm for 10 min. Phosphate buffer saline (PBS) solution was used to wash the harvested probiotic cells to obtain a target concentration of 10^8 to 10^9 CFU/g. The procedure was continued by resuspension of LGG in 25 mL of PBS solution and stored in a refrigerator until further use.

Probiotic edible films preparation

The formation of WPI film was completed according to methods described by Gouna *et al.* (2007), with slight modifications. The pH of film forming solutions in this study was controlled at pH 7, as studies reported neutral pH resulted in improved film strength (Limpan *et al.* 2010). The film-forming solution was prepared by dissolving 4.5 g of WPI powder in 100 mL of distilled water. Then, the solution was heated to 90 ± 2 °C for 30 min, under constant stirring. The solution was then cooled to room temperature, and 2.0% (w/v) glycerol was added as plasticizer. The development of NaCas film was prepared using the method reported by Sánchez-González *et al.* (2014), with slight modification. The NaCas film-forming solution was prepared by dissolving 2.0 g of NaCas powder in 100 mL distilled water and stirred for 1.5 h at room temperature. After dispersion, 0.25% (w/v) of glycerol was added.

An aliquot of 1% (v/v) of the LGG suspension was incorporated into the WPI and NaCas film-forming solution. The procedure was repeated for the control samples without the addition of LGG. Lastly, approximately 30 mL of film-forming solution was casted onto a 100 mm × 15 mm petri dish and dried at 40 °C for 20 h in a fan oven (1350 FX, SHEL LAB, Cornelius, OR, USA). The dried edible films were peeled off from the petri dish and stored in desiccators for at least 48 h to reach equilibrium prior to film analysis.

Viability of LGG in film-forming solution and dried film

The microbiology analysis of LGG was performed during the drying process and the storage of the film. The procedure used was as described by De Lacey *et al.* (2012) with

minor modification. The suspension was produced by mixing 0.5 g of the peeled probiotic film in 4.5 g of sterile PBS. Then, the mixture was vortexed (VTX-3000 L, LMS, Tokyo, Japan) for 30 s and left to hydrate under constant agitation in an orbital incubator (KS 4000 I Control, IKA, Staufen, Germany) at 37 °C for 1 h. Then, the obtained suspension was diluted and plated on MRS agar. The agar plates were incubated in an oven at 37 °C for 48 h to allow colonies to grow. During the film storage, the bacterial colonies formed were counted at day 0, 4, 7, 11, and 14, accordingly.

Thickness

Thickness of the edible films was measured using an electronic micrometer screw gauge (3109A, Insize, São Paulo, Brazil). Measurements were taken at five different locations of each film, and the average thickness was calculated (Chan *et al.* 2020).

Moisture content

The moisture content of edible films was determined by using the oven-drying method (Hashemi and Khaneghah 2017). The initial weight of each film was measured using an electronic weighing balance (XT 220A, Precisa, Dietikon, Switzerland). The films were then dried at 60 °C for 24 h (until the weight is constant after drying). The final weight of the dried edible films was measured and recorded. The moisture content of the edible films was obtained using Eq. 1,

$$\text{Moisture content (\%)} = [(W_0 - W_1) / (W_0)] \times 100 \quad (1)$$

where W_0 is the initial weight (g) and W_1 is the final weight (g).

Color

The color of the edible films was analyzed using a colorimeter (ColorFlex EZ, Hunter Associates Laboratory, Reston, VA, USA) according to the method described by Kuan *et al.* (2020). The color of edible films was generated in terms of L^* , a^* , and b^* . The L^* is the lightness component (where $L^* = 0$ indicates black and $L^* = 100$ yields diffuse white), while a^* (positive values = red and negative values = green) and b^* (positive values = yellow; and negative values = blues) are the green-red and blue-yellow components, respectively.

Mechanical properties

Tensile strength, elongation at break, and Young's modulus were measured using a tensile testing machine (LF1096, LLOYD Instruments, Bognor Regis, Ametek, England), in accordance with ASTM D882-00 (2001) standard method, as described by Remya *et al.* (2016), with slight modifications. Prior to the tests, the films were cut into dimensions of 60 mm length and 10 mm width using scissors and preconditioned for 48 h at 25 °C. The films were clamped parallel between grips with an initial separation of 40 mm, and the cross-head speed was set at 20 mm/min. Measurement was taken in three replicates for each film formulation and the average values of each sample were reported. Peak load (N) and peak extension (mm) readings were directly obtained from the screen of the machine. Tensile strength, elongation at break, and Young's modulus was calculated using the following Eqs. 2, 3 and 4:

$$\text{Tensile strength (MPa)} = \text{Peak load (N)} / \text{Cross-sectional area (mm}^2\text{)} \quad (2)$$

$$\text{Elongation at break (\%)} = [\text{Peak extension (mm)} / \text{Initial grip length (mm)}] \times 100 \quad (3)$$

$$\text{Young's modulus (MPa)} = \text{Tensile strength (MPa)} / \text{Elongation at break (\%)} \quad (4)$$

The puncture force of the films was determined according to Zivanovic *et al.* (2007). A texture analyzer (TA.XT Plus, Stable Mico Systems, Godalming, UK) equipped with a 2-mm diameter needle probe was used to determine puncture force of films. The probe moved at a constant rate of 1 mm/s. The results generated were expressed in terms of N and the puncture force was calculated using Eq. 5:

$$\text{Puncture force (N)} = \text{Maximum force at break (N)} / \text{Thickness at broken area (mm)} \quad (5)$$

Statistical analysis

The experimental data was analyzed using SPSS version 25.0 software (IBM Corp., Armonk, NY, USA). All of the analyses were conducted in triplicate, and the results were expressed as mean \pm standard deviation. Paired samples t-test and independent samples t-test were used to compare the means. Analysis of variance (ANOVA) followed by Tukey's *post hoc* test was performed to evaluate the antibacterial activity and the impact of storage time on the total viable count of probiotics. The P-value < 0.05 was considered statistically significantly different in this study.

RESULTS AND DISCUSSION

Physical Properties

Physical properties (thickness, moisture content, and color) of WPI and NaCas edible film incorporated with LGG were determined in this study. Table 1 represents the physical properties of WPI and NaCas edible films before and after the incorporation of LGG.

Table 1. Effect of the Incorporation of LGG on the Physical Properties

Edible Film	WPI	WPI-LGG	NaCas	NaCas-LGG	
Thickness (mm)	0.22 \pm 0.03 ^{ab}	0.27 \pm 0.00 ^{aB}	0.13 \pm 0.01 ^{aA}	0.15 \pm 0.01 ^{aA}	
Moisture Content (%)	16.34 \pm 0.09 ^{ab}	26.12 \pm 1.37 ^{bA}	13.89 \pm 0.07 ^{aA}	24.34 \pm 1.15 ^{bA}	
Color	<i>L</i> *	29.72 \pm 1.20 ^{ab}	32.31 \pm 1.21 ^{bB}	8.40 \pm 1.29 ^{aA}	12.32 \pm 1.67 ^{bA}
	<i>a</i> *	-1.14 \pm 0.06 ^{aA}	2.13 \pm 0.09 ^{bB}	-1.61 \pm 0.18 ^{aA}	-0.97 \pm 0.03 ^{bA}
	<i>b</i> *	15.51 \pm 2.37 ^{ab}	17.00 \pm 2.43 ^{aB}	-2.93 \pm 0.05 ^{aA}	0.06 \pm 0.00 ^{bA}

^{ab} Mean \pm standard deviation followed by various superscripts within the same row indicate significant differences between respective edible films formulations (P < 0.05);

^{AB} Mean \pm standard deviation followed by various superscripts within the same row indicate significant differences between edible films (P < 0.05)

Thickness is a significant parameter related to the mechanical properties, water vapor permeability, and transparency of the film (Galus and Lenart 2013; Wulandari and Warkoyo 2019). According to Table 1, the incorporation of probiotics did not affect the thickness of both WPI and NaCas film (P > 0.05). The NaCas was able to form a thinner

film. According to Nemet *et al.* (2010), a higher concentration of glycerol resulted in a higher dry matter content to the film.

The moisture content of edible film should be in a similar range with the food products to reduce the diffusion from each other (Singh *et al.* 2014). Table 1 shows that the addition of probiotics elevated the moisture content of the films. This might be due to the ability of biopolymer materials to affect the hydrogen bond formation with water molecules (Soukoulis *et al.* 2017). Table 1 showed that the moisture content in WPI film was higher than NaCas which might be influenced by the amount of glycerol added into edible films. As observed in this study, approximately 2.0% (w/v) of glycerol was added into WPI while 0.25% (w/v) of glycerol was added into NaCas. As a result, the moisture content of WPI was higher than NaCas due to a higher amount of glycerol incorporation.

The physical appearance of food packaging is a crucial parameter, as it directly affects consumer preference towards the food products (Soukoulis *et al.* 2017). Figure 1 illustrates the higher transparency of NaCas film as compared to WPI film. As observed from Table 1, the addition of probiotic culture into the WPI and NaCas improved the appearance of films. This is because a more transparent film was formed with higher lightness (Abdollahzadeh *et al.* 2018). A difference in brightness was observed in various locations for all WPI films. According to Ramos *et al.* (2013), this brightness difference was caused by phase separation that occurred in the filmogenic solution during drying. The negative value and positive value of a^* revealed the exhibition of greenness and redness, respectively. The addition of LGG increased the a^* value of both edible films, resulting in a lower intensity of green color. The higher positive value of b^* indicated the higher intensity of yellowness. The WPI film prepared in this study was light yellow; therefore the b^* value of WPI film was positive and it was not affected ($P > 0.05$) by the addition of LGG. In contrast, in the NaCas film the b^* value increased to a positive value after the addition of LGG. Total color difference (ΔE) values with addition of LGG were about 4.4 and 4.9 respectively, for WPI and NaCas, which is quite near to the minimum value that human eye could differentiate (5) (Obón *et al.* 2009).

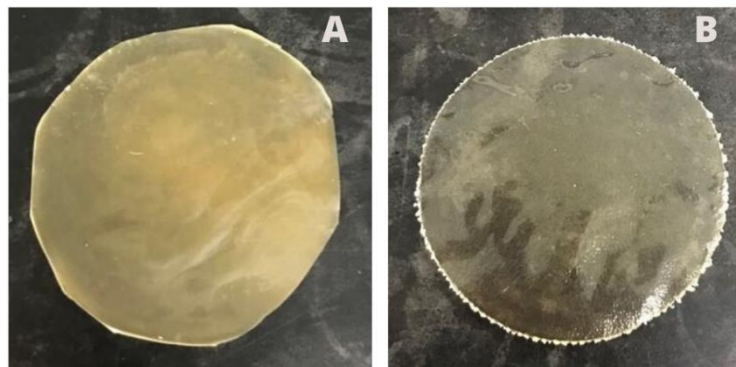


Fig. 1. The physical appearance of WPI (A) and NaCas (B) edible films

Mechanical Properties

An evaluation of the mechanical properties demonstrated the durability and potential ability of the edible films. Table 2 shows the mechanical properties of WPI and NaCas edible films incorporated with LGG. During the mechanical testing, the films were stretched and pulled at a constant head speed until it was broken.

Based on Table 2, tensile strength of WPI films was lowered after the addition of LGG. The tensile strength of WPI films was in the range between 4.07 ± 0.32 and 2.40 ± 0.06 MPa. However, Ramos *et al.* (2013) reported that the tensile strength of WPI films were less than 1.0 MPa. This could have been attributed to the addition of a higher amount of glycerol, from 40% to 60%, which reduced the intermolecular forces between polymers (Jouki *et al.* 2013). Elongation at break of films was not significantly affected ($P > 0.05$) by the incorporation of LGG. Water content in the NaCas films exhibited a vital role in increasing film flexibility. In the study conducted by Gialamas *et al.* (2010), at a moisture level of 20%, the measured elongation at break of film was approximately 15% to 25%, which was in line with present study. In contrast, Young's modulus of WPI was reduced with the addition of LGG. The film with lower Young's modulus was more flexible as less force is required to stretch the material (Briones *et al.* 2004) which has improved the flexibility of the WPI films. The NaCas films were more resistant to fracture and more stretchable due to the larger values of tensile strength, elongation at break, and Young's modulus than the WPI films.

Table 2. Effect of the Incorporation of LGG on the Mechanical Properties of WPI and NaCas Edible Films

Edible Films	WPI	WPI-LGG	NaCas	NaCas-LGG
Tensile Strength (MPa)	4.07 ± 0.32^{bA}	2.40 ± 0.06^{aA}	4.12 ± 0.44^{aA}	4.02 ± 0.32^{aB}
Elongation at Break (%)	23.81 ± 1.59^{aA}	22.73 ± 0.49^{aA}	24.47 ± 0.31^{aA}	23.93 ± 0.29^{aB}
Young's Modulus (MPa)	0.17 ± 0.00^{bB}	0.11 ± 0.02^{aA}	0.17 ± 0.02^{aA}	0.17 ± 0.01^{aB}
Puncture Force (N)	5.80 ± 0.41^{aA}	9.26 ± 0.25^{bB}	4.36 ± 0.18^{aA}	5.50 ± 0.10^{bA}

^{ab} Mean \pm standard deviation followed by various superscripts within the same row indicate significant differences between respective edible film formulations ($P < 0.05$);

^{AB} Mean \pm standard deviation followed by various superscripts within the same row indicate significant differences between edible films ($P < 0.05$)

An important attribute for a mechanically good film is being ductile but not brittle. Puncture force is generally defined as the maximum force required to penetrate or break a material (Maran *et al.* 2013). Based on the results shown in Table 2, WPI-LGG film possessed the highest value of puncture force, which was 9.26 MPa. The highest puncture force of WPI-LGG film was associated with its highest thickness (Hafnimardiyanti *et al.* 2014). Both WPI and NaCas films loaded with probiotics showed higher puncture force compared to the control samples. This phenomenon showed that the addition of probiotics positively improved the mechanical properties of the milk protein films to withstand higher external force.

Survivability of LGG During Drying Process

The viability of LGG added to the WPI and NaCas edible films was studied, and the result is shown in Fig. 2. The purpose of this analysis was to determine if the drying condition affects the growth of LGG.

To be a good carrier for bioactive compounds, the viability of probiotics in WPI and NaCas edible films at the drying endpoint must be sufficiently high (Soukoulis *et al.* 2017). According to Fig. 2, the viable count of LGG in WPI and NaCas was reduced at the end of drying from 11.4 to 10.5 log CFU/g and 11.7 to 10.7 log CFU/g, respectively. The

result obtained was comparable to previous studies by Sánchez-González *et al.* (2013) and Soukoulis *et al.* (2016), who reported there was a decrease in probiotics count after the drying process. When the amounts of probiotic cells keep declining, the osmotic pressure developed can result in osmolytic sub-lethal effects on probiotic cells (Soukoulis *et al.* 2017). Furthermore, the probiotic cells might experience heat shock related cellular injuries during the transfer from room temperature to certain drying temperature. However, at the end of drying, the viable count observed for both edible films were above the satisfying range, which was of 10^8 to 10^9 CFU/g. Thus, there was no overall lethal effect caused by the drying process as the viability was under the desired concentration for storage analysis.

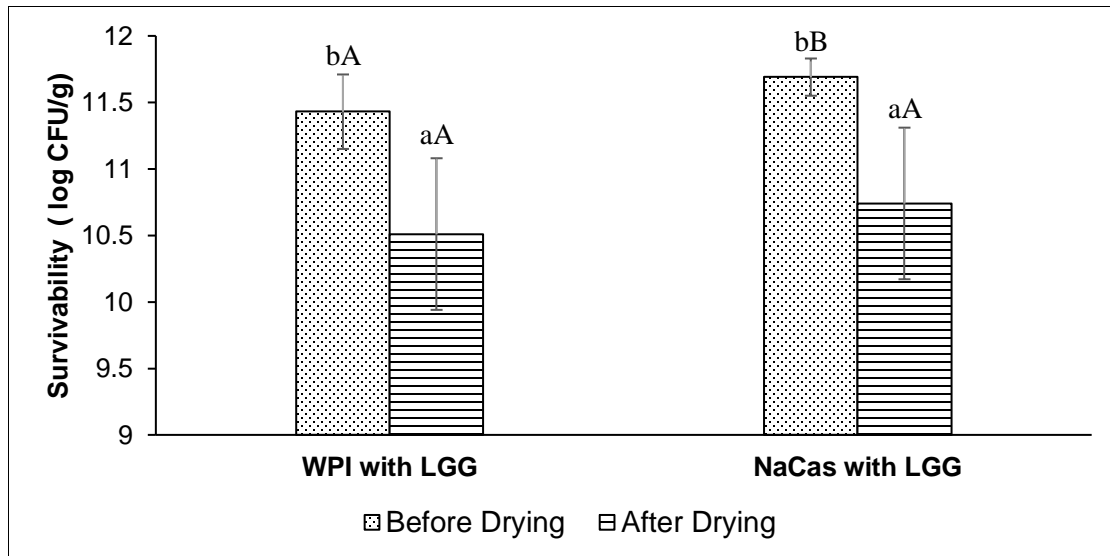


Fig. 2. The survivability of LGG throughout oven drying at 40 °C for 20 h; ^{ab} Mean ± standard deviation followed by various superscripts indicate significant differences between before and after drying ($P < 0.05$); ^{AB} Mean ± standard deviation followed by different superscripts indicate significant differences between LGG edible films ($P < 0.05$).

Total Viable Count of LGG During Storage

The prominent factors on the viability of probiotics include the strain dependency, storage temperature and condition, the presence of protective agents, and oxidative damage (Ferdousi *et al.* 2013; Tripathi and Giri 2014). The viability of dried WPI-LGG and NaCas-LGG films were investigated for 14 days of storage under two different temperatures, which were at refrigeration temperature of 4 °C (Fig. 3a) and room temperature of 25 °C (Fig. 3b).

According to Fig. 3, a decrease in the LGG viability throughout storage time was observed, regardless of the type of edible film and storage temperature. The sharp decline during the first 4 days could be due to the adaptation to a new substrate and low storage temperature (De Lacey *et al.* 2012). The data reported here appears to support the assumption that NaCas provide a better environment to retain LGG viability. Thus, the LGG might possess better tolerance to detrimental conditions in NaCas when compared to WPI. As described previously by Soukoulis *et al.* (2016), greater probiotic viability was obtained in NaCas film than in films from gelatin and soy protein concentrate. In the end of 14 days of storage, approximately 4 and 6 logs cycle of LGG were lost in WPI films, at temperatures of 4 °C and 25 °C, respectively.

During the end of storage, 63% and 65% of LGG were maintained in WPI and NaCas, respectively. The final viability obtained for both edible films was higher than the recommended level, which is 10^6 log CFU/g. The better viability of the LGG strain in the NaCas films can be explained by the positively charged protein chains are more likely to support the LGG metabolic pathway (Ly *et al.* 2008). The constituents of edible films are also one of the factors of providing good nutrients for strains that are beneficial to extend their viability as well as metabolic activity.

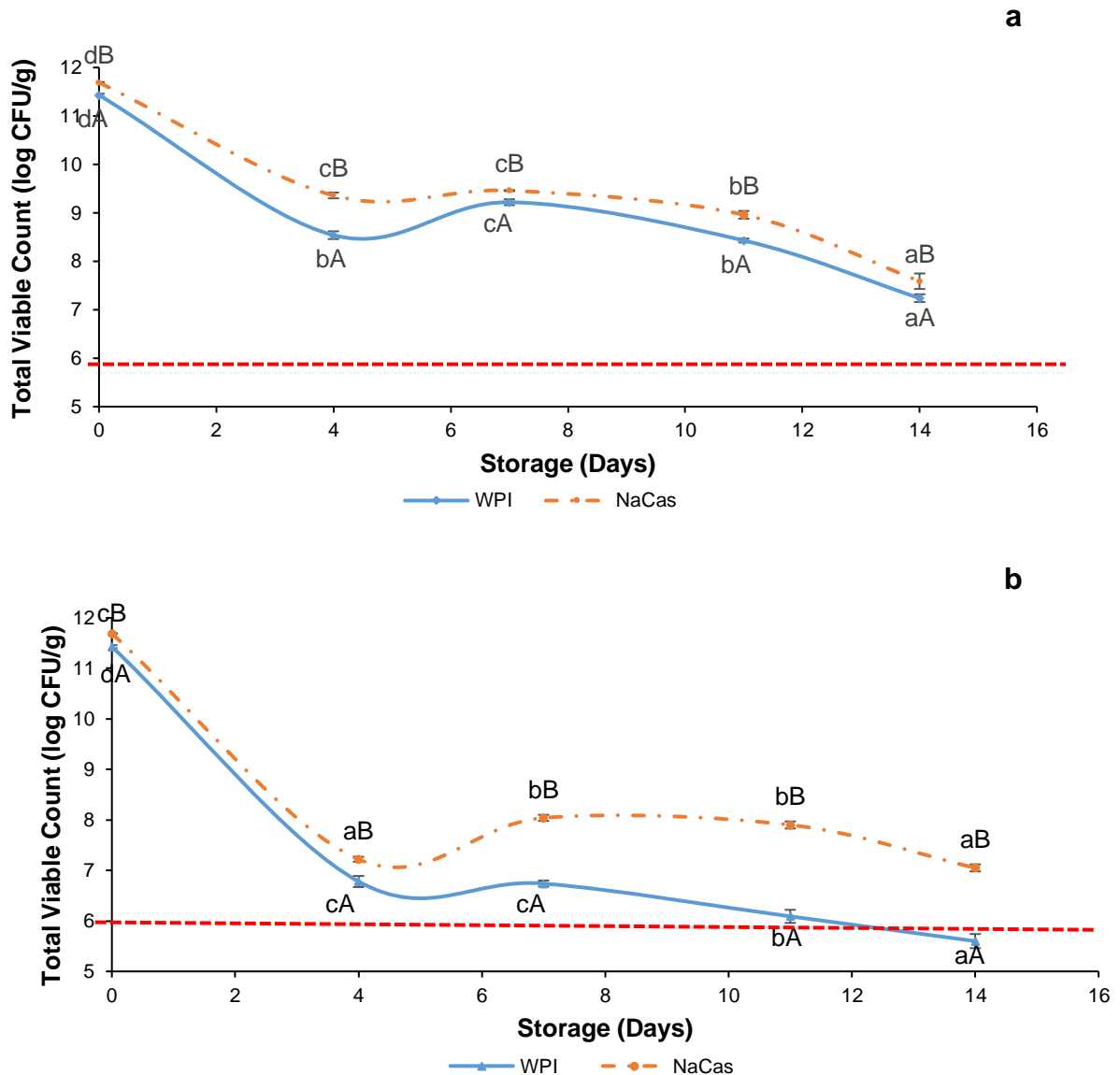


Fig. 3. Total viable count of LGG in edible films during 14 days of storage at 4 °C (a) and 25 °C (b); ^{abcd} Mean \pm standard deviation followed by various superscripts indicate significant differences during storage ($P < 0.05$) according to Tukey's test; ^{AB} Mean \pm standard deviation followed by different superscripts indicate significant differences between formulations ($P < 0.05$)

The shelf-life of the films is also another key factor on the viability of LGG. A study of Soukoulis *et al.* (2014b) reported that shelf-life of the edible films ranged respectively from 63 to 100 days and 17 to 30 days under chilled (4 °C) and room

temperature (25 °C) storage. However, the results obtained were in contrast with those reported in the study conducted by Kanmani and Lim (2013), in which the higher moisture content helped better retain probiotic cell viability. As shown in Table 1, the moisture content of WPI was higher than NaCas, however the probiotic viability in WPI was lower.

Probiotic food products are preferable to be stored at a temperature of 4 °C (Tripathi and Giri 2014). Nag *et al.* (2011) found that temperatures near 0 °C improved the cell viability rate due to the reduction in the oxidation rate. Similarly, the loss of LGG viability at 4 °C was lesser than the loss of LGG at 25 °C in this study. The LGG viabilities observed on days 4, 7, and 11 were lower at 25 °C. On the first and the last days of storage, lesser viability of LGG was found at room temperature, but it was not statistically significant ($P > 0.05$).

This research study showed that the type of edible film biopolymer was a significant factor for bacterial viability. The result from this study suggested that storage temperature affects the probiotic viability. Furthermore, the structural and physical state of the biopolymers can also affect the stability of probiotics. To provide better protection for probiotics, low residual water-glassy biopolymers with low gas exchange ability have been reported as an efficient strategy (Dong *et al.* 2013; Soukoulis *et al.* 2014a).

CONCLUSIONS

1. The edible film formed from NaCas possessed better physical properties compared to WPI edible film in terms of thickness and color. Incorporation of LGG increased the lightness and moisture content of both WPI and NaCas edible films.
2. The NaCas edible films exhibited stronger mechanical properties than WPI edible film, as the tensile strength, elongation at break, and Young's modulus of NaCas were higher. Incorporation of LGG increased the puncture force of edible films to withstand higher external force.
3. Drying is an essential process, as the survival of probiotics after experiencing heat and osmotic pressure are the key factors for further storage analysis. The drying of edible films resulted in the loss of probiotics in both WPI and NaCas films, and about 1 log CFU/g reduction of viability was observed. The NaCas resulted as a better substrate to hold the LGG during the drying and formation of films.
4. A decrease in the LGG viability throughout storage time was observed, regardless of the type of the film and the storage temperature. The better viability of the LGG strain in the NaCas film was observed in both storage temperatures. In contrast, the loss of LGG viability at 4 °C was less pronounced.
5. All of the edible films in various storage conditions exhibited 10^6 log CFU/g at the end of storage, which is the minimum value required for a probiotic efficiency, except for WPI-LGG edible film stored at 25 °C.

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