

Electrosprayed mucoadhesive alginate-chitosan microcapsules for gastrointestinal delivery of probiotics

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34 alginate-starch formulations was better protected against simulated gastric conditions than in
35 alginate ones, but not better than in the chitosan-coated ones.

36 **Keywords:** electrospraying; microcapsules; probiotics; mucoadhesion; alginate; chitosan

37

38 **1. Introduction**

39 Probiotics are living microorganisms present in human gastrointestinal tract that can promote
40 health by preventing or alleviating disorders and diseases when administered in sufficient (live)
41 amounts (FAO/WHO, 2001). These specific microorganisms are usually consumed as dietary
42 supplements or as fortified foods, due to their proposed beneficial effects on human
43 gastrointestinal health and immune system. For exerting their therapeutic functions, the most
44 generally suggested minimal amount of viable probiotics to be consumed is 10^6 - 10^7 CFU (colony
45 forming unit) per g/mL of a probiotic product (Nazir et al., 2018; Yao et al., 2020). Minelli and
46 Benini (2008) demonstrated a necessary probiotic survival at a minimum of 10^6 CFU/mL of
47 digesta after the gastric challenge and a minimum presence of 10^8 CFU/g of end digesta in the
48 colon.

49 However, probiotics incorporated in food products tend to lose a significant number of their viable
50 cells during processing, storage and the passage through the gastrointestinal tract. In this context,
51 the therapeutic functionality of probiotic supplements has also been shown to be questionable for
52 this same reason (Corona-Hernandez et al., 2013; Doodoo et al., 2017; Fredua-Agyeman et al.,
53 2015; Heidebach et al., 2012). Microencapsulation technology has emerged to support the survival
54 of probiotics against harsh environmental factors encountered during processing and their transit
55 through the gastrointestinal tract and to target their delivery to the colon to ensure probiotic
56 colonisation (Yao et al., 2020).

57 There has been a significant progress on probiotic microencapsulation using extrusion,
58 emulsification and spray drying (Chavarri et al., 2012; Cook et al., 2012; Das et al., 2014; Solanki
59 et al., 2013). However, some shortcomings in these methods are still present in terms of producing
60 particles with all the necessary characteristics, using technologies that are suitable for industrial
61 applications. In recent years, the concept of using electrospraying (electrohydrodynamic
62 atomisation) for microencapsulation has been reported a few times (Coghetto et al., 2016; Gómez-
63 Mascaraque et al., 2017; Librán et al., 2017; Zaeim et al., 2017, 2018). In this technique a polymer
64 solution, while passing it through a needle, becomes charged by the presence of high potential
65 electric field applied at the tip of the needle and is consequently atomised into finer liquid droplets.
66 These electrified droplets then fall towards an oppositely charged metallic collector, with which
67 microparticles can be formed.

68 Probiotic microencapsulation using electrospraying can offer several advantages, considering its
69 mild processing conditions, high production yield and good industrial scalability as compared to
70 the above-mentioned conventional methods. This technique also allows the preparation of particles
71 in micron-size range, making them suitable for incorporation in some specific commercial
72 probiotic products (Bhushani et al., 2017; Wang et al., 2019).

73 Previous studies have reported excellent potential of calcium alginate as a material for
74 microencapsulating and protecting probiotics (Cook et al, 2012). However, several considerable
75 limitations have also been identified for alginate-based microcapsules, including their high
76 porosity (Chen and Chen, 2007; Gombotz and Wee, 2012; Martín et al., 2015; Smidsrød and Skjåk-
77 Bræk, 1990).

78 Coating alginate with chitosan has been explored in some studies to resolve these problems
79 (Chávarri et al., 2010; Cook et al., 2011; Nualkaekul et al., 2012). In addition, inclusion of prebiotic
80 compounds (often oligosaccharides) into the alginate matrix or co-encapsulation with a range of
81 biopolymers have been proposed as alternative for enhancing the functional effects of encapsulated
82 probiotics (Ashwar et al., 2018; El-Abd et al., 2018; Krasaekoopt et al., 2003; Krasaekoopt and
83 Watcharapoka, 2014; Sabikhi et al., 2011; Samedi and Charles, 2019; Sultana et al., 2000).
84 Prebiotics are the group of non-digestible food nutrients that can serve as selective substrates for
85 the probiotics (and beneficial gut microbiota), thereby conferring positive effects on human health
86 (Davani-Davari et al., 2019).

87 Additional approach for enhancing the efficiency of gastrointestinal delivery of probiotics is to
88 formulate microcapsules with mucoadhesive coating/matrix materials, ensuring their sufficiently
89 longer residence time, thus better bioavailability of probiotics in the gastrointestinal tract (Alli et
90 al., 2011; Cook et al., 2012; Singh et al., 2012; van Tassell and Miller, 2011).

91 In this study, we have used the electrospraying technique to produce microcapsules containing
92 *Lactobacillus plantarum* for gastrointestinal delivery. Several types of microcapsules were
93 prepared and evaluated, including chitosan-coated and uncoated calcium alginate capsules as well
94 as microcapsules formulated as alginate mixture with resistant starch (prebiotic). These capsules
95 were characterized using laser light diffraction and fluorescent microscopy and assessed in terms
96 of the encapsulated bacteria survival, *in vitro* simulated gastric digestion and *in vitro* gastric
97 mucosal retention. Additionally, a novel, simple and cheap fluorescence imaging-based set-up was
98 designed to test the mucoadhesive properties of these microcapsules.

99

100 **2. Materials and methods**

101

102 *2.1. Materials*

103 The model probiotic strain of *Lactobacillus plantarum* NCDO 1752 used in this study was from
104 the culture stock collection of National Collection of Dairy Organisms (NCDO), UK. Sodium
105 alginate was obtained from SAFC Supply Solutions (St. Louis, MO, USA). Resistant starch
106 (unmodified, raw potato-originated) was purchased from a local health supplement retailer
107 (Reading, UK). Chitosan (low molecular weight, 75-85% deacetylated, 50-190 kDa), sodium
108 fluorescein (Na-Fluo), fluorescein isothiocyanate (FITC), fluorescein isothiocyanate-dextran
109 (FITC-dextran; MW 3000-5000 Da) and phosphate-buffered saline (PBS) in tablet form were
110 purchased from Sigma Aldrich (Gillingham, UK). Calcium chloride and sodium chloride were
111 received from Fisher Scientific (Loughborough, UK). De Man, Rogosa and Sharp broth (MRS
112 broth) and bacteriological agar were from Oxoid (Hampshire, UK). Deionised water was used for

113 the preparation of all solutions. All solutions (without containing microorganism) and glassware
114 related to microbiological experiments were sterilised at 121°C for 15 min with an autoclave.

115

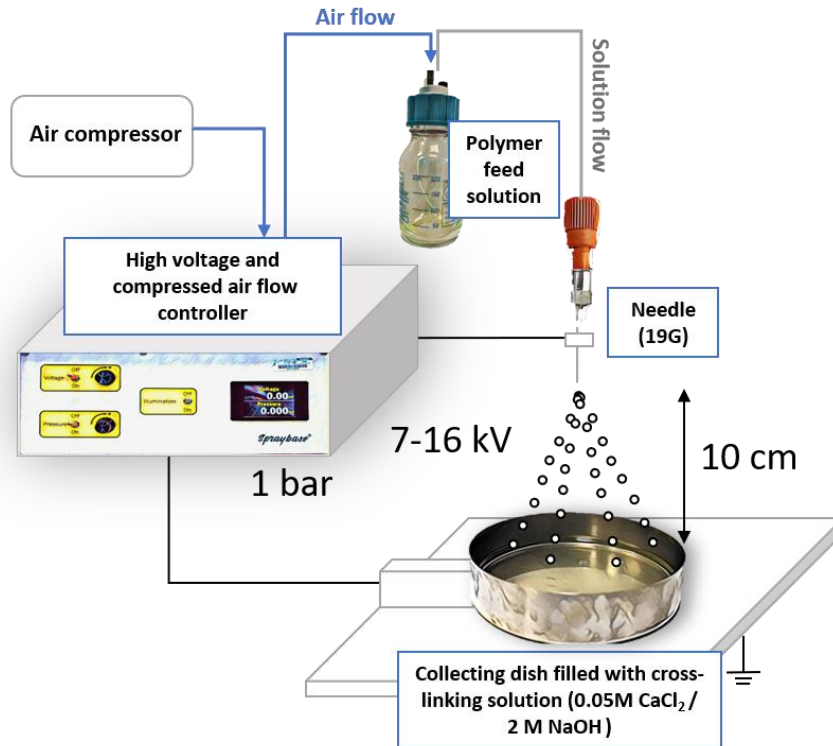
116 2.2. Bacterial culture preparation and cell enumeration

117 Fresh *Lactobacillus plantarum* cultures were prepared by transferring a stock culture from an agar
118 slant into MRS broth and reactivating it for 16-24 h at 37°C. Bacterial growth was checked using
119 turbidity (optical density, $\lambda = 600$ nm) of this culture medium. After this period, the cells were
120 collected by centrifugation at 10,000 rpm for 10 min at 4°C (Thermo Scientific Multifuge
121 Refrigerated Centrifuge, UK), and stored in sterile phosphate-buffered saline (PBS) solution at
122 4°C until further usage. Viable numbers of *L. plantarum* cells in any cultures (or samples) were
123 determined according to the plating method described by Miles et al. (1938). Briefly, 3×20 μ L
124 droplets of the bacterial culture were placed on a sufficiently dried MRS agar plate and left to
125 absorb for around 15-20 min. CFUs (colony forming units) of the inoculated bacteria were counted
126 on the MRS agar plate after incubating these agar plates for 1-2 days at 37 °C. All the CFU values
127 were expressed as log values.

128

129 2.3. Preparation of unloaded microcapsules

130 Gel microparticles were produced by the electrospraying technique using a Spraybase® device
131 (Avectas Ltd., Ireland) consisting of an air compressor, high voltage and flow rate controlling
132 compartments (**Figure 1**). Applied voltage was within the range of 7-12 kV, depending on the
133 lowest possible level that induced spraying. The pressure was set at 1 bar and the distance between
134 the tip and the collector was 10 cm. The feed solutions were composed of 2% (v/w) alginate and
135 2%-2% (v/w) alginate-resistant starch. These solutions were pneumatically pumped through the
136 high voltage emitter needle (19 G) and electrosprayed into a 0.05 M CaCl₂ solution. The particles
137 were then left to harden in this solution for 30 min before their isolation. The particles were washed
138 with and kept in deionised water. Pure chitosan particles were also fabricated by electrospraying
139 of 2% (w/v) chitosan (in 0.1 M acetic acid) solution into 2 M NaOH solution (at 16 kV). The
140 particles were collected by centrifugation at 10,000 rpm (4 °C for 10 min) and were handled with
141 a help of a syringe with sterile 30G needle. Pure chitosan particles were not used for encapsulation
142 of probiotic bacteria but were useful as a positive control in the mucosal retention studies.



143
144

145 **Fig. 1.** Schematic diagram of the electro spray system used

146 *2.4. Coating of alginate microcapsules with chitosan*

147 To form chitosan coating layer, 0.5 g of alginate microcapsules was placed and agitated in 0.2%
148 (w/v) chitosan solution (acidified with 0.1 M glacial acetic acid) for 30 - 60 min at 100 rpm. In the
149 case of the microcapsules loaded with bacteria, the pH of the chitosan solution was adjusted to pH
150 6 with 1 M NaOH and then microfiltered through a Whatman® Grade 4 filter paper. This resultant
151 solution was autoclaved before the coating step.

152 *2.5. Preparation of fluorescently-labeled microcapsules*

153 Fluorescently-labeled alginate and alginate-starch microcapsules were prepared by electro spraying
154 polysaccharide solutions containing 0.1% (w/v) sodium fluorescein; in the case of the
155 mucoadhesion study, 0.1% (w/v) FITC-dextran was used. Chitosan was labeled with FITC using
156 the protocol described in our previous study (Cook et al., 2011). Chitosan-based particles used in
157 the mucoadhesion study were prepared from chitosan labeled with 0.1 % (w/v) FITC.

158 *2.6. Microencapsulation of bacteria*

159 In order to produce the above-mentioned microcapsules loaded with *L. plantarum*, the viable
160 bacterial culture, prepared as described in Section 2.2, was first mixed with sterile polymer solution
161 at a volume ratio of 1:9. The mixture was then brought to electro spray (7 kV, 1 bar, 10 cm),
162 followed by harvesting with centrifugation (10,000 rpm, 4°C, 10 min) and double washing with

163 sterile PBS. The bacteria-loaded microcapsules were suspended in sterile PBS and stored at 4°C
164 for further use.

165 2.7. Characterisation of microcapsules

166 In order to image and assess the properties of the produced microcapsules fluorescence microscopy
167 (Leica MZ10F, UK) was carried out using an ET-GFP filter. Images were taken with an exposure
168 time of 57 ms for sodium fluorescein-loaded samples and 100 ms for FITC-labelled samples, using
169 the pseudo colour wavelength of 520 nm and adjusting a slight black/white level correction. The
170 ImageJ software (version 1.52a) was used to analyse the images in terms of fluorescent pixel
171 intensity and physical dimensions related to the particles.

172 The particle size distributions and sizes of each microcapsule type were based on 5 replicates of
173 laser light diffraction analysis using a Metasizer 3000 instrument (Malvern Instruments, Malvern,
174 UK) with the help of a Hydro Medium Volume dispersion unit. Analyses were conducted with
175 particles dispersed in deionised water [10 % (w/v)]. The particle refractive index, dispersant
176 refractive index and absorption index value were 1.4, 1.33 and 0.1, respectively. The volume-
177 weighted mean diameter (D [4,3]) and the width of the size distribution (span) were determined
178 according to the following equations (ASTM Standard E799-03, 2015; Resch-Genger, 2008):

$$179 \quad D[4,3] = \frac{\sum d_i^4 \cdot n_i}{\sum d_i^3 \cdot n_i} \quad (1)$$

$$180 \quad \text{Span} = \frac{d_{v0.9} - d_{v0.1}}{d_{v0.5}} \quad (2)$$

181

182 where d_i is the diameter and n_i is the number of the i -th particles; $d_{v0.9}$, $d_{v0.1}$ and $d_{v0.5}$ (median)
183 represents the diameter, below which the 90%, 10% and 50% of the population lies, respectively.

184 Microscopic images of particles were also generated using the Malvern Morphologi 4 static
185 automated image characterisation device.

186 2.8. Viability of encapsulated bacteria and encapsulation yield

187 Encapsulation yield, representing a measurement of the efficacy of entrapment and survival of
188 viable cells during the electrospray-based encapsulation process, was calculated as suggested by
189 Martin et al. (2013):

$$190 \quad \text{Encapsulation yield (\%)} = \frac{N}{N_0} \cdot 100 \quad (3)$$

191

192 where N is the number of viable cells detected in the resultant microcapsules and N_0 is the initial
193 number of viable (unencapsulated) cells mixed with the feed polymer solution used for
194 electrospraying process. To determine the N value, entrapped bacteria were released by placing
195 and agitating (15 min, 1000 rpm) the microcapsules in 0.1 M phosphate buffer (pH 7.5), then the
196 released bacteria were enumerated using the surface drop-based plating method described in
197 Section 2.2.

198

199 *2.9. Viability of free and microencapsulated bacteria after exposure to simulated gastric fluid*

200 This study was carried out based on the procedure described by Cook et al. (2011), with some
201 modifications. First, 0.05 g bacteria-loaded microparticles or 0.1 mL of free cell suspension were
202 added to 0.9 mL microfiltered (0.22 µm) simulated gastric fluid (SGF) which was prepared by
203 dissolving 0.2% (w/v) NaCl in deionised water, then adjusted to pH 2 with HCl solution. The
204 samples were then incubated for 1 h and 2 h at 37°C. After each incubation time, SGF was
205 separated using centrifugation (10,000 rpm, 4°C, 10 min), followed by resuspending bacteria or
206 agitating (1000 rpm, 15 min) the microcapsules in 1 mL PBS until their complete disintegration.
207 Survived bacterial numbers of each sample were enumerated using the method described in
208 Section 2.2.

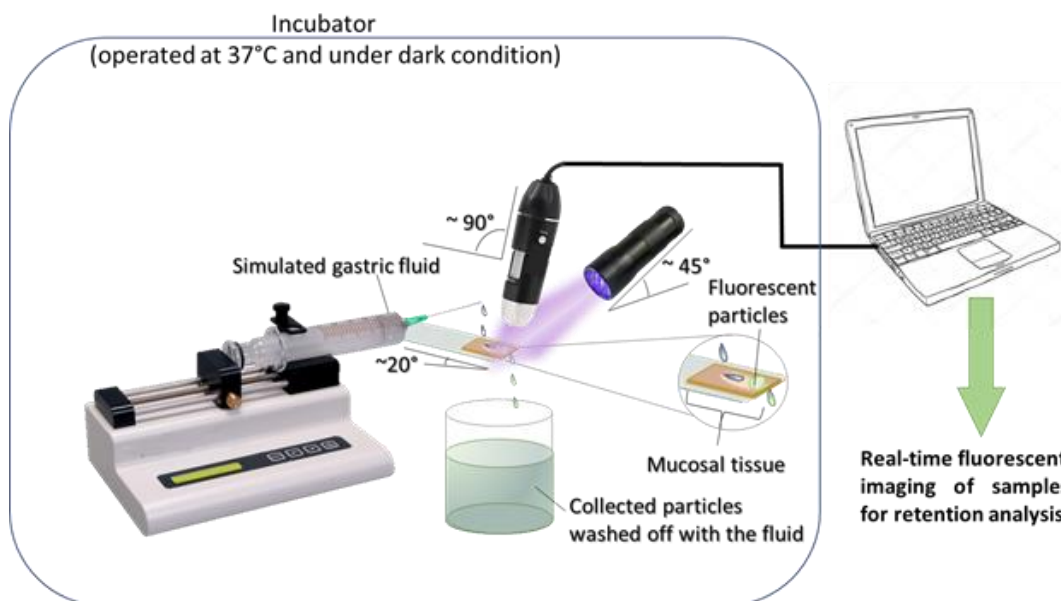
209 *2.10. Retention of microcapsules on mucosal surfaces*

210 The mucoadhesive properties of microcapsules on gastric mucosal surface were examined by using
211 the modified fluorescence flow-through retention test developed earlier by the Khutoryanskiy
212 group (Cook et al., 2018; Kaldybekov et al., 2018; Porfiriyeva et al., 2019). Retention on mucosal
213 surfaces in this case depends on mucoadhesive properties of microcapsules and represents their
214 ability to retain on the target mucosal surface over some period of time while washed with
215 simulated gastric fluid. This retention was monitored and investigated through the microscopic
216 imaging of the fluorescently labelled microcapsules on the mucosal surface at regular time
217 intervals. The main modification made in our study was that the retention was observed under a
218 1080P 1000X Zoom HD 8LED Digital USB Microscope Magnifier Endoscope Video Camera. In
219 this case, a Winzwon UV Torch was used as an external light source to illuminate the
220 fluorescently-labelled microparticles, whereas the internal light source of the microscope itself
221 was switched off for the whole duration of experiment to increase the fluorescent intensity. The
222 AmCap ver. 9.0 software was used for recording the images of the samples. The retention studies
223 were conducted using the experimental set-up shown in **Figure 2**.

224 Porcine stomachs were collected from a local abattoir (North Camp, UK) in a cold storage box (~
225 4°C). The stomach was dissected, then smaller and smooth rectangular tissue pieces
226 (approximately 1 × 1.5 cm) were carefully ablated from the mucosal fold part (rugae) of the organ,
227 using a surgical scalper. Simulated gastric fluid pre-warmed in a 37°C water bath was used for
228 modelling the wash-off process of the test microparticles from the mucosal surface.

229 The retention study was carried out firstly by spreading 0.02 g aliquot of fluorescent particles over
230 one edge of *ex vivo* pre-rinsed mucosal surface of the tissue piece fixed on one end of a microscope
231 slide. The microscope slide was fixed at an angle of 20 ° to the ground to ensure the consistent
232 flow of simulated gastric fluid through the microparticles, while the portable microscope was
233 oriented perpendicularly to the tissue surface, pointing the objective lens in the direction of
234 particles on the mucosal tissue. The UV torch was set to be sloped towards the tissue at 45° angle
235 and at a distance of 40 mm. With this arrangement, the aim was to ensure that the UV light intensity
236 provides an optimally exposed and threshold imaging of the fluorescent particles (distinguishing
237 them from the background fluorescence). After placing the tissue into the incubator, SGF was

238 allowed to drip through a needle onto the tissue surface using a syringe pump at 5 mL/min. This
239 specific flow rate mimics the average *in vivo* gastric secretion rates reported for both fasted and
240 active digestion periods (Versantvoort et al., 2004). These series of droplets were directed to fall
241 from a height of 15 mm to exclude the needle from the microscope field of view, and ~ 5 mm away
242 from the mucoadhesive formulation to ensure efficient wash off. All the fluid passed through the
243 mucosal tissue was simultaneously collected in a container. The tissue area of interest was captured
244 using the camera of the microscope at specific time points after interrupting the washing process
245 and the liquid was totally drained off for 2 min. In order to capture the entire particle mass on the
246 tissue, we used a 40× magnification on the microscope and a distance of around 15 mm between
247 the objective lens and the tissue surface (particle mass) during the whole test. All the acquired
248 images underwent the same light intensity correction and were analysed using ImageJ software to
249 quantify the intensity of fluorescence after each wash. All these experiments were performed in
250 triplicate for each formulation using an incubator at 37°C and under dark conditions.



251
252 **Fig. 2.** Experimental set-up for the retention study of particles on gastric mucosa. The microscope,
253 flashlight torch and microscopic slide were fixed at specified positions by using lab stands. Applied
254 distance of objective lens – mucosal surface was 15 mm, flashlight – mucosal surface was 40 mm,
255 needle tip – mucosal surface was 15 mm, and fluid was flowing from ~5 mm away to particles.
256 Simulated gastric fluid was adjusted to flow at 5 mL / min.

257
258 **2.11. Statistical analysis**

259 Statistical analyses of all the results generated in this study were performed with GraphPad Prism
260 software (version 8.0). One-way (or two-way where specified) analysis of variance (ANOVA)
261 with a significance level of $\alpha = 0.05$ was used to determine the statistical differences among any
262 independent variables, whereas multiple comparisons of these ANOVA results were based on

263 Tukey's post-hoc test. Final data were reported as the average value of three replicates, along with
264 their corresponding standard error of the mean value.

265

266 3. Results and discussion

267

268 3.1. Preparation and characterisation of microparticles

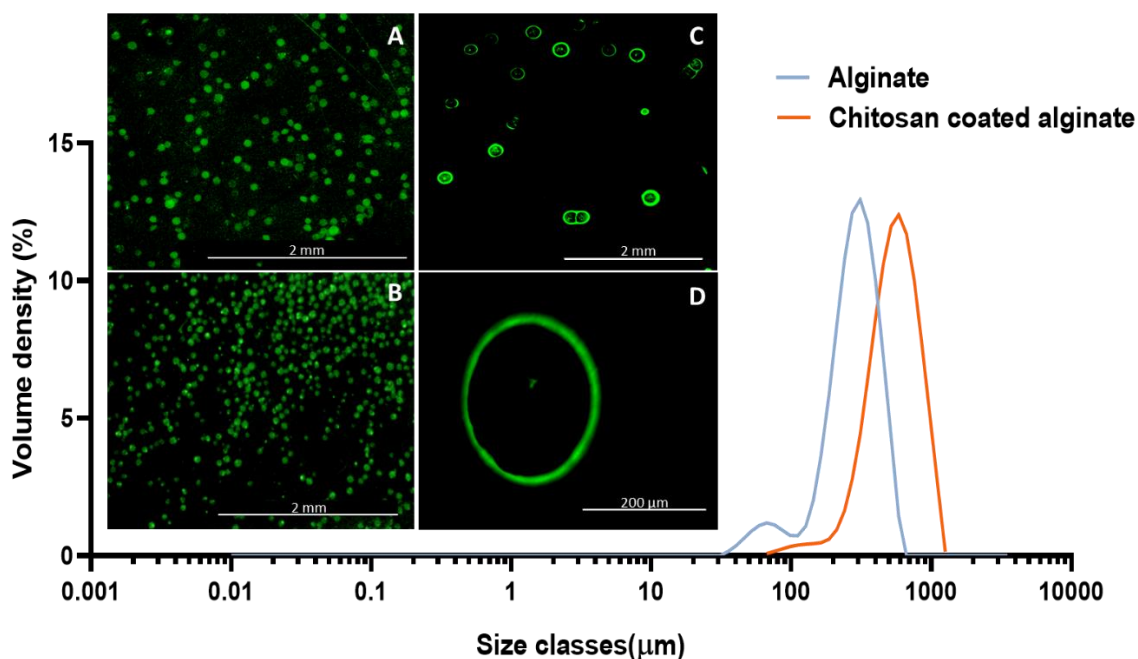
269 Gel particles were successfully prepared using sodium alginate, resistant starch and chitosan as
270 matrix-forming agents. Firstly, pure calcium alginate and a blend of alginate-starch particles were
271 produced by electrospraying of 2% (w/v) sodium alginate and 2 % (w/v) / 2 % (w/v) alginate –
272 resistant starch solution, respectively, into 0.05 M CaCl₂ solution. A third type of particles was
273 generated by coating the alginate particles with chitosan. Additionally, pure chitosan particles were
274 prepared by electrospraying 2 % (w/v) chitosan solution (in 0.1 M acetic acid) into 2 M NaOH
275 solution (these particles will be later used as a positive control to study mucoadhesive properties).

276 Fluorescence microscopy images of the particles prepared using different polymeric constituents
277 are presented in **Figure 3**. These microscopic observations confirmed that the electrospraying
278 process yielded spherically shaped particles. A chitosan layer was successfully formed on the
279 alginate bead surfaces, with an average thickness of 18.5 µm; this was measured using the ImageJ
280 analysis software. The laser light diffraction analysis revealed that alginate microparticles were
281 produced with a wide size distribution ranging from 30 to 600 µm (and span = 1.069, indicating
282 the width of the distribution) and with the most part (12.95%) of the microparticles population
283 measured at 310 µm. This wide size range can be caused by low viscosity sodium alginate solution
284 applied for the microcapsule formation (Zaeim et al., 2017). Very similar size distribution (and
285 range) of alginate microcapsules has also been generated with the emulsion-based formation
286 technique (Dikit et al., 2015). The wide distribution shifted to a greater size range of 60 – 1300
287 µm (and a width of span = 1.060) when chitosan coating was applied on the alginate microparticles.
288 In this case, the most frequent size (12.39%, similar to the alginate microcapsules without coating)
289 detected in the whole microparticle distribution increased to 586 µm (**Figure 3**). Based on the
290 volume (or mass)-based mean diameter value (D [4,3], derived from the center of the volume (or
291 mass) distribution (Resch-Genger, 2008), the mean sizes of the whole particle population are
292 estimated to be 309 µm and 607 µm for uncoated alginate and coated alginate particles,
293 respectively. However, it should be noted that some swelling and thus size expansion could have
294 occurred while the alginate microparticles were stirred in the chitosan solution for the coating
295 process; this could be attributed to the acidic conditions of the chitosan solution (pH 2-3), similarly
296 to our previous report (Cook et al., 2011). Furthermore, both particle size distribution curves as
297 seen in the volume density plot showed that the resultant particle size was not evenly distributed
298 in the population as they spread out more towards the larger size range. A bimodal distribution is
299 seen especially for the uncoated microparticles.

300 Such small sized microcapsules prepared with the electrospraying method are needed when they
301 are intended to be incorporated into food products, as large particles can negatively affect the

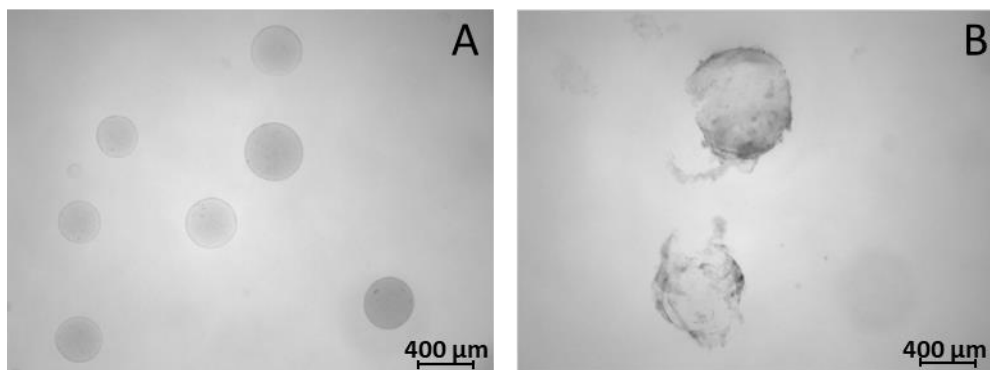
302 sensory and textural characteristics (generating some undesirable grittiness feeling) of the certain
303 food (Gbassi and Vandamme, 2012). If a post-drying (e.g. freeze- or spray-drying) step is applied
304 the particle size can be further reduced, although, at the same time, this might result in a lower
305 bacteria-loading yield, aggregation and cracking of the capsule gel matrix (Dianawati et al., 2016;
306 Cook et al., 2012).

307 The morphology related results are in agreement with the images generated using Morphologi 4
308 system (**Figure 4**). All particles have spherical shape and uniform size distribution.



309
310 **Fig. 3.** Particle size distribution of alginate and chitosan-coated alginate microcapsules. Inserts
311 show fluorescent microscopy images representing alginate (A), alginate-starch (B) and chitosan
312 coating layer on alginate (C and D) particles. Applied magnifications (and scale bars): 0.8× (2 mm)
313 for A, B, C and 8× (200 μm) for D images.

314



315

316 **Fig. 4.** Light microscopic images (scale bar = 400 µm) derived from Morphologi 4 automated
317 particle image analyser for uncoated (A) and chitosan-coated alginate (B) particles.

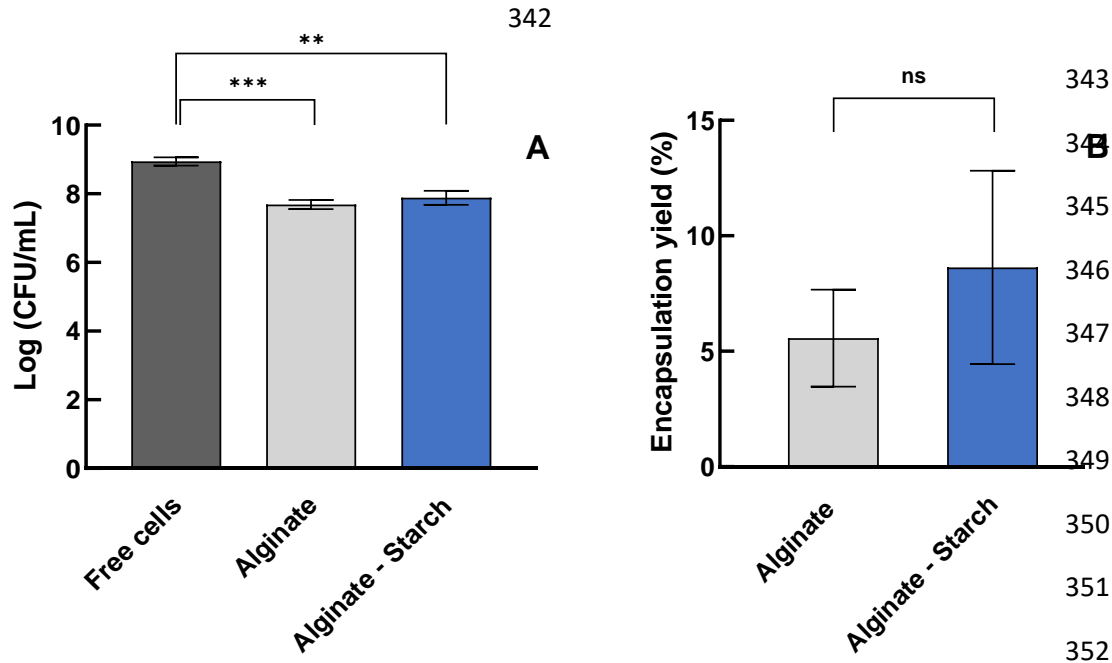
318

319 3.2. Bacterial survival yield after electrospray-based microencapsulation

320 The initial cell count of *L. plantarum* prior to electrospraying was 8.94 ± 0.12 log CFU/mL.
321 Significant ($p < 0.05$), but slightly less (~ 1.16 log CFU/mL) viable bacterial count could be
322 detected in the microcapsules produced right after the electrosprayed-based microencapsulation.
323 As can be seen in **Figure 5**, the incorporation of resistant starch did not affect the encapsulation
324 yield (EY) of alginate-based microcapsules significantly. In particular, the microcapsules with
325 alginate showed a decrease by 1.25 log CFU/mL (EY = 5.6 ± 1.7 %), while alginate-starch particles
326 resulted in viability loss of 1.06 log CFU/mL (EY = 8.6 ± 4.2 %).

327 Gómez-Mascaraque et al. (2017), who encapsulated bacteria by electrospraying, achieved a greater
328 bacterial survival of 32% for *L. plantarum* with inclusion of acidified gelatin-whey protein
329 concentrate. However, they used coaxial approach and their EY was lower compared to our results.
330 In the case of other microencapsulation techniques, the EY was generally found to be even higher
331 on average with extrusion method (around 72 %) and with encapsulation in calcium alginate matrix
332 [2 % (w/v)] (Afzaal et al., 2019; Gul and Dervisoglu, 2017; Lotfipour et al., 2012). Moreover,
333 bacterial survival of 47 % was yielded after spray drying-based microencapsulation with mixed
334 alginate and soy protein isolate (Hadzieva et al., 2017). However, a comparable result as with the
335 present alginate-resistant starch microcapsules could be found for those formed with the
336 emulsification method using calcium alginate (EY ~ 10 %) (Gul and Dervisoglu, 2017). Here, the
337 low yield especially for the alginate-entrapped bacteria resulted with electrospraying technique
338 may be due to their potential sensitivity to the combined stress effect of the high voltage electric
339 field, rapid water evaporation and high shearing force operated throughout the whole encapsulation
340 process (Coghetto et al., 2016).

341



353 **Fig. 5.** Number of bacterial cells survived after the microencapsulation process by electro spraying
 354 procedure (A) and the calculated percentage yields (B) of survived cells encapsulated in alginate
 355 and alginate-starch microcapsules. Data are shown as mean \pm standard error of mean (n=3).
 356 Significant differences are denoted by ** (p < 0.01) and 'ns' signifies no significant differences (p
 357 > 0.05).

358

359 3.3. Survival of free and microencapsulated bacteria exposed to simulated gastric condition

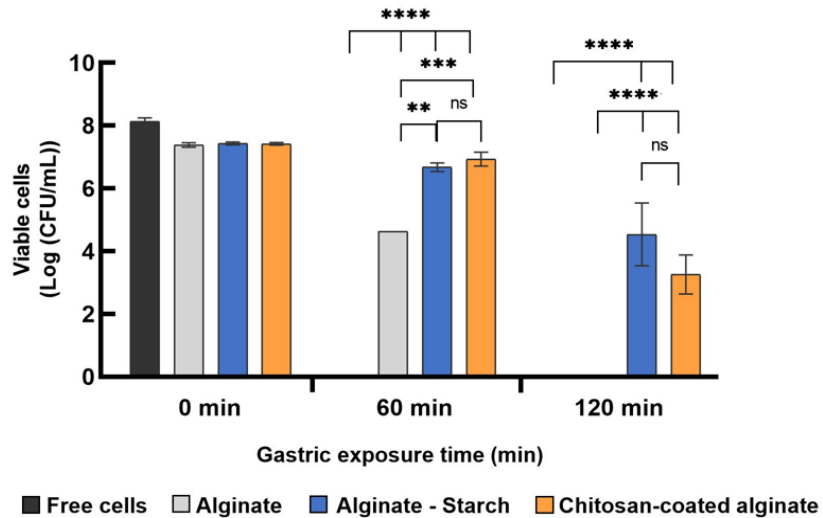
360 The viability results of free and microencapsulated *L. plantarum* in simulated gastric fluid (SGF)
 361 [0.2% (w/v) NaCl, pH 2] over different exposure times are summarised in **Figure 6**. In view of
 362 these results, the microcapsules prepared by electro spraying provided significantly enhanced
 363 survival rates (p < 0.05) for bacteria within all formulations as no free cells were found even after
 364 1 h of incubation, initiating from viable counts of log 8.14 CFU/mL. Pure alginate microcapsules
 365 significantly underperformed in terms of bacterial protection, compared to other types of alginate-
 366 based microcapsules with either blending with resistant starch or chitosan coating (p < 0.01).
 367 Electro sprayed alginate-starch and chitosan-coated alginate formulations retained the viability of
 368 *L. plantarum* with lower losses, i.e. by 0.76-2.14 log CFU/mL and 0.49-3.68 log CFU/mL after 1
 369 h and 2 h in SGF compared to alginate microparticles, respectively.

370 Applying chitosan coating has been reported to improve the protection abilities of alginate-based
 371 microcapsules (Chávarri et al., 2010; Cook et al., 2011; Nualkaekul et al., 2012). This can arise
 372 from the decreased pore size of the calcium alginate hydrogels after the application of coating
 373 (Pestovsky and Martínez-Antonio, 2019), with which the contact of the bacteria with the gastric
 374 fluid could be limited. Interestingly, incorporating resistant starch into the alginate matrix resulted
 375 in a statistically similar protection with chitosan coating throughout the digestion process. The
 376 former one could be explained by the direct presence of the resistant starch component within the

377 alginate matrix, which can serve as an energy and carbon source for the probiotic bacteria (Sultana
378 et al., 2000; Zaman and Sarbini, 2015). Some previous studies have also reported enhanced
379 bacteria protection in the microcapsules after exposure to gastric juice when resistant starch was
380 blended with alginate but the microencapsulation itself was conducted with other, more common
381 methods (Ashwar et al., 2018; Krasaekoopt et al., 2003; Muthukumarasamy et al., 2006). Zaeim et
382 al. (2017), who likewise assessed electrospray-based microencapsulation of *L. plantarum* in
383 alginate and consecutive coating with chitosan, showed similar viability reductions of around 2
384 and 3 log CFU/mL after 1 and 2 h gastric (pH 2.5) exposure, respectively. In another paper from
385 the same authors, chitosan coated alginate microcapsules additionally incorporated with resistant
386 starch, were reported to give a slightly weaker protection for *L. plantarum* than our resistant starch-
387 containing microcapsules during the 2 h simulated gastric digestion (Zaeim et al., 2019). No
388 notable alginate gel matrix disintegration was observed for any formulations after the end of the
389 gastric incubation, which can be associated with the fact that alginate exhibits an acid gel attribute
390 at pK_a below ~ 3.5 (Nualkaekul et al., 2012; Onsoyen, 1999).

391 There has been an observation suggesting that an enhanced viability in a strong acidic condition
392 (e.g., typical of gastric fluid) can be generally achieved with an increased microcapsule
393 size/diameter (Chandramouli et al., 2004; Cook et al., 2012; Ding and Shah, 2009; Ferreira Grosso
394 and Fávoro-Trindade, 2004; Liu et al., 2020; Muthukumarasamy et al., 2006). Our results were
395 found only partially in agreement with this observation. As presented earlier, our microcapsules
396 could be formed with a size range of 30 - 600 µm by using the electrospraying technique, with
397 which we experienced a cell viability reduction of more than 7 log CFU/mL (to an undetectable
398 level) in alginate microparticles after 2 h exposure to simulated gastric fluid. On the contrary, a
399 much greater survival of *L. plantarum* in gastric condition (pH 1.5; 2 h) was reported by
400 Nualkaekul et al. (2012) with their larger sized alginate capsules (2.9 mm). Likewise, a much better
401 gastric protection of probiotics was also reported when alginate capsules with an average size of
402 2.37 mm were used (Muthukumarasamy et al., 2006). Similar result was also reported for resistant
403 starch reinforced alginate by Sultana et al. (2000). However, in some cases, the viability of
404 probiotics under the same gastric conditions could be maintained better when the cells were
405 entrapped in microcapsules with a smaller or similar size range than our electrosprayed ones (Chun
406 et al., 2014; Dikit et al., 2015; Muthukumarasamy et al., 2006). As these microcapsules were
407 prepared under mild conditions, this may be due to the fact that the application of the electrostatic
408 field during the microencapsulation process could further weaken the subsequent acidic stress
409 tolerance of probiotics, possibly in combination with the effect of the small microcapsule size.

410



411

412 **Fig. 6.** Viable numbers (CFU/mL) of free and microencapsulated *L. plantarum* bacteria with
 413 alginate, alginate-starch and chitosan-coated alginate microcapsules over 2 h of exposure to
 414 simulated gastric fluid [0.2% (w/v) NaCl, pH 2] at 37°C. Significant p-values are denoted by **
 415 ($p < 0.01$), *** ($p < 0.001$), **** ($p < 0.0001$), and ns ($p > 0.05$) is for indicating non-significant
 416 differences, determined using two-way ANOVA with Tukey’s multiple comparison post-hoc tests.
 417 Data are all shown as mean \pm standard error of mean (n=3).

418 **3.4. Mucoadhesion study of microcapsules on gastric mucosa**

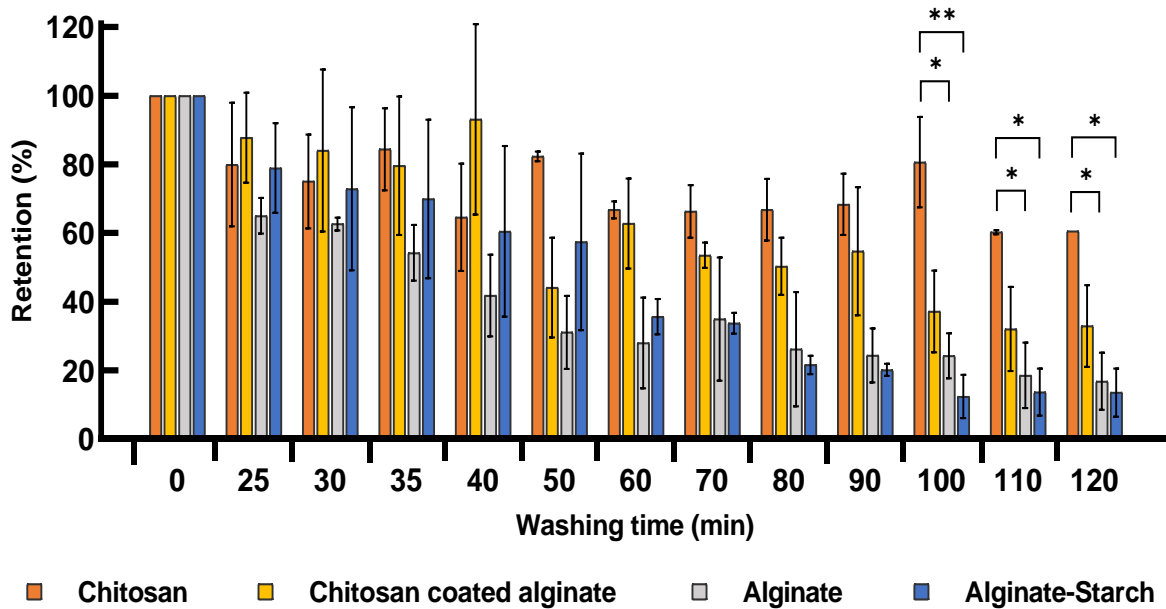
419 Aside from keeping an adequate bacterial survival rate, several studies have also highlighted the
 420 importance of mucosal retention of microcapsules within the gastrointestinal tract for appreciably
 421 longer time, in the context of designing effective delivery systems for probiotics (Alli et al., 2011;
 422 Cook et al., 2012; van Tassell and Miller, 2011). For instance, retention on gastric epithelium may
 423 potentially improve the chance of some probiotics to curb gastric ulcers and gastric cancer diseases
 424 induced by *Helicobacter pylori* and to contribute gastric mucosal barrier protection. Furthermore,
 425 it is also reported that gastric mucus itself can provide an additional potential protective function
 426 for gastric survival of probiotics (Butel, 2014; Khoder et al., 2016; Koga et al., 2019; Singh et al.,
 427 2012). Accordingly, the mucoadhesive properties of unloaded alginate, alginate-starch and
 428 chitosan-coated alginate microparticles were evaluated using an *in vitro* fluorescence imaging-
 429 based flow-through test on *ex vivo* porcine gastric epithelial mucosa, following the protocol
 430 described previously by the Khutoryanskiy group (Cook et al., 2018; Kaldybekov et al., 2018;
 431 Porfiryeva et al., 2019). As some recent reports also suggested the potential utilisation of a low-
 432 cost commercial USB microscope in different imaging-related assays (Bracker and Stender, 2019;
 433 Tortajada-genaro et al., 2019), here, we attempted to adapt this device for imaging the samples in
 434 the present mucoadhesion study, with assessing the usability of that as an alternative imaging tool
 435 for this study.

436 The retention on the mucosa was observed based on the detected intensity of the fluorescent
 437 particles labelled with the agents mentioned in Section 2.5. To ensure the excitation of the
 438 fluorophores a portable UV LED flashlight torch was applied. SGF with pH 2 was used to wash

439 the particles off the mucosal surface. To avoid the leakage of fluorescent tracers, both alginate and
440 alginate-starch particles were labelled with the greater molecular weight FITC-dextran instead of
441 sodium fluorescein. A positive control experiment was also undertaken with pure chitosan
442 particles.

443 **Figure 7** presents the retention profiles observed for different microparticles on gastric mucosa
444 through the series of captured fluorescent photomicrographs. It should be noted that the lowest
445 available magnification of 40× was needed for evaluating the whole particle mass. According to
446 the analysis using ImageJ software, it is confirmed that every type of microcapsules could remain
447 to some extent on the gastric mucosa even up to 2 h. Among them, chitosan-coated alginate
448 particles exhibited excellent retention ability, comparable to that of pure chitosan control ($p >$
449 0.05). For this formulation, around 62% and 32% of remaining fluorescence intensity could still
450 be observed after 60 min and 120 min of washing, respectively. On the other hand, more rapid
451 removal was observed in case of pure alginate, especially over the last 50 min of the 2 h
452 experiment. Slightly weaker retention of alginate-starch particles was observed compared to pure
453 alginate microcapsules. Improved mucoadhesion, however, can be feasible via some chemical
454 modifications or addition of specific functional groups (Jelkmann et al., 2019; Kaldybekov et al.,
455 2018). It should be noted that these weak gastric-mucoadhesive characteristics can be preferable
456 if the primary site of therapeutic action of the particular probiotic strain is the intestinal tract. The
457 exemplary series of fluorescent images representing the retention rate of each examined
458 formulation are shown in **Figure 8**.

459 It is well known that chitosan exhibits strong mucoadhesive properties due to its cationic nature
460 (Khutoryanskiy, 2011). Therefore, we have used chitosan particles as a positive control in these
461 experiments. As it was expected, these particles show the greatest retention on mucosal surfaces
462 in our experiments. The alginate capsules, coated with chitosan, exhibited poorer retention than
463 pure chitosan particles but better mucoadhesive properties than uncoated alginate capsules. This
464 is explained by the presence of chitosan on their surface. Starch is a non-ionic polysaccharide and
465 it is expected to exhibit poor mucoadhesive properties (Khutoryanskiy, 2011). Indeed, an addition
466 of starch to alginate makes the microcapsules less retentive on the mucosa.

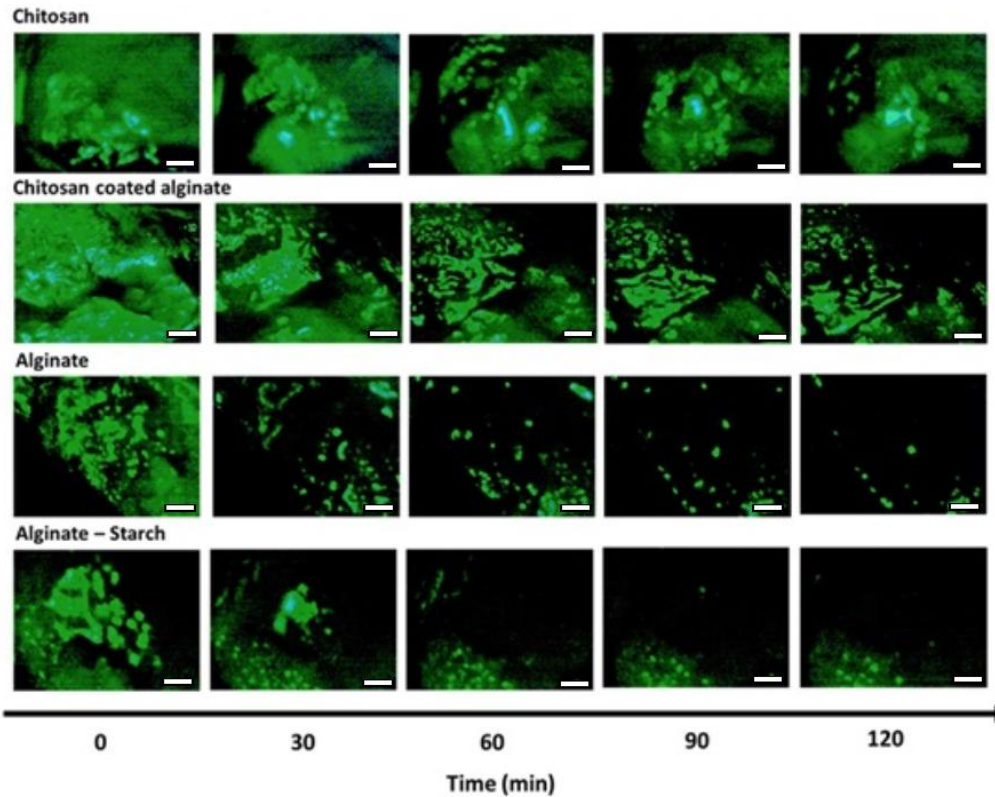


467

468 **Fig. 7.** *In vitro* retention profiles of each microcapsule variation on *ex vivo* porcine gastric mucosa
 469 over 2 h of washing process with simulated gastric fluid (0.2 % (w/v) NaCl, pH 2) at 37°C.
 470 Statistical differences are denoted by * (p < 0.05) and ** (p < 0.01). Values are represented as
 471 mean ± standard error of mean (n = 3).

472

473



474

475 **Fig. 8.** Example fluorescence images showing retention of each variation of microcapsules on
 476 porcine gastric mucosa after the indicated time of washing with simulated gastric fluid (0.2 %
 477 (w/v) NaCl, pH 2). Scale bar is 1000 μm .

478

479 Overall, the experiments with a portable digital USB microscope and UV light torch indicate the
 480 suitability of this low cost approach for performing fluorescence flow-through test to evaluate
 481 mucoadhesive properties of microcapsules. The use of this experimental setup can offer a number
 482 of advantages over the traditional fluorescent microscopy method, including the possibility for
 483 real-time imaging and detection capability in micro-scale resolution, video recording capability,
 484 user-friendliness, portability, increased affordability and availability of analysis.

485

486 4. Conclusions

487 In this study, alginate, blend of alginate-resistant starch and chitosan-coated alginate-based
 488 microcapsules were successfully prepared and loaded with *Lactobacillus plantarum* probiotic
 489 bacteria using the electrospray technique. Each type of microcapsules was characterized using
 490 laser light diffraction, encapsulation yield of survived bacteria, fluorescent microscopy, *in vitro*
 491 gastric digestion and *in vitro* gastro-retention analysis. The electrospraying resulted in the
 492 production of spherically shaped microcapsules with a size range of 30-600 μm (and a volume-
 493 based mean diameter of 309 μm), which increased to 60 – 1300 μm (and a volume-based mean

494 diameter of 607 μm) with chitosan coating. No statistically significant difference was found in the
495 encapsulation yield of viable cells between alginate and alginate-starch formulations, but some
496 significant losses in bacteria viability occurred following the encapsulation process. Microcapsules
497 with alginate-starch matrix provided one of the most effective viability protection for bacteria in
498 simulated gastric conditions (for 2 h) with a viable loss of 2.14 log CFU/mL, comparable to that
499 of chitosan coated alginate particles. On the contrary, this formulation exhibited weak retention on
500 gastric mucosa compared to particles with alginate only and especially alginate-chitosan
501 microparticles. This research demonstrated that electrospraying could be successfully used for
502 preparation of microcapsules with viable probiotic bacteria. These microcapsules provide adequate
503 protection to these bacteria against harsh environment in the stomach. Although a high bacteria
504 loading capacity (~ 8 log CFU/mL) was achieved with this technology, further investigations may
505 be needed with regard to their storage stability within these microcapsules.

506 Additionally, it was demonstrated that a low-cost handheld consumer USB microscope, under
507 some specific conditions, can be used as an alternative device to perform imaging of fluorescent
508 samples involved in the retention test for mucoadhesion analysis. Potentially this approach could
509 be used in laboratories, where researchers do not have access to expensive fluorescence
510 microscopes. This gives an opportunity to a wider range of research groups to use fluorescence
511 flow-through assay to evaluate mucoadhesive properties and retention of formulations *ex vivo*.

512

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