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RESEARCH ARTICLE

Protecting the viability of encapsulated *Lactobacillus rhamnosus* LGG using chocolate as a carrier

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ABSTRACT

The novel probiotic encapsulation approaches in snacks have not been thoroughly investigated. This study examined the viability of encapsulated *Lactobacillus rhamnosus* LGG using chocolate as a carrier. Various encapsulants, including cocoa powder, Na-alginate, fructooligosaccharides, whey protein concentrate, hi-maize starch and skim milk powder were tested using a freeze-drying technique. The encapsulation efficiency of *L. rhamnosus* reached 91.82% using cocoa powder and Na-alginate formulations. The encapsulated probiotic survived at thermal exposure maintaining more than 9 logs at 60°C. Chocolate was proven as a good carrier for encapsulated probiotic maintained viability above the therapeutic level (10^7 log) up to 180 and 120 days stored at 4°C and 25°C, respectively. Additionally, encapsulated *L. rhamnosus* in chocolate showed higher survival number (8.47 log cfu/g) at the end of gastrointestinal digestion. Hence, cocoa powder with Na-alginate as an encapsulation agent has potential applications in the development of healthy probiotic chocolate.

Keywords: Cocoa powder; Fructooligosaccharides; Gastrointestinal digestion; Microencapsulation; Probiotics

INTRODUCTION

Probiotics are 'live microorganisms which, when administered in adequate amounts, confer a health benefit on the host' (FAO/WHO, 2002). In order to exhibit the health benefits, it is mandatory to maintain the minimum number of live probiotics (7 log cfu/mL or g) at the time of consumption (Tripathi and Giri, 2014). Currently, there is a growing interest in the food industry to develop some promising probiotic functional foods (Champagne et al., 2018; Ranadheera et al., 2018). The consumption of probiotic enriched functional foods can assist in maintaining a healthy intestinal microbiota in the host (Hill et al., 2014; Wasilewski et al., 2015). Dairy products, such as yogurt, cheese and ice cream have been proved to be effective carriers for the delivery of probiotics in the gastrointestinal tract (Afzaal et al., 2019). Several non-dairy products such as fermented meat, pickles, fruit and vegetable juices have also been used as carriers of microencapsulated probiotics (Hossain et al., 2020). More recently, there has been an increasing interest in the development of novel snacks such as fruit bars and chocolate enriched with probiotics. However, the delivery of sufficient numbers of probiotics through foods is challenging due to various reasons such as processing conditions, storage and the harsh conditions after consumption, including stomach acid, bile salts and digestive enzymes in the GI tract (Mani-López et al., 2014; Vaziri et al., 2018).

Microencapsulation is a well accepted technique to enhance the viability of probiotics in stressful conditions. This employs the encapsulating matrixes which can preserve the probiotics during food processing, storage and release into a live and metabolically active state in the human intestine (Martín et al., 2015). A range of encapsulation materials have been exploited for this purpose including skim milk powder (Wang et al., 2016), alginate (de Araújo Etchepare et al., 2016), inulin (Krasaekoopt and Watcharapoka, 2014), pectin (Zhang et al., 2015), chitosan and starch (Noshad et al., 2015) and whey proteins (Jiang et al., 2016). Additionally, alginates, whey proteins and pectin have been used in combination with various types of biopolymers

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such as soy protein, chitosan, starch to further upgrade the protection of microorganisms (Gerez et al., 2012; Ragavan and Das, 2018). However, the application of cocoa powder in combination with fructooligosaccharides (FOS) as probiotic carries have not been previously investigated. The selection of the appropriate encapsulant matrixes and applying a suitable encapsulation technique are essential for a successful outcome. Various encapsulation techniques including spray drying, fluidized bed coating, freeze drying and extrusion are currently available (Soukoulis et al., 2014). Although the freeze drying encapsulation technique is more expensive than all other techniques due to the high energy consumption, it is considered as one of the most suitable techniques when encapsulating microorganisms and heat-sensitive ingredients, because moisture removes by sublimation through the technique without exposure to severe heat treatment (Ragavan and Das, 2018).

The probiotic *Lactobacillus rhamnosus* LGG has been successfully used in functional food formulations and its beneficial health effects have been well documented (Champagne et al., 2015; Succi et al., 2017). However, *L. rhamnosus* LGG has not been widely used in formulation of functional chocolate snacks and its functionality in chocolates are yet to be analysed. This study investigated the efficacy of cocoa powder and fructooligosaccharides (FOS) mixture as an encapsulant of probiotic bacteria and compared that mixture with other encapsulating agents (whey protein concentrate, hi-maize resistant starch, Na-alginate, skim milk powder). *L. rhamnosus* LGG was used as a probiotic and freeze-drying technique was applied for drying before adding this encapsulated probiotic to the chocolate. The survival of encapsulated probiotics in chocolate was assessed during storage and 4°C and 25°C (180 days) and when subjected to *in-vitro* gastrointestinal digestion and colonic fermentation. The thermal tolerance of encapsulated probiotics and total polyphenols and flavonoids in chocolate were also analysed.

MATERIALS AND METHODS

Materials

The *L. rhamnosus* LGG strain was kindly provided by Chr. Hansen, Bayswater, VIC, Australia. Na-alginate, Fructooligosaccharides-FOS, hi-maize resistant starch, skim milk powder and enzymes (salivary α-amylase, porcine pepsin, pancreatin), acetone, HCl, acetic acid were bought from Sigma Aldrich (NSW, Australia). Whey protein concentrate was gifted by Murray Goulburn Co-operative Co. Limited (Victoria, Australia). The nutrient agar & broth, selective media DeMan, Rogosa and Sharpe (MRS), AnaeroGen sachets, beef extract, yeast extract, protease peptone, bile salts, and trichloroacetic acid were purchased from Thermo Fisher (Thermo Fisher Scientific Pty Ltd, 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 acetate, aluminium chloride, potassium persulfate were procured from the Chem-Supply Pty Ltd, (Melbourne, Australia)

METHODS

Inoculum preparation

The *L. rhamnosus* LGG was inoculated in MRS broth (100 mL) and incubated anaerobically at 37°C for 22±2 h. The cells were collected after centrifugation for 15 min at 4°C and 5000×g (ALLEGRA X-12R, Beckman Coulter centrifuge, Australia), and washed twice using 0.85% saline before mixing with the encapsulating formulations and freeze drying (Hossain et al., 2021).

Blend formulations

The selected encapsulation formulations included: A_0 = Whey protein concentrate: Sodium alginate at 10:1 ratio; A_1 = Hi-maize starch: Sodium alginate (10:1); A_2 = Skim milk powder: Sodium alginate (10:1); A_3 = Cocoa powder: Sodium alginate (10:1) and A_4 = Cocoa powder: Sodium alginate: FOS (10:1:2). In the later section of this study, probiotic chocolate products were prepared and fortified with free and encapsulated *L. rhamnosus* LGG and designated as CA_0, CA_1, CA_2, CA_3 and CA_4 based on the formulations.

Encapsulation process

L. rhamnosus LGG was encapsulated in different types of formulations and an emulsion based freeze-drying technique was to freeze dry the sample according to method described by Hossain et al. (2021).

Viability of encapsulated probiotics

L. rhamnosus LGG viability was tested before and after encapsulation and during storage at 4°C and 25°C for 180 days. Samples were serially diluted with 0.1% sterile peptone water, plated on MRS selective medium and incubated anaerobically at 37°C for 48 h. The results were reported as log cfu/g (Hossain et al., 2021).

Encapsulation efficiency

The encapsulation efficiency calculates the survival rate of the probiotics after the encapsulation process (de Araújo Etchepare et al., 2020). The percentage encapsulation efficiency (% EE) was calculated as follows:

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

where A is the number of viable counts (log cfu/g) released after encapsulation and A0 is the number of counts (log cfu/g) before encapsulation.

Preparation of chocolate fortified with encapsulated probiotic

Chocolates were fortified with the encapsulated probiotic according to the method described by Hossain et al. (2021). Chocolate enriched with probiotic *L. rhamnosus* LGG and control samples were stored at 4°C and 25°C for 90 days. Probiotic counts, physical and chemical properties of probiotic chocolate and *in-vitro* bioaccessibility of *L. rhamnosus* LGG in probiotic chocolate were examined at 0, 30, 60 and 90 days.

Thermal tolerance of encapsulated probiotics

The thermostability of the encapsulated probiotics during chocolate processing was investigated according to the methods of Hossain et al. (2021). Inoculums of various encapsulated *L. rhamnosus* LGG was added to 70% dark chocolates at 1% concentration and mixed at 40°C, 50°C and 60°C and viable counts were recorded.

Scanning electron microscopy of the encapsulated *L. rhamnosus* LGG before and after adding to chocolate

The size and shape of the encapsulated probiotics in the freeze-dried formulation before and after added to chocolates were examined using a Field Emission Electron Probe Microanalyzer (FE-EPMA) (Hyperprobe JXA-8530F, JEOL Ltd., Japan) and a double-thickness gold coat (30 nm) to assist removal of heat over the imaged area according to the methods of Hossain et al. (2021).

Total polyphenols and flavonoids in chocolate enriched with probiotics

The freshly prepared chocolates (control and probiotic enriched) were grounded and approximately 0.5g of sample was taken and defatted three times with 5 mL of hexane and the residues were dried at 60°C following the method of Cooper et al. (2007). The final extracted supernatants were filtered through Whatman No. 1 filter papers and analysed for total polyphenols and flavonoids.

Total polyphenols and flavonoids assay

The total polyphenolic contents were estimated using the Folin–Ciocalteu method (Silva et al., 2017). A standard curve was generated using gallic acid following the same procedures, and results were calculated as mg of gallic acid equivalent per gram. The total flavonoids were also determined in the final filtrate following the methods of Kemsawasd et al. (2016) and Silva et al. (2017).

Survival of probiotics during simulated *in-vitro* gastrointestinal digestion of probiotic chocolate

Preparation of digestion fluids

The salivary, gastric and intestinal fluids for simulated gastrointestinal digestion were prepared following the method of Minekus et al. (2014). Each stock digestion fluid was prepared using the mixture of same electrolytes (Cl^-, K^+, Na^+, H_2PO_4, HCO_3, Mg^{2+}, NH^+ and Ca^{2+}) but at different concentrations. The pH of each fluid solution was adjusted using NaOH (1 mol L^{-1}) or HCl (6 mol L^{-1}).

In-vitro gastrointestinal digestion and colonic fermentation

The survival of probiotic bacteria during simulated gastrointestinal digestion of probiotic chocolate and free cultures (control) was evaluated using an *in-vitro* digestion model. The *in-vitro* digestion model consists of three-steps of gastrointestinal digestion involved sequentially digestion in mouth, stomach and small intestine as described by Minekus et al. (2014) with some modifications (Hossain et al., 2021). Samples were taken out at each stage of digestion to estimate probiotic bacteria and total counts. To avoid any kind of destruction during the digestion stages, triplicate samples were maintained for each treatment. The sample replicates were used for individual microbial analyses.

Colonic fermentation

Freshly voided feces were collected for fecal slurry preparation, basal medium were prepared according to the method described by Hossain et al. (2021). Colonic fermentation was carried out by mixing the gastrointestinal digested samples with fecal slurry at a 1:1 (v/v) ratio and incubated anaerobically at 37°C for 72 h. Aerobic and anaerobic counts were estimated after mixing (t=1 h) and every 24 h interval up to 72h of fermentation. The control sample was prepared using fecal slurry and basal medium 5 mL each only. Fig 1. represents a summary of the main steps during the preparation of chocolates fortified with probiotics and their analyses.

Statistical analysis

All the experiments were performed in triplicate and results were subjected to one way ANOVA using Minitab®19 statistical software, 2019. The means were separated using Tukey Honest significant difference (HSD) at 95% confidence level and demonstrated as mean ± standard deviation.

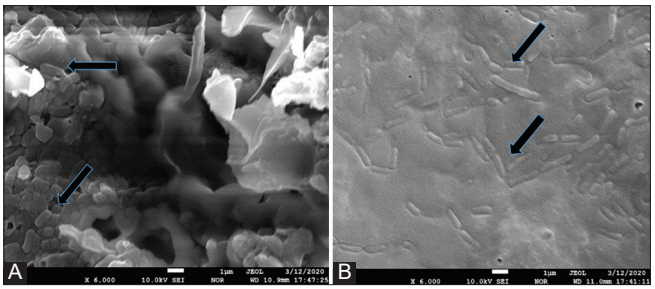

Fig 1. FE-EPMA photomicrographs of encapsulated bacteria (A) inside the chocolates (B) in the encapsulation formulations after freeze-drying.

RESULTS AND DISCUSSIONS

Percentage encapsulation efficiency of the probiotic L. rhamnosus LGG

The microencapsulation of *L. rhamnosus* LGG with various encapsulation formulations showed promising results in protecting the encapsulated bacteria (Table 1). The encapsulant mixture of cocoa powder: Sodium alginate: fructooligosaccharides at 10:1:2 ratio exhibited 84.60±0.28% encapsulation efficiency (EE), which was similar to the previous findings of Yasmin et al. (2019) who reported 85.49 % EE using whey protein concentrate with pectin. Additionally, results in Table 1 revealed that the combination of formulations using cocoa powder: Sodium alginate at 10:1 ratio showed significantly ($P < 0.05$) the highest encapsulation efficiency (91.82±0.52). (Xu et al. (2016) indicated that EE higher than 85% could be considered as successful and microencapsulation efficiency mostly depends on the amount of carbohydrates biopolymers and protein in the encapsulating materials. Consequently, most of the formulations listed in Table 1 meet these reported observations and could be considered good enough to provide a protective effect towards the probiotic cultures. The only exception was the cocoa, alginate and fructooligosaccharides formulation with 84.60% EE. This % EE was significantly lower ($P < 0.05$) than other formulations. However, the two new formulations used in this study [cocoa powder: Sodium alginate (10:1) and cocoa powder: Sodium alginate: FOS (10:1:2)] could be successfully used for probiotic encapsulation. The whey protein concentrate, skim milk and cocoa powder contain enough biopolymer and along with the above combinations, the cocoa powder could be used for probiotic bacteria microencapsulation.

Table 1: Encapsulation efficiency of various encapsulant formulations

Formulations	Compositions	% Encapsulation efficiency (EE)
*A_0	Whey protein concentrate: Na-alginate (10:1)	91.63 ± 0.30^{A}
A_1	Hi-maize starch: Na-alginate (10:1)	87.42 ± 0.57^{B}
A_2	Skim milk powder: Na-alginate (10:1)	91.62 ± 0.26^{A}
A_3	Cocoa powder: Na-alginate (10:1)	91.82 ± 0.52^{A}
A_4	Cocoa powder: Na-alginate: FOS (10:1:2)	84.60 ± 0.28^{C}

Means followed by different superscript letters indicate significant differences ($P<0.05$).
Sample number for each formulation, n=5
*A_0 - A_4 refers to types of encapsulant formulations

Viability of probiotic in various encapsulant mixtures during an extended storage time

The viability of encapsulated probiotic *L. rhamnosus* LGG was evaluated under two different temperatures (4°C and 25°C) upto 180 days of storage (Table 2). The results showed that irrespective of formulations, the viable count persisted above 8.0 log cfu/g for 180 days when stored at 4°C (Table 2A). These results meet the guidelines proposed by the International Dairy Federation (10^7 cfu/g or mL) to reveal the health benefits (Dong et al., 2013). However, Table 2B indicated the samples stored at 25°C sustained the required viable number of $>10^7$ cfu/g, but upto 120 days only. It was obvious that storage condition specially temperature can significantly ($P < 0.05$) affected the viability of encapsulated probiotics. These findings

Table 2: Impact of various encapsulants on *L. rhamnosus* LGG counts during storage at different temperatures

Duration (days)	(A) Log CFU/g at 4 ºC					
	Free Cell (FC)	*A_0	A_1	A_2	A_3	A4
0	12.66 ± 0.19^{Aa}	11.60 ± 0.31^{Ab}	11.07 ± 0.09^{Ac}	11.60 ± 0.27^{Ab}	11.63 ± 0.06^{Ab}	10.71 ± 0.17^{Ad}
30	10.72 ± 0.07^{Bcd}	10.86 ± 0.06^{Bbc}	10.59 ± 0.05^{Bd}	11.02 ± 0.13^{Bb}	11.27 ± 0.06^{Ba}	10.28 ± 0.39^{Be}
60	7.44 ± 0.36^{Ce}	10.32 ± 0.31^{Cc}	10.28 ± 0.11^{Cc}	10.59 ± 0.25^{Cb}	10.96 ± 0.09^{Ca}	10.00 ± 0.10^{Cd}
90	4.28 ± 0.40^{Dd}	9.81 ± 0.23^{Dbc}	9.89 ± 0.09^{Dbc}	9.92 ± 0.49^{Db}	10.16 ± 0.44^{Da}	9.70 ± 0.57^{Dc}
120	1.59 ± 0.28^{Ec}	9.70 ± 0.53^{Da}	9.63 ± 0.23^{Ea}	9.59 ± 0.08^{Da}	9.69 ± 0.42^{Ea}	9.18 ± 0.13^{Eb}
150	<1	9.55 ± 0.09^{Da}	9.45 ± 0.35^{Ea}	9.56 ± 0.11^{Da}	8.77 ± 0.10^{Fb}	8.61 ± 0.10^{Fb}
180	<1	8.97 ± 0.14^{Ea}	9.00 ± 0.10^{Fa}	9.16 ± 0.20^{Ea}	7.77 ± 0.08^{Gc}	8.36 ± 0.40^{Gb}
Duration (days)	**(B) Log CFU/g at 25 ºC**					
	Free Cell (FC)	*A_0	A_1	A_2	A_3	A_4
0	12.12 ± 0.22^{Aa}	11.60 ± 0.10^{Ab}	11.05 ± 0.12^{Acd}	11.50 ± 0.07^{Abc}	$11.43\pm^{Abc}$	10.78 ± 0.14^{Ad}
30	8.68 ± 0.46^{Ba}	9.21 ± 0.06^{Ba}	9.22 ± 0.09^{Ba}	9.29 ± 0.13^{Ba}	9.36 ± 0.08^{Ba}	9.18 ± 0.07^{Ba}
60	6.13 ± 0.30^{Cab}	8.81 ± 0.11^{Ba}	8.92 ± 0.06^{Ba}	8.91 ± 0.11^{BCa}	8.97 ± 0.09^{Ba}	8.85 ± 0.06^{Ba}
90	3.83 ± 0.32^{Db}	8.45 ± 0.10^{Ba}	8.34 ± 0.14^{BCa}	8.66 ± 0.25^{BCa}	8.44 ± 0.19^{BCa}	8.25 ± 0.36^{Ba}
120	1.65 ± 0.32^{Eb}	7.10 ± 0.07^{Ca}	7.73 ± 0.51^{Ca}	7.89 ± 0.49^{Ca}	7.88 ± 0.20^{Ca}	7.16 ± 0.18^{Ca}
150	<1	5.78 ± 0.70^{Dab}	5.79 ± 0.50^{Dab}	6.56 ± 0.70^{Da}	5.44 ± 0.49^{Dab}	4.28 ± 0.50^{Db}
180	<1	2.05 ± 0.27^{Eb}	2.67 ± 0.48^{Eab}	3.82 ± 0.35^{Ea}	3.10 ± 0.54^{Eab}	3.03 ± 0.48^{Eab}

Means followed by different uppercase letters within the same column indicate significant differences ($P < 0.05$).
Means followed by different lowercase letters within the same row indicate significant differences ($P < 0.05$).
*A_0 - A_4 refers to the types of encapsulant formulations in Table 1

were in agreement with the report by de Araújo Etchepare et al. (2020) who indicated that whey protein concentrate with alginate protect the probiotic cells and maintained more than 9.0 log cfu/g for upto 120 days. The another observation by Yasmin et al. (2019) indicated that more than 7.0 log cfu/g viability encapsulated with whey protein concentrate. The reduction rate of encapsulated *L. rhamnosus* LGG during storage for 180 days disclosed a smaller log decline at 4°C compared with 25°C. The numbers of log reduction after 180 days of storage at 4°C for A_0, A_1, A_2, A_3 and A_4 encapsulants were 2.63, 2.06, 2.44, 3.86 and 3.35 log cfu/g respectively. A similar phenomenon of storage temperature on the survival of encapsulated probiotics was published by Lalicic-Petronijevic et al. (2015). The data in Table 2 showed also that the decline in the number of probiotics in the control samples (unencapsulated) was significantly ($P < 0.05$) higher than the encapsulated bacteria. At least a two-thirds decline in the count of control sample was recorded after 90 days of storage at 25°C, and the count reached <1 cfu after 180 days of storage (Table 2A).

Comparing the protective effects of all formulations within the same treatments and storage conditions indicated that all encapsulants performed well and were able to protect the probiotics. However, the most protecting formulations that maintained the highest count after 180 days of storage at 4°C were A_2, followed by A_1, A_0, A_4 and lastly A_3. Similarly, observations recorded at 25°C revealed that A_2 encapsulant was the best-protecting encapsulant. Although skim milk with Na-alginate (A_2) was the best encapsulant at both storage temperatures (4°C and 25°C), but these results proved that cocoa powder and sodium alginate with and without FOS could be a promising probiotic encapsulant when adding probiotics to chocolate (Wu et al., 2015).

Thermostability of encapsulated probiotic in chocolates

The thermal tolerance of the probiotics in chocolate with 45% and 70% cocoa powder was almost identical, hence only the data for 70% dark chocolates were presented in this manuscript. Chocolate preparation usually involves treating the mixture at a temperature >45°C for melting and mixing purposes (Klindt-Toldam et al., 2016; Silva et al., 2017). To cover a wide range of thermal stability on encapsulated *L. rhamnosus* LGG, all formulations were added to chocolate at 1% and examined at 37°C, 40°C, 50°C and 60°C temperatures. *L. rhamnosus* LGG culture (not encapsulated) was used as a control. Results in Table 3 indicated that the encapsulated *L. rhamnosus* LGG counts were not influenced at 37°C and 40°C ($P > 0.05$). Besides, the encapsulated *L. rhamnosus* LGG tolerated heat at 50°C and 60°C during chocolate preparation. The survived counts at 60°C were 9.41±0.13, 9.13±0.5, 10.15±0.24, 9.56±0.15 and 10.24±0.10 log cfu/g for CA_0, CA_1, CA_2, CA_3, and CA_4, respectively. The maximum reduction in the *L. rhamnosus* LGG counts at 60°C was for CA_1 formulation and compared with the counts of free culture was significant ($P < 0.05$) and reached a maximum of 8.93 log cfu/g. Table 3 shows that the initial count in the free culture (12.39±0.14 log cfu/ml) was reduced to 3.40±0.52 when subjected to 60°C. These results confirmed the efficacy of the applied formulations in protecting *L. rhamnosus* LGG during chocolate processing and were supported by previous findings of Kemsawasd et al. (2016) and Rad et al. (2016). Thes authors indicated that using carboxymethylcellulose (CMC), pectin or starch, and Na-alginate coating materials improved the viability of immobilised probiotics in chocolates and proved that chocolates could be used as a carrier for probiotics. The thermal stability at 60°C using encapsulation formulations CA_2, CA_3 and CA_4 were significantly greater ($P < 0.05$) than other combinations of encapsulation formulations. These findings were corelated with previous study by de Araújo Etchepare et al. (2020) who reported that the layer of encapsulating formulation around the cells wasn't damaged while mixing at 60°C. These results ensured that the encapsulated probiotic could be tolerated stress while processing chocolates at temperature as high as 60°C.

Morphology of the encapsulated probiotics in chocolate using Scanning Electron Microscopy (FE-EPMA)

The SEM images (x6000) of the encapsulated probiotic clearly showed that the bacterial cultures were incorporated into chocolates (Fig 2-A) and in the matrices of the freeze-dried formulation (Fig 2-B). The rod shape bacterial cultures were homogeneously distributed and despite of the formulations, the morphology of encapsulated bacteria was almost the same. These results were disagreed

Table 3: Thermostability of *L. rhamnosus* LGG in various encapsulants added to 70% dark chocolates at different temperatures

Temp (ºC)	Log CFU/g					
	Free Cell (FC)	*CA_0	CA_1	CA_2	CA_3	CA_4
37	12.39±0.14^{Aa}	10.67±0.07^{Ca}	10.49±0.06^{Ca}	11.51±0.07^{Ba}	10.67±0.04^{Ca}	11.20±0.20^{Ba}
40	12.03±0.12^{Aa}	10.53±0.14^{CDab}	10.21±0.16^{Dab}	11.08±0.13^{Bab}	10.29±0.10^{Db}	10.93±0.13^{BCa}
50	8.25±0.10^{Db}	10.06±0.24^{BCb}	9.92±0.06^{Cb}	10.92±0.16^{Ab}	9.94±0.05^{Cc}	10.42±0.07^{Bb}
60	3.40±0.52^{Cc}	9.41±0.13A^{Bc}	9.13±0.15^{Bc}	10.15±0.24^{Ac}	9.56±0.15A^{Bd}	10.24±0.10^{Ab}

Means followed by different uppercase letters within the same row indicate significant differences (P < 0.05).
Means followed by different lowercase letters within the same column indicate significant differences (P < 0.05).
*CA_0-CA_4 refers to prepared chocolates fortified with encapsulated L. rhamnosus LGG using formulations in Table 1

with the research de Araújo Etchepare et al. (2020) who noted that the freeze drying process caused the shrinkage of the bacterial cell due to water reduction during the process. These SEM images confirm the fact that cocoa powder as an encapsulants could be appropriate for probiotic carriers.

Impact of different levels of cocoa powder in chocolates on the viability of probiotics during storage

Chocolates containing 70% cocoa were fortified with 1% encapsulated *L. rhamnosus* LGG and stored at temperatures 25°C and 4°C for 90 days. The results in Table 4 showed that more than 7.0 log cfu/g viable probiotic remained at both temperatures up to 90 days in chocolates. These results meet the recommendation of the International Dairy Federation (10^7 logs cfu/ml or g) for a good probiotic product (Dong et al., 2013). The log number reduction during the 90 days of storage at 4°C was 3.62, 2.82, 2.83, 2.95 and 2.93 in CA_0, CA_1, CA_2, CA_3 and CA_4, respectively. The formulation A_2 in chocolate (CA_2) revealed the highest count after 90 days of storage at 4°C with 7.99±0.29 log cfu/g. On the contrary, CA_0 had more pronounced effects on the reduction of bacterial counts at both 4°C and 25°C and showed the smallest counts of 7.41±08 and 6.98±0.24 respectively after 90 days of storage. Consequently, the decline rate in chocolate containing free bacteria (FC) was very significant ($P < 0.05$) and declined to 3.4±0.56 and 2.73±0.1 at both storage conditions.

The persistence of probiotic counts in chocolates with 45% cocoa powder had a similar trend to those reported with 70% cocoa powder (Table 4C,4D). The viable counts after 90 days of storage at 4°C were the highest (7.99±0.43 log cfu/g) for CA_2 as compared to the highest for CA_4 (7.37±0.10 log cfu/g) at 25°C. Those results showed that the viable counts at 25°C were slightly smaller than that at 4°C. However, as the highest difference in final counts at 4°C and 25°C was < 1 log after 90 days of storage, it can be exhorted that chocolates fortified with encapsulated probiotics can be stored at both temperature. Additionally, all the two types of chocolates (45% & 70% cocoa powder) conserved total probiotic counts ($P > 0.05$) above the recommended level (10^7 cfu/g).

Total polyphenol and flavonoid contents in chocolate

Total polyphenols and flavonoids contents in chocolates fortified with encapsulated probiotic and control were compared also chocolates containing 70% and 45% cocoa powder (Table 5). The polyphenol contents in the control and the chocolate enriched with probiotics were similar ($P > 0.05$). The polyphenols contents in 70% dark chocolate were 4.45±0.85, 4.32±0.79, 4.31±0.78,

Table 4: Viable count of encapsulated *L. rhamnosus* LGG in chocolates (70% and 45% cocoa powder) during storage at 4 □C and 25 °C for 90 days

Duration (days)	(A) Log CFU/g at 4 ºC (70% cocoa)					
	Free Cell (FC)	*CA_0	CA_1	CA_2	CA_3	CA_4
0	11.08±0.07^{Aa}	11.03±0.20^{ABa}	10.24±0.09^{Da}	10.82±0.27^{ABCa}	10.38±0.13^{CDa}	10.52±0.08^{BCDa}
30	8.49±0.14^{Db}	10.09±0.20^{Ab}	9.22±0.15^{Cb}	9.80±0.19^{ABb}	9.47±0.80^{BCb}	9.34±0.17^{BCb}
60	5.78±0.54^{Bc}	8.98±0.19^{Ac}	8.57±0.17^{Ac}	9.35±0.23^{Ac}	8.85±0.15^{Ac}	8.75±0.14^{Ac}
90	3.40±0.56^{Bd}	7.41±0.18^{Ad}	7.42±0.17^{Ad}	7.99±0.29^{Ad}	7.43±0.52^{Ad}	7.58±0.20^{Ad}
Duration (days)	**(B) Log CFU/g at 25 ºC (70% cocoa)**					
	Free Cell (FC)	***CA_0**	**CA_1**	**CA_2**	**CA_3**	**CA_4**
0	11.08±0.07^{Aa}	11.03±0.20^{Aba}	10.24±0.09^{Da}	10.82±0.27^{ABCa}	10.38±0.13^{CDa}	10.52±0.08^{BCDa}
30	8.23±0.06^{Bb}	9.89±0.38^{Ab}	9.02±0.15^{ABb}	9.46±0.47^{Ab}	9.13±0.29^{ABb}	8.90±0.34^{ABb}
60	4.45±0.26^{Cc}	8.51±0.29^{ABc}	8.00±0.19^{Bc}	8.95±0.21^{Ac}	8.26±0.30^{ABc}	8.35±0.35^{ABc}
90	2.73±0.10^{Bd}	6.98±0.24^{Ad}	7.01±0.26^{Ad}	7.32±0.19^{Ad}	7.09±0.07^{Ad}	7.37±0.11^{Ad}
Duration (days)	**(C) Log CFU/g at 4 ºC (45% cocoa)**					
	Free Cell (FC)	***CA_0**	**CA_1**	**CA_2**	**CA_3**	**CA_4**
0	11.18±0.14^{Aa}	10.98±0.20^{ABa}	10.31±0.12^{Ac}	10.92±0.14^{Aba}	10.41±0.13^{Ac}	10.53±0.06^{Abc}
30	8.53±0.31^{Bb}	9.83±0.37^{Ba}	9.36±0.12^{Bab}	10.00±0.28^{Ba}	9.85±0.21^{ABa}	9.44±0.17^{Ba}
60	5.78±0.70^{Cb}	9.17±0.32^{Ba}	8.66±0.12^{Ca}	9.55±0.11^{Ba}	9.19±0.32^{Ba}	9.02±0.17^{Ba}
90	3.50±0.09^{Db}	7.38±0.20^{Ca}	7.45±0.17^{Da}	7.99±0.43^{Ca}	7.45±0.25^{Ca}	7.41±0.14^{Ca}
Duration (days)	**(D) Log CFU/g at 25 ºC (45% cocoa)**					
	Free Cell (FC)	***CA_0**	**CA_1**	**CA_2**	**CA_3**	**CA_4**
0	11.16±0.07^{Aa}	11.10±0.27^{Aa}	10.38±0.22^{Ab}	10.82±0.35^{ABa}	10.48±0.15^{Aab}	10.62±0.08^{Aab}
30	8.18±0.25^{Bb}	10.10±0.24^{Ba}	9.29±0.63^{ABa}	9.66±0.25^{Ba}	9.36±0.20^{Ba}	9.30±0.21^{Ba}
60	4.55±0.42^{Cb}	8.48±0.36^{Ca}	8.20±0.32^{BCa}	9.15±0.09^{Ba}	8.49±0.13^{Ca}	8.59±0.37^{Ba}
90	2.87±0.58^{Db}	7.21±0.11^{Da}	6.93±0.27^{Ca}	7.08±0.36^{Ca}	7.15±0.08^{Da}	7.37±0.10^{Ca}

Means followed by different lowercase letters within the same column indicate significant differences (P < 0.05).
Means followed by different uppercase letters within the same row indicate significant differences (P < 0.05).
*CA_0-CA_4 refers to prepared chocolates fortified with encapsulated L. rhamnosus LGG using formulations in Table 1

4.22±0.48, 4.49±0.83 and 4.49±0.82 mg GAE/g equivalent in the control, A_0, A_1, A_2, A_3 and A_4, respectively. However, the total polyphenols content in control and fortified chocolates samples were not differ significantly ($P > 0.05$). For example, the control chocolate samples showed 4.45±0.85 and 2.92±0.71 mg GAE/g equivalent in the 70% and 45% dark chocolate respectively. Similar differences were also observed in all chocolate samples containing 45% cocoa powder.

The flavonoids contents in 70% dark chocolates were 320.70±47.9, 283.3±23.9, 271.0±32.6, 270.0±28.24, 324.0±46.4 and 320.7±4.25 mg QE/100g equivalent in the control, A_0, A_1, A_2, A_3 and A_4, respectively (Table 5). Similar to the observations noted in polyphenol contents, no significant ($P > 0.05$) differences in flavonoid contents were detected among all treatments (control and chocolate enriched with probiotics). These results suggested that total polyphenols and flavonoids were not affected by enriching the chocolate with probiotics. Variations in the contents of polyphenols and flavonoids when comparing 70% and 45% dark chocolate can be attributed to the differences in cocoa contents as cocoa powder is the main ingredient in chocolate.

Impact of *in-vitro* gastrointestinal digestion and colonic fermentation on L. rhamnosus LGG survival in probiotic chocolate

The initial *L. rhamnosus* LGG counts in the chocolate samples (FC, CA_0, CA_1, CA_2, CA_3 and CA_4) before *in-vitro* digestion were 11.44±0.18, 11.19±0.09, 10.94±0.06, 11.35±0.19, 10.79±0.15 and 10.92±0.24 log cfu/g, respectively (Table 6). The count of probiotic in chocolates containing free cell (control) exhibited a significant ($P < 0.05$) decline of 6.0 log units when investigated to simulated gastric juice for 120 min whereas fortified chocolates presented a decrease of only 3.01, 2.62, 2.94, 2.59 and 2.61 log unit for CA_0, CA_1, CA_2, CA_3 and CA_4, respectively. The significantly lower log reduction of probiotics in chocolate indicated that the encapsulating formulations had a protective effect on the probiotic during the *in-vitro* digestion and agreed with some previous reports (Brinques and Ayub, 2011; Khorasani and Shojaosadati, 2017). These authors reported that immobilising probiotics using Carboxymethylcellulose (CMC), pectin with or with starch, and Na-alginate coating significantly improved the cell viability in SGI conditions. For the intestinal digestion, the count was quite persistant due to favourable growth conditions compared to adverse gastric environment. Among the formulations, the $CA_{2\ sample}$ (skim milk powder

Table 5: Total polyphenol (A) and flavonoid (B) contents in plain (control) and probiotic enriched chocolates

Chocolate type	(A) Total polyphenols reported as (mg GAE/g)					
	Control chocolates	*CA0	CA1	CA2	CA3	CA4
70% cocoa	4.45±0.85Aa	4.32±0.79Aa	4.31±0.78Aa	4.22±0.48Aa	4.49±0.83Aa	4.49±0.82Aa
45% cocoa	2.92±0.71Ab	2.81±0.64Ab	2.73±0.60Ab	2.94±0.24Ab	3.01±0.43Ab	2.82±0.71Ab
Chocolate type	**(B) Total flavonoids reported as (mg QE/100g)**					
	Control chocolates	***CA0**	**CA1**	**CA2**	**CA3**	**CA4**
70% cocoa	320.7±47.9Aa	283.3±23.9Aa	271.0±32.6Aa	270.0±28.24Aa	324.0±46.4Aa	320.7±4.25Aa
45% cocoa	206.6±24.9Ab	184.9±20.1Ab	173.5±7.9Ab	177.0±21.1Ab	207.2±24.2Ab	195.90±8.55Ab

Values represent the means (n = 3) followed by Sd
Means followed by different lowercase letters within the same column indicate significant differences ($P < 0.05$).
Means followed by different uppercase letters within the same row indicate significant differences ($P < 0.05$).
*CA_0-CA_4 refers to prepared chocolates fortified with encapsulated L. rhamnosus LGG using formulations in Table 1

Table 6: Viability (log CFU/g) of *L. rhamnosus* LGG in various encapsulants added to dark chocolates (70% cocoa powder) after in vitro gastrointestinal digestion and colonic fermentation

Time (h)	(A) Log CFU/g after gastrointestinal digestion					
	Free Cell (FC)	*CA0	CA1	CA2	CA3	CA4
0	11.44±0.18Aa	11.19±0.09ABa	10.94±0.06ABa	11.35±0.19Aa	10.79±0.15Ba	10.92±0.24ABa
2	5.36±0.27Bb	8.18±0.13Ab	8.31±0.22Ab	8.41±0.23Ab	8.19±0.13Ab	8.31±0.12Ab
4	4.70±0.43Bb	8.13±0.22Ab	8.28±0.18Ab	8.56±0.26Ab	8.47±0.22Ab	8.42±0.24Ab
Time (h)	**(B) Log CFU/g after colonic fermentation**					
	Free Cell (FC)	***CA0**	**CA1**	**CA2**	**CA3**	**CA4**
1	4.79±0.08Dd	8.29±0.06BCc	8.15±0.07Cc	8.61±0.10Ad	8.18±0.04Cc	8.47±0.09ABc
24	6.59±0.37Bc	10.47±0.20Ab	10.25±0.19Ab	10.70±0.06Ab	10.27±0.15Aab	10.11±0.12Abc
48	8.67±0.27Ba	11.21±0.21Aa	11.07±0.22Aa	11.24±0.19Aa	10.89±0.24Aa	11.08±0.42Aa
72	8.71±0.35Bb	10.07±0.19Ab	10.00±0.21Ab	10.17±0.16Ac	10.08±0.33Ab	10.23±0.14Ab

Means followed by different lowercase letters within same column indicate significant differences ($P < 0.05$).
Means followed by different uppercase letters within same row indicate significant differences ($P < 0.05$).
*CA_0-CA_4 refers to prepared chocolates fortified with encapsulated L. rhamnosus LGG using formulations in Table 1

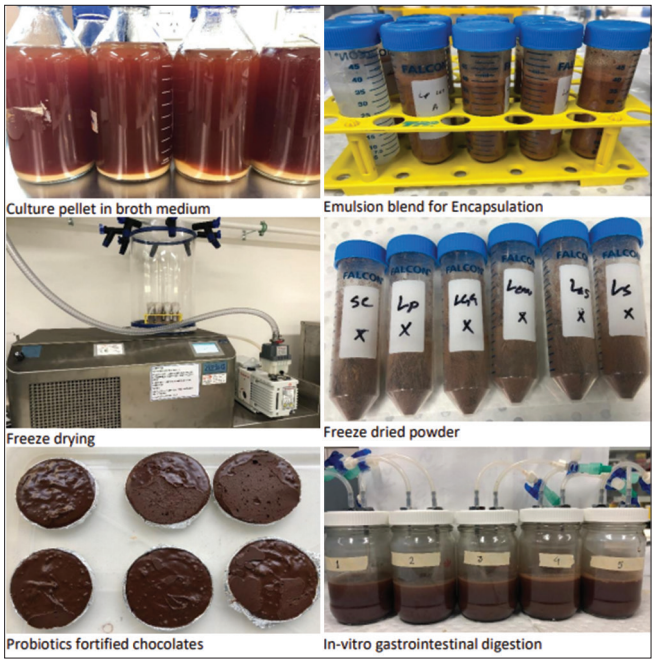

Fig 2. Photos showing the main steps in the preparation of probiotic fortified chocolates and analyses.

with Na-alginate) count was the highest during the *in-vitro* gastrointestinal digestion stage while cocoa powder, FOS and Na-alginate combination (CA_4) showed the second-highest count (Table 6).

Results from colonic fermentation showed an increased number of lactic acid bacteria by at least 2 log for the first 24 h of fermentation and counts reached highest at 48 h (11.24±0.19 cfu/g) for CA_2 formulation followed by a decline in counts after 72 h. Similar observations (Khorasani and Shojaosadati, 2016; Krunic et al., 2019) who reported increment during the colonic fermentation by 94.76% and 96%, respectively. The results demonstrated that partially damaged cultures during exposure to the gastric environment with extremely low pH (3.0) were able to recover when the pH increased to 7.0 during colonic fermentation. Consequently, all analysed encapsulant formulations were able to protect the encapsulated bacteria under the harsh acidic condition and prevent them from death. These results were corelated with some reported findings (Sandoval-Castilla et al., 2010; Zhang et al., 2015) who observed rapid recovery of bacteria upon improvement in the growth conditions. Whey protein concentrate, hi-maize resistant starch and skim milk powder with Na-alginate have been widely used previously for microbial encapsulation (Braber et al., 2020; de Araújo Etchepare et al., 2020). However, results from the current investigation confirmed that cocoa powder could be used for microencapsulation as all the tested encapsulant formulations exhibited similar results ($P > 0.05$).

CONCLUSION

The probiotic *L. rhamnosus* LGG was successfully encapsulated with cocoa powder along with sodium alginate and fructoseoligosccharide (FOS). The cocoa powder with sodium alginate and cocoa powder with sodium alginate and FOS showed 91.82% and 84.60% encapsulation efficacy, respectively. Additionally, the cocoa powder with sodium alginate combination exhibited a positive impact on the viability of the probiotics during storage, resisted thermal exposure and maintained the highest probiotic counts after the *in vitro* gastrointestinal digestion and fermentation. The probiotic viability in chocolate was higher than the recommended level (10^7 log) during the storage period of 180 days at 4°C and 90 days at 25°C. The thermal tolerance of the encapsulated probiotic was recorded at 60°C. All tested encapsulation materials were able to protect the probiotics during the *in-vitro* gastrointestinal digestion. The total polyphenol and flavonoid contents remained stable and were not affected by enriching the chocolate with probiotics. These findings suggested that cocoa powder with sodium alginate encapsulation blends could be a potential candidate in the development of functional probiotic chocolates.

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Author's contributions

The manin project conceptualization was done by MNH and SA. Methodology, formal analysis, investigation, data curation and original draft writing by MNH. Supervision, review and editing, and instructions by SA, CSR, GH and ZF. All authors have read and aggreed to the published version of the manuscript.

Conflicts of interests

The authors declare no conflict of interest.

Compliance with ethical standard

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved (ethical approval ID: 1954660.1) by the Human Ethics Advisory Group at the Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Australia.

REFERENCES

Afzaal, M., F. Saeed, M. U. Arshad, M. T. Nadeem, M. Saeed and T. Tufail. 2019. The effect of encapsulation on the stability of probiotic bacteria in ice cream and simulated gastrointestinal conditions. Probiotic. Antimicrob. 11: 1348-1354.

Braber, N. V., L. D. Vergara, Y. Rossi, C. Aminahuel, A. Mauri, L. Cavaglieri and M. Montenegro. 2020. Effect of microencapsulation in whey protein and water-soluble chitosan derivative on the viability of the probiotic *Kluyveromyces marxianus* VM004 during storage and in simulated gastrointestinal conditions. LWT. 118: 108844.

Brinques, G. B. and M. A. Z. Ayub. 2011. Effect of microencapsulation on survival of *Lactobacillus plantarum* in simulated gastrointestinal conditions, refrigeration, and yogurt. J. Food Eng. 103: 123-128.

Champagne, C. P., A. G. da Cruz and M. Daga. 2018. Strategies to improve the functionality of probiotics in supplements and foods, Curr. Opin. Food Sci. 22: 160-166.

Champagne, C. P., Y. Raymond, N. Guertin and G. Belanger. 2015. Effects of storage conditions, microencapsulation and inclusion in chocolate particles on the stability of probiotic bacteria in ice cream. Int. Dairy J. 47: 109-117.

Cooper, K. A., E. Campos-Giménez, D. Jiménez Alvarez, K. Nagy, J. L. Donovan and G. Williamson. 2007. Rapid reversed phase ultra-performance liquid chromatography analysis of the major cocoa polyphenols and inter-relationships of their concentrations in chocolate. J. Agric. Food Chem. 55: 2841-2847.

de Araújo Etchepare, M., G. L. Nunes, B. R. Nicoloso, J. S. Barin, E. M. M. Flores, R. de Oliveira Mello and C. Ragagnin de Menezes. 2020. Improvement of the viability of encapsulated probiotics using whey proteins. LWT. 117: 108601.

de Araújo Etchepare, M., G. C. Raddatz, É. M. de Moraes Flores, L. Q. Zepka, E. Jacob-Lopes, J. S. Barin, C. R. F. Grosso and C. R. de Menezes. 2016. Effect of resistant starch and chitosan on survival of *Lactobacillus acidophilus* microencapsulated with sodium alginate. LWT. 65: 511-517.

Dong, Q. Y., M. Y. Chen, Y. Xin, X. Y. Qin, Z. Cheng, L. E. Shi and Z. X. Tang. 2013. Alginate-based and protein-based materials for probiotics encapsulation: A review. Int. J. Food Sci. Technol. 48: 1339-1351.

FAO/WHO. 2002. Guidelines for the Evaluation of Probiotics in Food. Report of a Joint FAO/WHO Working Group on Drafting Guidelines for the Evaluation of Probiotics in Food. London, Ontario, Canada. Available from: https://www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf. [Last access on 2021 Aug 12].

Gerez, C. L., G. Font de Valdez, M. L. Gigante and C. Grosso. 2012. Whey protein coating bead improves the survival of the probiotic *Lactobacillus rhamnosus* CRL 1505 to low pH. Lett. Appl. Microbiol. 54: 552-556.

Hill, C., F. Guarner, G. Reid, G. R. Gibson, D. J. Merenstein, B. Pot, L. Morelli, R. B. Canani, H. J. Flint, S. Salminen, P. C. Calder and M. E. Sanders. 2014. Expert consensus document. The international scientific association for probiotics and prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat. Rev. Gastroenterol. 11: 506-514.

Hossain, M. N., C. S. Ranadheera, Z. Fang and S. Ajlouni. 2020. Healthy chocolate enriched with probiotics: A review. Food Sci. Tech. Brazil. 40: 1-13.

Hossain, M. N., C. S. Ranadheera, Z. Fang and S. Ajlouni. 2021. Impact of encapsulating probiotics with cocoa powder on the viability of probiotics during chocolate processing, storage, and *in vitro* gastrointestinal digestion. J. Food Sci. 86: 1629-1641.

Jiang, Y., Z. Zheng, T. Zhang, G. Hendricks and M. Guo. 2016. Microencapsulation of *Lactobacillus acidophilus* NCFM using polymerized whey proteins as wall material. Int. J. Food Sci. Nutr. 67: 670-677.

Kemsawasd, V., P. Chaikham and P. Rattanasena. 2016. Survival of immobilized probiotics in chocolate during storage and with an *in vitro* gastrointestinal model. Food Bioscience. 16: 37-43.

Khorasani, A. C. and S. A. Shojaosadati. 2016. Bacterial nanocellulose-pectin bionanocomposites as prebiotics against drying and gastrointestinal condition. Int. J. Biol. Macromol. 83: 9-18.

Khorasani, A. C. and S. A. Shojaosadati. 2017. Starch-and carboxymethylcellulose-coated bacterial nanocellulose-pectin bionanocomposite as novel protective prebiotic matrices. Food Hydrocolloid. 63: 273-285.

Klindt-Toldam, S., S. K. Larsen, L. Saaby, L. R. Olsen, G. Svenstrup, A. Müllertz, S. Knøchel, H. Heimdal, D. S. Nielsen and D. Zielińska. 2016. Survival of *Lactobacillus acidophilus* NCFM ® and *Bifidobacterium lactis* HN019 encapsulated in chocolate during in vitro simulated passage of the upper gastrointestinal tract. LWT. 74: 404-410.

Krasaekoopt, W. and S. Watcharapoka. 2014. Effect of addition of inulin and galactooligosaccharide on the survival of microencapsulated probiotics in alginate beads coated with chitosan in simulated digestive system, yogurt and fruit juice. LWT. 57: 761-766.

Krunic, T. Z., N. S. Obradovic and M. B. Rakin. 2019. Application of whey protein and whey protein hydrolysate as protein based carrier for probiotic starter culture. Food Chem. 293: 74-82.

Lalicic-Petronijevic, J., J. Popov-Raljic, D. Obradovic, Z. Radulovic, D. Paunovic, M. Petrusic and L. Pezo. 2015. Viability of probiotic strains *Lactobacillus acidophilus* NCFM (R) and *Bifidobacterium lactis* HN019 and their impact on sensory and theological properties of milk and dark chocolates during storage for 180 days. J. Funct. Foods. 15: 541-550.

Mani-López, E., E. Palou and A. López-Malo. 2014. Probiotic viability and storage stability of yogurts and fermented milks prepared with several mixtures of lactic acid bacteria. J. Dairy Sci. 97: 2578-2590.

Martín, M. J., F. Lara-Villoslada, M. A. Ruiz and M. E. Morales. 2015. Microencapsulation of bacteria: A review of different technologies and their impact on the probiotic effects. Innov. Food Sci. Emerg. Technol. 27: 15-25.

Minekus, M., M. Alminger, P. Alvito, S. Ballance, T. Bohn, C. Bourlieu, F. Carriere, R. Boutrou, M. Corredig, D. Dupont, C. Dufour, L. Egger, M. Golding, S. Karakaya, B. Kirkhus, S. Le Feunteun, U. Lesmes, A. Macierzanka, A. Mackie, S. Marze, D. J. McClements, O. Menard, I. Recio, C. N. Santos, R. P. Singh, G. E. Vegarud, M. S. Wickham, W. Weitschies and A. Brodkorb. 2014. A standardised static *in vitro* digestion method suitable for food an international consensus. Food Funct. 5: 1113-1124.

Noshad, M., M. Mohebbi, F. Shahidi and A. Koocheki. 2015. Effect of layer-by-layer polyelectrolyte method on encapsulation of vanillin. Int. J. Biol. Macromol. 81: 803-808.

Rad, A. H., M. M. Roudbaneh, V. G. Tabrizian, M. Javadi, N. Harati, H. N. Rad and Z. Kasaie. 2016. Chocolates as a probiotic carrier food a review. Int. J. Probiotic Prebiotics. 11: 37-43.

Ragavan, M. L. and N. Das. 2018. Process optimization for microencapsulation of probiotic yeasts. Front. Biol. 13: 197-207.

Ranadheera, C. S., N. Naumovski and S. Ajlouni. 2018. Non-bovine milk products as emerging probiotic carriers: Recent developments and innovations. Curr. Opin. Food Sci. 22: 109-114.

Sandoval-Castilla, O., C. Lobato-Calleros, H. García-Galindo, J. Alvarez-Ramírez and E. Vernon-Carter. 2010. Textural properties of alginate–pectin beads and survivability of entrapped *L. casei* in simulated gastrointestinal conditions and in yoghurt. Food Res. Int. 43: 111-117.

Silva, M. P., F. L. Tulini, J. F. Marinho, M. C. Mazzocato, E. C. De Martinis, V. Luccas and C. Favaro-Trindade. 2017. Semisweet chocolate as a vehicle for the probiotics *Lactobacillus*

acidophilus LA3 and *Bifidobacterium animalis* subsp. *lactis* BLC1: Evaluation of chocolate stability and probiotic survival under *in vitro* simulated gastrointestinal conditions. LWT. 75: 640-647.

Soukoulis, C., S. Behboudi-Jobbehdar, L. Yonekura, C. Parmenter and I. Fisk. 2014. Impact of milk protein type on the viability and storage stability of microencapsulated *Lactobacillus acidophilus* NCIMB 701748 using spray drying. Food Bioproc. Technol. 7: 1255-1268.

Succi, M., P. Tremonte, G. Pannella, L. Tipaldi, A. Cozzolino, R. Romaniello, E. Sorrentino and R. Coppola. 2017. Pre-cultivation with selected prebiotics enhances the survival and the stress response of *Lactobacillus rhamnosus* strains in simulated gastrointestinal transit. Front. Microbiol. 8: 1067.

Tripathi, M. K. and S. K. Giri. 2014. Probiotic functional foods: Survival of probiotics during processing and storage. J. Funct. Foods. 9: 225-241.

Vaziri, A. S., I. Alemzadeh, M. Vossoughi and A. C. Khorasani. 2018. Co-microencapsulation of *Lactobacillus plantarum* and DHA fatty acid in alginate-pectin-gelatin biocomposites. Carbohydr. Polym. 199: 266-275.

Wang, L., X. Yu, H. Xu, Z. P. Aguilar and H. Wei. 2016. Effect of skim milk coated inulin-alginate encapsulation beads on viability and gene expression of *Lactobacillus plantarum* during freeze-drying. LWT. 68: 8-13.

Wasilewski, A., M. Zielińska, M. Storr and J. Fichna. 2015. Beneficial effects of probiotics, prebiotics, synbiotics, and psychobiotics in inflammatory bowel disease. Inflamm. Bowel Dis. 21: 1674-1682.

Wu, J., M. Shi, W. Li, L. Zhao, Z. Wang, X. Yan, W. Norde and Y. Li. 2015. Pickering emulsions stabilized by whey protein nanoparticles prepared by thermal cross-linking. Colloid Surface B. 127: 96-104.

Xu, M., F. Gagné-Bourque, M. J. Dumont and S. Jabaji. 2016. Encapsulation of *Lactobacillus casei* ATCC 393 cells and evaluation of their survival after freeze-drying, storage and under gastrointestinal conditions. J. Food Eng. 168: 52-59.

Yasmin, I., M. Saeed, I. Pasha and M. A. Zia. 2019. Development of whey protein concentrate-pectin-alginate based delivery system to improve survival of *B. longum* BL-05 in simulated gastrointestinal conditions, Probiotics Antimicrob. 11: 413-426.

Zhang, Y., J. Lin and Q. Zhong. 2015. The increased viability of probiotic *Lactobacillus salivarius* NRRL B-30514 encapsulated in emulsions with multiple lipid-protein-pectin layers. Food Res. Int. 71: 9-15.