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Full title: Impact of encapsulating probiotics with cocoa powder on the viability of probiotics during chocolate processing, storage and *in vitro* gastrointestinal digestion

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ABSTRACT:

Chocolates can be formulated as a functional food *via* enrichment with probiotics. However, the added probiotics must overcome the challenges of processing and storage conditions and the harsh gastrointestinal environment. The study aimed to overcome these challenges using two different formulations of cocoa powder as alternative encapsulants along with Na-alginate (A_1) and Na-alginate & fructooligosaccharides (A_2). Seven different probiotic strains were encapsulated individually using the new formulations and viabilities of these encapsulated probiotics were assessed prior to and after they were added to chocolates. The highest achieved encapsulation efficiencies were 93.40% for formulation A_1 (with *Lactobacillus casei*) and 95.36% for formulation A_2 (with *Lactobacillus acidophilus* La5). The encapsulated probiotics with the new formulations maintained higher viability than the recommended therapeutic level (10^7 CFU/g) for up to 180 and 120 days of storage at 4 °C and 25 °C, respectively. The tested encapsulants improved probiotics survival when subjected to thermal stress and maintained about 9.0 Logs CFU/g at 60 °C. Additionally, the viable numbers of probiotics in fortified chocolates showed higher than 7 Logs CFU/g after 90 days of storage at 25 °C. Both formulations exhibited significantly ($P < 0.05$) high survivability of probiotics (8.0 Logs CFU/g) during the *in vitro* gastrointestinal digestion. This study demonstrated that cocoa powder along with Na-alginate and FOS has the potential to be used as a probiotic encapsulating material, and chocolates could be an excellent carrier for the development of healthy probiotic chocolate products.

Practical Application

The introduction of cocoa powder as an effective encapsulating agent to deliver probiotics could help the chocolate industry to develop healthy and attractive functional snacks for health-conscious consumers.

Keywords: Chocolates, cocoa powder, encapsulation, fructooligosaccharides, gastrointestinal digestion, Na-alginate, probiotics

Abbreviations

CFU - colony forming unit, FOS - fructooligosaccharides, FAO - Food and Agriculture Organization, WHO - World Health Organization, MRS - DeMan, Rogosa and Sharpe, VIC - Victoria, NSW - New South Wales, A1 - cocoa powder: Na-alginate at 10:1 ratio, A2 - cocoa powder: Na-alginate: FOS at 10:1:2 ratio , CA1 - cocoa powder: Na-alginate at 10:1 ratio in chocolates ,CA2 - cocoa powder: Na-alginate: FOS at 10:1:2 ratio in chocolates, EE - encapsulation efficiency, FE-EPMA - Field Emission Electron Probe Microanalyzer, SSF - simulated salivary fluid, SGF - simulated gastric fluid, SIF - simulated intestinal fluid, ANOVA - analysis of variance, HSD - honest significant difference, SEM - Scanning electronic microscope, FC - free culture, a_w - water activity, USDA - United States Department of Agriculture

1 Introduction

Probiotics are 'live microorganisms which, when administered in adequate numbers, confer a health benefit on the host (FAO/WHO, 2002). The International Dairy Federation recommended an adequate number of live probiotics (10^6-10^7 CFU/mL or g of food) at the time of consumption to exhibit the health benefits (Dong et al., 2013; Frakolaki, Giannou, Kekos, & Tzia, 2020). There is a growing interest in the industry to maintain recommended probiotic viability in popular food products through various food innovations (Granato et al., 2020; Ranadheera, Naumovski, & Ajlouni, 2018). The consumption of sufficient amounts of probiotics *via* food formulations can assist in maintaining a healthy gut due to the positive effects of probiotics on gut microbiota (Hill et al., 2014; Sharifi-Rad et al., 2020). The intestinal human microbiota is estimated at nearly 100 trillion and they have a symbiotic relationship with their host (Sommer & Bäckhed, 2013). Different dairy products are considered ideal carriers for the delivery of probiotic bacteria into the human gastrointestinal tract (Peng et al., 2020; Pourjafar, Noori, Gandomi, Basti, & Ansari, 2020), while there is a growing demand for non-dairy food matrixes for probiotic delivery as well. However, the delivery of probiotics *via* oral administration is challenging due to harsh gastrointestinal conditions such as stomach acid, bile and various digestive enzymes (Frakolaki et al., 2020; Vaziri, Alemzadeh, Vossoughi, & Khorasani, 2018) as well as during processing and storage (Mani-López, Palou, & López-Malo, 2014).

To extend the survivability and shelf life of probiotics under stressful conditions, microencapsulation is considered an excellent technique (Braber et al., 2020; Tolve et al., 2020). The majority of previous studies concluded that the encapsulating matrixes preserve probiotics during processing, storage and permit their release into a metabolically active state in the human intestine (Peng et al., 2020; Pourjafar et al., 2020). Several physical and chemical approaches have been investigated for the microencapsulation of probiotics (Pech-

93 Canul, Ortega, García-Triana, & González-Silva, 2020). The basic encapsulating materials
94 include protein (gelatin and whey protein), (Braber et al., 2020), polysaccharides (chitosan,
95 alginates and pectin), (Zhang, Lin, & Zhong, 2015) and fructooligosaccharides (FOS) (Nami,
96 Lornezhad, Kiani, Abdullah, & Haghshenas, 2020), or a combination of various types of
97 these biopolymers to further improve the protection of microorganisms (Ragavan & Das,
98 2018; Yasmin, Saeed, Pasha, & Zia, 2019).

99 It is well-known that the selection of encapsulant matrixes and applying a suitable technique
100 is essential to successful microencapsulation (Frakolaki et al., 2020). Various techniques have
101 been successfully used for probiotics microencapsulation including spray drying, spray
102 chilling and cooling, freeze-drying (lyophilization), fluidized bed drying, emulsification and
103 extrusion (Frakolaki et al., 2020; Pourjafar et al., 2020). Freeze-drying could be considered as
104 the best drying method for probiotic microencapsulation because this technique removes
105 moisture by sublimation under vacuum without exposing the microorganisms to heat stress
106 (Tolve et al., 2020; Chávez & Ledeboer, 2007), although it is more expensive than other
107 methods. Nevertheless, considering the fact that viable cell numbers in the final encapsulated
108 products play a significant role in its functionality, freeze-drying can be considered as a
109 suitable choice to guarantee the maximum viable number, where the extra cost of this
110 technique can be recovered by the best quality of the final encapsulated probiotics (Pech-
111 Canul et al., 2020).

112 As the demand for probiotic food products from both dairy and non-dairy sources continue to
113 grow, chocolates, one of the most appealing products among the consumers, is anticipated to
114 be an excellent probiotics carrier. Chocolate possesses a wide range of potent antioxidants
115 and other nutrients that can positively benefit human health. In addition, chocolate may serve
116 as a suitable carrier for probiotic delivery to the human gut (Hossain, Ranadheera, Fang, &

Ajlouni, 2020). Cocoa powder, one of the major ingredients in chocolate production, contains a complex structure of proteins, polysaccharides and lipids (Oracz, Nebesny, Zyzelewicz, Budryn, & Luzak, 2020; Sorrenti et al., 2020) which could form a good encapsulating admixture along with alginate or FOS. In this study, the probiotics encapsulated with cocoa powder were used in chocolate preparation at the laboratory scale and the viability of probiotics during the *in vitro* gastrointestinal digestion was examined. As cocoa powder has not been previously used as an encapsulating material, it is anticipated that these tested novel encapsulants and techniques could be helpful to the chocolate industry.

2 Materials and Methods

2.1 Materials

Seven different strains of probiotic bacteria were used in this study: *Lactobacillus acidophilus* (La5), *L. rhamnosus* (LGG), *L. sanfranciscensis*, *L. plantarum*, *L. casei* 431, *Bifidobacterium animalis* subspp. *lactis* (Bb12) and *Streptococcus thermophilus*. The *L. rhamnosus* (LGG), *B. animalis* subspp. *lactis* (Bb12) and *L. casei* probiotics were kindly provided by Chr. Hansen, Bayswater, VIC, Australia and all other probiotics were obtained from the stock culture collections in the Food Chemistry & Microbiology Laboratory at the University of Melbourne. FOS, Na-alginate, skim milk powder, hi-maize resistant starch and enzymes (salivary α-amylase, porcine pepsin, pancreatin), HCl, acetone, acetic acid were bought from Sigma Aldrich (Castle Hill, NSW, Australia). Whey protein concentrate was kindly provided by Murray Goulburn Co-operative Co. Limited (Melbourne, VIC, Australia). 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 bought from Thermo Fisher (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 bought from the Chem-Supply Pty Ltd. (Melbourne, VIC, Australia).

2.2 Methods

2.2.1 Preparation of formulation blends

Encapsulating materials were selected based on preliminary work to identify the best combinations of the encapsulants. The selected encapsulation formulations included cocoa powder: Na-alginate at 10:1 ratio (A_1) and cocoa powder: Na-alginate: FOS at 10:1:2 ratio (A_2).

2.2.2 Cell resuscitation and inoculum preparation

The freeze-dried probiotic strains were resuscitated and inoculated individually in a selective medium (100 ml) and incubated anaerobically using a BB-16 incubator (Heraeus Instruments, Hanau, Germany) at 37 °C for 22 ± 2 h. The probiotics 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 mixed with the new encapsulating formulations (de Araújo Etchepare et al., 2020). When these encapsulated probiotics were added to the chocolate, the chocolate probiotic samples were designated as CA_1 and CA_2.

2.2.3 Encapsulation of probiotics using a freeze-drying technique

The probiotic strains were encapsulated with two types of encapsulation formulations (A_1 and A_2) that were mentioned in section 2.2.1 using emulsion based freeze-dried technique. The formulation ingredients were dissolved in Milli-Q water followed by homogenization for 15

166 min at 10000 rpm using a homogenizer (IKA T25 digital Ultraturrax, Germany) and the
167 mixture was then kept at room temperature for 2 h for complete hydration. The formulations
168 admixture was pasteurized at 75 °C for 30 min in a water bath and cooled to 42 °C (Gebara et
169 al., 2013). The harvested cell pellets (approximately 12 Logs CFU/g) were suspended in the
170 relevant formulations and homogenized using a homogenizer. It was then left for 1 h at room
171 temperature to allow the interaction between encapsulants biopolymers and the probiotics (de
172 Araújo Etchepare et al., 2020). The suspended admixture was distributed into 50 ml sterile
173 falcon tubes (35ml each) and frozen at -20 °C overnight. The frozen samples were freeze-
174 dried at -50 °C using a benchtop freeze dryer (Dynavac Engineering FD3, NSW, Australia).
175 The freeze-dried encapsulated probiotics were stored at 4 °C until used.
176
177 **2.2.4 Viability of encapsulated probiotics**
178 The probiotics viability was assessed prior to and after encapsulation. The encapsulated
179 probiotic powders were aseptically transferred into sterile falcon tubes and stored at 4 °C and
180 25 °C for 180 days. Samples were tested for cell viability on day 1 to 180 days of storage.
181 Samples (1 g) were serially diluted using 0.1% sterile peptone water, plated on MRS selective
182 medium and incubated anaerobically at 37 °C for 48 h. Results were reported as Log CFU/g
183 (Favaro-Trindade & Grosso, 2002).
184
185
186
187
188 **2.2.5 Encapsulation efficiency**

189 The encapsulation efficiency indicated the survival rate of the probiotic strains after the
190 freeze-drying (de Araújo Etchepare et al., 2020; Doherty et al., 2011). The percentage
191 encapsulation efficiency (% EE) was calculated as the following:

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

192 where A is the number of viable cultures (Log CFU/g) released after encapsulation, and A0 is
193 the number of total free cultures (Log CFU/g) before encapsulation.
194
195 **2.3 Scanning electron microscopy of the encapsulated probiotics before and after mixing**
196 **with chocolate**
197 Encapsulated *L. rhamnosus* LGG was used as a representative sample for scanning electron
198 microscopy in this study. Briefly, the morphology of the encapsulated probiotics, both in
199 powder and chocolate forms, were analyzed using a Field Emission Electron Probe
200 Microanalyzer (FE-EPMA) (Hyperprobe JXA-8530F, JEOL Ltd., Tokyo, Japan) and a
201 double-thickness gold coat (30 nm) to assist with removal of heat over the imaged area. The
202 samples were gently spread out over the carbon tape on a glass slide and very gently tapped
203 down into the tape using a spatula. The prepared slide was then gold coated in an Emitec
204 K550X sputter coater to a thickness of 30 nm. Using very low accelerating voltages (5-7kV)
205 under a high vacuum helped in the preparation of extremely small spot size and enabled the
206 instrument to analyze submicron areas with ease (Zhang et al., 2014).
207
208
209
210 **2.4 Thermal tolerant of encapsulated probiotics**
211 The thermal tolerance of the encapsulated probiotics during chocolate processing was tested
212 according to the methods reported by Kemsawasd, Chaikham, & Rattanasena (2016) and

Silva et al. (2017). The various encapsulated probiotic cultures were added to the 70% dark and 45% white chocolates (100 g each) at 1% freeze-dried powder and mixed at 40 °C, 50 °C and 60 °C. The viable counts of the encapsulated cultures per gram in the chocolate mix were estimated by the spread plate technique (Favaro-Trindade & Grosso, 2002).

2.5 Preparation of chocolate enriched with encapsulated probiotic

Chocolate preparation and processing were conducted following the method of Erdem et al. (2014). The encapsulated probiotics were added to chocolate at the best pre-determined temperature (45 °C) to reach the recommended number of probiotic counts of at least 10^7 CFU/g in the final products (Dong et al., 2013). The freeze-dried probiotic cultures were added into the 45% and 70% commercial chocolates (500 g each) at 1% concentration following the procedures of Lalicic-Petronijevic et al. (2015). The probiotics were added to the chocolate at a temperature close to solidification conditions (Gadhiya, Shah, Patel, & Prajapati, 2018; Kemsawasd et al., 2016). Chocolate enriched with probiotics (probiotic chocolate) and controls (no added probiotic) were stored at 25 °C and 4 °C under aseptic conditions for 90 days. Probiotic counts, major important physical and chemical properties of the probiotic chocolate and *in vitro* bioaccessibility of probiotic chocolate assays were performed at 0, 30, 60 and 90 days.

2.6 Survival of probiotics during the *in vitro* gastrointestinal digestion of probiotic chocolate

2.6.1 Preparation of gastrointestinal digestion fluids

237 The *in vitro* gastrointestinal digestion was performed using salivary, gastric and intestinal
238 fluids. These fluids were prepared as described by Minekus et al. (2014). The stock digestion
239 fluids were prepared using a mixture of the electrolytes (K^+, Na^+, Cl^-, H_2PO_4, HCO_3, Mg^{2+},
240 NH^+ and Ca^{2+}) at different concentrations.
241
242 **2.6.2 Basal medium Preparation**
243 The basal medium was prepared following the methods of Zhang, Panozzo, Hall, & Ajlouni,
244 (2018). The composition of basal medium was as follows: 5.0 g soluble starch, 5.0 g peptone,
245 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
246 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
247 KH_2PO_4, 0.5 g K_2HPO_4, 0.4 g bile salts, 0.11g $CaCl_2$ and 1.0 mL tween 80 were dissolved in
248 1000 mL of Milli-Q water and autoclaved at 121 °C for 20 min (HANHSIN VD-3041
249 autoclave, VIC, Australia) and pH was adjusted to 7.0.
250
251 **2.6.3 *In vitro* gastrointestinal digestion and colonic fermentation**
252 **2.6.3.1 *In vitro* gastrointestinal digestion**
253 The probiotics counts during gastrointestinal digestion of probiotic-chocolate and free
254 cultures (control) were assessed using an *in vitro* gastrointestinal digestion model (Minekus et
255 al., 2014). The model consisted of three-steps involved sequentially simulated digestion in
256 the mouth, stomach and the small intestine as described by Minekus et al. (2014) with some
257 modification. Samples were collected at each stage of digestion for the estimation of bacteria
258 and total counts. To avoid any destruction, triplicate samples were prepared for each
259 treatment. The individual sample replicates were used for microbial colony counting.

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 mins at room temperature.

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.

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.

2.6.3.2 Colonic fermentation

The *in vitro* colonic fermentation was conducted with human feces, and ethical approval (ID: 1954660.1) was obtained from the Human Ethics Advisory Group at the Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Australia. Fresh feces were collected from a healthy male donor (32yr old) who had not ingested antibiotics for the last 3 months. Fecal 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 carried out by mixing the gastrointestinal digested sample residue to the fecal slurry at 1:1 (v/v) ratio and incubated anaerobically at 37 °C up to 72 h. Aerobic and anaerobic counts were enumerated

284 immediately after mixing and every 24 h interval successively up to 72h of fermentation. The
285 control sample was prepared using 5 mL fecal slurry and 5 mL basal medium only.
286
287 **2.7 Statistical analysis**
288 All experiments were carried out in triplicate with at least two measurements for each
289 parameter. Results were subjected to one-way ANOVA using Minitab®19 statistical software
290 (2019, USA). The means were separated using Tukey honest significant difference (HSD) at
291 95% confidence level. Final results were reported as means ± standard deviations.
292
293 **3 Results and Discussion**
294 **3.1 Percentage encapsulation efficiency of the probiotics**
295 The microencapsulation of seven probiotic strains with two formulations A_1: cocoa powder:
296 Na-alginate (10:1) and A_2: cocoa powder: Na-alginate: FOS (10:1:2) resulted in a higher
297 encapsulation efficiency (EE) that could potentially lead to maintaining and protecting the
298 bacterial cell (**Table 1, supplementary**). The formulation A_1 exhibited the highest
299 percentage of EE (93.40% ± 1.88) for *L. casei* and the lowest (82.15% ± 1.77) for *S.*
300 *thermophilus*. Additionally, formulation A_2 showed the highest EE 95.36% ± 1.87 for *L.*
301 *acidophilus* La5 and the lowest 84.83% ± 1.04 for *L. rhamnosus* LGG. Except for the two
302 lowest EE (82.15% ± 1.77 and 84.83 ± 1.04) for *S. thermophilus* and *L. rhamnosus* LGG,
303 respectively, the formulations for all other probiotics showed higher than 85% EE. These %
304 EE results were smaller than the value (98.4%) reported by Nami et al. (2020) using alginate-
305 persian gum, FOS and inulin mixture with extrusion method. However, the results were in
306 agreement with the % EE (85.69 ± 4.82) reported by Xu, Gagné-Bourque, Dumont, & Jabaji
307 (2016) who used a similar freeze-drying technique when encapsulated *L. casei* in a pea
308 protein isolate-alginate hydrogel. Consequently, these tested encapsulants could be

309 considered as successful formulations for probiotics encapsulation. The microencapsulation
310 efficiency depends on the content of carbohydrates biopolymers and protein in the materials
311 (Yasmin et al., 2019) which ensure enough protection to the core materials from the adverse
312 environment. Data in Table 1 (supplementary) also revealed a significant difference ($P < 0.05$) between the two tested formulations (A_1 & A_2). Formulation A_1 exhibited good
314 encapsulation efficiency for *L. rhamnosus* LGG, *L. casei*, *L. acidophilus* La5 and *L.*
315 *plantarum,* whereas formulation A_2 demonstrated better results for all the probiotics except *L.*
316 *rhamnosus* LGG. Based on the high EE, it can be concluded that the two new formulations
317 [cocoa powder: Na-alginate (10:1) and cocoa powder: Na-alginate: FOS (10:1:2)] could be
318 good materials for probiotic microencapsulation.

319 **3.2 Scanning electronic microscopic images of encapsulated probiotics**

320 SEM was used to observe the structure and interaction between chocolate and probiotics. The
321 SEM images (x6000) of the encapsulated probiotics *L. rhamnosus* LGG clearly showed the
322 probiotics were incorporated into the carrier chocolate (**Fig 1A**) and in the matrices of the
323 freeze-drying (**Fig 1B**). The rod shape probiotics were homogeneously distributed throughout
324 the blend. Despite the different types of encapsulation formulations, the morphology of
325 encapsulated bacteria was similar in all treated samples. These results were in disagreement
326 with some recent researchers (Chen et al., 2017; de Araújo Etchepare et al., 2020) who noted
327 that the freeze-drying process caused the shrinkage of the probiotic cell due to water loss
328 during the process. These SEM images in this study confirmed few morphological changes
329 hence encapsulation with cocoa powder and chocolate could be suitable probiotic carriers.
330

331 **3.3 Viability of various encapsulated probiotic strains**

332 The viability of seven probiotic strains encapsulated with two encapsulant formulations A_1
333 and A_2 were evaluated in two different storage temperatures 4 °C (**Figure 2**) and 25 °C

(**Figure 1, supplementary**) for up to 180 days of storage. The results revealed that for both encapsulation formulations, the viable count remained above 7.5 Logs CFU/g for the entire 180 days when stored at 4 °C. Additionally, there were no significant differences ($P > 0.05$) between the two formulations on each day of measurement. However, free culture (FC) counts decreased significantly ($P < 0.05$) after 120 days at 4 °C (**Figure 2**) and reached below the detectable level after 180 days of storage. These results showed that encapsulated probiotics with more than 7.0 Logs CFU/g can satisfy the guideline proposed by the International Dairy Federation (10^7 CFU/g or mL) to exhibit the health benefits (Dong et al., 2013). On the other hand, the viability of all the tested strains using both formulations and stored at 25 °C maintained the required viable number of > 7 logs CFU/g, but up to 120 days only (**Figure 1, supplementary**). For the non-encapsulated cell, the significant reduction ($P < 0.05$) happened at 90 days of storage duration. Thus, it was obvious that storage temperature can significantly ($P < 0.05$) affect the viability of encapsulated probiotics. These results were in agreement with de Araújo Etchepare et al. (2020) who indicated that whey protein concentrate with alginate maintained probiotics counts of more than 9 Logs CFU/g for up to 120 days. Additionally, a similar study by Yasmin et al. (2019) showed more than 7.0 Logs CFU/g viability of probiotics encapsulated with whey protein concentrate.

The highest count at the end of 180 days of storage at 4 °C for *L. casei*, *S. thermophilus*, *L. rhamnosus* LGG, and *L. acidophilus* La5 were 8.02±1.40, 7.92±1.66, 7.77±1.30 and 7.68±1.62 Logs CFU/g, respectively for the formulation of A_1. For the formulation of A_2, the highest counts at 180 days of storage at 4 °C for *L. casei, L. rhamnosus* LGG, *L. plantarum* and *S. thermophilus* were 8.27±1.30, 8.25±1.05, 8.12±1.35 and 7.87±1.67, respectively (**Figure 2**). The final counts in both formulations (A_1 and A_2) were not significantly different ($P > 0.05$) for the samples stored at 4 °C. Additionally, calculating the Log reduction in the numbers of encapsulated probiotics during storage for 180 days revealed a significant decline

359 ($P < 0.05$) at 25 °C (**Figure 1, supplementary)** compared to 4 °C (**Figure 2**). Similar
360 observations on the survival of encapsulated probiotics with dark chocolates were reported by
361 Lalicic-Petronijevic et al. (2015). These findings could lead to the conclusion that cocoa
362 powder along with alginate with or without FOS could be a good encapsulant admixture to
363 probiotic cultures usually stored at room temperature (González-Herrera, Bermúdez-Quiñones,
364 Ochoa-Martínez, Rutiaga-Quinones, & Gallegos-Infante, 2020; Wu et al., 2015) but for a longer
365 shelf life (180 days) the encapsulated probiotics could be stored at refrigerated (4 °C)
366 condition.

367 **3.4 Heat tolerance of various encapsulated strains**

368 The thermotolerant capacity of encapsulated probiotics was tested to ensure that an adequate
369 number of probiotics remain viable in chocolates throughout the processing steps. The
370 melting and tempering temperatures of different chocolates had been reported to be dark
371 chocolate at 46 – 48°C, milk chocolate at 40 - 45 °C and white chocolate at 37 - 43 °C
372 (Afoakwa, Paterson, & Fowler, 2007; Afoakwa, Paterson, Fowler, & Vieira, 2008). The
373 thermostability of the probiotics in dark chocolate containing 45% and 70% cocoa powder
374 was almost identical, hence only the data on 70% dark chocolates were presented here.
375 Chocolate preparation usually involves heating the mixture at a temperature > 45 °C for
376 melting and mixing purposes (Klindt-Toldam et al., 2016; Silva et al., 2017). Data in **Figure**
377 **3** revealed that the encapsulated cell counts were not affected at 37 °C and 40 °C ($P > 0.05$).
378 Similarly, the encapsulated probiotics of all the strains resisted the heat
379 treatment at 50 °C and 60 °C during chocolate preparation. It was noted that for both
380 encapsulant formulations, a sufficient number of encapsulated probiotics survived at 60 °C
381 whereas significant cell reduction ($P < 0.05$) occurred in the controls (free culture) which was
382 8.93 Logs CFU/g (**Figure 3**). The maximum recovery of counts for encapsulated *L.*
383 *rhamnosus* LGG at 60 °C were 9.56±0.97 and 10.25±0.95 Logs CFU/g, in formulation A1

and A2, respectively, whereas the *L. casei* and *L. acidophilus* La5 showed the second-highest count for A_1 formulation which was not differed significantly ($P > 0.05$). These results confirmed the efficacy of the applied encapsulants in protecting probiotics during chocolate processing. Similar observations related to the protective effects of encapsulants against heat treatments were reported in previous studies (Rad et al.; 2016; Anekella & Orsat, 2013; Jantzen, Göpel, & Beermann, 2013) which indicated that the layer of encapsulating formulation blends around the probiotics was not damaged while mixing at 60 °C. Such results can ascertain that the encapsulated probiotic cells can tolerate the heat stress while processing chocolates at the temperature as high as 60 °C.

3.5 Survivability of probiotics in chocolate during storage at two different temperatures

3.5.1 Chocolates containing 70% cocoa powder

The data in **Figure 4** showed the viability of encapsulated and free probiotics in chocolate containing 70% cocoa and stored at 4 °C and 25 °C for 90 days with 1% encapsulated probiotics. The Results demonstrated the positive effects of encapsulation formulation on the survival of probiotic culture in chocolate at 4 °C when compared with the free culture. Chocolate containing free culture probiotics showed a significant ($P < 0.05$) Log reduction (5.0 Logs CFU/g) as compared with encapsulated probiotics with only 2.0 Logs reduction under the same conditions. At the end of 90 days of storage, more than 7.0 Logs CFU/g of encapsulated probiotics survived in 70% cocoa containing chocolates in both formulations (A_1 & A_2) with all the seven probiotics. Additionally, no significant differences ($P > 0.05$) in probiotic counts were observed between A_1 and A_2 formulations. These results confirmed the fact that the applied formulations were sufficient in protecting the probiotics and meeting the recommendation of the International Dairy Federation for a healthy probiotic product (Dong et al., 2013). The same chocolate-probiotic formulations were stored at 25 °C and showed

similar results to those at 4 °C with some exceptions with a slightly higher cell number reduction (**Figure 2, supplementary**). The highest cell counts were recorded for *L. acidophilus* La5, *L. casei* and Bb12 were 8.08 ± 0.83, 7.94 ± 0.69 and 7.97 ± 0.99, respectively using formulation A_1, and 7.80 ± 0.48, 7.93 ± 0.70, 7.65 ± 0.93 Logs CFU/g in formulation A_2. Consequently, it was concluded that the final counts in all tested chocolate samples using both types of encapsulants formulation were able to maintain more than 7.0 Logs CFU/g after 90 days of storage at both 4 °C (**Figure 4**) and 25 °C (**Figure 2, supplementary**).

3.5.2 Chocolates containing 45% cocoa powder

The survival of probiotics in chocolates with 45% cocoa powder (**Figure 5**) showed similar trends to those of 70% cocoa powder. The viable probiotic counts at 4 °C were slightly greater than that at 25 °C (**Figure 3**, **Supplementary**). The difference in final counts at 4 °C and 25 °C was < 1.0 Log after 90 days of storage. Similar to the 70% cocoa containing chocolates, at the end of 90 days of storage the *L. casei* and Bb12 probiotic cell survival rates were the highest. It can be suggested that chocolates fortified with an encapsulated probiotic can be stored at either temperature with

good protection of probiotic survival. Additionally, both types of chocolates (45% & 70% cocoa powder), maintained total probiotic counts above the recommended level (10^7 CFU/g). Such a protective effect of chocolate with different cocoa powder contents could be attributed to low water activity (a_w about 0.5) and high polyphenols content of chocolate (USDA, 2019). These results were very promising and clearly showed the good survival of probiotics in fortified chocolate.

3.6 Delivery of probiotics *via* chocolate during *in vitro* gastrointestinal digestion and colonic fermentation

The *in vitro* gastrointestinal digestion and colonic fermentation were performed with dark chocolates containing 70% cocoa powder only since both types of chocolates (45% & 70% cocoa) showed similar probiotic counts (section 3.5). The count in chocolate containing free probiotic culture (control) showed a significant ($P < 0.05$) reduction of 6.0 Logs CFU/g when exposed to simulated gastric juice for 2 h, whereas chocolate fortified with encapsulated probiotic revealed on average only 2.5 Logs reduction during the gastrointestinal digestion (**Figure 6A**). The data in **Figure 6A** showed significantly ($P < 0.05$) less Log reduction in encapsulated probiotics in chocolate, indicating that encapsulating agents had a protective effect on the probiotics during the *in vitro* digestion. Both encapsulation formulations of A_1 and A_2 exhibited promising results in protecting the probiotics under such adverse gastrointestinal environment. These results were in agreement with previous studies (Brinques & Ayub, 2011; Sandoval-Castilla, Lobato-Calleros, García-Galindo, Alvarez-Ramírez, & Vernon-Carter, 2010) which reported similar protective effects of encapsulation during gastric digestion. The significant decline in the counts of encapsulated probiotic in chocolate reaching 2-3 Logs after 2 h of exposure to the gastric digestion might be due to the adverse effect of low pH (2-3) in the stomach environment (Khorasani & Shojaosadati, 2017). At the intestinal digestion phase, the colony count was quite stable due to favorable growth conditions compared to the gastric digestion phase, as the above 8.0 Logs CFU/g count existed (**Figure 6A)** during intestinal digestion. Therefore, in the simulated gastrointestinal digestion, cocoa powder along with all tested formulations had a positive effect on cell protection compared with free probiotics.

Data in **Figure 6B** presented the results of *in vitro* colonic fermentation. A gradual increase of probiotics number at least 2-4 Logs were detected during the first 48 h of fermentation,

458 followed by a decline in the probiotic counts between 48 and 72 h. The highest detected
459 counts for *L. casei* and *L. rhamnosus* LGG were 11.25±1.29, 11.08±1.48 and 11.00±1.52 and
460 10.89±0.68 Logs CFU/g for the formulation of A_2 and A_1, respectively. The probiotic counts
461 in both formulations were significantly larger ($P < 0.05$) than the non-encapsulated count,
462 whereas the formulation types (A_1 and A_2) did not show any significant difference ($P > 0.05$).
463 In the whole course of *in vitro* digestion, it was clear that the encapsulating formulations
464 protected the probiotics in an adverse environment such as the very low acidity (pH 2-3) in
465 the gastric condition. These findings were in agreement with those reported by Cielecka-
466 Piontek et al., 2020; Dala-Paula, Deus, Tavano, & Gloria, 2021 who demonstrated that dark
467 chocolate (70% cocoa) could protect probiotics from the adverse impact of the
468 gastrointestinal digestion process. However, in the colonic condition (pH 7), the coated
469 probiotics were benefited from such a favorable growth environment, thus rapidly increased
470 the number (Braber et al., 2020). However, the results from the present investigation revealed
471 that cocoa powder could be used as a good alternative for probiotics microencapsulation.
472
473

474 ## 4 Conclusion

475 The present study has demonstrated that all the seven tested probiotic strains were
476 successfully encapsulated using cocoa powder along with Na-alginate or Na-alginate-FOS
477 and maintained sufficient viable count when added to chocolates. Both tested formulations
478 exhibited high encapsulation efficiency (EE). The highest EE rates for cocoa: Na-alginate and
479 cocoa: Na-alginate: FOS were 93.40% (*L. casei*) and 95.36% (*L. acidophilus* La5),
480 respectively. These encapsulated probiotics resisted thermal exposure at 60 °C, suggesting
481 they are suitable for chocolate processing at the melting stage of 45 ºC. The encapsulated

probiotics showed higher viable counts than the recommended level (10^7 CFU/g) during the entire storage period of 180 days at 4 °C. Similarly, encapsulated probiotics in chocolate (probiotic-chocolate) maintained the recommended probiotic dose (10^7 CFU/g) for 90 days when chocolates were stored at both 4 °C and 25 °C. The tested two encapsulation formulations significantly improved the probiotics tolerance against the adverse gastrointestinal conditions in comparison with free probiotics. Encapsulated probiotics in chocolate maintained the highest probiotic counts after the *in vitro* gastrointestinal digestion and colonic fermentation, indicating high probiotics bioaccessibility. The probiotics survivability was around 8.0 Logs CFU/g during the gastrointestinal digestion stage, and the number was boosted up above 10.50 Logs CFU/g during the colonic fermentation. These findings confirmed that cocoa powder with Na-alginate or Na-alginate-fructooligosaccharides formulation could be a potential encapsulation admixture to develop functional probiotic chocolates.

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Author Contributions

MNH and SA planned the study. MNH performed all the experiments. SA, CSR and ZF provided necessary advice and guidelines in conducting the work. MNH wrote the first draft of the manuscript and all authors revised, read and approved the final manuscript.

Conflicts of Interests

The authors have no conflict of interest to declare.

Data Availability

All the data and materials are available in this manuscript.

References

Afoakwa, E. O., Paterson, A., & Fowler, M. (2007). Factors influencing rheological and textural qualities in chocolate–a review. *Trends in Food Science & Technologyy, 18*(6), 290-298. doi:https://doi.org/10.1016/j.tifs.2007.02.002

Afoakwa, E. O., Paterson, A., Fowler, M., & Vieira, J. (2008). Particle size distribution and compositional effects on textural properties and appearance of dark chocolates. *Journal of Food Engineering, 87*(2), 181-190. doi:https://10.1016/j.jfoodeng.2007.11.025

Anekella, K., & Orsat, V. (2013). Optimization of microencapsulation of probiotics in raspberry juice by spray drying. *LWT - Food Science and Technology, 50*(1), 17-24. doi:http://dx.doi.org/10.1016/j.lwt.2012.08.003

528 Braber, N. V., Vergara, L. D., Rossi, Y., Aminahuel, C., Mauri, A., Cavaglieri, L., &
529 Montenegro, M. (2020). Effect of microencapsulation in whey protein and water-
530 soluble chitosan derivative on the viability of the probiotic *Kluyveromyces marxianus*
531 VM004 during storage and in simulated gastrointestinal conditions. *LWT - Food*
532 *Science and Technology, 118*, 108844. doi:https://doi.org/10.1016/j.lwt.2019.108844
533 Brinques, G. B., & Ayub, M. A. Z. (2011). Effect of microencapsulation on survival of
534 *Lactobacillus plantarum* in simulated gastrointestinal conditions, refrigeration, and
535 yogurt. *Journal of Food Engineering, 103*(2), 123-128.
536 doi:http://dx.doi.org/10.1016/j.jfoodeng.2010.10.006
537 Chávez, B., & Ledeboer, A. (2007). Drying of probiotics: optimization of formulation and
538 process to enhance storage survival. *Drying Technology, 25*(7-8), 1193-1201.
539 doi:https://doi.org/10.1080/07373930701438576
540 Chen, H.Y., Li, X.Y., Liu, B.J., & Meng, X.H. (2017). Microencapsulation of *Lactobacillus*
541 *bulgaricus* and survival assays under simulated gastrointestinal conditions. *Journal of*
542 *Functional Foods, 29*, 248-255. doi:https://doi.org/10.1016/j.jff.2016.12.015
543 Cielecka-Piontek, J., Dziedziński, M., Szczepaniak, O., Kobus-Cisowska, J., Telichowska,
544 A., & Szymanowska, D. (2020). Survival of commercial probiotic strains and their
545 effect on dark chocolate synbiotic snack with raspberry content during the storage and
546 after simulated digestion. *Electronic Journal of Biotechnology, 48*, 62-71.
547 doi:https://doi.org/10.1016/j.ejbt.2020.09.005
548 Dala-Paula, B. M., Deus, V. L., Tavano, O. L., & Gloria, M. B. A. (2021). In vitro
549 bioaccessibility of amino acids and bioactive amines in 70% cocoa dark chocolate:
550 What you eat and what you get. *Food Chemistry, 343*, 128397.
551 doi:https://doi.org/10.1016/j.foodchem.2020.128397

552 de Araújo Etchepare, M., Nunes, G. L., Nicoloso, B. R., Barin, J. S., Moraes Flores, E. M., de
553 Oliveira Mello, R., & Ragagnin de Menezes, C. (2020). Improvement of the viability
554 of encapsulated probiotics using whey proteins. *LWT - Food Science and Technology,*
555 *117*. doi:https://doi.org/10.1016/j.lwt.2019.108601
556 Doherty, S., Gee, V., Ross, R., Stanton, C., Fitzgerald, G., & Brodkorb, A. (2011).
557 Development and characterisation of whey protein micro-beads as potential matrices
558 for probiotic protection. *Food Hydrocolloids, 25*(6), 1604-1617.
559 doi:http://dx.doi.org/10.1016/j.foodhyd.2010.12.012
560 Dong, Q. Y., Chen, M. Y., Xin, Y., Qin, X. Y., Cheng, Z., Shi, L. E., & Tang, Z. X. (2013).
561 Alginate- based and protein- based materials for probiotics encapsulation: a review.
562 *International Journal of Food Science and Technology, 48*(7), 1339-1351.
563 doi:https://doi.org/doi:10.1111/ijfs.12078
564 Erdem, Ö., Gültekin-Özgüven, M., Berktaş, I., Erşan, S., Tuna, H. E., Karadağ, A., . . .
565 Cutting, S. M. (2014). Development of a novel synbiotic dark chocolate enriched with
566 *Bacillus indicus* HU36, maltodextrin and lemon fiber: Optimization by response
567 surface methodology. *LWT - Food Science and Technology, 56*(1), 187-193.
568 doi:https://doi.org/10.1016/j.lwt.2013.10.020
569 FAO/WHO. (2002). Guidelines for the Evaluation of Probiotics in Food. *Report of a joint*
570 *FAO/WHO working group on drafting guidelines for the evaluation of probiotics in*
571 *food. London, Ontario, Canada*. doi:http://www.fao.org/es/ESN/Probio/probio.htm
572 Favaro-Trindade, C. S., & Grosso, C. R. (2002). Microencapsulation of *L. acidophilus* (La-
573 05) and *B. lactis* (Bb-12) and evaluation of their survival at the pH values of the
574 stomach and in bile. *Journal of Microencapsulation, 19*(4), 485-494.
575 doi:http://dx.doi.org/10.1080/02652040210140715

576 Frakolaki, G., Giannou, V., Kekos, D., & Tzia, C. (2020). A review of the
577 microencapsulation techniques for the incorporation of probiotic bacteria in functional
578 foods. *Critical Reviews in Food Science and Nutrition*, 1-22.
579 doi:https://doi.org/10.1080/10408398.2020.1761773
580 Gadhiya, D., Shah, N. P., Patel, A. R., & Prajapati, J. B. (2018). Preparation and shelf life
581 study of probiotic chocolate manufactured using *Lactobacillus Helveticus* Mtcc 5463.
582 *Acta Alimentaria, 47*(3), 350-358. doi:https://doi.org/10.1556/066.2018.47.3.11
583 Gebara, C., Chaves, K. S., Ribeiro, M. C. E., Souza, F. N., Grosso, C. R., & Gigante, M. L.
584 (2013). Viability of *Lactobacillus acidophilus* La5 in pectin–whey protein
585 microparticles during exposure to simulated gastrointestinal conditions. *Food*
586 *Research International, 51*(2), 872-878.
587 doi:http://dx.doi.org/10.1016/j.foodres.2013.02.008
588 González-Herrera, S. M., Bermúdez-Quiñones, G., Ochoa-Martínez, L. A., Rutiaga-
589 Quinones, O. M., & Gallegos-Infante, J. A. (2020). Synbiotics: a technological
590 approach in food applications. *Journal of Food Science and Technology*, 1-14.
591 doi:https://doi.org/10.1007/s13197-020-04532-0
592 Granato, D., Barba, F. J., Kovačević, D. B., Lorenzo, J. M., Cruz, A. G., & Putnik, P. (2020).
593 Functional foods: product development, technological trends, efficacy testing, and
594 safety. *Annual Review of Food Science and Technology, 11*.
595 doi:https://doi.org/10.1146/annurev-food-032519051708
596 Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., . . . Sanders, M. E.
597 (2014). Expert consensus document. The international scientific association for
598 probiotics and prebiotics consensus statement on the scope and appropriate use of the
599 term probiotic. *Nature Reviews I Gastroenterology and Hepatology, 11*(8), 506-514.
600 doi:https://doi.org/10.1038/nrgastro.2014.66

601 Hossain, M. N., Ranadheera, C. S., Fang, Z., & Ajlouni, S. (2020). Healthy chocolate
602 enriched with probiotics: a review. *Food Science and Technology, 40*(4), 1-13.
603 doi:https://doi.org/10.1590/fst.11420
604 Jantzen, M., Göpel, A., & Beermann, C. (2013). Direct spray drying and microencapsulation
605 of probiotic *Lactobacillus reuteri* from slurry fermentation with whey. *Journal of*
606 *Applied Microbiology, 115*(4), 1029-1036.
607 Kemsawasd, V., Chaikham, P., & Rattanasena, P. (2016). Survival of immobilized probiotics
608 in chocolate during storage and with an in vitro gastrointestinal model. *Food*
609 *Bioscience, 16*, 37-43. doi:https://doi.org/10.1016/j.fbio.2016.09.001
610 Khorasani, A. C., & Shojaosadati, S. A. (2017). Starch-and carboxymethylcellulose-coated
611 bacterial nanocellulose-pectin bionanocomposite as novel protective prebiotic
612 matrices. *Food Hydrocolloids, 63*, 273-285.
613 doi:http://dx.doi.org/10.1016/j.foodhyd.2016.09.002
614 Klindt-Toldam, S., Larsen, S. K., Saaby, L., Olsen, L. R., Svenstrup, G., Müllertz, A., . . .
615 Zielińska, D. (2016). Survival of *Lactobacillus acidophilus* NCFM ® and
616 *Bifidobacterium lactis* HN019 encapsulated in chocolate during *in vitro* simulated
617 passage of the upper gastrointestinal tract. *LWT - Food Science and Technology, 74*,
618 404-410. doi:https://doi.org/10.1016/j.lwt.2016.07.053
619 Lalicic-Petronijevic, J., Popov-Raljic, J., Obradovic, D., Radulovic, Z., Paunovic, D.,
620 Petrusic, M., & Pezo, L. (2015). Viability of probiotic strains *Lactobacillus*
621 *acidophilus* NCFM (R) and *Bifidobacterium lactis* HN019 and their impact on
622 sensory and theological properties of milk and dark chocolates during storage for 180
623 days. *Journal of Functional Foods, 15*, 541-550.
624 doi:https://doi.org/10.1016/j.jff.2015.03.046

Mani-López, E., Palou, E., & López-Malo, A. (2014). Probiotic viability and storage stability of yogurts and fermented milks prepared with several mixtures of lactic acid bacteria. *Journal of Dairy Science, 97*(5), 2578-2590. doi:http://dx.doi.org/10.3168/jds.2013-7551

Martín, M. J., Lara-Villoslada, F., Ruiz, M. A., & Morales, M. E. (2015). Microencapsulation of bacteria: A review of different technologies and their impact on the probiotic effects. *Innovative Food Science and Emerging Technologies, 27*, 15-25. doi:http://dx.doi.org/10.1016/j.ifset.2014.09.010

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., . . . Brodkorb, A. (2014). A standardised static in vitro digestion method suitable for food - an international consensus. *Food & Function, 5*(6), 1113-1124. doi:https://doi.org/10.1039/c3fo60702j

Nami, Y., Lornezhad, G., Kiani, A., Abdullah, N., & Haghshenas, B. (2020). Alginate-Persian Gum-Prebiotics microencapsulation impacts on the survival rate of *Lactococcus lactis* ABRIINW-N19 in orange juice. *LWT - Food Science and Technology, 124*, 109190. doi:https://doi.org/10.1016/j.lwt.2020.109190

Oracz, J., Nebesny, E., Zyzelewicz, D., Budryn, G., & Luzak, B. (2020). Bioavailability and metabolism of selected cocoa bioactive compounds: A comprehensive review. *Critical Reviews in Food Science and Nutrition, 60*(12), 1947-1985.

Pech-Canul, A. d. l. C., Ortega, D., García-Triana, A., & González-Silva, N. (2020). A brief review of edible coating materials for the microencapsulation of probiotics. *Coatings, 10*(3), 197. doi:doi:10.3390/coatings10030197

Peng, M., Tabashsum, Z., Anderson, M., Truong, A., Houser, A. K., Padilla, J., . . . Biswas, D. (2020). Effectiveness of probiotics, prebiotics, and prebiotic-like components in

649 common functional foods. *Compr Rev Food Sci Food Saf, 19*(4), 1908-1933.
650 doi:https://doi.org/10.1111/1541-4337.12565
651 Pourjafar, H., Noori, N., Gandomi, H., Basti, A. A., & Ansari, F. (2020). Viability of
652 microencapsulated and non-microencapsulated lactobacilli in a commercial beverage.
653 *Biotechnology Reports, 25*, e00432. doi:https://doi.org/10.1016/j.btre.2020.e00432
654 Rad, A. H., Roudbaneh, M. M., Tabrizian, V. G., Javadi, M., Harati, N., Rad, H. N., &
655 Kasaie, Z. (2016). Chocolates as a probiotic carrier food - A Review. *International*
656 *Journal of Probiotics and Prebiotics, 11*(1).
657 Ragavan, M. L., & Das, N. (2018). Process optimization for microencapsulation of probiotic
658 yeasts. *Frontiers in Biology, 13*(3), 197-207. doi:https://doi.org/10.1007/s11515-018-
659 1495-1
660 Ranadheera, C. S., Naumovski, N., & Ajlouni, S. (2018). Non-bovine milk products as
661 emerging probiotic carriers: recent developments and innovations. *Current Opinion in*
662 *Food Science 22*, 109-114. doi:https://doi.org/10.1016/j.cofs.2018.02.010
663 Sandoval-Castilla, O., Lobato-Calleros, C., García-Galindo, H., Alvarez-Ramírez, J., &
664 Vernon-Carter, E. J. (2010). Textural properties of alginate–pectin beads and
665 survivability of entrapped *Lb. casei* in simulated gastrointestinal conditions and in
666 yoghurt. *Food Research International, 43*(1), 111-117.
667 doi:http://dx.doi.org/10.1016/j.foodres.2009.09.010
668 Sharifi-Rad, J., Rodrigues, C. F., Stojanovic-Radic, Z., Dimitrijevic, M., Aleksic, A., Neffe-
669 Skocinska, K., . . . Calina, D. (2020). Probiotics: Versatile Bioactive Components in
670 Promoting Human Health. *Medicina (Kaunas), 56*(9), 433.
671 doi:https://doi.org/10.3390/medicina56090433
672 Silva, M. P., Tulini, F. L., Marinho, J. F., Mazzocato, M. C., De Martinis, E. C., Luccas, V.,
673 & Favaro-Trindade, C. (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 - Food Science and Technology, 75*, 640-647. doi:http://dx.doi.org/10.1016/j.lwt.2016.10.025

Sommer, F., & Bäckhed, F. (2013). The gut microbiota - masters of host development and physiology. *Nature Reviews Microbiology, 11*(4), 227-238. doi:https://doi.org/10.1038/nrmicro2974

Sorrenti, V., Ali, S., Mancin, L., Davinelli, S., Paoli, A., & Scapagnini, G. (2020). Cocoa polyphenols and gut microbiota interplay: bioavailability, prebiotic effect, and impact on human health. *Nutrients, 12*(7), 1908. doi:https://doi.org/10.3390/nu12071908

Tolve, R., Cela, N., Condelli, N., Di Cairano, M., Caruso, M. C., & Galgano, F. (2020). Microencapsulation as a Tool for the Formulation of Functional Foods: The Phytosterols' Case Study. *Foods, 9*(4), 470. doi:https://doi.org/10.3390/foods9040470

Tzounis, X., Vulevic, J., Kuhnle, G. G., George, T., Leonczak, J., Gibson, G. R., . . . Spencer, J. P. (2008). Flavanol monomer-induced changes to the human faecal microflora. *British Journal of Nutrition, 99*(4), 782-792. doi:http://dx.doi:10.1017/S0007114507853384

USDA. (2019). FoodData Central: Chocolate, dark, 70-85% cacao solids. Retrieved from https://fdc.nal.usda.gov/fdc-app.html#/food-details/170273/nutrients.

Vaziri, A. S., Alemzadeh, I., Vossoughi, M., & Khorasani, A. C. (2018). Co-microencapsulation of *Lactobacillus plantarum* and DHA fatty acid in alginate-pectin-gelatin biocomposites. *Carbohydrate Polymers, 199*, 266-275. doi:https://doi.org/10.1016/j.carbpol.2018.07.002

Wu, J., Shi, M., Li, W., Zhao, L., Wang, Z., Yan, X., . . . Li, Y. (2015). Pickering emulsions stabilized by whey protein nanoparticles prepared by thermal cross-linking. *Colloids*

699 *Surfaces B: Biointerfaces, 127*, 96-104.
700 doi:http://dx.doi.org/10.1016/j.colsurfb.2015.01.029
701 Xu, M., Gagné-Bourque, F., Dumont, M.-J., & Jabaji, S. (2016). Encapsulation of
702 *Lactobacillus casei* ATCC 393 cells and evaluation of their survival after freeze-
703 drying, storage and under gastrointestinal conditions. *Journal of Food Engineering,*
704 *168*, 52-59. doi:http://dx.doi.org/10.1016/j.jfoodeng.2015.07.021
705 Yasmin, I., Saeed, M., Pasha, I., & Zia, M. A. (2019). Development of whey protein
706 concentrate-pectin-alginate based delivery system to improve survival of *B. longum*
707 BL-05 in simulated gastrointestinal conditions. *Probiotics and Antimicrobial*
708 *Proteins, 11*(2), 413-426. doi:https://doi.org/10.1007/s12602-018-9407-x
709 Zhang, L., Wang, Y., Peng, B., Yu, W., Wang, H., Wang, T., . . . Wang, J. (2014).
710 Preparation of a macroscopic, robust carbon-fiber monolith from filamentous fungi
711 and its application in Li–S batteries. *Green Chemistry, 16*(8), 3926-3934.
712 doi:http://dx.doi.org/10.1039/c4gc00761a
713 Zhang, Y., Lin, J., & Zhong, Q. (2015). The increased viability of probiotic *Lactobacillus*
714 *salivarius* NRRL B-30514 encapsulated in emulsions with multiple lipid-protein-
715 pectin layers. *Food Research International, 71*, 9-15.
716 doi:http://dx.doi.org/10.1016/j.foodres.2015.02.017
717 Zhang, Y. Y., Panozzo, J., Hall, M. S., & Ajlouni, S. (2018). Bioaccessibility of some
718 essential minerals in three selected Australian pulse varieties using an *in vitro*
719 gastrointestinal digestion model. *Journal of Food Science, 83*(11), 2873-2881.
720 doi:https://doi.org/10.1111/1750-3841.14377
721

FIGURE 1 SEM images of encapsulated probiotics of *L. rhamnosus* LGG with formulation A_2 in chocolate (A) and in freeze-dried encapsulated probiotics (B)

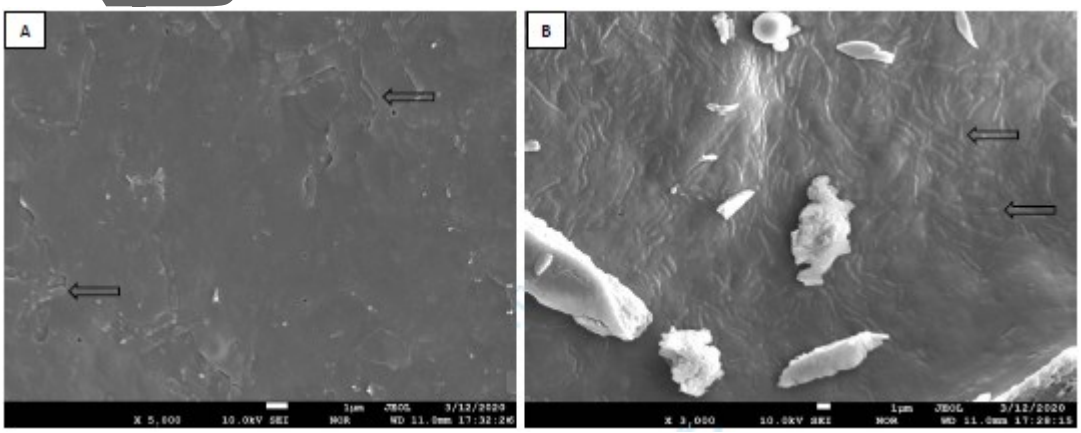


Figure 2: Encapsulated probiotics viability during the storage of 180 days at 4 °C

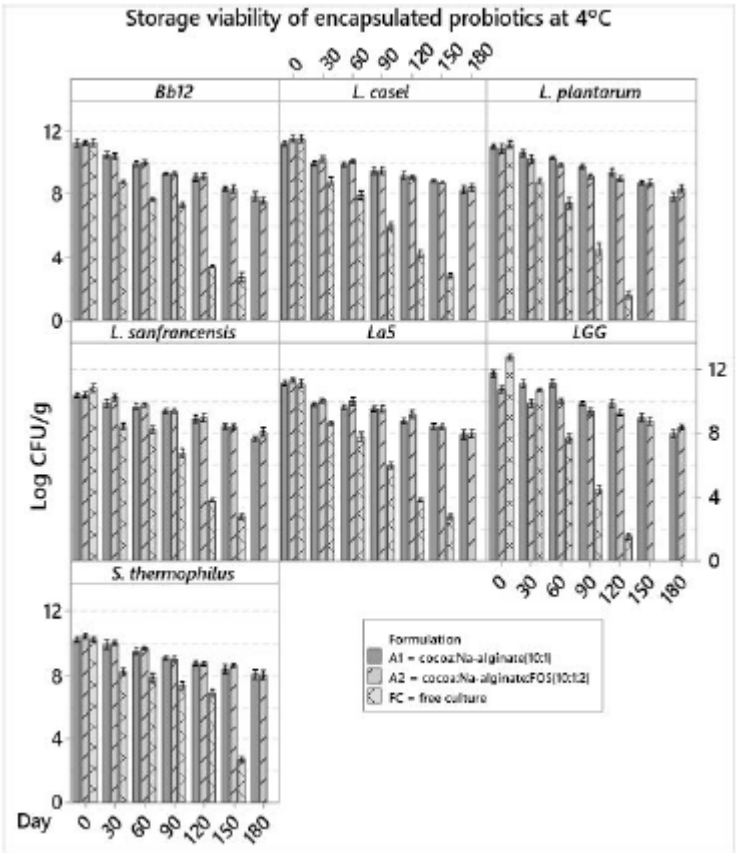
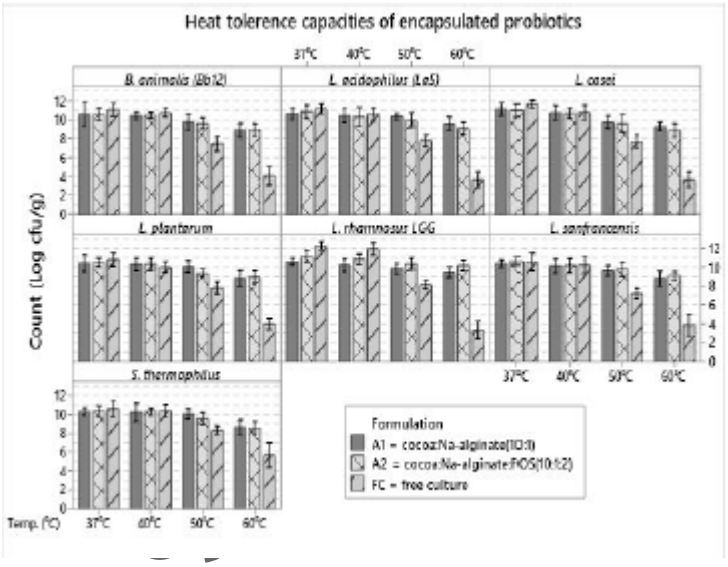
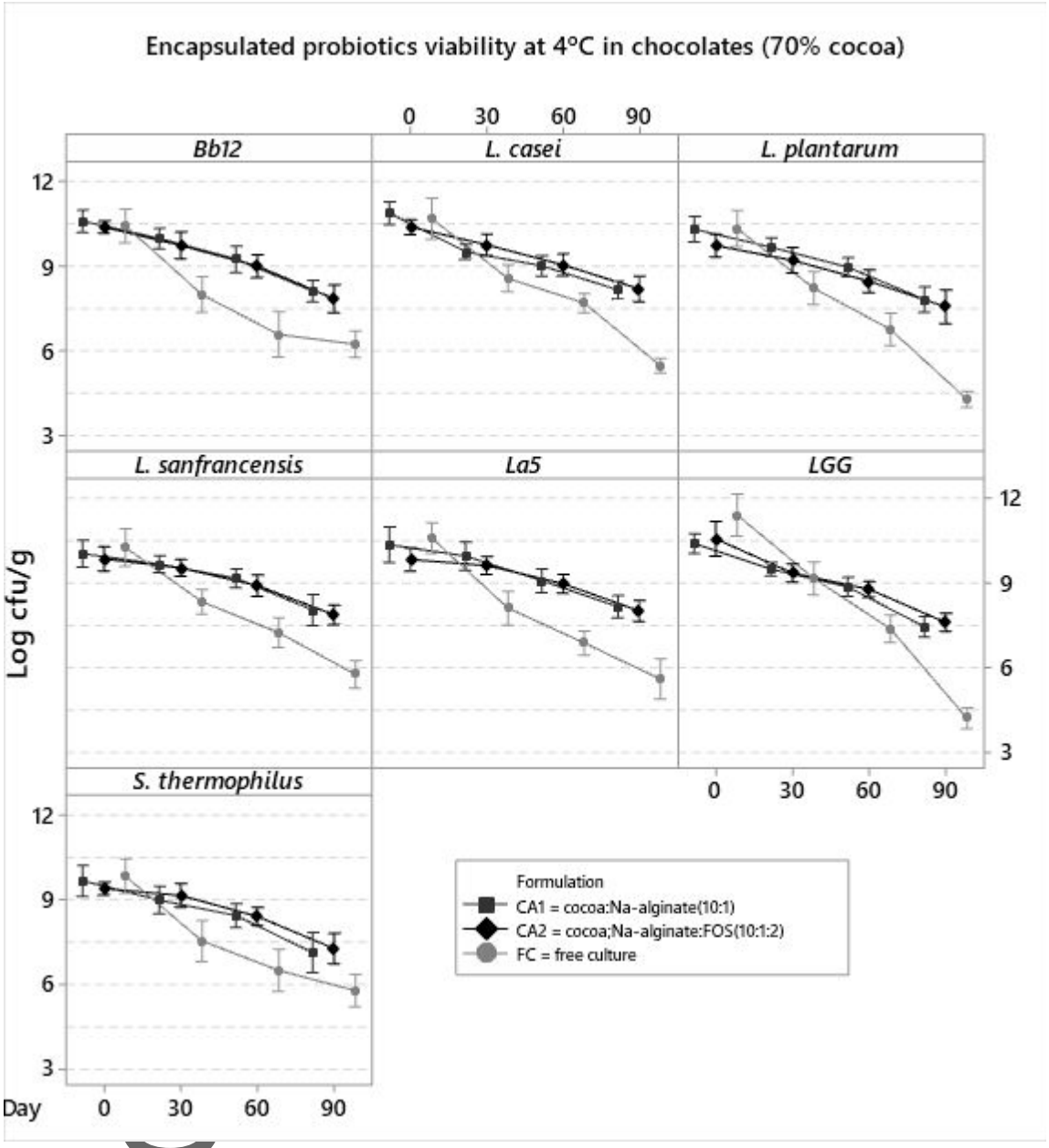


Figure 3: Effect of heat treatment on the viability of various encapsulated probiotic strains using two different encapsulants



731

732 **Figure 4:** Viability of encapsulated and free probiotics in chocolates (70% cocoa)
733 storage at 4
734 °C.



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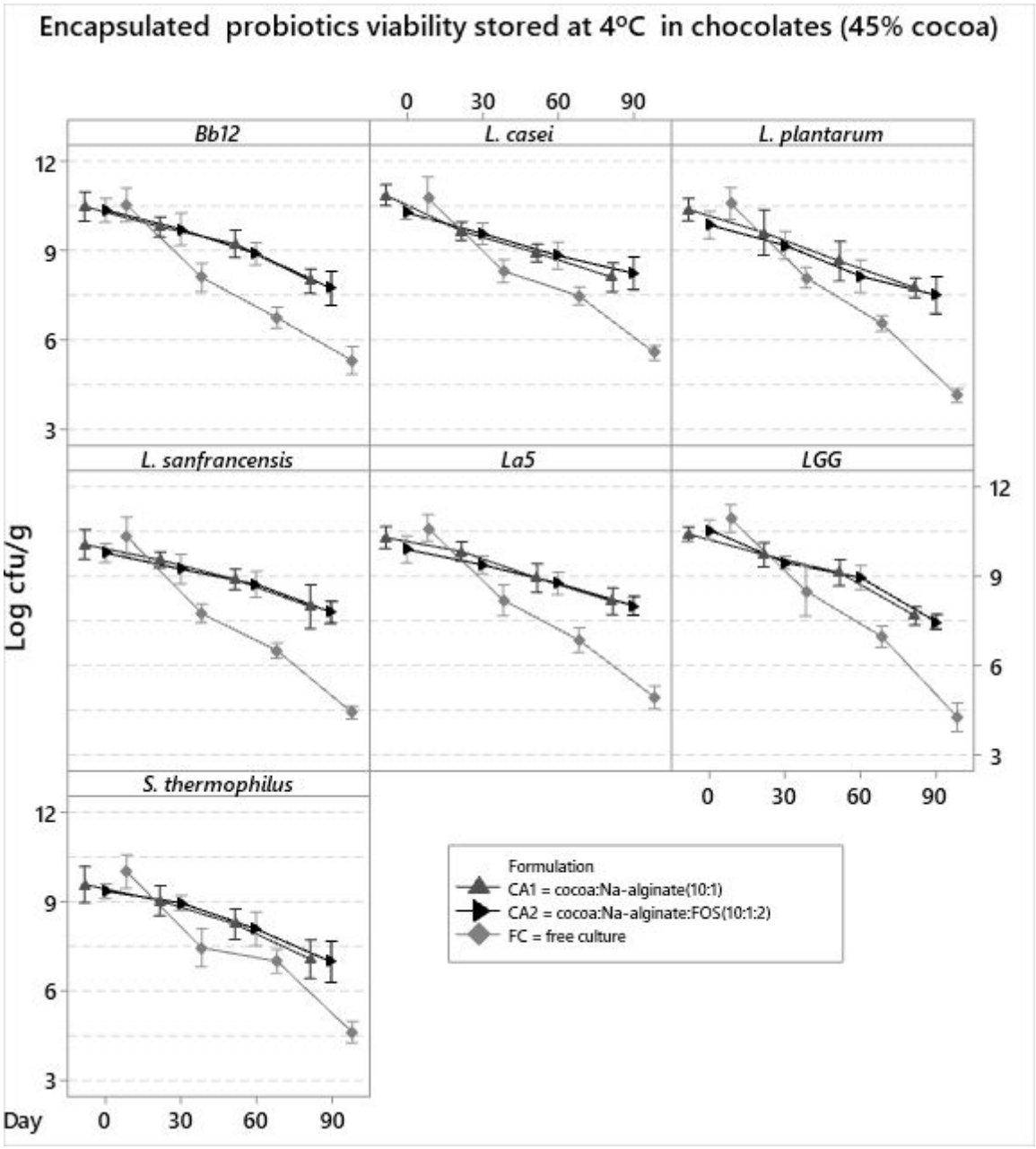
736 **Figure 5:** Viability of encapsulated and free probiotics in chocolates (45% cocoa)
737 storage at
738 4°C.



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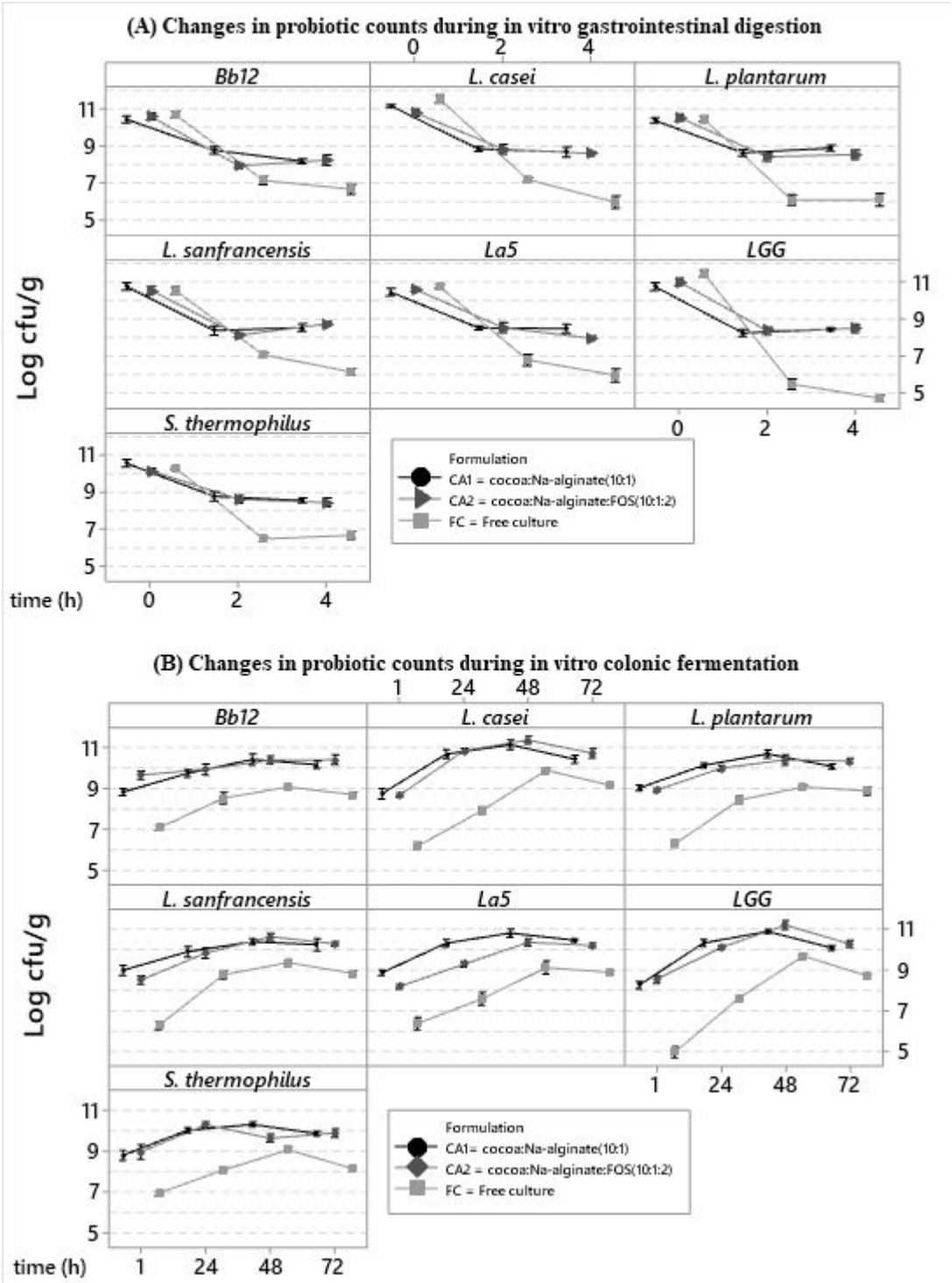
740 **Figure 6:** Effect of *in-vitro* gastrointestinal digestion (A) and colonic fermentation (B)
741 on
742 encapsulated probiotics in chocolates (70% cocoa).



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