PROFESSOR SAID AJLOUNI (Orcid ID : 0000-0002-6896-5509)

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Encapsulation increases the *in vitro* bioaccessibility of probiotics in yoghurt

Running Title: Improving yoghurt probiotic bioaccessibility

Said Ajlouni*, Chaminda Senaka Ranadheera and Ee Ling Chua

School of Agriculture & Food, Faculty of Veterinary & Agricultural Sciences, The
University of Melbourne, Melbourne VIC 3010, Australia

*Corresponding Author:

Said Ajlouni | Associate Professor | School of Agriculture and Food

Faculty of Veterinary and Agricultural Sciences

The University of Melbourne

Royal Parade, Parkville, Victoria 3010 Australia

The University of Melbourne, Victoria 3010 Australia

T: +61 3 8344-8620 **F:** +61 3 8344-5037 **E:** said@unimelb.edu.au |

www.unimelb.edu.au

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Abstract

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This study examined the encapsulation efficacy of probiotics in yoghurts, product physico-chemical properties, the best timing to add encapsulated probiotics during yoghurt manufacturing (before or after fermentation) and their *in vitro* bioaccessibility. Three different yoghurt types were produced in this study including plain and with encapsulated probiotics added before and after fermentation. A significant ($P<0.05$) reduction in total probiotic count was observed in yoghurts containing encapsulated probiotics after 21 days of refrigerated storage. However, the total probiotic count increased during the *in vitro* colonic fermentation when yoghurts containing encapsulated probiotics were used, indicating increased bioaccessibility of probiotics in the colon.

Keywords:

Probiotics, Fermentation, Gastrointestinal, Colonic fermentation, Bioaccessibility

Introduction

Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (FAO/WHO 2002). Probiotics can be consumed in the form of capsules, tablets, sachets or with food carriers, one of the most traditional and popular method. Fermented dairy products, soy products, fermented meat and fish products, and fruit and vegetable juices are well-developed and can be considered as popular probiotic food products in the market at present. However, the viability and the capability of probiotics to possess health effect are reliant on many factors such as the processing methods and type of carrier foods (Ranadheera *et al.* 2017). Among these probiotic foods, fermented dairy products such as yoghurt, cultured buttermilk and sour cream are more popular probiotic carriers because they possess other nutritional properties including naturally high calcium and vitamin D (Weichselbaum 2009).

For probiotics to sustain these therapeutic effects in human, they should be able to remain viable in the product and through the GI tract and present in adequate amount in the colon. In order to fulfil this requirement at least 10^6 cfu per mL or g of food product upon consumption is considered as the minimum therapeutic level (Kailasapathy and Rybka 1997). In addition, several essential characteristics have to be considered when choosing the probiotic strains in food applications. The selected probiotics should be non-toxic and safe. They should be able to withstand

66 technological stresses introduced during manufacturing and can be produced at large
67 scale. These strains should also exhibit resistance to adverse gastric and intestinal
68 conditions (acidic gastric juice, digestive enzymes and bile secretion in the intestine)
69 so that they can attach themselves to the gut epithelium for promoting health benefits
70 (Ranadheera *et al.* 2017).

71 Probiotics can be protected against the external stresses by a physicochemical
72 process named encapsulation. In this process the probiotics are entrapped into
73 miniature capsule-like particles that can protect them against food processing,
74 storage and harsh GI conditions while facilitating delivery to the large intestine/
75 colon alive and in sufficient numbers (Burgain *et al.* 2011). The protective capsules
76 are made up of bio-polymers which are digestible and allow probiotics to survive
77 under stressful environment and remain viable during the passage along GI tract.
78 Numerous studies showed that the viability of encapsulated probiotics was higher as
79 compared with free probiotics during products storage (Kailasapathy 2006) and
80 during *in vitro* digestion process (Fritzen-Freire *et al.* 2013). However, inappropriate
81 encapsulation materials and food preparation techniques may affect the level at
82 which probiotic cells release themselves from the encapsulant (Huq *et al.* 2013).
83 Picot and Lacroix (2004). It has been reported that whey protein polymers are the
84 most commonly used as coating material and able to produce food-grade material in
85 large scale at low cost. Another factor which affects the viability of probiotic is the
86 presence of inhibitory substance produced from the metabolisms of yoghurt starter
87 culture during fermentation (Hull *et al.* 1984). *Streptococcus thermophilus* and
88 *Lactobacillus delbrueckii* subsp. *Bulgaricus* are the common yoghurt starter
89 culture combination used in yoghurt manufacture due to their symbiotic relationship
90 (Lourens-Hattingh and Viljoen 2001). Study by Gilliland and Speck (1977)
91 demonstrated that the presence of starter culture *L. bulgaricus* caused instability in
92 probiotic *L. acidophilus*, leading to loss in its viability.

93 This project aimed to compare the bioaccessibility of free probiotics and whey
94 protein-encapsulated probiotics in yoghurt during the refrigerated storage.
95 Additionally, this study aimed to investigate the encapsulation stability of probiotic
96 *Lactobacillus acidophilus* LA-5 and *Bifidobacterium lactis* BB-12 in terms of
97 viability in yoghurt, the product physicochemical characteristics, the impact of
98 adding encapsulated probiotics before and after fermentation and their *in vitro*
99 bioaccessibility after colonic fermentation.

100 **Materials and Methods**

101 ***Materials***

102 The freeze-dried yoghurt starter cultures (*S. thermophilus* and *L. delbrueckii* subsp.
103 *bulgaricus*) and the probiotic bacteria (*Lactobacillus acidophilus* LA-5 and
104 *Bifidobacterium lactis* BB-12) were obtained from Chr. Hansen (Hørsholm,
105 Denmark). Whey protein isolate was obtained from Marry Goulburn Nutritionals,
106 VIC., Australia. Full cream milk (Woolworths Homebrand) was purchased from
107 Woolworths, a local supermarket in Melbourne, VIC, Australia. Sodium phosphate
108 (monobasic), sodium phosphate (dibasic), sodium chloride, sodium hydroxide,
109 hydrochloric acid, calcium chloride dihydrate, magnesium sulphate heptahydrate,
110 potassium phosphate (monobasic), potassium phosphate (dibasic), and sodium
111 hydrogen carbonate were purchased from the chem store in Bio21 Institute, the
112 University of Melbourne, VIC, Australia. Bile from bovine and ovine, casein, guar,
113 L-cysteine HCl, mucin from porcine stomach, pancreatin from porcine pancreas,
114 pectin from citrus peel, pepsin from porcine gastric mucosa, soluble potato starch
115 and yeast extract were purchased from Sigma-Aldrich, Castle Hill, NSW, Australia.
116 Nutrient Agar and de Man Rogosa Sharpe (MRS) Agar, AnaeroGen sachets, peptone
117 and tryptone were purchased from Thermo Fisher Scientific, Scoresby, VIC,
118 Australia. MRS agar supplemented with 0.05% L-cysteine HCl (MRS-L) was used
119 as a selective medium for LA-5 and BB-12 enumeration.

120

121 ***Methods***

122 ***Activation and preparation of cultures***

123 The yoghurt starter culture was prepared following the method of Mudannayake *et*
124 *al.* (2016). Each probiotic stock culture was prepared by adding 2.5g of the freeze-
125 dried probiotic powder into 100mL of pasteurized full cream milk at room
126 temperature. The probiotic-milk mixture was incubated at 37°C until the pH reached
127 6 (~2h). A mixed probiotic culture was prepared by mixing both inoculated cultures
128 at 1:1 ratio and stored at -20°C until used.

129

130

131 ***Preparation of whey protein solution for probiotic encapsulation process***

132 Whey protein solution (WPS) was prepared following the method of Picot and
133 Lacroix (2004). WPI (50g) was dissolved in 500mL of sterile Milli-Q water (Merk

134 Pty Ltd, Bayswater, Melbourne, Australia) and left overnight in the fridge at 4°C for
135 complete protein hydration. The WPS was then pasteurised at 65°C for 30min in a
136 water bath.

137

138 ***Encapsulation of probiotic***

139 The mixed probiotic cultures were suspended in 200mL of 10% w/v WPS and left
140 for about 1h at room temperature to allow the interaction between protein and
141 probiotic. The WPS was applied as an encapsulant for probiotics. The suspended
142 probiotics were distributed into 50mL Falcon tubes (35mL each), frozen at -20°C
143 overnight, followed by freeze-drying at -50°C for 72h using benchtop freeze dryer
144 (DynaVac FD3, United States). The freeze-dried encapsulated probiotic powder was
145 then stored at -20°C until used.

146

147 ***Yoghurt production***

148 This study involved three types of yoghurt, namely yoghurt with starter culture and
149 encapsulated probiotics added after yoghurt fermentation (A), yoghurt with starter
150 culture and encapsulated probiotics added before yoghurt fermentation (B) and
151 yoghurt with starter culture only (control). Yoghurt samples were produced
152 following the method of Mudannayake *et al.* (2016) with slight modifications.
153 Briefly, starter culture at a concentration of 0.2% (v/v) was added and the
154 encapsulated probiotic powder (0.4g) was added into only the respective samples
155 before and after fermentation. All samples were incubated at 43°C for up to 12h until
156 the pH reached 4.5, and then stored at 4°C for 21 days. Yoghurts samples were
157 prepared at least in two trials. In each trial, pasteurized full cream milk (3 litres)
158 were used to produce each type of yoghurts. In this experiment, microbial,
159 physicochemical properties were performed in duplicate on day 0, 7, 14 and 21 with
160 duplicates measurements of each parameter within each trial ($n=4$). The *in vitro*
161 gastrointestinal digestion and colonic fermentation were performed on days 0 and 21
162 only.

163

164 ***Enumeration of probiotics in yoghurt samples***

165 Viable counts of yoghurt samples were conducted using a spread plate technique as
166 described by Mudannayake *et al.* (2016) on MRS agar and expressed as log

167 colony forming unit per millilitre of yoghurt (log cfu/mL after anaerobic incubation
168 at 37°C for 48h.

169

170 ***Determination of physico-chemical properties***

171 The pH of yoghurt was determined by a pre-calibrated pH meter (Hanna HI5221)
172 using 20g of yoghurt sample mixed with equal amount of Milli-Q water. Titratable
173 acidity of yoghurt was determined by titrating 9.0 ± 1.0 g of sample in 10.0 ± 1.0 mL of
174 Milli-Q water against 0.1N NaOH using phenolphthalein as an indicator (Ryan *et al.*
175 2020). Viscosity was measured using DV1 Digital Viscometer (Brookfield, John
176 Morris Scientific) with spindle number 4 at 50 rpm Mudannayake *et al.* (2016). The
177 spindle was rotated in 50mL of yoghurt in a glass beaker and the values were
178 recorded in centipoise (cP) every 30 seconds for 2min. The mean value was
179 determined as the apparent viscosity of the yoghurt sample. The whey separation
180 (syneresis) was determined following the method of Amatayakul *et al.* (2006) where
181 10 ± 0.1 g of yoghurt was placed on the funnel lined with Whatman Filter Paper No. 1
182 and left for 2h. The amount (mL) of whey was determined by measuring the volume
183 of filtrate using a measuring cylinder

184

185 ***Simulated gastric and intestinal digestion and colonic fermentation***

186 Ethical approval was obtained from the Veterinary and Agricultural Sciences Human
187 Ethics Advisory Group, University of Melbourne (Ethics Approval ID: 1851148)
188 before conducting the colonic fermentation assay. Simulated gastrointestinal
189 digestion was carried out as described by Fu *et al.* (2016). Freshly voided faeces was
190 collected from a healthy donor who had not consumed antibiotics for 3 months.
191 Faecal slurry was prepared by mixing 20g faecal sample with 80 g sterilised 0.1 M
192 (pre-N₂ flushed) phosphate buffer in a stomacher bag to make 20% w/w faecal slurry
193 (Fu *et al.* 2016). The slurry was stomached for 5min and filtered through sterile
194 muslin cloth to remove particulate matter. Faecal slurry (5 mL aliquots) was then
195 distributed into 50mL sterile, pre-N₂ flushed tubes and stored in -80°C until used.
196 The basal medium was prepared by mixing 5g soluble starch, 5g peptone, 5g
197 tryptone, 4.5g yeast extract, 4.5g NaCl, 4.5g KCl, 2g pectin, 4g mucin, 3g casein,
198 1.5g NaHCO₃, 0.8g L-Cysteine HCl, 1.23g MgSO₄.7H₂O, 1.0g guar, 0.5g KH₂PO₄,
199 0.5g K₂HPO₄, 0.4g bile salts, 0.11g CaCl₂ and 1mL Tween-80 mixed with Milli-Q

200 water to make up to 1L. The pH was adjusted to 7 using 1M HCl or 1M NaOH
201 before autoclaving.
202 *In vitro* colonic fermentation of yoghurt samples (A, B and control) was conducted
203 by aseptically mixing known weight of sediment from the gastrointestinal-digested
204 samples (0.5 ± 0.1 g) with 5ml faecal slurry. Then 5mL of sterile basal medium was
205 added and mixed well. Blanks were prepared by mixing only 5mL faecal slurry and
206 5mL basal medium (no yoghurt was added). All tubes were flushed with O₂ free N₂
207 and lids were closed with hand-tight. The tubes were then arranged in anaerobic
208 incubation chambers containing anaerobic gas generator. The chambers were placed
209 in a shaking incubator at 100 rpm and 37°C for 24h of fermentation. The anaerobic
210 bacteria counts of the blank samples were performed at zero time (immediately after
211 preparation) and after 24h of colonic fermentation. Yoghurt samples (A, B and
212 control) from zero day and 21 days of refrigerated storage were tested only after 24 h
213 of colonic fermentation. Selected dilutions were spread plated in duplicate on
214 nutrient agar (for total anaerobic counts) and MRS agar supplemented with 0.05% L-
215 cysteine HCl (for probiotic counts). All plates were incubated anaerobically at 37°C
216 for 48h (Mudannayake *et al.* 2016).

217

218 ***Statistical analysis***

219 All experiments were repeated twice and data were subjected to one-way analysis of
220 variance (ANOVA) using Minitab 17. Treatments with significant differences were
221 separated using Tukey test at 95% confidence level ($P < 0.05$). All data were
222 presented as mean $\pm$ standard deviation.

223

224 **Results**

225 ***Viability of encapsulated probiotic bacteria***

226 Microbiological test results of the freeze-dried encapsulated probiotic showed Gram
227 positive morphological characteristics (results not shown) with a viable count of 10^8
228 cfu/g.

229 Probiotic counts in yoghurts were determined weekly during 21 days of storage at
230 4°C. Both yoghurt A and B showed a significant ($P < 0.05$) reduction in cell viability
231 over the three weeks of storage (Table 1). However, the probiotic viability in yoghurt
232 B was significantly ($P < 0.05$) greater than in yoghurt A throughout the storage time.

233 The decline in probiotic count in yoghurt A was significant ($P<0.05$) during the first
234 week of storage, and reduced to a count that was lower than the minimum
235 therapeutic level (10^7 cfu/g). However, the decline rate was slower starting from the
236 second week. Probiotic counts in control samples were <1 log cfu/g from the
237 beginning, since no probiotics were added.

238

239 ***Change in pH during yoghurt storage***

240 The pH values of all yoghurt samples were decreased during the storage. The initial
241 pH values in all yoghurt samples were significantly ($P<0.05$) different. Yoghurt B
242 had the highest pH, followed by the control and yoghurt A (Fig 1-a). The pH values
243 in B and control yoghurts showed continuous decline during storage. Similarly,
244 yoghurt A revealed a significant decline ($P<0.05$) after the 1st week of storage
245 followed by a significant increase and decrease on day 14 and 21, respectively.

246

247 ***Change in titratable acidity during yoghurt storage***

248 The titratable acidity of all yoghurt samples increased over the 3-week storage.
249 Yoghurt B and control shared a similar initial titratable acidity whereas yoghurt A
250 had significantly larger titratable acidity (Fig 1-b). The increases in titratable acidity
251 in all yoghurt samples were significant ($P<0.05$) after the 21-day storage

252

253 ***Viscosity of yoghurt during storage***

254 The viscosity of yoghurt A was significantly ($P<0.05$) lower than yoghurt B
255 throughout the storage periods (Table 2). The viscosity of all yoghurt samples
256 decreased significantly ($P<0.05$) in all yoghurt samples at 21 days of refrigerated
257 storage with the control showing the highest viscosity throughout the storage.

258

259 ***Syneresis during yoghurt storage***

260 The initial whey separation in yoghurt A and control are similar, whereas for yoghurt
261 B, it differed from both A and B samples significantly ($P<0.05$) (Table 3). However,
262 the whey separation of all yoghurt samples was not different significantly ($P>0.05$)
263 after being stored for 21 days.

264

265 ***Tolerance of probiotics to simulated gastrointestinal digestion***

Tolerance of probiotic in yoghurts to simulated gastrointestinal (GI) digestion was examined on days 0 and 21. The viability of probiotic was examined in both supernatant and sediment after centrifugation of digested yoghurt. Results showed no detectable viable probiotics counts in the supernatant of all yoghurt treatments after gastric digestion, consequently the results were not shown.

Bioaccessibility of probiotic after in vitro colonic fermentation

Change in probiotic counts in yoghurt samples on 0 day of storage when subjected to 24h in vitro colonic fermentation

The total probiotic count in the blank samples without any digested yoghurt increased by approximately 2 logs after 24h colonic fermentation. Additionally, the total probiotic count in the control was similar to the blank after 24 h fermentation (Fig 2-a). However, the probiotic counts in yoghurt A and B were significantly ($P<0.05$) larger than those in the control and blanks. Yoghurt B revealed the highest ($P<0.05$) counts among all other treatments.

Change in probiotic counts in yoghurt samples stored for 21 days followed by 24h colonic fermentation

Similar to the results of probiotic counts obtained after colonic fermentation of yoghurt stored refrigerated for zero day, yoghurt B that was refrigerated for 21 days revealed significantly ($P<0.05$) greater probiotic counts than yoghurt A and the control stored under same conditions. Additionally, blanks subjected to 24 h colonic fermentation showed significantly ($P<0.05$) greater counts than blank with 0 fermentation time (Fig 2-b).

Discussion

Effect of adding probiotics before and after yoghurt fermentation on the probiotic counts

Data in Table 1 showed that the initial probiotic count in yoghurt B (8.19 ± 0.06 log cfu/g) was significantly ($P<0.05$) higher than that of yoghurt A (7.14 ± 0.10 log cfu/g). Apparently adding the encapsulated probiotics and the starter culture before fermentation provided the bacteria with extra time to adapt and to multiply well during fermentation. In contrast to yoghurt B, the addition of encapsulated probiotics to yoghurt A after its fermentation limited the time of adaption and multiplication.

300 However, both yoghurts A and B had met the minimum therapeutic level of 10^6
301 cfu/g of yoghurt on the day of manufacturing (7.14 ± 0.10 and 8.19 ± 0.06 log cfu/g,
302 respectively). These results were in agreement with observations reported by Simões
303 *et al.* (2013), who reported that adding *L. casei* before the starter culture gave greater
304 probiotic counts than adding *L. casei* concurrently with the starter culture.
305 Data in Table 1 revealed also a decline of approximately 1 and 4 logs in probiotic
306 count of yoghurt B and A, respectively after 7 days of refrigerated storage. These
307 results were consistent with the finding by Hull *et al.* (1984), in which the decline in
308 viable count was greater in yoghurt with probiotic added after yoghurt fermentation.
309 Another study by Özer *et al.* (2005) showed that *L. acidophilus* was unable to
310 survive in yoghurt when yoghurt starter culture bacteria (*L. bulgaricus*) was present
311 in large number. The loss of probiotics, mainly *L. acidophilus*, was attributed to the
312 production of hydrogen peroxide by *Lactobacillus* starter culture. Such significant
313 decrease in *L. acidophilus* was named as acidophilus death (Lourens-Hattingh and
314 Viljoen 2001). Refrigeration temperature is another factor that can negatively affect
315 probiotic viability during yoghurt storage. Falade *et al.* (2015) reported a significant
316 decrease in lactic acid bacteria counts of both soy and Bambara yoghurts after 9 days
317 of storage at 7 °C. Probiotic bacteria grew well at temperatures between 20 and 40
318 °C with an optimum temperature range of 30–32 °C (Falade *et al.*, 2015).
319 Consequently, the growth of probiotic bacteria in yoghurt was not favourable during
320 cold storage, resulting in the decline in probiotic population. The growth of
321 bifidobacteria was substantially retarded during low temperature storage as
322 compared to *L. acidophilus* due to the low resistance of bifidobacteria cells to low
323 refrigeration temperatures (Tripathi and Giri 2014). Evidently, in this study the
324 decrease in total probiotic count in all yoghurt samples could be possibly due to the
325 loss in bifidobacterial viability.
326 In contrast to the negative effect of temperature on probiotics growth, synergistic
327 growth-promoting effects between *L. acidophilus* and *Bifidobacterium* in yoghurt or
328 yoghurt-like products were reported by Prasanna *et al.* (2014). Such synergism was
329 attributed to the role of proteolytic activity of *L. acidophilus* in supplying utilizable
330 nitrogen for *Bifidobacterium* spp. The *L. acidophilus* not only can consume peptides
331 and free amino acids for its own growth and acidification, but also can generate more
332 low molecular mass nitrogen, particularly peptides, *via* milk caseins hydrolysis to
333 sustain the growth of *Bifidobacterium* (Timmerman *et al.* 2004). Thus, any decrease

334 in cell count of *L. acidophilus* could lead to indirect decline in cell count of
 335 *Bifidobacterium*, leading to lower total probiotic count in the yoghurt.
 336 The better survival of probiotics in yoghurt B (only 1 log reduction) was probably
 337 due to transient tolerance of the starter culture *L. acidophilus* to hydrogen peroxide.
 338 Lourens-Hattingh and Viljoen (2001) reported that *L. acidophilus* cultures may
 339 develop and exhibit the ability of breaking down hydrogen peroxide during their
 340 growth in yoghurt fermentation. Most of the lactic acid bacteria, including *L.*
 341 *acidophilus*, have the ability of creating and separating hydrogen peroxide by the
 342 action of NADH oxidase and NADH peroxidase, respectively (Tripathi and Giri,
 343 2014). Similar to the reported developed tolerance against hydrogen peroxide, the
 344 results of this current study showed that the higher probiotic count in yoghurt B
 345 could be attributed to the developed tolerance and better survival of *L. acidophilus*.
 346 Consequently, adding encapsulated probiotics with starter cultures simultaneously
 347 before fermentation is suggested for better probiotics survival.
 348 Data in Table 1 revealed also a gradual decline in probiotics counts in yoghurt A and
 349 yoghurt B during the refrigerated storage. These results were in agreement with
 350 those reported by Falade *et al.* (2015) who noted similar decline in probiotic counts
 351 during storage. However, yoghurt B maintained $>10^6$ cfu/g until day 14 of storage,
 352 while yoghurt A showed significant decline in probiotic counts to $<10^5$ cfu/g starting
 353 day 7. Consequently, adding probiotics before fermentation (yoghurt B) is the
 354 recommended method since it can maintain the recommended minimum therapeutic
 355 probiotic counts during refrigerated storage.
 356 No probiotic counts were detected in the control samples (Table 1) due to the fact
 357 that only starter cultures were added to the control yoghurts before fermentation.
 358

359 **Effect of adding probiotic before and after fermentation on yoghurt pH**

360 The pH values of all yoghurt samples were significantly ($P<0.05$) different on day
 361 zero of fermentation (Fig 1-a). Yoghurt A had the smallest pH value (4.35 ± 0.02),
 362 followed by the control (4.40 ± 0.01) and yoghurt B (4.48 ± 0.01). The lower pH in
 363 yoghurt A could be due to the intense metabolic activity of the starter culture *S.*
 364 *thermophilus* and *L. bulgaricus* in the absence of the probiotics. It has been
 365 suggested by Lourens-Hattingh and Viljoen (2001) that during fermentation, *S.*
 366 *thermophilus* grows first using the amino acids released by *L. bulgaricus* and
 367 actively produces lactic acid to reduce the pH and to make the condition favourable

for the growth of *L. bulgaricus*. It can be speculated that adding encapsulated probiotics to yoghurt A after fermentation would allow them to carry out further lactose fermentation and cause more decline in pH without any competition with the starter culture. In contrast, probiotics added before fermentation (yoghurt B) must compete with the starter *L. bulgaricus* to convert lactose to lactic acid. Some of these lactic acid bacteria may not be able to survive during such competition and die off. Consequently, lower rate of lactic acid production and hence less acidic pH was detected in yoghurt B. The decline in pH values in all yoghurt samples was very rapid during the first week of storage. This was mostly the consequence of a natural phenomenon called post or over-acidification (Simões *et al.* 2013). This post or over-acidification is a mechanism that occurred mainly because of the unmanageable growth of the starter culture *L. bulgaricus* in acidic environment and even at refrigerated temperatures. As a result, D(-)-lactic acid would be accumulated in the product and lead to the decline in pH value of yoghurt after fermentation and during refrigerated storage. However, the pH values of yoghurts A (4.29) and B (4.33) after 21 days of storage (Fig 1-a) were within the acceptable pH range (3.7 to 4.3) of the commercial yoghurt (Lourens-Hattingh and Viljoen 2001). The pH values of the control samples were significantly ($P<0.05$) greater than yoghurt A and smaller than yoghurt B. These results were consistent with the initial pH values and showed similar pattern of continuous decline throughout the storage period, except for day 14 when yoghurt A showed a sudden increase in pH value. Such a variation on day 14 could be due to an experimental error, since it was not consistent with the titratable acidity measurements (Fig. 1-b).

Effect of adding probiotics before and after yoghurt fermentation on the titratable acidity of yoghurt

Titratable acidity represents a simple estimate of total acid content in a food (Sadler and Murphy 2010). Lactic acid, formic acid and some of the fatty acids and amino acids contribute to the total acid content in yoghurt. In this study, titratable acidity was expressed as percentage lactic acid. Any increase in titratable acidity was assumed as an increase in lactic acid production. The results in Figure 1-b showed that the titratable acidity of yoghurt A was significantly ($P<0.05$) higher than yoghurt B and the control on the day of production. There was no statistically significant difference ($P>0.05$) between the titratable acidity of yoghurt B and control as the rate

of lactic acid production was about the same in both samples. The titratable acidity of all yoghurt samples increased significantly ($P<0.05$) after 21 days of storage at 4°C, indicating that lactic acid was produced continuously during storage. These results were consistent with most changes in pH values, which showed continuous decline during storage, except yoghurt A on day 14, (Fig. 1-a) as discussed before under changes in pH values.

Effect of adding probiotics before and after yoghurt fermentation on yoghurt viscosity

The viscosity of yoghurt A (1293 ± 68 cP) was significantly ($P<0.05$) lower than that of yoghurt B (2130 ± 87 cP) and control (2413 ± 237 cP) on day zero of storage (Table 2). Such variations in viscosity could be attributed to the higher level of acidity in yoghurt A. The production of lactic acid increased immediately when probiotic were added after yoghurt fermentation. Consequently, yoghurt protein is denatured and broken down when acidity increased (Đurđević-Denin *et al.* 2002) leading to more water flow and a lower viscosity. This could explain the lower viscosity in yoghurt A.

Viscosity is an important parameter of yoghurt quality because it provides desirable mouthfeel and minimise syneresis Jafarpour *et al.* (2017). The viscosity decreased with storage time in all yoghurt samples. This could be attributed to an increase in yoghurt acidity. The results of this study were consistent with the findings of Jafarpour *et al.* (2017). They reported that viscosity of flavoured drinking yoghurt samples decreased during storage at 4°C over 28 days.

Effect of adding probiotics before and after yoghurt fermentation on whey separation in yoghurt

The percentage (v/w) whey separation in yoghurt B (33.55 ± 0.84) was significantly ($P<0.05$) lower than in yoghurt A (37.00 ± 0.80) and the control (37.87 ± 0.37) on day zero of storage (Table 3). Such differences in whey separation could be due to exopolysaccharides production by probiotics during fermentation. Patel *et al.* (2010) explained that probiotic exopolysaccharides showed an ample deviation in molecular structures and action complexity, which resulted in physical alteration of food components. The change in whey separation (syneresis) during the 21-day of refrigerated storage was negligible in all yoghurt samples. Similar findings were

reported by Jafarpour *et al.* (2017) who confirmed that there were no changes in total solid content or whey separation of various flavoured drinking yoghurt samples during refrigerated storage for up to 28 days.

Tolerance of probiotics to simulated gastrointestinal digestion

In vitro digestion using simulated gastric and intestinal fluids at 37°C resulted in a dramatic loss in viable probiotic counts in all yoghurt samples on day zero and after 21 days of refrigerated storage (data are not shown). Exposing the yoghurt samples to acidic gastric fluid (pH 2) for 2h, followed by 3h of incubation with pancreatin and bile salts, apparently damaged all *L. acidophilus* and *B. lactis* in both supernatant and sediment of digested yoghurt. Such significant decline in the probiotic counts may be attributed to microorganisms partial damages and entrance the state of viable but non-culturable (VBNC) under the extremely acidic conditions in gastric fluid. Previous studies by Lourens-Hattingh and Viljoen, (2001) and Tripathi and Giri (2014) indicated that growth of *Bifidobacterium* and *L. acidophilus* were retarded at pH lower than 4.6 and 4.0, respectively. The pH of gastric fluid is usually around 2.0, which is extremely low for the growth of probiotic cells. Similar observations were reported by Picot and Lacroix (2004), in which almost all the encapsulated bifidobacteria lost their viability following the exposure to simulated gastric fluid for 30min. These authors suggested that probiotic cells were experiencing low pH stress which led to temporary damages. The same study demonstrated also that the viable number of bifidobacterial in the digestion mixture increased significantly after 6h incubation at pH 7.5 in the presence of simulated intestinal fluid. It was concluded that the increase in probiotic population was due to the recovery of VBNC probiotics and multiplication at pH 7.5 due to the suitable pH conditions. Encapsulation of probiotic bacteria in whey protein-based microbeads offered a progressive release of living cells during simulated intestinal phase and hence retained viability during simulated gastric digestion by forming a physical barrier against both acidity and gastric enzymes around probiotic cells (Picot and Lacroix, 2004). However, results from the current study did not show good recovery of probiotics after 3h of incubation with simulated intestinal fluid at pH 6.8. Such results could be attributed to the short time incubation (3h only) in comparison with the 6 h reported by Picot and Lacroix (2004).

Bioaccessibility of probiotics in yoghurt after *in-vitro* colonic fermentation

The yoghurt samples were tested for probiotics bioaccessibility on day zero (Fig 2-a) and after 21 days of refrigerated storage (Fig 2-b). In order to possess health benefits to the host body, probiotics have to be viable and to reach the human colon in sufficient number to facilitate colonization and proliferation (Vasiljevic and Shah, 2008). Results from yoghurt samples refrigerated for day zero and then subjected to 24 h of colonic fermentation revealed an increase in total probiotic counts which reached 10^9 and 10^{10} cfu/g in yoghurt A and B, respectively as compared to 10^7 cfu/g in the control (Fig 2-a). The same results (Fig 2-a) showed also that 24 h *in vitro* fermentation of the blank samples (no added yoghurt) increased the total anaerobic count by approximately 2 log cycles in comparison with 0 h (no colonic fermentation). These results were in agreement with those reported by Sirisena *et al.* (2018), where the faecal anaerobic count increased significantly after *in vitro* fermentation. The availability of nutrients required for growth and multiplication during the colonic fermentation, can help probiotics cells divide and increase in number.

The higher probiotic counts in yoghurt B than A stored for zero day followed by 24 h colonic fermentation could be attributed to greater initial probiotic count in yoghurt B (8.19 ± 0.06) than yoghurt A (7.14 ± 0.10) before the *in vitro* digestion (Table 1). Additionally, the significant increase in the probiotic counts of all tested yoghurt samples after the *in vitro* colonic fermentation (Fig 2) could be attributed to recovery of the VBNC cells. As discussed before, probiotics will enter the VBNC state during the gastrointestinal (GI) digestion due to the high acid conditions (pH 2). While colonic fermentation under optimal pH and good supplement of nutrients from fermentation medium provides good recovery conditions for those damaged probiotics.

Results from probiotic counts of yoghurt samples stored for zero day (Fig 2-a) and 21 days (Fig 2-b) followed by 24 h colonic fermentation revealed similar pattern of recovery and final counts. The highest probiotic counts under both storage periods (zero and 21 days) and 24 h of colonic fermentation was detected in B followed by A and the control yoghurt samples. Consequently, it was concluded that adding probiotics before fermentation should be recommended.

Conclusions

504 Storing yoghurt for 21 days at refrigeration temperature caused significant decline in
505 the number of encapsulated probiotics despite the inoculation time: before or after
506 fermentation. However, the final probiotic count after two weeks of storage was still
507 above the recommended minimum therapeutic level (10^6 CFU/mL) when probiotics
508 were added before yoghurt fermentation. Post acidification seems responsible for not
509 only decline in probiotic counts over the storage but also major changes in
510 physicochemical properties in all yoghurt samples. Further, 24 h of colonic
511 fermentation improved the number of probiotics in both yoghurts A and B even after
512 21 days of storage, which revealed final counts of 7.14 ± 0.10 and 8.19 ± 0.06 log
513 CFU/mL, respectively. Hence, incorporation of probiotics into the yoghurt mixture
514 before fermentation could be more beneficial in terms of maintaining satisfactory
515 probiotic viability during product storage and bioavailability in the gut.

516
517 Data Availability Statement:

518 The data that support the findings of this study are available from the corresponding
519 author upon reasonable request.

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606 Legend:

607 **Table 1.** The viability (cfu/g) of total probiotics (*L. acidophilus* LA-5 and *B. lactis*
608 BB-12) in yoghurt A, B and control during 21 days of storage.

609

610 **Table 2.** The viscosity (cP) of yoghurt B, A and control measured over 21 days of
611 refrigerated storage.

612

613 **Table 3.** The percentage (v/w) whey separation determination in yoghurt B, A and
614 control during 21-day storage.

615

616 **Figure 1.** The overall pH (a) and titratable acidity (b) of yoghurt A, B and control on
617 days 0, 7, 14 and 21 of refrigerated storage. Means within each day of measurement
618 followed by different capital letters indicate significant differences ($P<0.05$) between
619 yoghurt samples. Means within each type of yoghurt followed with different small
620 letters indicate significant differences ($P<0.05$) over time.

621

622 A (yoghurt with starter culture and encapsulated probiotics added after
623 fermentation),

624 B (yoghurt with starter culture and encapsulated probiotics added before
625 fermentation and Control (yoghurt with starter culture only).

626

627 **Figure 2.** Total anaerobic counts in blanks after zero ($t=0h$) and 24h ($t=24h$) of
628 colonic fermentation (CF), and probiotic counts in yoghurts samples (A, B and

629 control) stored refrigerated for zero and 21 days and subjected to 24h CF. Data
630 represent means (n=4) with error bars as standard deviation. Columns with different
631 letters are significantly ($P<0.05$) different.
632 Blanks (faecal slurry and basal medium only)
633 A (yoghurt with starter culture and encapsulated probiotics added after yoghurt
634 fermentation), B (yoghurt with starter culture and encapsulated probiotics added
635 before yoghurt fermentation and
636 Control (yoghurt with starter culture only).

Table 1.

Yoghurt	Storage time (days)			
	0	7	14	21
A	7.14±0.10 ^{Aa}	3.66±0.09 ^{Ab}	3.54±0.17 ^{Abc}	3.33±0.09 ^{Ac}
B	8.19±0.06 ^{Ba}	7.26±0.16 ^{Bb}	6.62±0.10 ^{Bc}	5.71±0.05 ^{Bd}
Control	<1.00 ^{Ca}	<1.00 ^{Ca}	<1.00 ^{Ca}	<1.00 ^{Ca}

Values are expressed as the mean (log cfu/ml) ± standard deviation (n=4).

Means followed with different capital letters within each day of testing indicate significant differences ($P<0.05$) between yogurt samples. Means followed with different small letters within a row indicate significant differences ($P<0.05$) over storage time.

A (yoghurt with starter culture and encapsulated probiotics added after fermentation),

B (yoghurt with starter culture and encapsulated probiotics added before fermentation and

Control (yoghurt with starter culture only).

Table 2.

Yoghurt	Storage time (days)			
	0	7	14	21
A	1293±68 ^{Aa}	1182±33 ^{Ab}	1225±17 ^{Aab}	1050±33 ^{Ac}
B	2130±87 ^{Ba}	1948±22 ^{Bb}	1986±42 ^{Bb}	1765±55 ^{Bc}
Control	2413±237 ^{Ba}	1990±51 ^{Bb}	2237±47 ^{Cab}	2025±81 ^{Cb}

Values are expressed as the mean viscosity(cP) ± standard deviation (n=4).

The different capital letters indicate statistically significant differences between samples on each day ($P < 0.05$). The different small letters indicate statistically significant differences between days within each sample ($P < 0.05$).

A (yoghurt with starter culture and encapsulated probiotics added after fermentation),

B (yoghurt with starter culture and encapsulated probiotics added before fermentation and

Control (yoghurt with starter culture only).

Table 3.

Yoghurt	Storage time (days)			
	0	7	14	21
A	37.00±0.80 ^{Aa}	35.60±1.06 ^{Aa}	36.62±1.89 ^{Aa}	37.41±0.47 ^{Aa}
B	33.55±0.84 ^{Ba}	34.61±0.74 ^{Aa}	35.45±0.97 ^{Aa}	35.66±1.81 ^{Aa}
Control	37.87±0.37 ^{Aa}	35.58±0.39 ^{Aab}	34.94±1.88 ^{Ab}	35.80±1.17 ^{Aab}

Values are expressed as the mean percentage weight of whey (%) ± standard deviation (n=4).

The different capital letters indicate statistically significant differences between samples on each day ($P < 0.05$). The different small letters indicate statistically significant differences between days within each sample ($P < 0.05$).

A (yoghurt with starter culture and encapsulated probiotics added after fermentation),

B (yoghurt with starter culture and encapsulated probiotics added before fermentation and

Control (yoghurt with starter culture only).

Figure 1.

Figure 2.

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Author/s:

Ajlouni, S;Ranadheera, CS;Chua, EL

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