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Electrosprayed mucoadhesive alginate-chitosan microcapsules for gastrointestinal delivery of probiotics

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Abstract

Besides viability protection, a sufficiently prolonged gastrointestinal retention of probiotics has emerged as critically important in improving the functional effectiveness of gastrointestinal delivery of these microorganisms. In this work, we formulated pure, resistant starch-reinforced and chitosan-coated alginate microparticles using an electrospray technique and evaluated their performance as mucoadhesive probiotic formulations for gastrointestinal delivery. In addition, we designed and successfully validated a novel experimental set-up of *in vitro* wash-off mucoadhesion test, using a portable and low-cost USB microscope for fluorescence imaging. In our test, pure chitosan microparticles (positive control) exhibited the greatest mucoadhesive property, whereas the alginate-resistant starch ones (negative control) were the least retentive on a gastric mucosa. These electrosprayed formulations were spherically shaped, with a size range of 30 - 600 µm (60 - 1300 µm with chitosan coating). Moreover, model probiotic *Lactobacillus plantarum* loaded in

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

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

1. Introduction

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

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

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

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

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

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

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

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

2. Materials and methods

2.1.Materials

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

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

2.2. Bacterial culture preparation and cell enumeration

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

2.3.Preparation of unloaded microcapsules

Gel microparticles were produced by the electrospraying technique using a Spraybase® device (Avectas Ltd., Ireland) consisting of an air compressor, high voltage and flow rate controlling compartments (**Figure 1**). Applied voltage was within the range of 7-12 kV, depending on the lowest possible level that induced spraying. The pressure was set at 1 bar and the distance between the tip and the collector was 10 cm. The feed solutions were composed of 2% (v/w) alginate and 2%-2% (v/w) alginate-resistant starch. These solutions were pneumatically pumped through the high voltage emitter needle (19 G) and electrosprayed into a 0.05 M $CaCl_2$ solution. The particles were then left to harden in this solution for 30 min before their isolation. The particles were washed with and kept in deionised water. Pure chitosan particles were also fabricated by electrospraying of 2% (w/v) chitosan (in 0.1 M acetic acid) solution into 2 M NaOH solution (at 16 kV). The particles were collected by centrifugation at 10,000 rpm (4 °C for 10 min) and were handled with a help of a syringe with sterile 30G needle. Pure chitosan particles were not used for encapsulation of probiotic bacteria but were useful as a positive control in the mucosal retention studies.

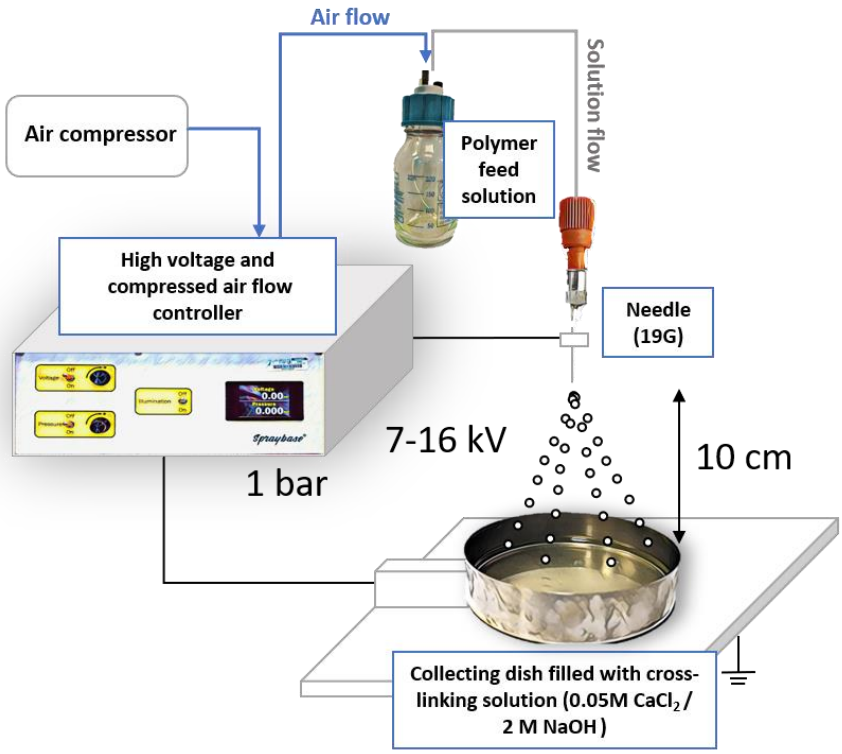

Fig. 1. Schematic diagram of the electrospray system used

2.4. Coating of alginate microcapsules with chitosan

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

2.5. Preparation of fluorescently-labeled microcapsules

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

2.6. Microencapsulation of bacteria

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

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

2.7. Characterisation of microcapsules

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

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

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

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

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) represents the diameter, below which the 90%, 10% and 50% of the population lies, respectively.

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

2.8. Viability of encapsulated bacteria and encapsulation yield

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

$$\text{Encapsulation yield (\%)} = \frac{N}{No} \cdot 100 \qquad (3)$$

where N is the number of viable cells detected in the resultant microcapsules and No is the initial number of viable (unencapsulated) cells mixed with the feed polymer solution used for electrospraying process. To determine the N value, entrapped bacteria were released by placing and agitating (15 min, 1000 rpm) the microcapsules in 0.1 M phosphate buffer (pH 7.5), then the released bacteria were enumerated using the surface drop-based plating method described in Section 2.2.

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

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

2.10. Retention of microcapsules on mucosal surfaces

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

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

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

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

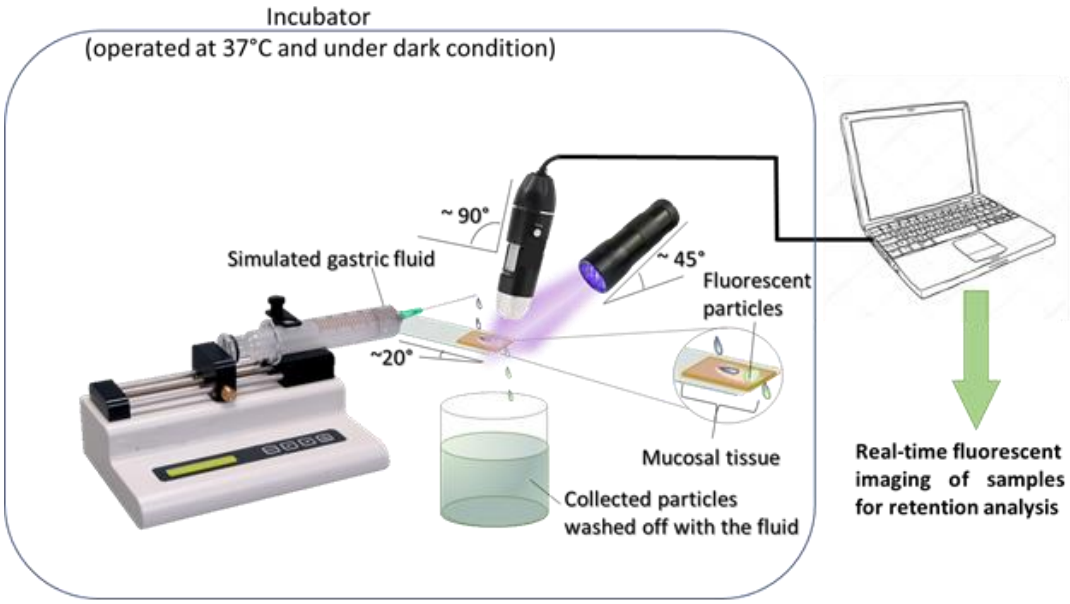

Fig. 2. Experimental set-up for the retention study of particles on gastric mucosa. The microscope, flashlight torch and microscopic slide were fixed at specified positions by using lab stands. Applied distance of objective lens – mucosal surface was 15 mm, flashlight – mucosal surface was 40 mm, needle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

2.11. Statistical analysis

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

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

3. Results and discussion

3.1. Preparation and characterisation of microparticles

Gel particles were successfully prepared using sodium alginate, resistant starch and chitosan as matrix-forming agents. Firstly, pure calcium alginate and a blend of alginate-starch particles were produced by electrospraying of 2% (w/v) sodium alginate and 2 % (w/v) / 2 % (w/v) alginate – resistant starch solution, respectively, into 0.05 M $CaCl_2$ solution. A third type of particles was generated by coating the alginate particles with chitosan. Additionally, pure chitosan particles were prepared by electrospraying 2 % (w/v) chitosan solution (in 0.1 M acetic acid) into 2 M NaOH solution (these particles will be later used as a positive control to study mucoadhesive properties).

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

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

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

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

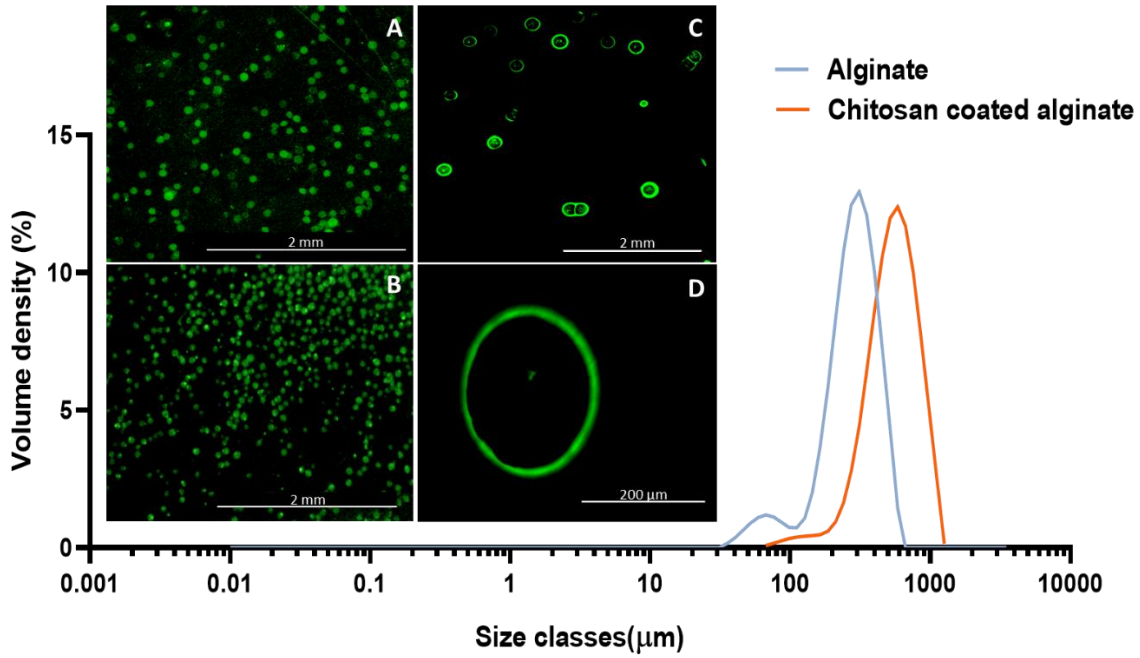

Fig. 3. Particle size distribution of alginate and chitosan-coated alginate microcapsules. Inserts show fluorescent microscopy images representing alginate (A), alginate-starch (B) and chitosan coating layer on alginate (C and D) particles. Applied magnifications (and scale bars): 0.8× (2 mm) for A,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

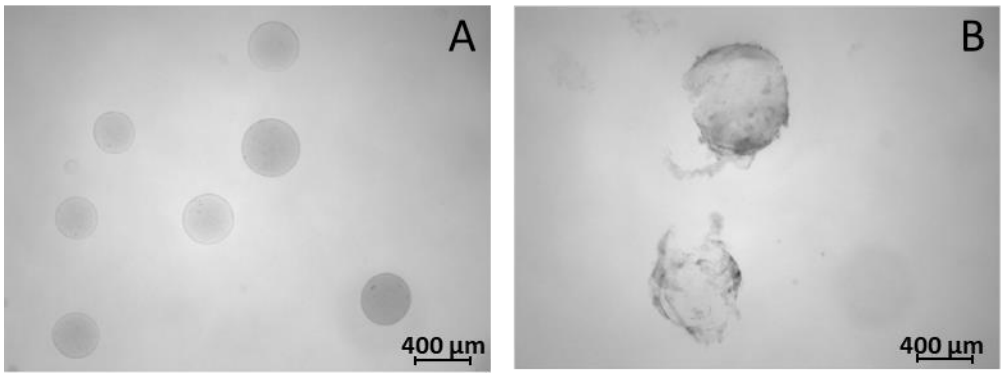

Fig. 4. Light microscopic images (scale bar = 400 µm) derived from Morphologi 4 automated particle image analyser for uncoated (A) and chitosan-coated alginate (B) particles.

3.2. Bacterial survival yield after electrospray-based microencapsulation

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

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

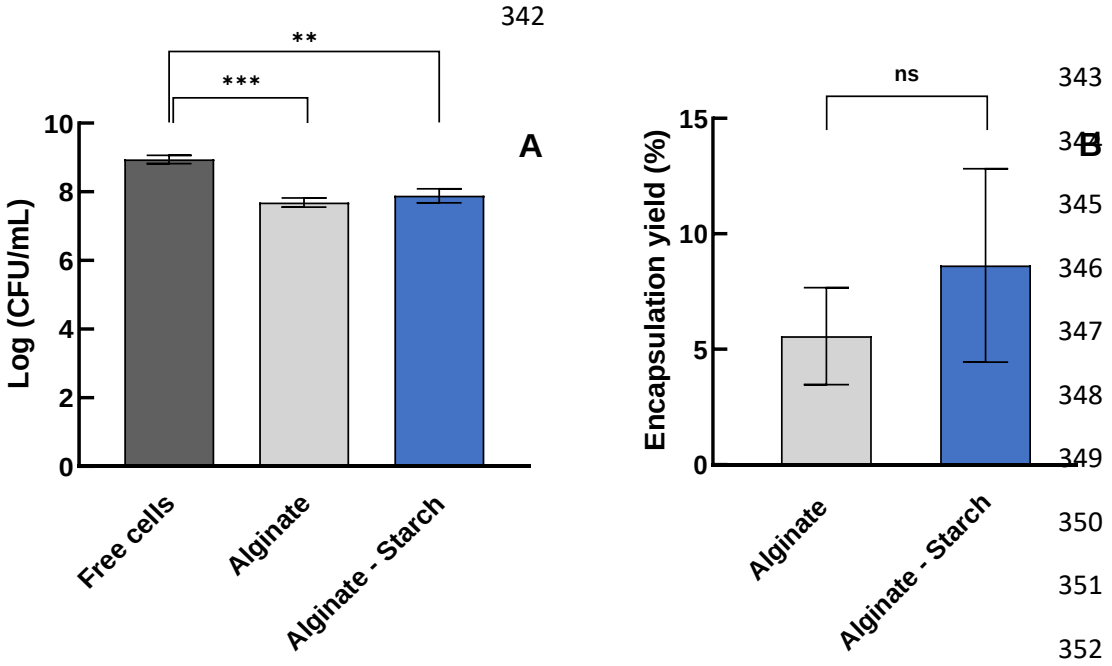

Fig. 5. Number of bacterial cells survived after the microencapsulation process by electrospraying procedure (A) and the calculated percentage yields (B) of survived cells encapsulated in alginate and alginate-starch microcapsules. Data are shown as mean ± standard error of mean (n=3). Significant differences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

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

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

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

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

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

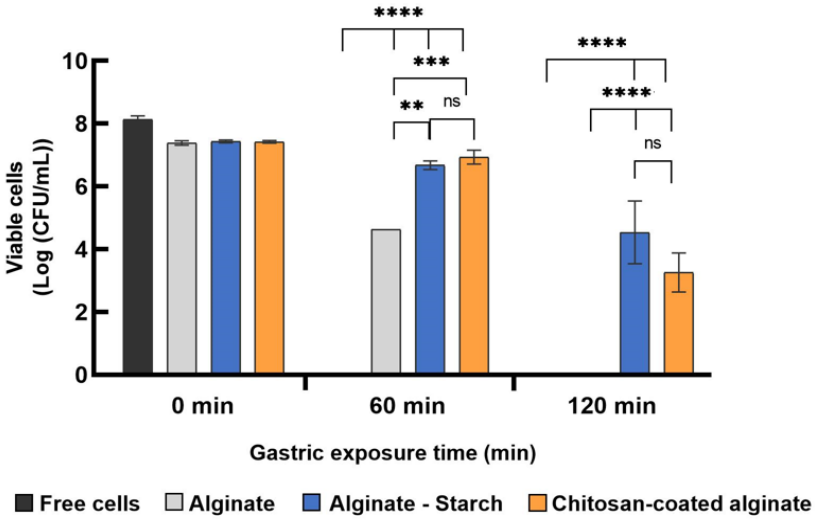

Fig. 6. Viable numbers (CFU/mL) of free and microencapsulated *L. plantarum* bacteria with alginate, alginate-starch and chitosan-coated alginate microcapsules over 2 h of exposure to simulated gastric fluid [0.2% (w/v) NaCl, pH 2] at 37°C. Significant p-values are denoted by ** ($p < 0.01$), *** ($p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

3.4. Mucoadhesion study of microcapsules on gastric mucosa

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

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

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

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

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

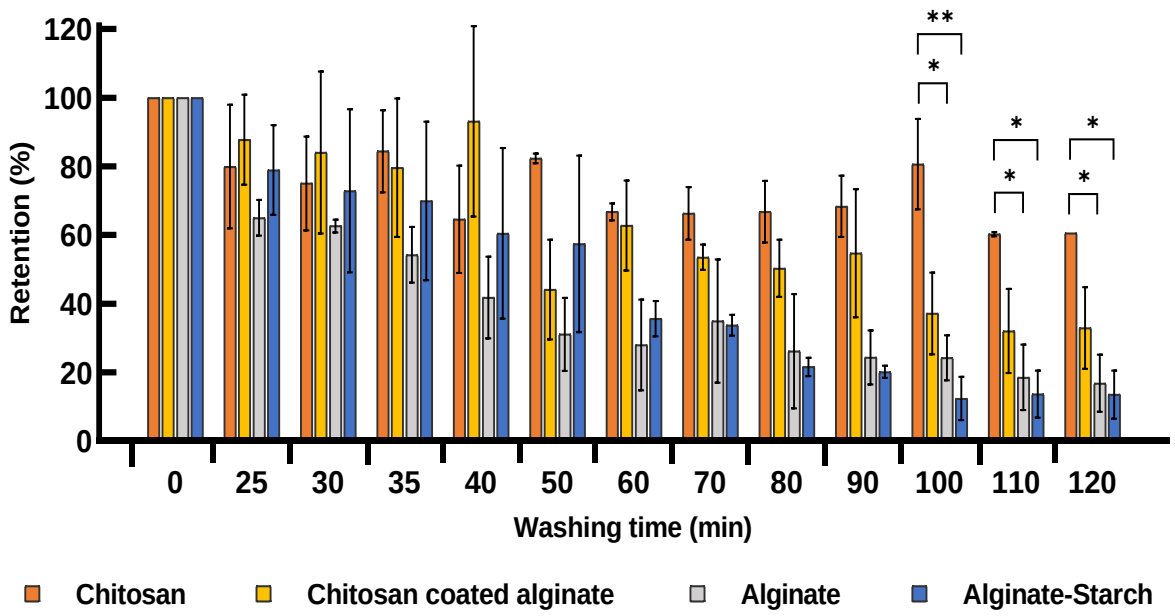

Fig. 7. *In vitro* retention profiles of each microcapsule variation on *ex vivo* porcine gastric mucosa over 2 h of washing process with simulated gastric fluid (0.2 % (w/v) NaCl, pH 2) at 37°C. Statistical differences are denoted by * ($p < 0.05$) and ** ($p < 0.01$). Values are represented as mean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

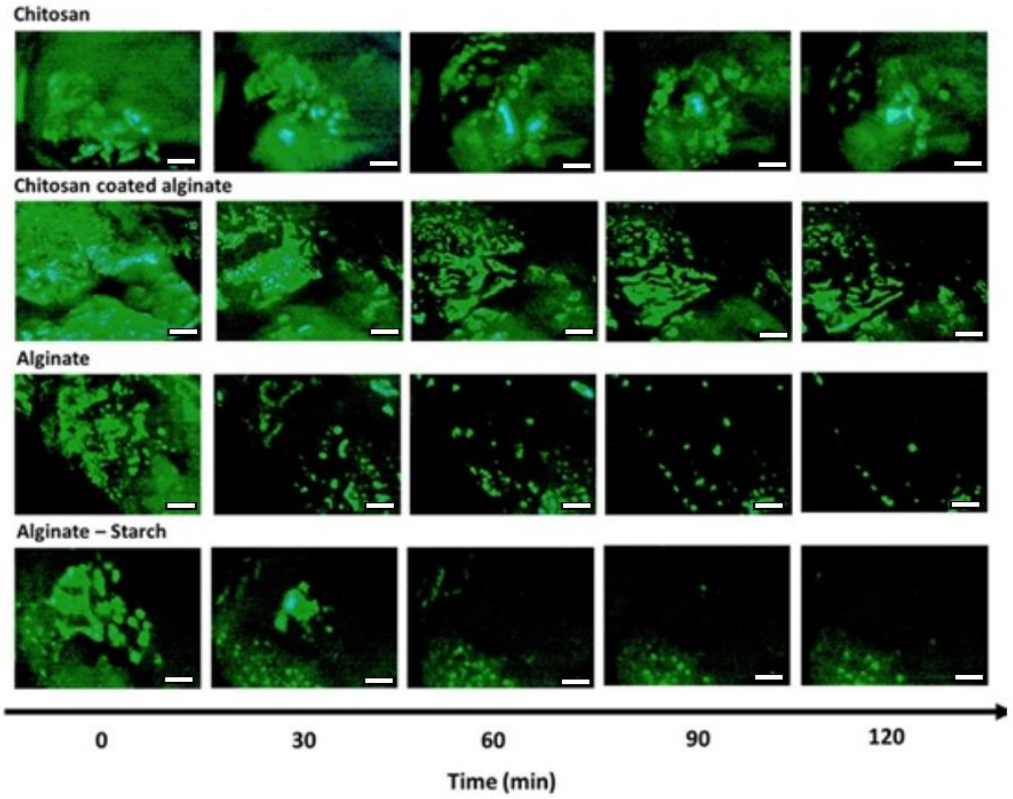

Fig. 8. Example fluorescence images showing retention of each variation of microcapsules on porcine gastric mucosa after the indicated time of washing with simulated gastric fluid (0.2 % (w/v) NaCl, pH 2). Scale bar is 1000 μm.

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

4. Conclusions

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

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

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

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