

1 **Targeted delivery of probiotics to enhance gastrointestinal stability**
2 **and intestinal colonisation**

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13 **Abstract**

14 The aim of this work was to assess the viability of some commercial probiotics after
15 exposure to gastric acid and the possibility of modifying these formulations for
16 delivery into the distal parts of the intestines. Gastrointestinal tolerance testing was
17 conducted for three commercial probiotics and an in-house freeze-dried
18 *Lactobacillus acidophilus* strain. The contents of the commercial products and the in-
19 house freeze-dried strain were then loaded into capsules for site-specific delivery
20 into the colon using the Phloral® coating technology; the viability upon release was
21 then ascertained. An assessment of the potential of these products to adhere to
22 intestinal cells was also conducted. The results showed that all the commercial
23 products contained the minimum number of probiotic strains as indicated on their
24 respective packages. When gastric acid tolerance tests were performed on these
25 products, all the commercial probiotics and the prepared freeze-dried strain
26 demonstrated over 10^6 CFU reductions within 5 minutes. When these were
27 encapsulated for site-specific delivery into the distal parts of the gut, viabilities of
28 approximately 90% were obtained after these capsules had been initially deposited
29 in gastric acid for 2 hours. An evaluation of the ability of the probiotic formulations to
30 adhere to intestinal cells demonstrated adhesion in the range 64-76% for the
31 products evaluated. The need to target the delivery of probiotics into the intestines
32 has been demonstrated here as this offers a greater potential for colonisation of the
33 intestines once the harshness of the stomach has been overcome.

34

35 **Keywords:** Probiotics; Gastric acid tolerance tests; Phloral® coating technology;
36 Intestinal colonisation.

37 1 Introduction

38 In recent years, there has been an increased interest in the use of probiotics with
39 several probiotic products on the market with various delivery mechanisms. The
40 concept of probiotics emerged from the early 20th century when the Russian
41 immunologist Elie Metchnikoff observed that Bulgarian peasant farmers had long life-
42 spans. He suggested this was due to the consumption of large quantities of
43 fermented milk, rich in lactobacilli. Metchnikoff suggested that pathogens present in
44 the intestine released toxins which were poisonous to the body and the consumption
45 of fermented milk helped alleviate the effects of these pathogens and their toxins
46 (Fuller, 1991, Vasiljevic and Shah, 2008, Dixon, 2002). The Food and Agriculture
47 Organisation of the United Nations, and World Health Organisation jointly describe
48 probiotics as live microorganisms that when administered in adequate amounts
49 confer a health benefit on the host (FAO/WHO, 2002).

50 For an organism to be considered as a probiotic, it must have been documented to
51 have a health benefit (Sanders, 2009). Several health claims have been attributed to
52 probiotics, some of these health claims are towards gastrointestinal health whereas
53 others are intended for systemic benefits contributing towards overall general
54 wellbeing. Gastrointestinal health claims attributed to probiotics include the
55 alleviation of lactose intolerance, prevention of antibiotic-associated diarrhoea, and
56 management of inflammatory bowel disease (Tung et al., 2009, Vasiljevic and Shah,
57 2008, Gismondo et al., 1999, Guslandi et al., 2003, Mimura et al., 2004). Probiotics
58 have also been reported to have roles in immune stimulation and modulation as well
59 as in the management of obesity (Morita et al., 2002, Kato et al., 1999). Some
60 probiotics have been reported to have anti-carcinogenic and cholesterol-lowering
61 properties (Couteau et al., 2001, Tabuchi et al., 2003).

62 The recent increased interest in probiotics also stems from the greater awareness of
63 the human microbiome and its potential applications. An increased realisation of the
64 need for alternatives to antibiotics has partly contributed to this. Even though
65 antibiotics have been around for over 50 years, hospital infection rates are not
66 declining and multi-drug resistant bacteria continue to emerge creating a major
67 public health problem as a result (Broeckx et al., 2016, Teughels et al., 2011).

68 Ideally, a probiotic must have viable organisms at the point of consumption,
69 minimum numbers of 10^6 , 10^7 , and 10^8 CFU/g at the point of consumption have been
70 reported (Shah, 2007, Douglas and Sanders, 2008, Krishnakumar and Gordon,
71 2001). The FAO/WHO, however, recommends that minimum viable numbers of each
72 probiotic strain in a product at the end of the product's shelf-life should be indicated
73 on the product label. Probiotic formulations, upon consumption, must withstand
74 gastrointestinal transit and colonise the intestines for benefits to be observed
75 (FAO/WHO, 2002, Vasiljevic and Shah, 2008, Sanders, 2009). Dairy products
76 supplemented with probiotics are a natural means of probiotics administration,
77 however, for the purposes of prevention or treatment of diseases, specifically
78 targeted applications, formulations, devices, or carriers with a slow release of
79 probiotics might be needed (Meurman and Stamatova, 2007). There have been
80 reports of commercial products exhibiting significant drops in viability after exposure
81 to gastric fluids; other products have been reported with probiotic populations that do
82 not correspond to numbers indicated on products (Hoa, 2000, Hamilton-Miller et al.,
83 2007, Fredua-Agyeman and Gaisford, 2015, Temmerman et al., 2003, Huff, 2004, de
84 Vos et al., 2010, Masco et al., 2005, Charteris et al., 1998, Caillard and Lapointe,
85 2017).

86 Formulating probiotics can be challenging since product viability must be maintained
87 during formulation and after consumption by consumers. Formulating products that
88 can withstand the harshness of the gastrointestinal tract and target them to the
89 intestines is currently receiving great interest as are formulation processes that are
90 not deleterious to organisms (Kailasapathy, 2002, Mortazavian et al., 2007). The aim
91 of this work was, therefore, to assess the viability of some commercial probiotics
92 after exposure to gastric acid and the possibility of modifying these formulations. The
93 modification approach used here was the Phloral® coating technology to target
94 probiotics to the colon. Phloral® consists of a blend of bacteria-activated (resistant
95 starch) and pH-activated (Eudragit® S) components. The independent triggers of a
96 bacterially-triggered component within a pH-responsive polymer are effective,
97 complementary, and act as failsafe mechanisms for each other in drug delivery
98 (Ibekwe et al., 2008, McConnell et al., 2008, D'Haens et al., 2017). An assessment
99 of the potential of these products to adhere to intestinal cells was also conducted.

100 2 Materials and methods

101 2.1 Materials

102 Pepsin, trehalose, xylitol, sucrose, and triton X-100 were purchased from Sigma, UK.
103 Dulbecco's Modified Eagle Medium, heat-inactivated foetal bovine serum,
104 phosphate-buffered saline (PBS), 1% non-essential amino acid, and trypsin-ethylene
105 diamine tetraacetic acid were from Gibco, UK. de Man, Rogosa and Sharpe (MRS)
106 agar and broth were from Oxoid, UK. Hydrochloric acid was purchased from VWR,
107 UK. Sodium chloride was purchased from Fisher Scientific, UK.

108 Three commercial probiotic products were bought from a health food shop and
109 analysed. The choice of probiotics was limited to products that contain only
110 lactobacilli strains; these are represented here as Products A, B, and C and their
111 composition is indicated in Table 1. These probiotics were selected because they
112 contain strains common to most probiotic products, and so the results have wide
113 applicability. An in-house probiotic was also prepared by freeze-drying *Lactobacillus*
114 *acidophilus* LA 5.

115 Table 1: Composition of probiotic products used

Product	Formulation	Composition	Stated minimum content per capsule (x 10 ⁸ CFU)
Product A	Encapsulated dried powder	<i>Lactobacillus acidophilus</i> PXN 35 <i>Lactobacillus plantarum</i> PXN 47	5
Product B	Encapsulated dried powder	<i>Lactobacillus plantarum</i> LP 299v	200
Product C	Encapsulated dried powder	<i>Lactobacillus plantarum</i> CECT 7527 <i>Lactobacillus plantarum</i> CECT 7528 <i>Lactobacillus plantarum</i> CECT	12

116

117 **2.2 Preparation of freeze-dried probiotic**

118 An in-house freeze-dried probiotic formulation was prepared for comparison with the
119 commercial products. *Lactobacillus acidophilus* LA5 was used as the model probiotic
120 strain with 10% sucrose or 10% trehalose used as protectants.

121 2.2.1 Cultivation of microorganism and preparation of bacterial culture

122 *Lactobacillus acidophilus* was initially grown on MRS (de Man, Rogosa, and Sharpe)
123 agar and incubated under anaerobic conditions for 48 hours at 37 °C. A few colonies
124 were taken and used to inoculate 7 mL of MRS broth to create a starter culture and
125 incubated for 24 hours. 99 mL of fresh MRS broth was inoculated with 1 mL of
126 starter culture to create a 1: 100 dilution and this was incubated for 24 hours. The
127 culture was then mixed using a magnetic stirrer and 30 mL each was dispensed into
128 3 falcon tubes (Tubes A, B, and C). Centrifugation was done at 9500 rpm and 4 °C
129 for 10 minutes to harvest the cells after which supernatant was removed carefully by
130 suction. The cells were washed with PBS and centrifuged at 9500 rpm and 4 °C for
131 10 minutes. The supernatant was removed by suction and the washing process
132 repeated. The cells in tube A were resuspended in ¼ Ringer's solution only and
133 tubes B and C resuspended in 10% sucrose and 10% trehalose in ¼ Ringer's
134 solution respectively. The bacterial cultures were mixed continuously using a
135 magnetic stirrer and 2 mL aliquoted into sterile 5 mL glass vials for freeze-drying.

136 2.2.2 Freeze-drying

137 Freeze-drying was done with VirTis-Advantage freeze-dryer (UK). The samples were
138 initially submerged under dry ice for about 60 minutes. Once frozen, the vials were
139 transferred onto pre-cooled shelves in the freeze-dryer. The freezing and condenser
140 temperatures were -40 °C and -60 °C respectively. The primary drying step was at -
141 20 °C for 48 hours. The vacuum pressure was maintained below 200 mBar.

142 Secondary drying was done at 20 °C for 3 hrs. Enumeration after freeze-drying was
143 conducted and percentage recovery obtained.

144 **2.3 Evaluating viability of probiotic products**

145 The contents of one capsule of each commercial probiotic were suspended in 5 mL
146 of PBS and vortexed for about 10 seconds and allowed to stand for 10 minutes and
147 vortexed once more for homogeneity. These were then serially diluted and plated
148 onto MRS agar. The agar plates were incubated at 37 °C under anaerobic conditions
149 for 48 hours after which colonies were counted. 100 mg was used for the prepared
150 freeze-dried formulation.

151

152 **2.4 Evaluating the in vitro adhesion of probiotics to intestinal cells**

153 The evaluation done here was similar to work carried out by Forestier et al. (2001).
154 The growth medium used was Dulbecco's Modified Eagle Medium supplemented
155 with 10% of heat-inactivated foetal bovine serum and 1% non-essential amino acid.
156 The prepared medium was used to routinely grow Caco-2 cells in cell culture flasks
157 with surface area 75cm² or to seed cells in a well-plate. All cells were incubated at
158 37 °C in a humidified atmosphere of 5% CO₂.

159 Caco-2 monolayers were seeded at a concentration of 2 x 10⁵ cells per well in 12-
160 well plates and incubated at 37 °C with humidified atmosphere of 5% CO₂. Caco-2
161 cells were used in the late post-confluence stage with passage numbers between 39
162 and 54. Prior to adhesion testing, Caco-2 monolayers were washed twice with 1 mL
163 PBS; 1 mL of the cell culture growth medium was then added to each well. The
164 contents of one capsule of each commercial probiotic and 100 mg of the freeze-dried
165 *L. acidophilus* strain were suspended in 5 mL of PBS. These were serially diluted
166 such that the estimated bacterial numbers did not exceed the Caco-2 cell numbers.
167 1mL of each test sample was then added to 1mL of growth medium in each well and
168 plates incubated for an hour. The monolayers were then washed three times with 1
169 mL PBS to remove any non-adhered bacterial cells. The cells were lysed by addition
170 of 1 mL 0.1% triton X-100 solution and the number of viable adhering bacteria
171 determined by plating out serial dilutions onto MRS agar. The number of adhered

172 cells was then expressed as a percentage of the initial number. An estimate of the
173 number of Caco-2 cells in the wells was made each time adhesion tests were
174 performed. This was to ensure the available Caco-2 cells were not less in number
175 than the bacterial cells being added. The Caco-2 cells used for these tests were
176 between 2.9×10^5 – 1×10^6 cells per well.

177 **2.5 Evaluating tolerance to gastric fluids**

178 2.5.1 Preparation of Simulated Gastric fluid (SGF)

179 100 mL of sodium chloride and hydrochloric acid (HCl) mixture was first prepared by
180 dissolving 0.2 g of sodium chloride in 90 mL purified water. The pH was adjusted to
181 1.2 with HCl and volume made up to 100 mL. 0.32 g of pepsin was then added to 50
182 ml of the sodium chloride and HCl solution and stirred until complete dissolution. The
183 volume was adjusted to 100 mL afterwards. The solution was filter-sterilised and
184 used within 48 hours of preparation.

185 2.5.2 Tolerance testing

186 The contents of one capsule of each product were deposited into the SGF for 2
187 hours. All the media were pre-warmed to 37 °C prior to use and bacterial
188 enumeration was conducted at set times. To ensure the process was carried out at
189 37 °C with 50 rpm paddle movement as observed in USP dissolution testing, a hot
190 plate magnetic stirrer was used with temperature and speed set to 37 °C and 50 rpm
191 respectively. 50 mL of gastric media in sterile duran bottles were used. Aliquots were
192 taken at set times and enumeration of probiotic species conducted using MRS agar
193 plates. The plates were incubated for 48 hours afterwards under anaerobic
194 conditions. 100 mg was used for the prepared freeze-dried formulation.

195 **2.6 Encapsulation of probiotics for site-specific delivery**

196 Capsules were coated using Phloral® – a coating technology developed at University
197 College London that targets the release of drugs to the colon. The contents of one
198 capsule of each commercial probiotic and 100 mg of the in-house freeze-dried *L.*
199 *acidophilus* were then transferred into empty size 0 capsules (Qualicaps® USA) and
200 coated using the Phloral® coating technology as reported by Ibekwe et al. (2008).
201 These were then analysed using a modified dissolution testing method mimicking
202 standard USP dissolution testing.

203 Capsules were immersed in three consecutive media i.e., 0.1M HCl (2 hours),
204 phosphate buffer at pH 6.8 (2 hours), and phosphate buffer at pH 7.4 (1 hour). All the
205 media were pre-warmed to 37 °C prior to use. Bacterial enumeration was conducted
206 at hourly intervals in all media. To ensure the process was carried out at 37 °C with
207 50 rpm paddle movement as observed in USP dissolution testing; a hot plate
208 magnetic stirrer was used with temperature and speed set to 37 °C and 50 rpm
209 respectively. 50 mL media in sterile duran bottles were used and all capsules were
210 completely immersed with the aid of sinkers.

211 3 Results and Discussion

212 3.1 Freeze-drying *Lactobacillus acidophilus* LA5

213 The freeze-dried formulations with the protectants yielded recoveries over 90%; a
214 complete loss of viability was observed when no protectant was added (Table 2).

215

216 Table 2: Percentage recovery after freeze-drying *L. acidophilus* LA5 using sugars as protectants

Sample	Percentage Recovery (%)
Control	0
10% Sucrose	92.94 ± 1.08
10% Trehalose	94.22 ± 2.67

217

218 Freeze-drying is a procedure based on sublimation under high vacuum, this occurs
219 in three phases, i.e., freezing, primary drying, and secondary drying. During freeze-
220 drying, the formation of intracellular ice crystals and macromolecule denaturation
221 results in the loss of viability; hence, to preserve cell viability, protectants are added
222 (Santivarangkna et al., 2007, Zayed and Roos, 2004, De Giulio et al., 2005). There
223 are numerous protectants used to improve cell viability during such procedures.
224 Sugars have been reported to be beneficial to most lactobacilli during drying and
225 upon storage; 10% sugar concentrations have been used with high recoveries after
226 freeze-drying (Costa et al., 2000, Zhao and Zhang, 2005). In this study, 10%
227 trehalose or 10% sucrose was, therefore, used as protectants during freeze-drying.
228 Other additives like non-skimmed fat milk, polyols, polymers, amino acids have also
229 been used (Efiuvwevwere et al., 1999, Costa et al., 2000). There is no single additive
230 that fits all organisms, therefore, as best practice, a variety of excipients are explored
231 during freeze-drying and the best excipient with the most improved viability chosen.
232 Viabilities of 93% and 94% were obtained when 10% sucrose and 10% trehalose
233 were used respectively as compared to a complete loss of viability when no
234 protectant was included in freeze-drying *L. acidophilus*. De Giulio et al. (2005) also
235 observed viabilities over 90% after freeze-drying some lactobacilli; they, however,
236 used 32% sucrose and trehalose. The concentration of sugar used in freeze drying is

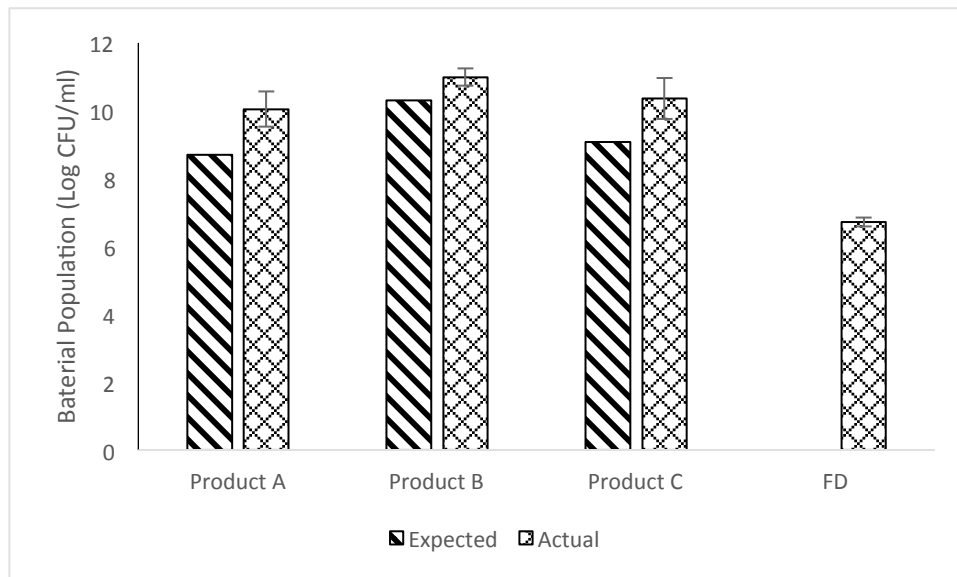
237 important; when Zayed and Roos (2004) used 4% sucrose and 4% trehalose as
238 protectants, they could recover only 13% and 34% viabilities respectively after
239 freeze-drying. They needed a combination of both amounts in addition to 18%
240 skimmed milk to obtain 83-85% survival rates.

241

242 **3.2 Evaluating viability of product content**

243 An evaluation of the content in each of the products analysed is shown in Figure 1.
244 All the products met the minimum indicated content on their packages, with Product
245 A having more than 10 times the microorganisms as stated on product package.

246



247

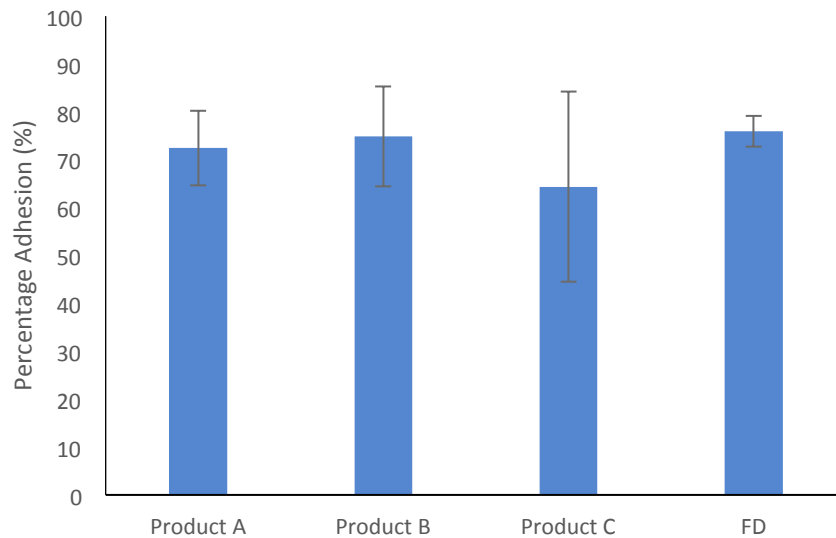
248 Figure 1: Bar chart illustrating the comparison between the expected and actual bacterial numbers per
249 dose – a capsule each for the solid dose probiotics and 100 mg for freeze-dried (FD) strain – obtained
250 after enumeration.

251

252 With several studies as indicated in the introduction reporting about the lack of viable
253 organisms or reduced numbers than what has been indicated on product packages,
254 this was a good indication as it confirmed the probiotics had been well preserved and
255 from the consumer's point of view, gives confidence that the formulations purchased
256 actually contain the stated number of bacteria. The presence of viable organisms
257 after product formulation is key to ensuring probiotic activity.

258 **3.3 *In vitro* adhesion of commercial probiotics to intestinal cells**

259 An enumeration of probiotic cells that adhered to intestinal cells for the products A, B
260 and the freeze-dried strain showed that over 70% of the administered probiotics
261 adhered to intestinal cells (Figure 2). Product C, however, had 64% of the
262 administered probiotics adhering.



263

264 Figure 2: An illustration of the percentage of administered probiotics that adhered to intestinal cells

265

266 For probiotics delivered into the gastrointestinal tract to act, these need to colonise
267 the intestines. Evaluation of probiotic colonisation *in vivo* is very challenging; a few
268 studies have used the presence of probiotics in faeces as an indication of
269 colonisation; this is, however, very speculative as faecal enumeration generally
270 represents unadhered strains (Saxelin et al., 1993, Saxelin et al., 1995). An *in vitro*
271 evaluation was demonstrated here whereby adhesion to Caco-2 intestinal cells was
272 used to evaluate probiotic adhesion. This method of evaluation that ascertains
273 probiotic organisms that adhere to intestinal cell lines as an *in vitro* model for has
274 been reported (Govender et al., 2014, Maragkoudakis et al., 2006, Forestier et al.,
275 2001, Gopal et al., 2001, Tuomola and Salminen, 1998). More than half the number
276 of the probiotic organisms administered adhered to intestinal cells, Products C which
277 exhibited the least adhesion of the products tested had 64% of administered
278 organisms adhering to Caco-2 cells. This indicated that all the dried probiotics

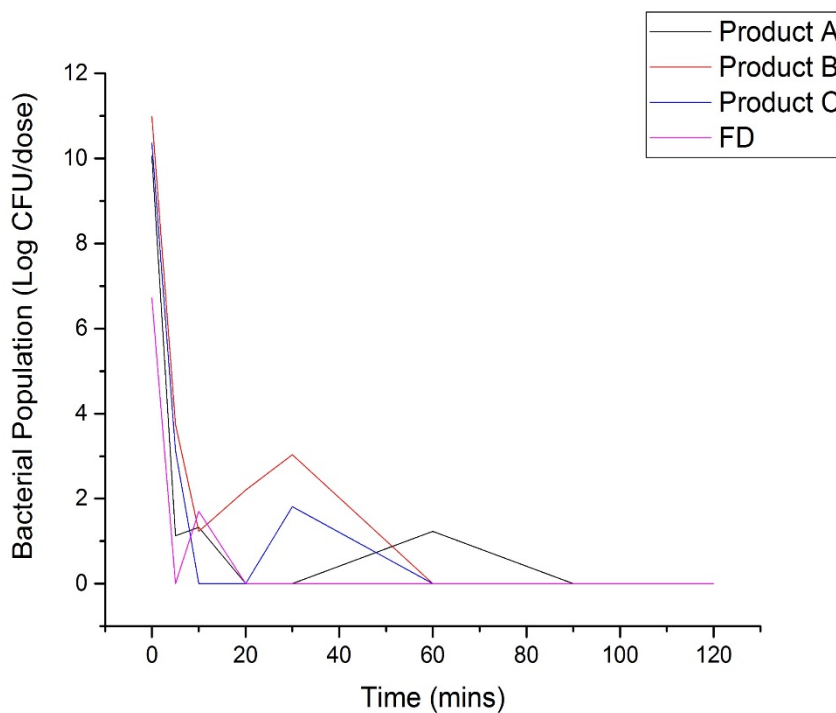
279 evaluated had the ability to adhere to intestinal cells and could possibly colonise the
280 intestines should these arrive there.

281 3.4 Evaluating tolerance to gastric fluids

282 When the probiotics were exposed to simulated gastric fluid as shown in Figure 3,
283 there was a sharp drop in viability within 5 minutes for all the solid dose probiotics
284 evaluated; this reduction was circ. over 6 Log CFU.

285

286



287

288 Figure 3: An illustration of probiotic population with time after exposure to fasted state simulated
289 gastric fluid for products A-C and freeze-dried strain, FD.

290

291 Gastric fluid is one of the barriers most biological preparations need to overcome to
292 exert their impact. Even though some reports have highlighted the harshness of this
293 medium to probiotics, others are of the perception that probiotics are acid-producing
294 organisms and can, therefore, withstand the acidity of the stomach (Govender et al.,
295 2014, Sahadeva et al., 2011, Picot and Lacroix, 2004). Probiotics produce lactic
296 acid, which is a weak acid and well tolerated by most probiotics as compared to
297 gastric acid which is, 0.1 M HCl, a strong acid. In a study conducted to evaluate

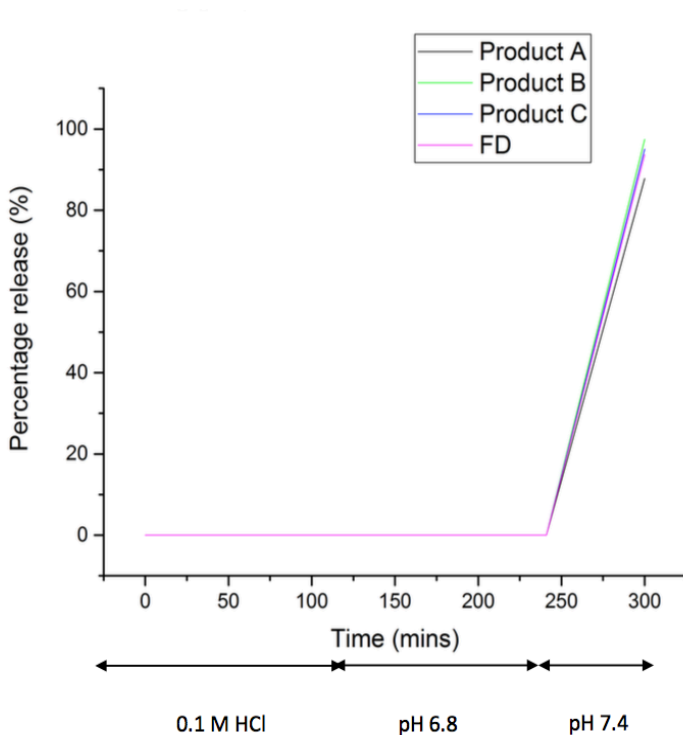
298 some probiotic strains to gastric acid, only 1 of 15 probiotic strains was considered to
299 be intrinsically resistant to gastric acid, the remaining strains were all killed (Charteris
300 et al., 1998).

301 When the probiotics were exposed to SGF, there was a significant drop in viability. It
302 was observed that all the solid probiotics were just dried probiotics with no protective
303 mechanisms against the gastric fluid. Such formulations have a low probability of
304 delivering viable organisms into the intestines when taken. Fredua-Agyeman and
305 Gaisford (2015) observed similar results when they evaluated the gastric acid
306 tolerance of some commercial solid dose formulations. It is important for probiotics to
307 be formulated with protective mechanisms against gastric fluid otherwise most
308 probiotic strains could be killed during transit. The outcome of this test was worrying
309 because 2 of the products, B and C, had inscriptions on their packages indicating the
310 tolerance of the contained probiotic strains to gastric acid, the results here, however,
311 demonstrated otherwise.

312 **3.5 Encapsulation of probiotics for site-specific delivery**

313 When the probiotics were encapsulated using the Phloral® coating technology, no
314 viable bacterial enumeration was obtained until after pH 7 when the threshold of
315 dissolution for the coating material was reached. Bacterial enumeration at the target
316 site was over 90% for all products, except for Product A which had a percentage
317 recovery of 88% (Figure 4).

318



319

320 Figure 4: An illustration of percentage recovery after Phloral® coated capsules (Products A – C and the
321 freeze-dried strain, FD) were immersed consecutively in 0.1 M HCl and pH 6.8 for 2 hours then in pH
322 7.4.

323

324 With the distal parts of the gut as target sites, Phloral® coating technology was used.
325 The main components of Phloral® are Eudragit® S, which is a synthetic polymer that
326 dissolves at pH > 7, and resistant starch, which is not digested by mammalian
327 amylase enzymes secreted by the pancreas but by colonic bacterial enzymes; this
328 makes the technology useful in drug targeting (Ibekwe et al., 2008, McConnell et al.,
329 2008, D'Haens et al., 2017). Upon capsule dissolution, it was observed that the
330 probiotics investigated all had high viabilities with the lowest recorded viability being

331 88%. This confirms the need for most dried probiotics to be incorporated into gastro-
332 resistant formulations and delivered to the distal parts of the intestines. Some
333 formulations have been reported whereby probiotics have been microencapsulated,
334 although the reported recoveries have been low (Poelvoorde et al., 2008, Sultana et
335 al., 2000). These formulations usually involve applying coating material directly to
336 probiotics strains. The reduced viabilities could be because these coating materials
337 themselves could have impacted directly on the organisms since organic solvents
338 are sometimes used as ingredients in capsule coating (Cole et al., 2002,
339 Huyghebaert et al., 2004). Optimisation of coating conditions may be needed to
340 enhance product viability of these microencapsulated formulations. The need to
341 protect probiotics was emphasised recently by Caillard and Lapointe (2017) when
342 they evaluated some commercial probiotics and found that only the enteric-coated
343 formulations had resistance after exposure to gastric acid at fasting and all the
344 unprotected formulations had high drops in numbers after exposure.

345

346

347

348 **4 Conclusion**

349 The commercial products and freeze-dried *L. acidophilus* LA5 evaluated here all
350 demonstrated poor tolerance to simulated gastric fluid even though some of these
351 commercial products had inscriptions on product packs indicating tolerance of the
352 contained probiotic strains to gastric acid. When the commercial and in- house
353 freeze-dried probiotics were encapsulated using the Phloral® to target the delivery
354 into lower small intestines or colon, viabilities of approximately 90% were observed
355 after these capsules had been initially deposited in gastric acid for 2 hours. These
356 products also demonstrated over 60% adhesion to intestinal cells *in vitro* highlighting
357 the potential for colonisation should these overcome the harshness of the stomach.
358 The need to target the delivery of probiotics into the intestines has been
359 demonstrated here as this offers a greater potential for colonisation of the intestines
360 once the harshness of the stomach has been overcome.

361

362 Declaration of Interest: This work was supported by the Commonwealth Scholarship
363 Commission.

364

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