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Research Paper

Viability of *Lactobacillus delbrueckii* in chocolates during storage and *in-vitro* bioaccessibility of polyphenols and SCFAs

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ABSTRACT

This study evaluated the viability of encapsulated *Lactobacillus delbrueckii* subsp. *bulgaricus* in chocolate during storage and *in-vitro* gastrointestinal transit. Flavonoid contents and short chain fatty acids (SCFAs) production during gastrointestinal transit were also assessed. Encapsulated L. *delbrueckii* subsp. *bulgaricus* survived well in chocolates >7 logs both after 120 days of storage at 4 °C and 25 °C, and during *in-vitro* gastrointestinal transit. The release of SCFAs through *in-vitro* gastrointestinal digestion and colonic fermentation revealed that probiotic-chocolates could be an excellent source of nutrients for the gut microbiota. Encapsulated probiotic in chocolates with 70% cocoa produced significantly ($P < 0.05$) more acetic, propionic, isobutyric, butyric and isovaleric acids than that with 45% cocoa. The bioconversion results of a specific polyphenol by *L. delbrueckii* subsp. *bulgaricus* exhibited that chocolate polyphenols could be utilized by probiotics for their metabolism. These findings confirmed that chocolate could be successfully fortified with *L. delbrueckii* subsp. *bulgaricus* encapsulation to improve health promoting properties of chocolates.

1. Introduction

Interests in functional foods which provide additional health benefits beyond its nutritional value continue to grow and gain the attention of both consumers and the scientific community (Hossain et al., 2021). Probiotics are such important functional ingredients, and the consumption of sufficient amounts of live probiotics *via* food formulations can assist in maintaining a healthy gut due to their positive physiological effects on gut microbiota (Hill et al., 2014; Wasilewski et al., 2015). In order to achieve the health benefits, the International Dairy Federation recommends an adequate number of live probiotics (10^6-10^7 cfu/mL or g of food) at the time of consumption (Dong et al., 2013; Frakolaki et al., 2020). Consequently, there is a growing interest in the food and pharmaceutical industries to maintain the recommended probiotics viability through various innovation methods (Granato et al., 2020; Ranadheera et al., 2018). However, this is challenging and need further scientific investigations.

Lactobacillus delbrueckii subsp. *bulgaricus* is one of the mostly studied bacteria with probiotic characteristics. It is a Gram-positive, facultatively anaerobic, non-motile, non-spore-forming, and rod-shaped bacterium belongs to lactic acid bacteria (Tang et al., 2020). *L. delbrueckii* subsp. *bulgaricus* is a component of thermophilic starter cultures used in the manufacture of yogurts. *L. delbrueckii* subsp. *bulgaricus* is considered as an excellent probiotic in modulating the human gut environment and the production of essential metabolites such as lactic, acetic, propionic, and butyric acid (Tang et al., 2020; Zeng et al., 2018). Currently, the demand for functional food matrixes for probiotic delivery is growing, however, the delivery of probiotics *via* oral administration is challenging

Abbreviations: cfu, colony forming unit; FOS, fructooligosaccharides; MRS, DeMan, Rogosa and Sharpe; VIC, Victoria; NSW, - New South Wales; EE, encapsulation efficiency; SCFA, short chain fatty acid; BSCFA, branched short chain fatty acid; SEM, scanning electron microscope; SSF, simulated salivary fluid; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; HPLC, high performance liquid chromatography; PDA, photodiode array; GC FID, gas chromatography-flame ionization detector; HSD, honest significant difference; a_w, water activity; Pro B1, procyanidin B1; Pro B2, procyanidin B2.

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due to harsh gastrointestinal conditions such as stomach acid, bile and various digestive enzymes (Frakolaki et al., 2020; Vaziri et al., 2018) which are detrimental factors for probiotic survival during the gastrointestinal transit. In addition, there are challengers associated with probiotic survival during processing and storage of carrier food products (Mani-López et al., 2014).

Encapsulation is one of the most simple and effective technique which improves the endurance of probiotics during processing, storage as well as in GIT system (Afzaal et al., 2019; Yeung et al., 2016). Selection of encapsulant matrixes and applying a suitable encapsulation technique are essential for the successful delivery of targeted probiotics. Cocoa powder, one of the major ingredients in chocolate production, contains a complex composition of proteins, polysaccharides, and lipids (Oracz et al., 2020; Sorrenti et al., 2020) which could form a good encapsulating mixture along with alginate or FOS to encapsulate probiotics. In addition, chocolates is considered as a functional food product and rich antioxidant polyphenols, mainly flavanols such as (−)-epicatechin (EC), (+)-catechin and their dimers procyanidins B1 (Socci et al., 2017; Davinelli et al., 2018; Martín and Ramos, 2016). Hence, encapsulation of probiotics in chocolate products could provide additional nutritional benefits for the consumers. Currently much research has not been focused on the encapsulated probiotic fortified chocolate products, with *L. delbrueckii* subsp. *bulgaricus*. Furthermore, freeze-drying could be considered as the best drying method for probiotic encapsulation (Capela et al., 2006; Chávez and Ledeboer, 2007) which can be considered as a suitable choice to guarantee the maximum viable number, where the extra cost of this technique can be recovered by the best quality of the final encapsulated probiotics (Pech-Canul et al., 2020). Hence, this study focused on the evaluation of the viability of freeze-dried encapsulated *L. delbrueckii* subsp. *bulgaricus* in chocolate during storage and *in-vitro* gastrointestinal transit. Additionally, flavonoid contents and short chain fatty acids (SCFAs) production during gastrointestinal transit and colonic fermentation *in-vitro* were also assessed. A well-established *in-vitro* model with human faecal samples was used in this study (Hossain et al., 2021).

2. Materials and methods

2.1. Materials

The L. *delbrueckii* subsp. *bulgaricus* probiotic bacteria were used from the stock culture collections in the Food Microbiology Laboratory at the University of Melbourne. Fructooligosaccharides (FOS), Na-alginate, enzymes (salivary α-amylase, porcine pepsin, pancreatin), HCl, acetone, acetic acid, epicatechin, catechin, procyanidin B1, procyanidin B2, quercetin 3-*O*-galactoside, quercetin 3-*O*-glucoside were purchased from Sigma Aldrich (Castle Hill, NSW, Australia). A standard mixture of short chain fatty acids (SCFAs) was purchased from Cayman Chemical (Ann Arbor, MI, USA). The selective media DeMan, Rogosa and Sharpe (MRS), nutrient agar & broth, AnaeroGen sachets, yeast extract, beef extract, protease peptone, n-hexadecane, L-cysteine hydrochloride, bile salts, and trichloroacetic acid were purchased from Thermo Fisher Scientific Pty Ltd (Melbourne, VIC, Australia). NaOH, phosphate-buffered saline, $CaCl_2$, dextrose, K_2HPO_4, $(NH_4)_2SO_4$, $MgSO_4{\cdot}7H_2O$, NaCl, KCl, $NaHCO_3$, $MgCl_2(H_2O)_6$, $(NH_4)_2CO_3$, potassium persulfate, potassium acetate, aluminum chloride were ordered from the Chem-Supply Pty Ltd. (Melbourne, VIC, Australia). Chocolates with 45% and 70% cocoa mass, cocoa powder (pure), cocoa liquor, cocoa butter, sugar, soy lecithin were purchased from local supermarket (VIC, Australia).

2.2. Methods

2.2.1. Preparation of encapsulation formulation blend and cell inoculum

Encapsulating materials were selected based on preliminary work to identify the best combinations of the encapsulants. The selected encapsulation formulation included cocoa powder: Na-alginate: FOS at 10:1:2 ratios. The probiotic strain was cultured and inoculated in a selective MRS medium and incubated anaerobically using a BB-16 incubator (Heraeus Instruments, Hanau, Germany) at 37 °C for 22 ± 2 h. The bacterial cells were harvested by centrifugation in a refrigerated centrifuge (Allegra X-12R, Beckman Coulter, NSW, Australia) at 5000×*g*, 4 °C for 15 min. The probiotics were washed twice with 0.85% saline solution and encapsulated by the following method of Hossain et al. (2021a,b,c).

2.2.2. Encapsulation of probiotics using a freeze dryer and encapsulation efficiency

The prepared probiotic strain was encapsulated using an emulsion based freeze-dried technique. The encapsulation formulation ingredients were dissolved in Milli-Q water followed by homogenization for 15 min at 10000 rpm using a homogenizer (IKA T25 digital Ultraturrax, Germany) and the mixture was then kept at room temperature for 2 h for complete hydration. The formulation mixture was pasteurized at 75 °C for 30 min in a water bath and cooled to 42 °C (Gebara et al., 2013). The harvested cell pellets (approximately 12 Logs cfu/g) were suspended in the formulation and homogenized for 5 min at 4000 rpm. The homogenate was then left for 1 h at room temperature to allow the interaction between encapsulants biopolymers and the probiotics (de Araújo Etchepare et al., 2020). The final homogenized mixture was distributed into 50 ml sterile falcon tubes (35 mL each) and frozen at −20 °C overnight. The frozen samples were freeze-dried at −50 °C using a benchtop freeze dryer (Dynavac Engineering FD3, NSW, Australia). The freeze-dried encapsulated probiotics were stored at 4 °C until used.

The percentage encapsulation efficiency (% EE) was calculated following the method of de Araújo Etchepare et al. (2020) using the following equation:

$$\%\ EE = \frac{A}{A0} \times 100$$

where A is the number of viable cultures (Log cfu/g) released after encapsulation, and A0 is the number of total free cultures (Log cfu/g) before encapsulation.

2.2.3. Preparation of probiotic-chocolate (PCh)

Two types of chocolates (A & B, with 45% and 70% cocoa mass, respectively) were prepared and used in this study. The compositions of chocolate A involved cocoa mass- 45%, cocoa butter- 7%, sugar- 27%, milk solids- 20.7%, and soy lecithin- 0.30% with a total fat content of 27%, while chocolate B contained cocoa mass-70%, cocoa butter-7%, sugar- 22.5%, and soy lecithin- 0.30% with a total fat content of 43% following some previous works (Glicerina et al., 2016; Hinneh et al., 2019). The encapsulated probiotic was added to chocolate at the best pre-determined temperature (45 °C) close to solidification at 1% (w/w) concentration (Gadhiya et al., 2018; Kemsawasd et al., 2016) to reach the recommended number of probiotic counts of at least 10^7 cfu/g in the final products (Dong et al., 2013). Chocolate enriched with encapsulated probiotics (probiotic-chocolate) and control (chocolate with non-encapsulated probiotic) were stored at 25 °C and 4 °C under aseptic conditions for 120 days.

2.2.4. pH and water activity (α_w) measurement

The pH changes in probiotic chocolates were determined using a pH meter (Hl 9125, Hanna Instruments, USA). Water activity (a_w) was determined using the a_w-meter (LabMaster -aw, Novasina AG, Switzerland). Analyses of these two parameters were performed during the entire storage period of 120 days at 30 days interval (Kobus-Cisowska et al., 2019) as these parameters could directly affect the viability of probiotics and their performances.

2.2.5. Scanning electron microscopy of the encapsulated probiotics before and after mixing with chocolate

A scanning electron microscope (SEM, Phenom Pro, Thermo Fisher Scientific, The Netherlands) was used to study the surface microstructure of freeze-dried encapsulated *L. delbrueckii* subsp. *bulgaricus* and probiotic-chocolate powders. The freeze-dried powder particles were mounted on an aluminium stub using a both-sided adhesive carbon tape and the excess of powder particles were removed using a jet of dry air. The sample was then coated with platinum (at 18 mA for 60s) using a sputter coater. The SEM images were taken at 10 kV accelerating voltage with different magnifications to visualize the surface microstructure of the encapsulated probiotic bacteria (Masum et al., 2019).

2.2.6. Viability of encapsulated probiotic in chocolates

The probiotic viability was assessed during the entire storage period of 120 days at 30 days interval. Samples (25 g) were mixed with 225 mL of sterile 0.1% peptone water and serially diluted, plated on MRS selective medium and incubated anaerobically at 37 °C for 48 h. Results were reported as Log cfu/g (Hossain et al., 2021).

2.2.7. Testing the survival of probiotics during the in-vitro gastrointestinal digestion of probiotic chocolate

2.2.7.1. Preparation of gastrointestinal digestion fluids and basal medium. The *in-vitro* gastrointestinal digestion was performed using salivary, gastric and intestinal fluids. These fluids were prepared as described by Minekus et al. (2014). The stock digestion fluids were prepared using a mixture of the electrolytes (K^+, Na^+, Cl^-, H_2PO_4, HCO_3, Mg^{2+}, NH^+ and Ca^{2+}) at different concentrations. The basal medium was prepared following the methods of Zhang et al. (2018). The composition of basal medium was as follows: 5.0 g soluble starch, 5.0 g peptone, 5.0 g tryptone, 4.50 g yeast extract, 4.5 g NaCl, 4.5 g KCl, 2.0 g pectin, 2.0 g mucin, 3.0 g casein, 1.5 g $NaHCO_3$, 0.8 g L-cysteine HCl, 1.23 g $MgSO_4{\cdot}7H_2O$, 1.0 g guar gum, 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 0.4 g bile salts, 0.11g $CaCl_2$ and 1.0 mL tween 80 dissolved in 1000 mL of Milli-Q water and autoclaved at 121 °C for 20 min (HANSIN VD-3041 autoclave, VIC, Australia) and pH was adjusted to 7.0.

2.2.7.2. In-vitro gastrointestinal digestion. The probiotics counts during gastrointestinal digestion of probiotic-chocolate and the control (non-encapsulated probiotics) were assessed using an *in-vitro* gastrointestinal digestion model (Minekus et al., 2014) at 1 & 90 days of storage. The model included three-steps sequentially simulated digestion in the mouth, stomach, and the small intestine. Samples were collected at each stage of digestion for the estimation of probiotics and total plate counts. To avoid any destruction, triplicate samples were prepared for each treatment. The individual sample replicates were used for colony counting. The *in-vitro* study involved chocolate samples with nonencapsulated probiotics (A1 and B1, 45% and 70% cocoa mass, respectively), probiotic chocolates (enriched with encapsulated probiotics) with 45% (A2) and 70% (B2) cocoa mass, positive control (fresh culture of *L. delbrueckii* subsp. *bulgaricus*), and negative control (faecal slurry).

1. **Mouth mastication**: The chocolate samples (2.5 g) were mixed with 1.75 mL of simulated salivary fluid (SSF), 0.25 mL of the salivary α-amylase solution of 1500 U mL^{-1}, 12.5 μL of 0.3 M $CaCl_2$ and 487.5 μL of Milli-Q water and vortexed for 2 min at room temperature.
2. **Gastric digestion**: Mouth masticated samples were mixed with 3.75 mL of simulated gastric fluid (SGF), 0.8 mL of porcine pepsin (3200–4500 U mg^{-1}), 2.5 μL of 0.3 M $CaCl_2$ and 0.375 mL of Milli-Q water. The pH was adjusted to 3.0 using 1 M HCl and incubated in a shaking incubator anaerobically for 2 h at 37 °C.
3. **Intestinal digestion**: The gastric digested samples were mixed with 5.5 mL of simulated intestinal fluid (SIF), 2.5 mL of porcine pancreatin (800 U ml^{-1}), 1.25 mL fresh bile (160 mM), 20 μL of 0.3M $CaCl_2$ and 0.655 mL of Milli-Q water. The pH was adjusted to 7 using NaOH (1 M) and the samples were digested for 2 h at 37 °C in a shaking incubator. The digested samples were centrifuged at 2500×*g*, 4 °C for 5 min and the residues were collected to continue the colonic fermentation.

2.2.7.3. Colonic fermentation. The *in-vitro* colonic fermentation was conducted with human faeces, after an ethical approval (ID: 1954660.1) was obtained from the Human Ethics Advisory Group at the University of Melbourne, Australia. Fresh faeces were collected from a healthy male donor (32yr old) who had not ingested antibiotics for the last 3 months. Faecal slurry preparation was performed as described by Tzounis et al. (2008) and used on the same day. The basal medium pH was adjusted to 7.0 using 1M HCl or 1M NaOH before autoclaving. The *in-vitro* colonic fermentation was conducted by mixing the gastrointestinal digested sample residue with the faecal slurry at 1:1 (v/v) ratio and incubated anaerobically at 37 °C up to 72 h. Aerobic and anaerobic counts were enumerated immediately after mixing and every 24 h interval successively up to 72 h of fermentation. The control sample was prepared using 5 mL faecal slurry and 5 mL basal medium only.

2.2.8. Extraction of polyphenols from the digested/fermented chocolate samples

2.2.8.1. Sample preparation. To avoid excessive fat of chocolates, the samples (approximately 0.5 g each) were defatted three times with 5 mL of hexane and the residues were dried at 60 °C to evaporate hexane completely (Cooper et al., 2007). The residues were then extracted three times with 2.5 mL of acetone: water: acetic acid (70:28:2 v/v/v) by sonication and centrifugation at 2500×*g* using a refrigerated centrifuge (Allegra X-12R, Beckman Coulter, NSW, Australia) and supernatants were collected. The polyphenols were extracted from the supernatants after completing the gastric digestion and colonic fermentation of all treatments (samples, positive and negative controls). Each individually collected supernatants were concentrated to 2–3 mL by vacuum evaporator (G3B, Hei-VAP, Germany) and diluted to 10 mL in Milli-Q water. The diluted supernatants were filtered through 0.22 μm membrane cartridge (Millipore, Sigma Aldrich, NSW, Australia) before the analyses of individual phenolic compounds.

2.2.8.2. Analyses of individual phenolic compounds using a HPLC technique. The identification and quantitation of individual flavonoids in the extracted supernatants were performed using a Waters 2690 Alliance HPLC machine, equipped with a Waters 2998 photodiode array (PDA) detector (Waters, NSW, Australia). A Gemini C18 Silica 250 × 4.6 mm, 5 μm column was used (Phenomenex, NSW, Australia). The filtered sample extracts (20 μL) were injected into the system with a binary phase A: Milli Q water with 0.1% formic acid and phase B: acetonitrile with 0.1% formic acid using flow rate of 0.3 mL/min and 55 min gradient elution at a wavelength of 280 nm (Sirisena et al., 2018). A standard curve was generated using six standard phenolic compounds (epicatechin, catechin, procyanidin B1, procyanidin B2, quercetin 3-*O*-galactoside, quercetin 3-*O*-glucoside) that were previously identified in cocoa powder and chocolates (Katz et al., 2011). The detected individual phenolic compounds were quantitated using the retention time of the matching peak area in the external standard along with the generated linear regression equation and expressed as μg/g of probiotic-chocolate.

2.2.9. Identification and quantification of SCFAs using GC-FID

2.2.9.1. Sample preparation. The samples from *in-vitro* colonic fermentation (100–150 μL) were transferred into a 2 mL screw cap test tube with ceramic beads (KT03961-1, Bertin Technologies, France). The

samples were homogenized with 1 mL of 10% isobutanol at 6000×*g* for 30 s three times with a 30 s interval (IKA ULTRA-TURRAX, Sigma-Aldrich, NSW, Australia). The samples were then centrifuged at 21000×*g* for 5 min and all supernatant (675 μL) were transferred into a new test tube and subjected to methylation following the method of Furuhashi et al. (2018). The supernatant was mixed with 20 μL aliquot of 3-methylpentanoic acid, followed by the addition of 125 μL NaOH (20 mM) and 400 μL chloroform. This sample mixture was then vortexed and centrifuged at 21000×*g* for 2 min. An aliquot of 400 μL from the upper phase was transferred into a new test tube and 80 μL isobutanol and 100 μL pyridine were added to the tubes. The final volume of the tubes was adjusted to 650 μL using Milli-Q water. One boiling chip was added to minimize foaming. For the sample derivatization process, a 50 μL aliquot of isobutyl chloroformate was added carefully to the 650 μL sample tube and uncapped for 1 min to release the gases. Following the vortexing, 150 μL of hexane was added to the tube and centrifuged at 21000×*g* for 2 min. The upper hexane-isobutanol phase was transferred to the autosampler for GC analysis.

2.2.9.2. Analyses of SCFA in the prepared samples using GC-FID. The SCFAs identification and quantification were performed on an Agilent 7890B GC-FID and Agilent 7693 autosampler. Nukol capillary 15 m × 0.53 mm × 0.5 μm film thickness column (Sigma-Aldrich, NSW, Australia) was used with column gas flow of 1.0 mL/min at 155 °C. Split (50:1) injection mode was used and oven temperatures was 100 °C, ramped at 10 °C/min to 220 °C. Helium gas was used as a carrier and run time was 12 min (Furuhashi et al., 2018).

2.3. Statistical analysis

All experiments were conducted in triplicate with at least two measurements for each parameter. Results were subjected to one-way ANOVA using Minitab®19 statistical software (Pennsylvania, USA). The means were separated using Tukey honest significant difference (HSD) at 95% confidence level. Results were reported as means ± standard deviations.

3. Results

3.1. Percentage encapsulation efficiency of the probiotics

In the current study, *L. delbrueckii* subsp. *bulgaricus* was encapsulated with a mixture of cocoa powder: Na-alginate: fructooligosaccharides at 10:1:2 ratio. The encapsulating mixture exhibited 89.32 ± 2.03% encapsulation efficiency (EE) and these encapsulated probiotics were used for the entire study.

3.2. pH and water activity (a_w) of probiotic-chocolates

Changes in pH and water activity (a_w) of probiotic-chocolates during the 120 days of storage at 25 °C were presented in Table 1. The pH of probiotic-chocolates for each group (A & B) increased gradually during the storage period. The initial pH for 45% cocoa probiotic-chocolates were 5.31 ± 0.08 (A1) and 5.29 ± 0.03 (A2), and final pH were 6.08 ± 0.13 (A1) and 5.79 ± 0.09 (A2) at day 120 (Table 1). The pH values in 70% cocoa were 5.10 ± 0.12 (B1) and 5.13 ± 0.05 (B2) at the beginning and 5.72 ± 0.03 (B1) and 5.83 ± 0.11 (B2) at the end of 120 days of storage. These results indicated significant ($P < 0.05$) increment in pH values during storage at 25 °C for 120 days in both chocolate samples (45% and 70% cocoa), and in the presence and absence of probiotics. However, the increment in pH values in chocolate containing 45% cocoa with and without probiotic were similar and reached 0.5 and 0.77, respectively. A similar pattern can also be observed in chocolate with 70% cocoa (Table 1).

No significant differences ($P > 0.05$) in water activity were detected upon comparing chocolate with and without probiotics within each type of chocolate (45% and 70% cocoa). However, significant differences ($P<0.05$) in a_w were observed when comparing chocolates with 45% and chocolate containing 70% cocoa (Table 1). The water activity declined gradually in all treatments during the storage time for 120 days at 25 °C. The initial a_w in plain (A1) and probiotic-chocolates (A2) with 45% cocoa were 0.302 ± 0.02 and 0.31 ± 0.01, respectively. These a_w values were declined to 0.291 ± 0.03 (A1) and 0.241 ± 0.02 (A2) after 120 days of storage. For 70% cocoa probiotic-chocolates, the a_w was 0.246 ± 0.03 in B1 and 0.256 ± 0.08 in B2 at the beginning of storage and dropped significantly ($P < 0.05$) to 0.215 ± 0.05 (B1) and 0.183 ± 0.06 (B2) at end of 120 days of storage.

Table 1
pH and a_w of probiotic-chocolates during storage at 25 °C.

Storage duration (days)					
Sample	0	30	60	90	120
			pH		
A1	5.31 ± 0.08^{Ba}	5.39 ± 0.11^{Bb}	5.43 ± 0.04^{Cbc}	5.56 ± 0.04^{Bd}	6.08 ± 0.13^{De}
A2	5.29 ± 0.03^{Bb}	5.18 ± 0.04^{Aa}	5.32 ± 0.03^{Bb}	5.66 ± 0.15^{Cc}	5.79 ± 0.09^{Bd}
B1	5.10 ± 0.12^{Aa}	5.19 ± 0.05^{Ab}	5.14 ± 0.04^{Aab}	5.47 ± 0.11^{Ac}	5.72 ± 0.03^{Ad}
B2	5.13 ± 0.05^{Aa}	5.18 ± 0.11^{Ab}	5.34 ± 0.10^{Bc}	5.44 ± 0.07^{Ad}	5.83 ± 0.11^{BCe}
			a_w		
A1	0.302 ± 0.02^{Bb}	0.295 ± 0.04^{Bb}	0.30 ± 0.03^{Cb}	0.261 ± 0.07^{Ba}	0.291 ± 0.03^{Cb}
A2	0.31 ± 0.01^{Bd}	0.305 ± 0.03^{Bd}	0.275 ± 0.03^{Bc}	0.263 ± 0.6^{Bb}	0.241 ± 0.02^{Ba}
B1	0.246 ± 0.03^{Ab}	0.242 ± 0.04^{Ab}	0.205 ± 0.04^{Aa}	0.263 ± 0.02^{Bd}	0.215 ± 0.05^{Bc}
B2	0.256 ± 0.08^{Ad}	0.241 ± 0.05^{Ac}	0.212 ± 0.03^{Ab}	0.21 ± 0.03^{Ab}	0.183 ± 0.06^{Aa}

*A1: 45% cocoa chocolate without probiotic, A2: 45% cocoa chocolate with probiotic, B1: 70% cocoa chocolate without probiotic, B2: 70% cocoa chocolate with probiotic.
**Means within each column followed by the different uppercase letters were significantly different ($p < 0.05$).
***Means in a row followed by different lowercase letters were significantly different ($p < 0.05$).

3.3. Scanning electronic microscopic images of encapsulated probiotics

The scanning electronic images (x10000 and x20000) of the *L. delbrueckii* subsp. *bulgaricus* probiotic showed the encapsulated probiotic cells were entrapped in the cocoa powder (Fig. 1A) and in the fortified chocolate matrices (Fig. 1B). The rod shape cells were homogeneously distributed, and the cell appearance of encapsulated probiotics were same in all samples.

3.4. Viability of encapsulated and free L. delbrueckii subsp. bulgaricus in chocolates

The shelf life of chocolates fortified with encapsulated and free *L. delbrueckii* subsp. *bulgaricus* was evaluated at two different temperatures (4 °C and 25 °C) up to 120 days of storage (Fig. 2). Results showed that the initial probiotic counts in all chocolate treatments were similar (>11 logs) on the first day of storage. Most importantly, chocolate enriched with encapsulated probiotics and containing 45% (A2) and 70% (B2) cocoa powder remained above 7.5 log cfu/g for the entire 120 days at both 4 and 25 °C.

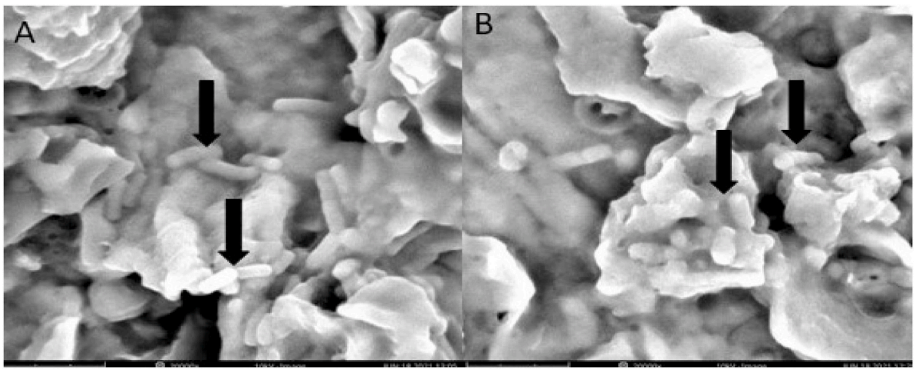


Fig. 1. SEM images of encapsulated *L. delbrueckii* subsp. *bulgaricus* in (A) freeze dried form and (B) inside the chocolates.

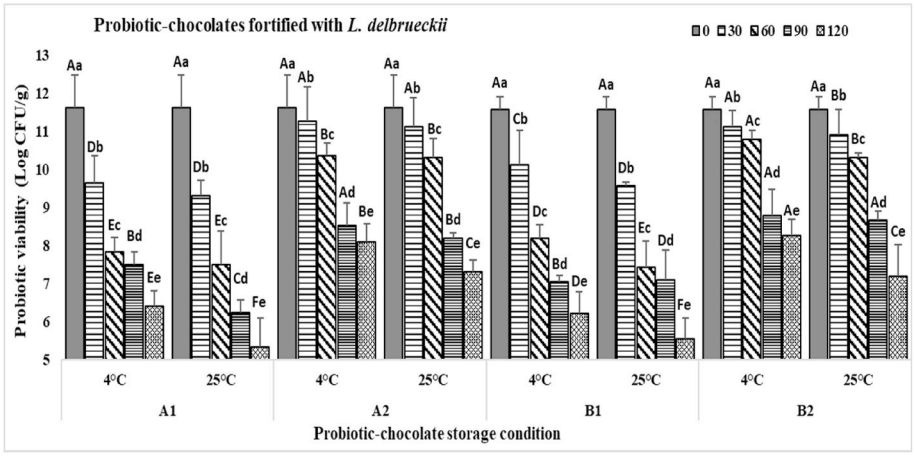


Fig. 2. *L. delbrueckii* subsp. *bulgaricus* viability in chocolates during storage up to 120 days.
*(A1: 45% cocoa probiotic-chocolate with nonencapsulated probiotic, A2: 45% cocoa probiotic-chocolate with encapsulated probiotic, B1: 70% cocoa probiotic-chocolate with nonencapsulated probiotic, B2: 70% cocoa probiotic-chocolates with encapsulated probiotic)
**Means within each storage time followed by the different uppercase letters were significantly different ($P < 0.05$).
***Means within each temp. followed by the different lowercase letters were significantly different ($P < 0.05$).

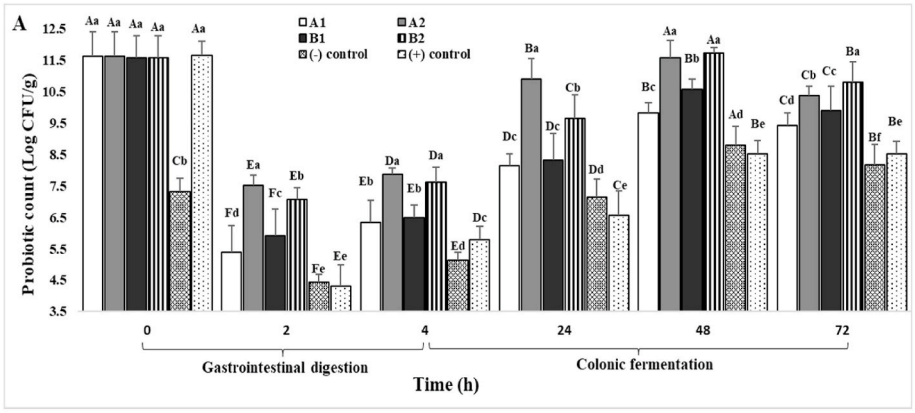
3.5. Survival of *L. delbrueckii subsp. bulgaricus* in chocolates during in-vitro gastrointestinal digestion and colonic fermentation

The gastrointestinal digestion and colonic fermentation of chocolates fortified with encapsulated *L. delbrueckii* subsp. *bulgaricus* after day one and day 90 of storage at 25 °C were performed (Fig. 3). Results from probiotic chocolate were compared with chocolate enriched with non-encapsulated *L. delbrueckii* subsp. *bulgaricus*, the negative control (faecal slurry) and the positive control (free probiotic culture). *In-vitro* analysis after one day of storage at 25 °C (Fig. 3A) revealed a significant ($P< 0.05$) reduction of 7.33 ± 0.18 log in chocolate fortified with non-encapsulated probiotics when exposed to 2 h of gastric digestion (pH 3). However, chocolate with encapsulated probiotics showed only 4.08 ± 0.09 and 4.51 ± 0.31 log reduction in the sample containing 45% and 70% cocoa, respectively. At the same time, the probiotic reduction in the positive control (free probiotics) and the negative control (faecal slurry) reached 7.30 ± 0.24 and 3.11 ± 0.30 logs, respectively. It should be noted that the initial count in the negative control was only 7.31 ± 0.43, as compared to about 11.60 logs in all other treatments. This means that the percentage reduction in the negative and positive controls were 42.54% and 62.85%, respectively, as compared to 35.17% and 38.99% in the probiotic chocolates with 45% and 70% cocoa powder, respectively. As no bacterial counts were detected in the positive or negative control samples after 90 days of storage, the simulated gastrointestinal digestion and colonic fermentation were performed on formulated samples only (A1, A2, B1 & B2) at 90 days of storage (Fig. 3-B). Results were similar to the gastrointestinal digestion and colonic fermentation at 1 day (Fig. 3-A). The initial counts in both A2 and B2 treatments (chocolate 45% and 70% cocoa, respectively) and encapsulated probiotics were above 8.5 Logs. However, those counts declined significantly ($P > 0.05$) during gastric digestion (4 h digestion) and increased later to reach the maximum counts during the colonic fermentation (48 h fermentation).

3.6. In-vitro bioaccessibility of individual phenolic compounds from the probiotic-chocolates

The individual phenolic compounds in both types of chocolates (45% and 70% cocoa content) fortified with *L. delbrueckii* subsp. *bulgaricus*, non-encapsulated (A1 & B1) and encapsulated (A2 & B2), and in the faecal slurry as a negative control at 3rd day of sample preparation are presented in Table 2. All the samples were analyzed after *in-vitro* gastrointestinal digestion and colonic fermentation. The negative control samples did not show any detectable peaks against the standard flavonoid compounds. The major identified and quantified flavonoids in chocolates with 70% and 45% cocoa powder included epicatechin, catechin, procyanidin B1, procyanidin B2, quercetin-3-*O*-galactoside and quercetin-3-*O*-glucoside. As expected, the largest percentage of the quantitated phenolic compounds were bioaccessible during the gastrointestinal digestion stage in all treatments (Table 2). For example, the percentage bioaccessibility of epicatechin, catechin, procyanidin B1, procyanidin B2, quercetin-3-*O*-galactoside and quercetin-3-*O*-glucoside were 75.68%, 79.61%, 77.62%, 85.55%, 90.56% and 92.79% in A1 sample and 80.71%, 81.75%, 78.89, % 87.49%, 92.66% and 96.75% in A2 sample, respectively. The same data showed also that flavonoids (polyphenols) contents in chocolate containing 70% cocoa (A2 & B2) were significantly ($P < 0.05$) larger than in chocolate with 45% cocoa. Such results could be attributed to the larger amounts of cocoa in chocolate with 70% cocoa.

The amounts of bioaccessible polyphenols in all treatments (A1, A2,



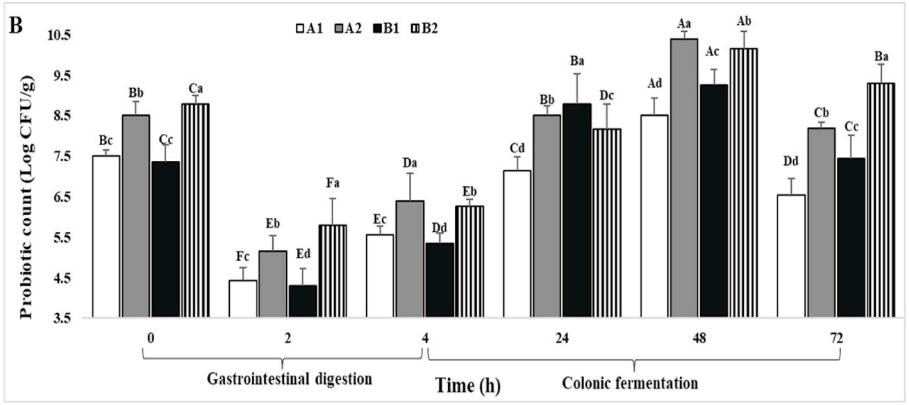


Fig. 3. *L. delbrueckii* subsp. *bulgaricus* survivability in chocolate subjected to *in-vitro* digestion and fermentation after storage for 1 day (A) and 90 days (B) at 25 °C.
*(A1: 45% cocoa probiotic-chocolate with nonencapsulated probiotic, A2: 45% cocoa probiotic-chocolate with encapsulated probiotic, B1: 70% cocoa probiotic-chocolate with nonencapsulated probiotic, B2: 70% cocoa probiotic-chocolates with encapsulated probiotic)
**Means within each digestion time followed by different uppercase letters were significantly different at 95% confidence level.
***Means within each sample followed by different lowercase letters were significantly different at 95% confidence level.

Table 2
Major flavonoids (μg/g) in probiotic-chocolates during *in-vitro* gastrointestinal digestion and colonic fermentation.

Samples	Digestion stages	EC	C	Pro B1	Pro B2	QC gal	QC glu
A1	Before digestion	195.65 ± 4.03^{Aa}	77.32 ± 1.82^{Aa}	261.53 ± 4.05^{Aa}	512.33 ± 6.52^{Aa}	421.10 ± 2.43^{Aa}	364.79 ± 5.43^{Ba}
	After gastrointestinal digestion	148.07 ± 2.87^{Bb}	61.56 ± 1.08^{Bb}	203 ± 2.08^{Ab}	438.32 ± 7.68^{Bb}	381.37 ± 5.59^{Aa}	338.50 ± 4.01^{Ba}
	After colonic fermentation	42.92 ± 1.80^{Ac}	11.67 ± 0.3^{Ac}	53.3 ± 0.28^{Ac}	58.08 ± 2.24^{Ac}	**nd	nd
A2	Before digestion	200.71 ± 6.22^{Aa}	79.57 ± 2.42^{Aa}	259.43 ± 2.05^{Aa}	517.43 ± 1.52^{Aa}	418.31 ± 3.15^{Aa}	381.79 ± 6.23^{Aa}
	After gastrointestinal digestion	162.01 ± 3.6^{Ab}	65.05 ± 2.81^{Ab}	204.67 ± 3.11^{Ab}	452.75 ± 2.07^{Ab}	387.63 ± 3.05^{Aa}	369.40 ± 1.28^{Aa}
	After colonic fermentation	27.33 ± 0.55^{Bc}	9.93 ± 0.11^{Bc}	51.73 ± 1.12^{Ac}	47.67 ± 1.15^{Bc}	nd	nd
B1	Before digestion	300.72 ± 6.89^{Aa}	114.56 ± 2.53^{Aa}	378.78 ± 7.05^{Aa}	791.73 ± 5.52^{Aa}	641.76 ± 8.55^{Aa}	578.06 ± 7.93^{Aa}
	After gastrointestinal digestion	201.33 ± 6.77^{Bb}	76.43 ± 3.8^{Bb}	287.73 ± 6.83^{Bb}	655.71 ± 6.71^{Bb}	611.83 ± 4.18^{Aa}	541.78 ± 4.18^{Aa}
	After colonic fermentation	94.46 ± 3.51^{Ac}	31.30 ± 1.52^{Ac}	69.47 ± 3.67^{Ac}	78.03 ± 5.78^{Ac}	nd	nd
B2	Before digestion	302.66 ± 3.2^{Aa}	117.35 ± 5.56^{Aa}	383.18 ± 3.05^{Aa}	793.92 ± 5.52^{Aa}	644.47 ± 2.76^{Aa}	581.27 ± 4.19^{Aa}
	After gastrointestinal digestion	231.31 ± 6.59^{Ab}	88.82 ± 1.22^{Ab}	307.67 ± 3.60^{Ab}	676.03 ± 5.17^{Ab}	619.31 ± 6.57^{Aa}	548.12 ± 2.28^{Aa}
	After colonic fermentation	58.76 ± 4.16^{Bc}	23.26 ± 1.3^{Bc}	63.70 ± 2.12^{Bc}	76.93 ± 1.33^{Ac}	nd	nd

*(A1: 45% cocoa probiotic-chocolate with nonencapsulated probiotic, A2: 45% cocoa probiotic-chocolate with encapsulated probiotic, B1: 70% cocoa probiotic-chocolate with nonencapsulated probiotic, B2: 70% cocoa probiotic-chocolates with encapsulated probiotic).
** EC: Epicatechin, C: Catechin, Pro B1: Procyanidin B1, Pro B2: Procyanidin B2, QC gal: Quercetin 3-*O*-galactoside, QC glu: Quercetin 3-*O*-glucoside.
***Means within each digestion stage for two individual sample group (A & B) followed by the different uppercase letters were significantly different ($p < 0.05$).
****Means in a column for each sample group (A & B) followed by the different lowercase letters were significantly different ($p < 0.05$).

B1 and B2) appeared in the following order from the largest to smallest: procyanidin B2 > quercetin 3-*O*-galactoside > quercetin 3-*O*-glucoside, procyanidin B1 > epicatechin > catechin. For example, the largest and smallest detected amount of bioaccessible flavonoids in A1 sample were procyanidin B2 (438.32 ± 7.68 μg/g) and catechin (61.56 ± 1.08 μg/g). Such a great bioaccessibility of procyanidin (Pro B2) could be attributed to the enormous amounts of Pro B2 (512.33 ± 6.53 μg/g) in the original sample before digestion (Table 2). Similar trends with greatest amounts of bioaccessible flavonoids detected in Pro B2 were also seen in all other treatments.

3.7. Quantification of SCFAs during the in-vitro colonic fermentation

Results in Fig. 4 illustrated the amounts of SCFAs produced during the colonic fermentation of probiotic-chocolate (PCh) fortified by *L. delbrueckii* subsp. *bulgaricus* in comparison with the positive and negative controls. The data revealed that probiotic-chocolates with 70% cocoa (B2) produced the largest quantities ($P < 0.05$) of all five tested SCFAs (acetic, propionic, isobutyric, butyric and isovaleric acid). For example, the amounts of acetic acid produced after 48 h and 72 h of fermentation of B2 samples (15.64 ± 1.79 mmol and 15.32 ± 1.35 mmol, respectively) were significantly ($P < 0.05$) greater than those in the positive (1.13 ± 0.07 and 2.98 ± 0.09 mmol, respectively) and

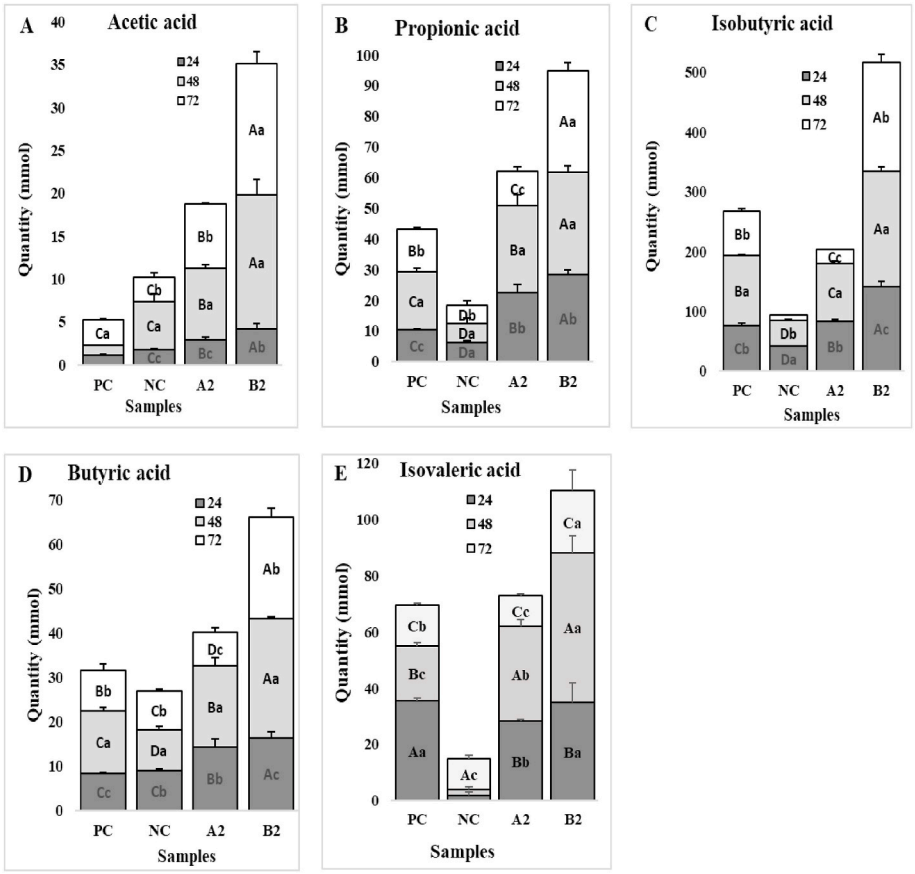


Fig. 4. SCFAs production by probiotic-chocolates fortified by *L. delbrueckii* subsp. *bulgaricus*.
*(A2: 45% cocoa with encapsulated probiotic, B2: 70% cocoa with encapsulated probiotic, NC: Negative control (fecal slurry), PC: Positive control (fresh culture of *L. delbrueckii* subsp. *bulgaricus*)
**Means within each fermentation time followed by an uppercase letter are not significantly different at 95% confidence level.
***Means within each sample followed by a lowercase letter are not significantly different at 95% confidence level.

negative (5.56 ± 0.90 and 2.78 ± 0.55 mmol, respectively) controls and chocolate with 45% cocoa (8.32 ± 0.45 and 7.56 ± 0.91, respectively). The highest amount propionic acid was detected at 48 h of colonic fermentation in both A2 (28.24 ± 3.56 mmol) and B2 (33.57 ± 1.87) samples. The positive control also produced much larger quantity (18.85 ± 1.09 mmol) of propionic acid than the negative control (6.29 ± 1.52 mmol) indicating that *L. delbrueckii* subsp. *bulgaricus* is a potential health modulating probiotic.

Similarly, regarding to the amounts of isobutyric, butyric, and isovaleric acids produced during the colonic fermentation, the largest quantity was released in the presence of probiotic chocolate containing 70% cocoa (B2), followed by probiotic chocolate with 45% cocoa (A2). Interestingly, the positive control produced more isobutyric acid than A2 samples after 48 h of fermentation (Fig. 4C). While the amount of isovaleric acid was larger than that in A2 probiotic chocolate after 72 h of fermentation (Fig. 4E). Both isobutyric and isovaleric are branched short chain fatty acids (BSCFAs) and usually produced in smaller amounts than their SCFAs counterparts.

4. Discussions

In this study we have been examined the cocoa powder along with Na-alginate and FOS as an encapsulating mixture and the impact of encapsulated probiotic fortified to chocolates through *in-vitro* gastrointestinal digestion. The previous findings of Yasmin et al. (2019) reported 85.49% EE using whey protein concentrate and pectin as encapsulating materials. Xu et al. (2016) indicated that EE higher than 85% could be considered as successful and the encapsulation efficiency is depended on the content of carbohydrates biopolymers and protein in the encapsulating materials. The reported EE in this study was higher than the previous findings and could be considered satisfactory to provide a protective effect towards the probiotic cultures. The results presented in Table 1 concluded that the increment in pH during storage at 25 °C was not caused by the added probiotics. Factors like hydrolysis, oxidation, and sugar transformation might be responsible to such a pH change during chocolate storage. The results were supported by Kobus-Cisowska et al. Kobus-Cisowska et al. (2019) who reported similar increases in pH of chocolates containing *Bacillus coagulans* bacteria.

The a_w results (Table 1) in chocolate with 45% cocoa (A1 and A2) were significantly ($P < 0.05$) larger than those with 70% cocoa probiotic-chocolates (B1 and B2), which might be due to more cocoa mass in 70% chocolate samples. The larger cocoa mass in 70% chocolate could facilitate more water binding and leave less free water that usually contribute to a higher a_w. Decline of a_w might occur due to water migration because of product damage, cracking of external portions or dying out of internal portion (Kobus-Cisowska et al., 2019). As water activity is one of the major internal factors that affect microbial growth, and most microorganisms require high water activity ($a_w > 0.8$) to grow (Hiura et al., 2021; Tapia et al., 2020), it could be concluded that all tested chocolate samples with a_w <0.4 will not facilitate microbial growth during storage. As dark chocolate contains high fat, rancidity of fat might be another reason which affect the increment pH and a_w during storage. The SEM images indicated that the regular size and shape of the probiotic bacteria during freeze drying with this encapsulating mixture and also in the fortified chocolates did not changed which agreed with some previous results (de Araújo Etchepare et al., 2020; Chen et al., 2017). These SEM images confirm the fact that cocoa powder as an encapsulants could be suitable for probiotic carriers to fortify its functional property.

The shelf life results (Fig. 2) confirmed that chocolate fortified with encapsulated probiotics can meet the recommended therapeutic minimum (10^7 cfu/g or mL) to exhibit the health benefits (Dong et al., 2013). On the contrary, chocolates fortified with non-encapsulated probiotics

(A1 and B1) revealed significant ($P < 0.05$) and gradual decline in the probiotic counts during storage in both chocolate with 45% and 70% cocoa contents (Fig. 2). The results also demonstrated clear and significant ($P < 0.05$) impact of storage temperature on survival of probiotic in chocolate in general, with more significant decline in the probiotic counts at 25 °C. A similar phenomenon regarding the effect of storage temperature on the survival of probiotics in chocolates was reported by Lalicic-Petronijevic et al. (2015), who reported that refrigerated milk chocolates could maintain more than 8.0 cfu/g of bacteria during the storage time of 180 day. The same authors also reported that the survivability in 75% cocoa chocolates was also acceptable. In comparison with the protective effects of encapsulations, the A2 and B2 samples have a greater number (at least 2.0 logs) of probiotics at the end of 120 days compared to non-encapsulated probiotics in chocolates (A1 and B1) which proved that the encapsulants performed well and were able to protect the probiotics at both storage conditions.

The lower log reduction of probiotics in chocolates (Fig. 3) indicated that both encapsulation and using chocolate as carriers having a protective effect on the probiotics during the *in-vitro* digestion. Monitoring the changes in the probiotic counts in all samples during the intestinal digestion (after 4 h) showed stable counts with only a slight ($P > 0.05$) increase. These changes could be attributed to the more suitable pH (7) for bacterial growth in the intestine, as compared with harsh gastric conditions of pH 3. The data from colonic fermentation exhibited gradual increases in the probiotic counts until 48 h of digestion and plateaued thereafter. Such increases in the probiotic counts during the colonic fermentation could be attributed to favourable growth conditions and the utilisation of cocoa mass materials as prebiotics. Makivuokko et al. (2007) reported that the anaerobic conditions during colonic fermentation might create optimum conditions to hydrolyse and fermentation of the cocoa soluble fibre, such as hemicellulose and unabsorbed long chain fatty acids. The number of *L. delbrueckii* subsp. *bulgaricus* increased by at least 2 logs at 24 h of colonic fermentation and reached the highest counts (11.71 ± 0.18 log cfu/g for B2 and 11.56 ± 0.31 log cfu/g for A2) after 48 h of fermentation, followed by insignificant ($P > 0.05$) decline after 72 h (Fig. 3A).

Further, data in Fig. 3 showed that chocolate with 70% cocoa contents and encapsulated probiotic was able to maintain significantly higher probiotic counts than all other treatments and throughout the gastric and colonic fermentation. Some previous findings (Khorasani and Shojaosadati, 2016; Krunic et al., 2019) reported that the increment during the colonic fermentation with nanocellulose pectin, whey protein hydrolysate by 94.76% and 96%, respectively. Another study by Kobus-Cisowska et al. [30] confirmed the phenomena that dark chocolate (70% cocoa content) provided more protection and prebiotc effect. The same authors reported that the counts of *B. coagulans* passed through the large intestine increased to 2.2×10^{10} cfu/g. Data in Fig. 3B showed the results of the *in-vitro* study of probiotic chocolate and control sample after 90 days of storage. These results presented the similar trends and changes in the probiotic counts to those reported after 1 day of storage (Fig. 3A). These results clearly demonstrated that probiotic chocolate fortified with encapsulated probiotics can maintain the minimum therapeutic limits during long-term storage. It was concluded also that complex mixture of chocolate ingredients is a good nutrient source for probiotics growth in gastrointestinal track.

The bioaccessibility of individual polyphenols in chocolates during the in-vitro gastrointestinal digestion and colonic fermentation (Table 2). These findings were supported by those reported by Hii et al. (2009), who indicated that proanthocyanin was the major phenolic compound in cocoa and accounted for 58% of the total polyphenols. At the colonic fermentation stage, significantly ($P < 0.05$) less amount of phenolic compound was detected in A2 & B2 samples compared to A1 & B1 samples, and no detectable amount of quercetion-3-*O*-galactoside and quercetin-3-*O*-glucoside were found during the colonic fermentation stage. These results demonstrated the fact that the majority of the chocolate polyphenols (75–96%) are soluble and could be absorbed during the gastrointestinal digestion stage. The small proportion (4–25%) remaining in the indigestible faction of chocolate could be fermented by the colon microbiota and release the entrapped lesser amounts of polyphenols. Similar observations were reported by Cantele et al. (2020) that bioaccessible polyphenolic compounds were available at both phase of gastrointestinal digestions (*in-vitro* gastrointestinal digestion & colonic fermentation) but most polyphenols were absorbed in gastrointestinal digestion. Rios-Covian et al. (2020) reported that BSCFAs, were produced by the gut microbiota *via* the fermentation of the branched amino acids, such as valine and leucine. As all treatments in this study including the positive and negative controls contained the same conditions and composition of ingredients, the detected results of isobutyric and isovaleric acids indicated that *L. delbrueckii* subsp. *bulgaricus* is a good BSCFAs producing probiotic under these conditions.

Other studies reported that the maximum amounts of SCFAs were produced between 24 and 48 h of colonic fermentation (Granado-Serrano et al., 2019; Horiuchi et al., 2020; Zheng et al., 2020). These results agreed with the present findings which showed the highest efficacy of SCFA production at 48 h of fermentation indicated that abundant number of gut bacteria, host metabolism, available sources of indigestible carbohydrates would be responsible for higher production of SCFAs between 24 and 48 h of digestion. SCFAs are considered as the predominant end-product of gut bacterial fermentation of indigestible dietary carbohydrates and proteins. Chocolates with 70% cocoa contains more undigested nutrient sources which can act as prebiotics and contribute to the production of more SCFAs (Tran et al., 2020). The release of SCFAs in gastrointestinal tract is positive to human health. SCFAs have been reported to increase the absorption of main minerals such as calcium, iron, and magnesium, maintain the integrity and function of the intestine, and contribute to the anti-inflammatory activities (Markowiak-Kopeć and Śliżewska, 2020). The findings of the present study confirmed that probiotic chocolate enriched with *L. delbrueckii* subsp. *bulgaricus* could be considered as a functional snack with a good nutrient source and potential prebiotic function for human health.

5. Conclusion

In the current study, 45% and 70% cocoa chocolates were enriched successfully with probiotic strain *L. delbrueckii* subsp. *bulgaricus*. The fortification of chocolate with the encapsulated probiotic to chocolate did not affect chocolate quality parameters such as water activity, pH, and polyphenol content. Incorporation of L. *delbrueckii* was effective in preserving a prominent level of cell viability, above the therapeutic minimum during 120 days of storage at 25 °C. These findings suggested that the probiotic-chocolate could be stored at room temperature without compromising the probiotic functionality. The tolerance of gastrointestinal environment was satisfactory with the encapsulated probiotic bacteria in both type of chocolates in terms of high survival rate of bacteria, especially given the low viability of *L. delbrueckii* subsp. *bulgaricus* in the gastrointestinal environment. The results also indicated that chocolates with 70% cocoa mass content can provide a suitable matrix for *L. delbrueckii* subsp. *bulgaricus* probiotic bacteria and facilitate the interaction between chocolates carbohydrates and polyphenols with probiotic bacteria and gut microbiome in the way to production of beneficial and bioaccessible SCFAs. These findings on the storage stability and the simulated digestion conditions of the gastrointestinal tract confirmed that chocolates could be fortified with *L. delbrueckii* subsp. *bulgaricus* to formulate a new functional probiotic-chocolates.

Credit author statement

Md Nur Hossain conducted all the laboratory work, collected the data and prepared the 1st draft; Senaka Ranadheera and Zhongxiang Fang revised the 2nd draft and contributed to regular discissions. Said Ajlouni conceptualised the research idea, revised the 1st and last draft

and supervised the student work.

Data availability

All the data and materials are available in this manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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