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Development of enteric polymer-based microspheres by spray-drying for colonic delivery of *Lactobacillus rhamnosus* GG

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Abstract

Antibiotics are well-known disruptive elements of the intestinal microbiota and antibiotic-associated diarrhea appeared as the most common complication related with post-antibiotic dysbiosis. *Lactobacillus rhamnosus* GG (LGG) strain is very effective in preventing antibiotic-associated diarrhea in children and adults. However, as any probiotics, it is concerned by the loss of viability during storage and gastrointestinal transit. The aim of this study was to develop an encapsulation system suitable for the specific colonic delivery of LGG strain after oral administration. For this purpose, spray-dried Eudragit® S100 microparticles encapsulating LGG bacteria were developed by using an aqueous based spray-drying approach, avoiding the use of organic solvents. Carbohydrates were added to the formulation since they are widely used as protective agents of bacteria against the harmful effect of dehydration stress. Here, both Surface Enhanced Raman Scattering (SERS) and conventional plate count methods showed that carbohydrates increased the survival ratio of bacteria after spray-drying from 3 to more than 50%. Moreover, these protective agents ensured low residual moisture content thus providing great stability of the cells in the spray-dried powder during storage. Significant improvement of the cell viability in simulated gastro intestinal fluid (SGIF) was observed for encapsulated cells as compared with free LGG bacteria for which no viable cell was detectable after 1h incubation in gastric fluid only. As a consequence, 4.5×10^7 CFU/g of encapsulated LGG were found viable after incubation of microparticles 1 h in Simulated Gastric Fluid followed by 6 h in Simulated Intestinal Fluid, corresponding to less than 3 log reduction of viable cells during the 7 h incubation in Simulated Gastro Intestinal Fluid. These results attested that the developed encapsulation system is suitable for its use as a bacteria carrier for specific colonic delivery.

Keywords: *Lactobacillus rhamnosus* GG, colonic delivery, microencapsulation, oral delivery, Enteric polymers, spray-drying, SERS, probiotics

1. Introduction

Probiotics are defined as 'live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host', as proposed by the Joint FAO/WHO Expert Consultation in 2001 (FAO/WHO, 2001). Lactic acid bacteria (members of the genera *Lactobacillus*, *Bifidobacterium* and *Streptococcus*) are widely used as probiotics because they are important and normal constituents of the human gut microbiota. Some yeasts such as *Saccharomyces boulardii* are not naturally present in the colon but can also be employed as probiotics (Madsen, 2001; Holzapfel and Schillinger, 2002). *Lactobacillus rhamnosus* GG (LGG) strain is a Gram-positive bacteria isolated from the human digestive system for the first time in 1985 by Sherwood Gorbach and Barry Goldin. LGG appeared to be very effective in preventing antibiotic-associated diarrhea in children and adults (McFarland, 2006; Videlock and Cremonini, 2012; Szajewska and Kołodziej, 2015). This strain is often incorporated in encapsulation systems in order to improve its stability and preservation against adverse environmental conditions related to oral administration (Doherty, *et al.*, 2011; Sohail *et al.*, 2012; Burgain *et al.*, 2013; Cheow and Hadinoto, 2013; Zhu *et al.*, 2013; Abbaszadeh *et al.*, 2014; Li *et al.*, 2016). Indeed, before reaching their target site of activity, which is generally the large intestine, probiotic bacteria have to go through the highly acidic conditions of the stomach and enzymes and bile salts in the small intestine (Vandamme *et al.*, 2002; Liu *et al.*, 2003; Begley *et al.*, 2005). Microencapsulation systems should be also able to release bacteria in the colon lumen in response to changes in the environment such as pH variations or enzymatic activity of the colonic microbiota (Vandamme *et al.*, 2002; Liu *et al.*, 2003).

Milk proteins (Doherty *et al.*, 2011; Burgain *et al.*, 2013) and polysaccharides such as alginate (Sohail *et al.*, 2012; Abbaszadeh *et al.*, 2014; Pirarat *et al.*, 2015), pectin (Li *et al.*, 2016), carrageenan (Cheow and Hadinoto, 2013) or xanthan, gellan and pullulan gums (Jiménez-Pranteda *et al.*, 2012) are mainly used as polymeric materials for targeted colonic delivery of probiotics. Their gelation properties are exploited by the most often used probiotic microencapsulation processes, i.e prilling and emulsification/gelation. However, these processes present some difficulties for large-scale production of food-grade microencapsulated microorganisms in contrast to the spray-drying technique (Anal & Singh, 2007; Burgain *et al.*, 2011). Synthetic polymer with enteric properties such as Eudragit® S100 and L100, anionic copolymers based on methacrylic acid and methyl methacrylate, are frequently used for the design of pH-dependent colonic delivery systems. Indeed, their free carboxylic acid groups make these polymers pH sensitive: Eudragit® S100 and L100 are respectively soluble at pH > 7 and at pH between 6 and 7 (Agyilrah and Banker, 1991). Thus, the use of Eudragit® S100 or L100 microparticles as a carrier for specific colonic delivery of probiotic bacteria should be a suitable approach since such system is expected to protect cells from the acidic gastric fluid while allowing their release at the pH of the distal part of the intestine near the ileo-caecal junction. However, Eudragit® S100 or L100 polymers are barely used as matrix for the microencapsulation of probiotics and are mainly used as enteric coating material of preexistent microspheres containing bacteria (De Barros *et al.*, 2015; Ansari *et al.*, 2017) or associated with other polymers as matrix in formulated tablets (Villena *et al.*, 2015). Their solubility features often require the use of the

emulsion/solvent evaporation technique to produce microparticles but this process remains inappropriate for the encapsulation of living cells due to the presence of organic solvents (Lee *et al.*, 1999; Jain *et al.*, 2005; Obeidat and Price, 2006; Rawat *et al.*, 2007; Rawat and Saraf, 2009; Villamizar *et al.*, 2010; Mundargi *et al.*, 2011). In this context, spray-drying appears to be a promising and more appropriate encapsulation method since avoiding the use of organic solvents. Aqueous-based spray-drying process, using Eudragit® S100 or L100 soluble salt form in solution, has already been explored and applied for the microencapsulation of small molecules like prednisolone (Alhnan *et al.*, 2011) or sodium pantoprazole (Raffin *et al.*, 2006, 2007). However, to our knowledge, such systems have never been developed for the encapsulation of probiotics.

Spray-drying is far less used for probiotics encapsulation than the previously mentioned gelation-based methods. The spray-drying process consists of the atomization of a liquid feed (encapsulation material and active agent) into a hot drying gas allowing the formation of dried particles separated from the drying gas by means of a cyclone that deposits them in a glass collector (Sosnik and Seremeta, 2015). This technique offers many benefits: it is a rapid one-step procedure, relatively low cost and easily scalable to industrial levels without major modifications (Sosnik and Seremeta, 2015) while ensuring high reliability, reproducibility and a control of particle size (Alhnan *et al.*, 2011). But, during the spray-drying process, cells are logically subjected to heat stress and to other stresses related to dehydration, oxidation phenomena or even osmotic stress. Heat stress inactivation of cells is mainly due to the damage inflicted on macromolecules such as DNA, RNA, proteins or supramolecular assemblages such as ribosomes or cell membranes. However, the stress related to dehydration appears as the main critical in the inactivation of cells. Indeed, the water removal imposes important physiological constraints on cells due to the major contribution of the bound water to the stability of proteins, DNA molecules as well as bacterial membranes (Santivarangkna *et al.*, 2008). To enhance cell protection during drying step, protective agents can be added to the surrounding medium. Carbohydrates such as maltodextrins (Reddy *et al.*, 2009; Broeckx *et al.*, 2017; Bustamante *et al.*, 2017; Nunes *et al.*, 2018), lactose (Su *et al.*, 2018), mannitol (Broeckx *et al.*, 2017), trehalose (Sunny-Roberts and Knorr, 2009; Su *et al.*, 2018; Nunes *et al.*, 2018) or dextran (Broeckx *et al.*, 2017) are commonly used for this purpose. Skim milk and milk proteins (i.e whey protein, casein) also exhibit good protective effect (Sunny-Roberts and Knorr, 2009; Páez *et al.*, 2013; Liu and Liu, 2015; Khem *et al.*, 2016; Reddy *et al.*, 2009; Huang *et al.*, 2016).

The development of an encapsulation system of probiotics requires a reliable quantification method of the bacteria to determine formulation characteristics such as encapsulation efficiency, release kinetics or microorganism viability. The conventional counting method, using plating and culturing, is the most widely used method for microorganism quantification although it remains tedious and above all time consuming. Surface Enhanced Raman Scattering (SERS) technique appears as a highly attractive alternative to limit the duration of the bacteria quantification process and was introduced to offset the low sensitivity of the conventional Raman spectroscopy limited to the analysis of highly concentrated samples by the weakness of the inelastic light scattering phenomena. This is achieved by means of a high enhancement of Raman scattering from molecules adsorbed on a nanostructured

noble metal surface (silver, gold) (Le Ru and Etchegoin, 2009). Electromagnetic effect (EE) and chemical effect (CE) are the two well documented mechanisms used to explain the SERS effect. EE exploits the optical phenomenon of localized surface plasmon resonance at the surface of the metallic nanostructure leading to the generation of intense electromagnetic fields at “hot-spot” zones (Stiles *et al.*, 2008). CE is induced by a charge-transfer between the chemisorbed analyte and the metallic nanostructure which can cause an increase of the molecular polarizability of the analyte and thus an increase of the Raman scattering (Schlücker, 2014). Due to its simplicity and sensitivity while retaining the high molecular specificity of the conventional Raman spectroscopy (Schlücker, 2014), numerous studies have reported the use of Surface Enhanced Raman Scattering (SERS) technique for the detection, identification or quantification of pathogenic or probiotic bacteria (Premasiri *et al.*, 2005; Jarvis *et al.*, 2006; Mircescu *et al.*, 2014; Zhou *et al.*, 2014; Ankamwar *et al.*, 2016; Lemma *et al.*, 2016; Akanny *et al.*, 2019).

In this context, the objectives of this study were first to develop a pH-dependent colonic delivery system of LGG probiotics based on Eudragit® S100 microparticles and secondly to investigate the potential application of SERS technique to quantify encapsulated bacteria in microparticles. To this end, we implemented the SERS method dedicated to the label-free quantification of LGG that was developed in a previous work (Akanny *et al.*, 2019). LGG were encapsulated using an aqueous-based spray-drying approach. The influence of five protective agents (mannitol, trehalose, maltodextrin (Glucidex® IT6) and poly(vinylpyrrolidone) (PVP)) on the viability of the encapsulated strain after spray drying was studied. The microparticles obtained were characterized by their size and morphology, chemical composition and residual moisture content. The viability of encapsulated LGG in the optimal formulation was finally determined during storage and after incubation in simulated gastric and intestinal fluids.

2. Materials and Methods

2.1. Materials

Sodium chloride, sodium hydroxide, hydrogen tetrachloroaurate (III) hydrate (HAuCl₄) were purchased from Alfa Aesar. MRS agar, MRS broth, trehalose, hydroxylamine solution 50 wt% were obtained from Sigma Aldrich. Eudragit® S100 was bought from Evonik. PVP K30 (Kollidon® 30) was obtained from BASF. Mannitol and Glucidex® IT6 were obtained from Roquette. Hydrochloric acid (HCl), sulfuric acid 98% (H₂SO₄) and nitric acid 70% (HNO₃) were purchased from Carl Roth. *Lactobacillus rhamnosus* GG strain (ATCC 53103) was obtained from American Type Culture Collection (ATCC). All glassware used for gold nanoparticles synthesis was previously washed with freshly prepared aqua regia (3:1 HCl: HNO₃) followed by extensive rinsing with Milli Q water. All glassware, centrifuge tubes, pipet tips and solutions (prepared using Milli-Q water) used were sterilized at 120 °C for 15 min before use.

2.2. Preparation of bacterial suspensions

LGG cells were cultured on MRS agar plates at 37 °C for 48 h to reach the stationary phase in aerobic conditions. Bacteria were then collected with sterile plastic inoculating loops from solid culture plates and washed twice by centrifugation using NaCl (0.9% w/v) aqueous solution and re-suspended in NaCl (0.9% w/v) aqueous solution. The average LGG concentration of the bacterial suspension was approximately 4.5×10^9 CFU/mL.

2.3. Preparation of feed solutions for spray-drying

Five different feed solutions (F1-F5) were formulated by adding to Eudragit® S100 aqueous solutions various protective agents. Their compositions are detailed in Table 1. Eudragit® S100 6.25 % (w/v) was first dispersed in phosphate-buffered saline solution (PBS; pH 7.4). The pH of the polymer suspension formed (pH 6.5) was then adjusted to 7.6 by adding 1 M NaOH aqueous solution at a volume ratio of polymer suspension to NaOH solution of 4.55:1 in order to generate the soluble salt form of Eudragit® S100. The resulting Eudragit® S100 5.125 % (w/v) solution was finally mixed with the bacterial suspension under magnetic stirring at a volume ratio of polymer solution to bacterial suspension of 4:1 (F1 solution). The final concentration of Eudragit® S100 was 4.1 % (w/v). The same procedure was followed to prepare F2 to F5 feed solutions including protective agents. PVP, mannitol, trehalose or Glucidex® IT6 were dissolved in the Eudragit® S100 5.125 % (w/v) solution, before adding the bacterial suspension (Fig. 1). The protective agent final concentration was set at 5 % (w/v) in the case of PVP and 10 % (w/v) for carbohydrates. The average LGG concentration in the final F1-F5 feed solutions was 9×10^8 CFU/mL.

2.4. Spray-drying process

The feed solutions were spray-dried using a laboratory scale spray dryer (Buchi B-191 Mini Spray Dryer). Experiments were carried out under the following conditions: inlet air temperature of 105 °C, outlet air temperature of 75-80 °C, feed rate of 3 mL/min and air pressure of 5 bars.

2.5. Characterization of spray-dried powders

2.5.1. Determination of the solid yield after spray-drying

The Solid Yield (SY) of the spray-dried powder was determined for the 5 formulations (F1-F5) tested and was calculated as follows:

$$SY(\%) = \frac{M_f(g)}{M_0(g)}$$

with M_f the mass of collected microparticles and M_0 the mass of solid content in feed suspension.

2.5.2. Determination of the probiotic survival after spray-drying

For each formulation, the survival ratio of LGG after spray-drying was determined and calculated as follows:

$$S (\%) = \frac{N}{N_0} \times 100$$

N is the CFU per g (CFU/g) of the spray-dried powder quantified immediately after drying and N_0 is the CFU/g of dry matter in the initial suspension before drying.

To determine N, 1 g of spray-dried microparticles were dissolved in 40 mL of PBS to dissolve microparticles and release LGG bacteria. The latter were then washed twice by centrifugation using PBS. N and N_0 were determined by both conventional plate count method and Surface Enhanced Raman spectroscopy (SERS) analysis as described in part 2.6.

2.5.3. Morphology and size distribution of microparticles

Microparticle morphology was observed by scanning electron microscopy (SEM) using a Vega 3 (TESCAN) microscope. Powders were deposited and fixed on an aluminum stub with a carbon conductive tape and then coated with a thin layer of gold (15 nm) deposited by sputtering deposition (compact rotary-pumped sputter coater Quorum150RS) to produce a conductive surface.

Atomic Force Microscopy (AFM) image of LGG was obtained using an Agilent M5500 instrument operating in tapping mode (Agilent Technologies, Palo Alto, CA, USA). The average resonance frequency of tips was 300 kHz.

The particle size distribution was determined by light scattering (Mastersizer 3000, Malvern Instruments, UK) with Aero S dry powder dispersion unit. The particle size distribution was determined from Mie theory. Polydispersity was given by a span parameter calculated as (Huang *et al.*, 2016):

$$Span = \frac{d_{90} - d_{10}}{d_{50}}$$

Where d_{10} , d_{50} and d_{90} are the maximum diameters of 10%, 50% and 90% of the particles, respectively.

2.5.4. Moisture content

The residual moisture content of spray-dried samples was determined by thermogravimetric analysis with a TA Instruments Q50 thermogravimetric analyzer using 10°C/min ramp rate from room temperature to 600 °C in air atmosphere.

2.5.5. Chemical composition analyses of Eudragit S100 – mannitol microparticles by FT-IR

In order to study the chemical composition of microparticles and determine the ratio between polymer and protective agent (mannitol), infrared spectroscopy analyses were performed with a transformed Fourier Infra-Red (FT-IR) spectrometer model NEXUS (Nicolet-ThermoFisher, UK) equipped with a DTGS detector with a 4 cm^{-1} resolution. Quantitative determination of Eudragit® S100 and mannitol in the MF3 final powder (Table. 1) was achieved using a KBr compressed pellet method in the transmission mode. Solid mixture of mannitol and lyophilized powder of the Eudragit® S100 solution in PBS were mixed with KBr (2 mg of solid sample for 200 mg of KBr) and pressed into KBr pellets. FT-IR spectra were then recorded in a frequency range of $4000\text{--}400\text{ cm}^{-1}$ and with a total of 32 scans per sample. Qualitative analysis of the Eudragit® S100 powder, Eudragit® S100 in PBS solution, MF3 microparticles and mannitol were carried out in the attenuated total reflectance (ATR) mode with a Thunderdome (Spectratech) accessory containing Germanium crystal with a mono reflection at 45° . ATR-FTIR spectra were acquired in a frequency range of $4000\text{--}680\text{ cm}^{-1}$ and with a total of 256 scans per sample.

2.6. Quantification of LGG probiotics by the plate count method and by SERS analysis

In the case of the enumeration of LGG living cells using the plate count method, subsequent serial dilutions were made in a 0.9% (m/v) NaCl aqueous solution and plated in duplicate on MRS agar. Colony forming units (CFU) were determined after 48 h of incubation at 37°C in aerobic conditions.

Concerning the quantification by SERS, uncoated spherical gold nanoparticles were first prepared according to a previously published method (Akanny *et al.*, 2019). Briefly, gold nanostar precursors were first produced by a one-step reduction process based on the reduction of a gold precursor in a basic environment using hydroxylamine as a reducing agent. $65\text{ }\mu\text{L}$ of 0.2 M hydroxylamine aqueous solution were added to 10 mL of 0.01 M sodium hydroxide aqueous solution. Then $200\text{ }\mu\text{L}$ of 0.02 M HAuCl_4 aqueous solution were quickly added under vigorous stirring. The color of the solution changed instantaneously from transparent to deep blue. Suspension of star-like nanoparticle precursors was then diluted to a fifth and H_2SO_4 aqueous solution was added to reach the final concentration of 0.55 mM . The color of the solution changed gradually from deep blue to pink under 10 min due to the formation of uncoated spherical gold nanoparticles (AuNPs) used as SERS substrate. $50\text{ }\mu\text{L}$ of the bacterial suspension were finally added to 5 mL of the nanoparticle suspensions and SERS signals were then recorded after an incubation time of 10 min . The determination of the unknown LGG concentrations from the measured SERS signals at 735 cm^{-1} was achieved using a calibration curve obtained by plotting the intensity of the SERS signal of LGG bacteria at 735 cm^{-1} versus the concentration of LGG from $7.6\times 10^5\text{ CFU/mL}$ to $9.2\times 10^6\text{ CFU/mL}$ (Akanny *et al.*, 2019). RamanRxn1 spectrometer (Kaiser Optical Systems, Ann Arbor, USA), equipped with a thermoelectrically cooled CCD detector, was used in combination with a non-contact probe. Bacteria SERS spectra were acquired under 785 nm irradiation (400 mW) in 1 cm quartz-cell with 15 sec data acquisition and two spectral accumulations. Data collection was operated using the Holoreact™ software.

2.7. Resistance to gastrointestinal conditions

The resistance in simulated gastro-intestinal fluids of free and microencapsulated LGG was evaluated for each formulation. Simulated gastric fluid (SGF) consisted of 0.1 M HCl aqueous solution (pH 1.5). Simulated intestinal fluid (SIF) consisted of FASSIF-V2 (Fasted State Simulated Intestinal Fluid) from Biorelevant. It was composed of taurocholate 3 mM, phospholipids (lecithin) 0.2 mM, sodium 106 mM, chloride 69 mM, maleic acid 19 mM (pH 6.5).

The resistance to gastric fluid was first studied by adding 1 g of microparticles or 1 mL of the bacterial suspension into 10 mL of SGF for 60 and 120 min at 37°C with constant agitation. After incubation the volume was adjusted to 40 mL with PBS to raise the pH to about 6, thus interrupting the acidic treatment. In order to investigate the LGG survival in microparticles after their gastric incubation, the pH of the suspension was adjusted to 7.6 with 1M NaOH aqueous solution to dissolve the polymer and release the LGG. The enumeration of LGG living cells was done using the plate count method described in section 2.6.

The effect of SIF was then tested consecutively to the incubation in SGF. 1 g of microparticles were first mixed with 10 mL of SGF for 60 min at 37°C under stirring. After 1h of incubation, the microparticles were removed, placed in 10 mL of SIF and incubated at 37°C for 1h, 4h or 6h with constant agitation. After incubation, microparticles were finally removed and placed in 40 mL of PBS adjusted to pH 7.6 to release LGG bacteria. The effect of SIF on free LGG bacteria was also evaluated by mixing 1 mL of bacterial suspension with 10 mL of SIF for 1h, 4h and 6h at 37°C with constant agitation. In both cases, the enumeration of LGG living cells was done using the plate count method (section 2.6).

Decrease of viable cells with respect to counts after treatment were expressed as a logarithmic reduction of viable cells (LRVC) calculated as:

$$LRVC = \log\left(\frac{Q}{Q_0}\right)$$

where Q_0 and Q refer to the bacterial count before and after treatment, respectively.

2.8. Viability of encapsulated cell during storage

The encapsulated LGG in MF3 microparticles were stored at 5°C in a 50 mL sterile Falcon tubes. The viability of encapsulated LGG in the powders was determined after 1, 2, 4 and 8 weeks of storage. For each control, 1 g of microparticles were dissolved in 40 mL of PBS to release LGG bacteria. The enumeration of LGG living cells was done using the plate count method (section 2.6). LRVC was calculated as mentioned in section 2.7 with Q and Q_0 referring to the bacterial count at a particular storage period and before storage, respectively.

3. Results and discussion

3.1. Microparticle preparation by spray-drying

As previously mentioned, Eudragit® S or L microparticles are mainly prepared by emulsion/solvent evaporation technique but this process is highly harmful for probiotic bacteria. Here, we decided to perform for this purpose a spray-drying method that avoids the use of organic solvents. The obtained Eudragit® S or L microparticles should be used to develop pH-dependent colonic delivery systems of probiotics. To our knowledge such systems have already been developed for the encapsulation of small molecules but not for that of bacteria. Raffin *et al.* (2006, 2007) encapsulated sodium pantoprazole in spray-dried particles obtained from the water soluble salt form of Eudragit® S100. This was made using alkaline sodium hydroxide solution to neutralize the acidic groups in Eudragit® S100 polymer. However, it should be noted that the retained sodium hydroxide can lead to an increase of the local pH near the microparticles when dispersed in water or gastric fluid. A partial dissolution of the polymer matrix may occur thus limiting the protection of bacteria against acidic conditions (Alhnan *et al.*, 2011). Alhnan *et al.* (2011) developed an alternative approach for the encapsulation of prednisolone using ammonium hydrogen carbonate instead of sodium hydroxide. Unlike sodium hydroxide, ammonium salts can be eliminated under elevated temperature inducing the donation of a proton from the ammonium group to the carboxylate group of the polymer restoring its acidic form. However, this elimination is only partially achieved in the drying chamber and the total removal of ammonium residues requires the incubation of the microparticles at elevated temperatures (60-70 °C) for several hours. This could be inconsistent with the survival of probiotics that are already highly stressed by the spray-drying process. In this study, we chose to follow the approach developed by Raffin *et al.* (2006, 2007) with some modifications to develop a pH-dependent colonic delivery system for LGG. The feed aqueous solutions for spray drying were obtained by solubilizing Eudragit® S100 in PBS solution adjusted at a pH above the polymer solubility conditions (pH 7.6) using sodium hydroxide, instead of NaOH solution only. Here, we suggest that the presence of phosphate buffer might limit the pH increase. In case of incubation in acidic conditions, this should prevent the early dissolution of the Eudragit matrix before the acidic form of the polymer is restored. The formation of the soluble salt form of the Eudragit® S100 can be observed in the ATR-FTIR spectra (Fig. 2) measured for the dry deposit of the Eudragit® S100 solution in PBS and MF3 microparticles: an IR band at about 1560 cm⁻¹ appeared corresponding to the stretching vibration of carboxylic acid salts. The loss of this band in the ATR-FTIR spectra of the microparticles collected after 1h incubation in acidic environment (HCl solution at pH 1.5) confirmed the restoration of the acidic form of the polymer.

3.2. Microparticle size distribution, morphology and solid yield

The size of the microparticles is an important issue since it has to be suitable for the encapsulation of probiotics which generally exhibit relatively large size comprised between 1 and 5 µm. The microparticles have to be designed to ensure a high loading of microorganisms as well as their

protection against adverse gastro-intestinal conditions (Anal and Singh, 2007). LGG bacteria used in this study had a size between 1 and 2 μm (Fig. 3a) that can be slightly reduced after spray drying (Sunny-Roberts and Knorr, 2009). As observed in Fig. 3b, the size distribution of the spray-dried microparticles was not really influenced by the presence nor the type of protective agent in the feed solutions. The microparticles presented a relatively high polydispersity characterized by a span parameter between 1.6 and 1.9. Mean diameters (d_{50}) of about 4.4 μm without protective agent and varying from 5.2 to 7.3 μm with protective agents were observed (Table 2). This slight increase of the microparticle size might be due to the increase of the viscosity of the feed solutions in the presence of the protective agents. Solid yields of 50-60% were obtained in the absence or presence of carbohydrate protective agents (Table 2), that appeared as satisfying for a lab-scale spray-drying experiment (Sosnik and Seremeta, 2015). The higher yield was obtained using mannitol as a protective agent with 59% of solid content retrieved after drying. However, a sharp decrease of the solid yield to 30% was observed with PVP in the spray-dried formulation and might be related to its sticky behavior (Horaczek and Viernstein, 2004).

The morphology of the obtained spray-dried microparticles was observed by SEM (Fig. 4). Particles exhibited a spherical shape and, as previously mentioned, a relatively high heterogeneity in size ranging from 1 to 20 μm . Wrinkles can be observed at the particle surface in the case of formulations containing mannitol (Fig. 4b) and trehalose (Fig. 4c) as protective agent. Such a phenomenon was commonly described and encountered in cases of particles obtained with feed preparations including carbohydrates (Su *et al.*, 2017; Bustamante *et al.*, 2018; Nunes *et al.*, 2018). Concavities were also observed at particle surface and may be related to a slight collapse of particles during the drying process due to the quick water evaporation from the droplet (Bustamante *et al.*, 2018). This phenomenon was more pronounced for particles containing carbohydrates. In all cases, no free LGG were visible at the particle surface suggesting that they were successfully encapsulated in the microparticles.

3.3. Cell survival after spray-drying and during storage

During the spray-drying process, bacterial cells are logically subjected to heat and dehydration stresses that might impact their viability. The survival of LGG after spray-drying in the presence or absence of protective agent was studied. As previously described, five protective agents were tested: three carbohydrates namely mannitol, trehalose, maltodextrin (Glucidex® IT6) that are commonly used to this end, and also poly(vinylpyrrolidone) (PVP K30) which shows protective properties against stresses encountered by cell strains during drying (Lodato *et al.*, 1999) and appears as an interesting formulation excipient for its adhesiveness and film forming properties (Horaczek and Viernstein, 2004; Liu *et al.*, 2009).

The survival ratio of LGG after spray-drying clearly demonstrated the protective effect of carbohydrates (Fig. 5a) since it increased significantly from 3 ± 2 % without protective agent to over

20% with carbohydrates. Mannitol and trehalose allowed to reach the best survival ratio with respectively $63\pm 7\%$ and $55\pm 5\%$, versus $23\pm 6\%$ for maltodextrin. Here, PVP did not exhibit any protecting effect with a survival ratio of only $2\pm 1\%$. The protective effect of sugar-based components has been suggested to be due to their ability to replace the water in membranes and proteins, thus maintaining their structural and functional integrity (Crowe *et al.*, 1987, 1988, 1998; Ananta *et al.*, 2005). It was described in the literature that the removal of bound water induces a state transition of the phospholipid bilayer of the cytoplasmic membrane from a lamellar phase to a gel or an hexagonal ones leading to an increase in membrane permeability during rehydration and thus exposing to the leakage of intracellular components (Ananta *et al.*, 2005; Santivarangkna *et al.*, 2008; Khem *et al.*, 2015). The higher survival ratio obtained for mannitol and trehalose as compared to maltodextrin (Glucidex®) could be explained by the better protecting effect provided by small carbohydrate molecules such as polyols like mannitol or disaccharides like trehalose. Indeed, contrary to polysaccharides, these small molecules are most likely to interact directly with the polar head groups of membrane phospholipids and thus to ensure membrane stability (Crowe *et al.*, 1996; Ananta *et al.*, 2005).

The outlet temperature, i.e the temperature at which the product leaves the drying chamber, is highly correlated to the cell inactivation due to heat stress (Santivarangkna *et al.*, 2008). Indeed, although the temperature of the spray-dried droplets increases, it remains relatively low and does not reach the inlet temperature due to an evaporative cooling effect relating to the instant removal of moisture. Afterward, exposure time of dried particles to the high temperature is very short (Santivarangkna *et al.*, 2008). Ananta *et al.* studied the influence of the outlet temperature over the range 70-100 °C on the survival of spray-dried LGG in skim milk-based preparations (20 % w/v). They reported a better survival rate of bacteria ($> 60\%$) for an outlet temperature between 70 and 80 °C associated with a residual moisture content between 3.5 and 4.5%. A similar study was achieved by Gardiner *et al.* encapsulating *L. paracasei* NFBC and *L. salivarius* UCC 118 in skim milk matrix at outlet temperatures ranging from 70 to 120 °C and from 60 to 90°C, respectively. In both cases, an increase of the survival rate was observed with the decrease of the outlet temperature. However, for temperatures below 80 °C, the residual moisture content in the final product increases and exceeds the optimal content required to ensure the proper cell survival during storage which is between 2 and 4 % (Zayed and Roos, 2004; Sunny-Roberts and Knorr, 2009; Khem *et al.*, 2016). Therefore, a compromise should be made between the outlet temperature and the residual moisture content in order to maintain the cell viability during and after the spray-drying process. Generally, both good cell viability and residual moisture content are obtained for an outlet temperature comprised between 70 and 90 °C (Gardiner *et al.*, 2000; Ananta *et al.*, 2005; Sunny-Roberts and Knorr, 2009; Khem *et al.*, 2016; Guerin *et al.*, 2017). In this study, the inlet temperature was set at 105°C in order to obtain an outlet temperature of about 75-80°C. However, as previously mentioned (see Fig. 5), LGG did not survive the encapsulation process without the addition of a protective agent. Moreover, the corresponding residual moisture content of 9.1% (w/w) obtained without protectant was well above the recommended values and thus too high to ensure a good cell viability during storage. The use of carbohydrates in the formulation led to a decrease in the residual moisture content in

microparticles from 9.1% (w/w) with no protective agent to 1.9, 3.3 and 5.2 (w/w) in the presence of mannitol, trehalose and Glucidex® IT6, respectively (Fig. 4a). The values obtained with mannitol and trehalose were in accordance with the recommendation mentioned above for optimal preservation of cell viability during storage.

As previously mentioned, the microparticles containing mannitol (MF3) provided the better solid yield after spray-drying. They also presented the highest survival ratio (63 ± 7 %) and a residual moisture content in accordance with the recommended range (<4 %). Moreover, the encapsulated LGG in these microparticles showed a great stability during storage with less than 0.4 log reduction in viable cells after 8 weeks of storage at 5°C (Fig. 4b). This formulation was thus retained for further investigations: *i*) IR and SERS analyses and *ii*) for studying the resistance of encapsulated LGG in simulated gastrointestinal conditions.

3.4. IR analyses for chemical characterization of MF3 microparticles

The quantitative analysis of the MF3 microparticles composition was performed by IR spectroscopy in transmission using KBr compressed pellet method and references made of solid mixtures of mannitol and lyophilized powder of the Eudragit® S100 solution in PBS. Linear relationships (ESM, Fig. S1) were observed between the proportion of Eudragit® S100 or mannitol and the area of the IR absorption band at 1720 cm^{-1} (-C=O stretching vibrations) (Fig. A.1) and 1020 cm^{-1} (-C-O vibrations) (ESM, Fig. S1b), respectively. These calibration curves allowed to determine a proportion of Eudragit® S100 of 24 ± 3 % (w/w) and mannitol of 56 ± 2 % (w/w) in the MF3 powder, for theoretical values of 26% and 65 %, respectively. The closeness between the determined and theoretical values of the MF3 powder composition indicated a relatively constant ratio Eudragit®/mannitol before and after spray-drying.

3.5. Application of the SERS technique to the quantification of encapsulated LGG in MF3 microparticles

The tedious and time consuming conventional plate count method is nearly always performed for microorganism quantification in order to characterize encapsulation systems. SERS technique appears as a promising alternative since it provides high sensitivity and specificity, easy and rapid sample preparation, and rapid analysis in aqueous matrices. In a previous work, we developed a SERS method based on uncoated spherical gold nanoparticles which was successfully used for the label-free quantification of Gram-positive *Lactobacillus rhamnosus* GG (LGG) (Akanny *et al.*, 2019). Here, the developed SERS method was applied and compared to the classical plate count method to quantify the probiotics encapsulated in microparticles. The LGG quantification by SERS analyses requires less than 1 h, from the synthesis of SERS substrate to the acquisition of LGG SERS spectra while the duration of the conventional plate method ranges from 24 to 48h.

An example of LGG SERS spectrum measured in the presence of gold nanoparticles is presented in Fig. 6a, where a major feature at 735 cm^{-1} can be observed. Raman signal enhancement of LGG might be especially due to the combination of anion-induced chemical enhancement and gold nanoparticle aggregation onto the bacteria cell wall (Akanny *et al.*, 2019). Due to the dependence of the SERS enhancement mechanisms on the metal surface proximity (Premasiri *et al.*, 2005), the main feature observed at 735 cm^{-1} is generally assigned to: *i*) the polysaccharide structures of the bacteria cell wall composed of peptidoglycan (Jarvis and Goodacre, 2004), *ii*) adenine-containing molecules, that might be located on or in the cell wall structures (e.g. DNA, RNA, ADP or ATP) (Zeiri *et al.*, 2004; Kahraman *et al.*, 2008, 2011), or *iii*) metabolites of the purine degradation pathway located in the extracellular regions near the outer cell walls, such as AMP, adenine, guanine, hypoxanthine, xanthine and uric acid (Kubryk *et al.*, 2016; Premasiri *et al.*, 2016). The LGG concentrations in the powder obtained after spray-drying (N) and in the suspension before drying (N_0) were determined from the calibration curve using LGG SERS signals at 735 cm^{-1} (section 2.6). As shown in figure 7b, $59\pm 8\%$ of the LGG initially introduced in the fed suspension was retrieved in MF1 microparticles and $58\pm 4\%$ in MF3 microparticles using the SERS method. In the case of MF3 microparticles including mannitol, this amount appeared as non-significantly different from the ratio of encapsulated LGG of $63\pm 7\%$ determined using the conventional plate count method (Fig. 6b). However, regarding the MF1 microparticles formulated without protective agent, the ratio of encapsulated LGG determined by the SERS method was far from the $3\pm 2\%$ measured with the conventional plate count method (Fig. 6b). The reason for this might be that the count plate method allows to quantify viable LGG only while the SERS method detects both live and dead bacteria. As a consequence, the comparison between the two quantification methods indicated that all the LGG entrapped in the MF3 microparticles remained viable whereas LGG encapsulated in MF1 were not enough protected during the encapsulation process to survive. The slight decrease in the survival rate for MF3 was mainly due to losses of the product in the spray-drier and not to an inactivation of LGG due to the harsh conditions of the encapsulation process.

SERS technique appeared as a promising method for the quantification of bacteria applied to the characterization of encapsulating systems in a faster and more convenient way than the conventional counting method. However, further investigations are required to try to discriminate alive from dead bacteria by SERS such as a deeper study of their SERS fingerprints. For example, in addition to the dominant band around 735 cm^{-1} , LGG SERS spectrum displays many other features that could be exploited (Fig. 6a). The exploitation of SERS spectra recorded for different bacterial strains using multivariate data analysis is an approach commonly encountered with the purpose of strain discrimination according to their SERS fingerprints (Jarvis & Goodacre., 2004 ; Chu *et al.*, 2008 ; Dina *et al.*, 2017 ; Zhang *et al.*, 2012 ; Mircescu *et al.*, 2014 ; Akanny *et al.*, 2020). A similar strategy could be used to analyze the SERS spectra acquired before and after submitting a bacterial strain to deleterious operating conditions. Such conditions have to be chosen so as to mimic those encountered during spray-drying or acidic treatment. The operating conditions that affect the physiological state of the microorganisms would modify their metabolic activity and thus the SERS spectrum since the metabolites secreted by bacteria highly contribute to their SERS signal (Kubryk *et al.*, 2016; Premasiri *et al.*, 2016).

3.6. Study of protective effect of Eudragit® S100 microparticles on LGG survival in simulated gastrointestinal conditions.

The resistance to simulated gastro-intestinal conditions of LGG free and microencapsulated in MF3 microparticles was studied using the plate count method.

Firstly, the survival in SGF only was tested. Free LGG showed a dramatic loss in viability after their incubation in the acidic environment (pH 1.5) since no viable cells were detectable after 1h exposure. In contrast, only 1.5 (± 0.1) and 1.5 (± 0.4) log reduction in viable cells were observed for encapsulated LGG after 1h and 2h exposure, respectively (Fig. 7). This corresponded to a number of living LGG loaded in the particles after 2h incubation in SGF of about $2.2 (\pm 0.9) \times 10^8$ CFU/g.

To the best of our knowledge, enteric soluble Eudragit® S100 has not been used as matrix polymer alone for the encapsulation of probiotics by spray-drying. As mentioned above, Eudragit® S100 was mainly used as enteric coating agent of microparticles obtained for example by extrusion-spheronisation or prilling process (De Barros *et al.*, 2015; Ansari *et al.*, 2017), or associated with other polymers in tablets or microparticles (Villena *et al.*, 2015; Rahmati *et al.*, 2020). Moreover, in this study Eudragit® S100 was used in aqueous solution to form microspheres by spray-drying in order to protect probiotics from denaturation during the encapsulation process. A recent study reported the development of Eudragit® RS 100 and Eudraguard® (maize starch-based polymer) microparticles for the colonic delivery of Gram-positive and Gram-negative bacteria mediated by pH-independent swelling (Yus *et al.*, 2019). However, this kind of polymers required the use of the emulsion/solvent evaporation technique potentially deleterious for probiotics viability during process. Microencapsulation in natural biopolymer microparticles is usually preferred for targeted colonic delivery of probiotics. These polymers should allow the possible achievement, through mild processes but difficult to be scale up (i.e. prilling and emulsification/gelation), of sufficiently large microparticles to provide a good protection in simulated gastric fluid (Koo *et al.*, 2001; Krasaekoopt *et al.*, 2004; Muthukumarasamy *et al.*, 2006; Sandoval-Castilla *et al.*, 2010; Qiang Zou *et al.*, 2011; Jiménez-Pranteda *et al.*, 2012; Sohail *et al.*, 2012; Cheow and Hadinoto, 2013; Abbaszadeh *et al.*, 2014; Li *et al.*, 2016). Hansen *et al.* (2002) reported that alginate beads with a size smaller than 100 μm did not significantly improve the survival of acid-sensitive probiotic bacteria in simulated gastric fluid. Moreover, improvements in survival of probiotics bacteria under simulated gastric conditions have also been reported with the increase of the size of alginate beads, with an optimal protection reported for sizes larger than 100 μm (Sheu *et al.*, 1993; Lee and Heo, 2000; Chandramouli *et al.*, 2004). SEM image of spray-dried MF3 microparticles after 2h of incubation in gastric fluid is shown in Fig. 8. In this study, a satisfactory LGG cell protection in SGF was ensured despite the relatively small size of the Eudragit® spray-dried particles with $d_{50} < 10 \mu\text{m}$.

The resistance of encapsulated LGG to SIF (FASSIF V2) consecutively to the SGF was then studied. MF3 microparticles were incubated into SGF for 1 h and then into FASSIF V2 for up to 6 h. After 1h in FASSIF V2, a 2.0 ± 0.2 log reduction in viable cells was observed, followed by a 2.1 ± 0.6 log reduction

after 4h and 2.4 ± 0.7 log reduction after 6h of exposure (Fig. 7). This corresponded to a number of living LGG loaded in the particles after 7h of incubation in simulated gastro-intestinal fluids of $4.5 \pm 2.5 \times 10^7$ CFU/g. This result is in accordance with the recommended amount of probiotic bacteria required in food products which should be at least 10^6 CFU/g or mL (FAO/WHO, 2002). It should be noted that free LGG were not very sensitive to the tested FASSIF V2, containing bile salts, and presented a good surviving rate after 6h of incubation with less than 0.3 ± 0.1 log reduction in viable cells observed (without previous incubation in SGF). However, considering the incubation in FASSIF V2 only, a 0.9 log reduction in viable cells was observed for encapsulated LGG; a loss slightly higher than for free cells. As previously described in literature, this could be explained by the cell membrane damages occurring during spray-drying and leading to an increased sensitivity of spray dried cells to NaCl (Gardiner *et al.*, 2000; Picot and Lacroix, 2004; Sunny-Roberts and Knorr, 2009) and enzymes (Sunny-Roberts and Knorr, 2009). Therefore, the higher reduction in viability observed in case of encapsulated cells compared with free cells after incubation in SIF might be due to a developed sensitivity of the spray-dried LGG to bile salts. However, in the case of encapsulated LGG, the incubation in FASSIV V2 was preceded by 1h incubation in SGF. The undergone pH variation might also participate in the sub-lethal damage and in the increased sensitivity to bile salts.

4. Conclusion

In this work, pH-responsive Eudragit® S100 microparticles were developed for the colonic delivery of *Lactobacillus rhamnosus* GG (LGG) using an aqueous-based spray-drying approach as an alternative of the classical but deleterious emulsification/solvent evaporation technique. One of the main drawbacks of the spray-drying is the thermal and dehydration stresses imposed on bacteria, which are the major causes of cell inactivation. The use of carbohydrates as protective agent allowed to increase the survival ratio after spray-drying from 3% to a maximum of 63 % using mannitol. SERS and IR characterizations indicated the viability of all entrapped LGG in microparticles containing mannitol with a constant ratio Eudragit/mannitol during the whole encapsulation process. Encapsulated LGG showed a great stability during storage at 5°C with less than 0.4 log reduction in viable cells after 2 months thanks to the low residual moisture content (1.9%) achieved in presence of mannitol. Moreover, encapsulating carrier was shown to greatly improve the survival of LGG in gastric medium *in vitro* since only 1.5 log reduction in viable cells was observed for encapsulated LGG after 2h exposure in SGF while no viable cell was detectable after 1h exposure in case of free LGG. This satisfactory cell protection was surprisingly ensured by Eudragit® spray-dried particles with a median diameter d_{50} smaller than 10 μm . 4.5×10^7 CFU/g of encapsulated cells were found viable after incubation of microparticles for 1 h in SGF followed by 6 h in SIF. This value, consistent with the recommended amount of probiotic bacteria required in food products ($>10^6$ CFU/g or mL), attests that the developed encapsulation system is suitable for its use as a carrier for colonic delivery of LGG. However, *in vivo* experimentations are required to confirm such the colonic targeting. From an analytical point of view, SERS appeared as a very promising alternative to the tedious and time consuming plate count method but further investigations are needed to evaluate its potential for a selective quantification of live and dead bacteria as mentioned above.

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Figure legends

Fig. 1. Schematic overview of the preparation of the feed solutions (F2 to F5) to be spray-dried.

Fig. 2. FTIR-ATR spectra of Eudragit® S100 powder, Eudragit® S100 in PBS solution, MF3 microparticles and mannitol, in the IR spectral region 1700-1300 cm^{-1} .

Fig. 3. (a) AFM image of LGG bacteria. (b) Spray-dried microparticle size distributions as a function of the protective agent used in the formulation.

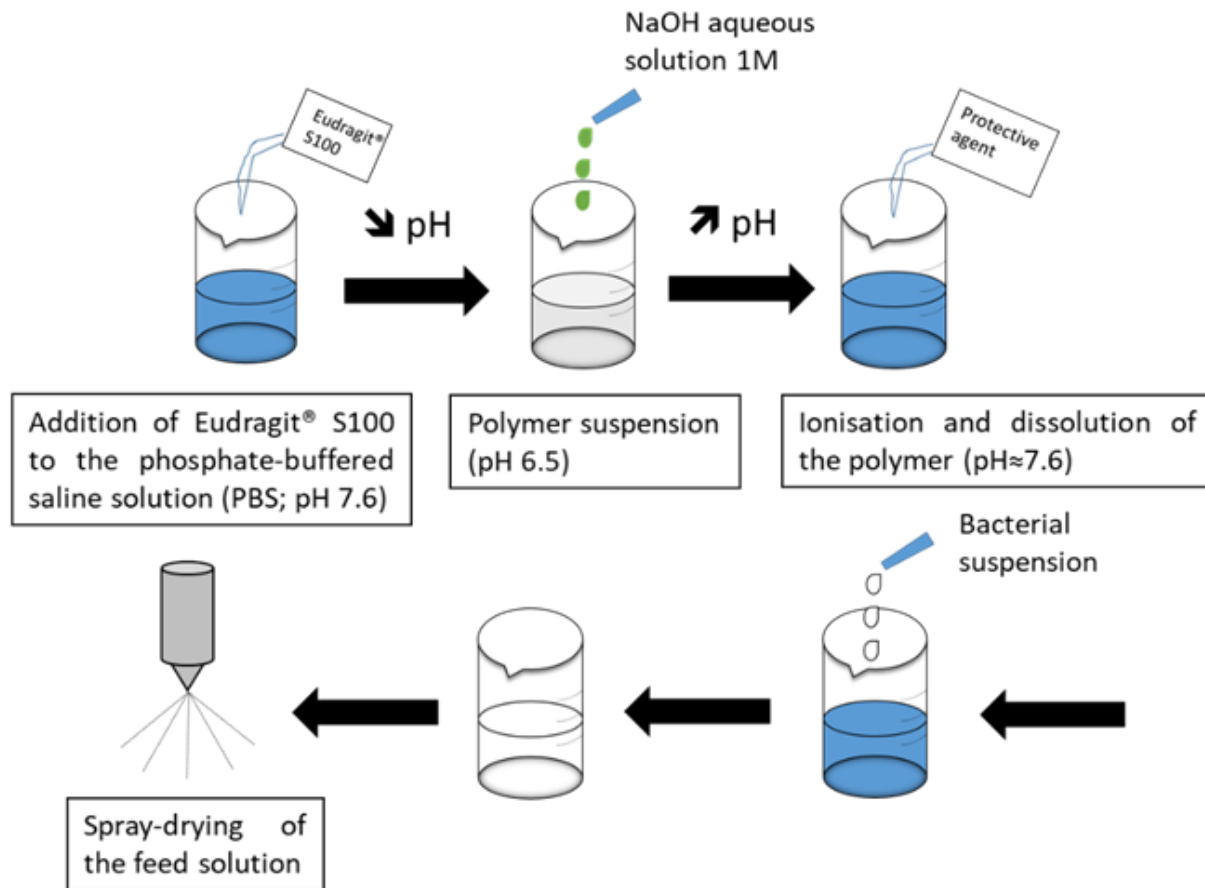
Fig. 4. SEM images of Eudragit® S100 spray-dried microparticles in the absence (a) or the presence of protective agents such as mannitol (b), trehalose (c), Glucidex® IT6 (d) and PVP (e)

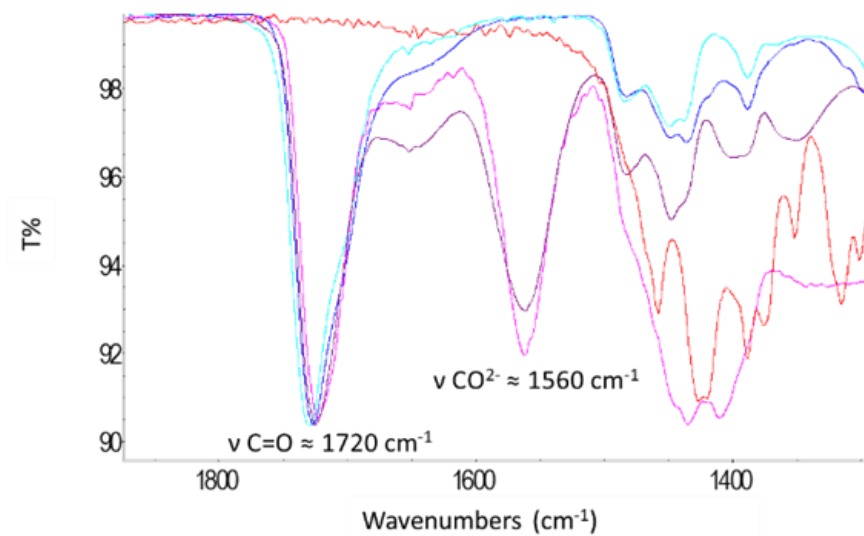
Fig. 5. (a) LGG survival after spray-drying with or without protective agent. Bars and error bars represent the mean \pm Standard Deviation estimated from triplicates. (b) Logarithmic reduction of viable cells in spray dried MF3 microparticles (Eudragit®S100/mannitol) during 1, 2, 4 and 8 weeks storage at 7°C. The results are means based on data from triplicate, and standard deviations (n=3) are indicated by vertical bars.

Fig. 6. (a) Example of SERS spectrum obtained from 3.1×10^6 CFU/mL LGG suspension with AuNPs (b) Ratio of encapsulated LGG (%) determined with plate count or SERS method for MF1 (no protective agent) and MF3 (10% w/w mannitol) microparticles. Bars and error bars represent the mean \pm Standard Deviation estimated from triplicates. Note that N is the CFU per g (CFU/g) of the spray-dried powder immediately after drying and N_0 is the CFU/g of dry matter in the initial suspension before drying.

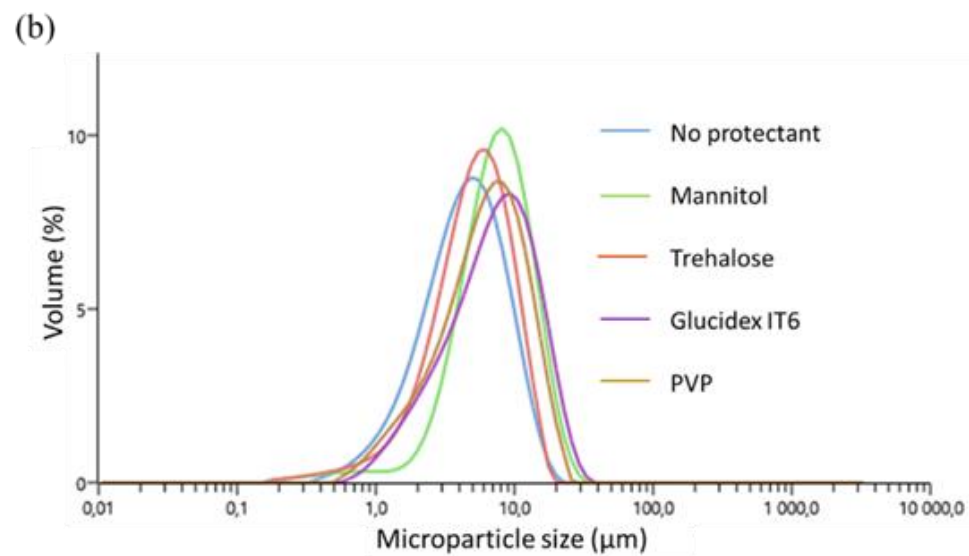
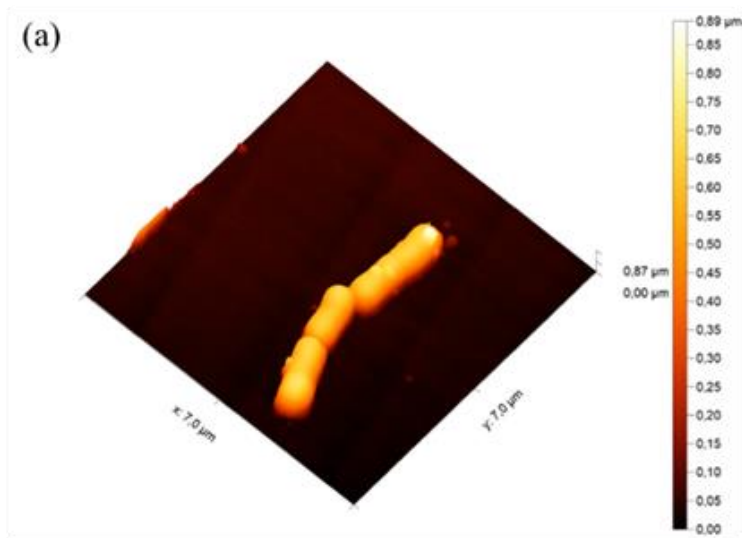
Fig. 7. Logarithmic reduction of viable cells for encapsulated LGG in MF3 microparticles after incubation in SGF and/or FASSIF V2. Bars and error bars represent the mean \pm Standard Deviation estimated from triplicates.

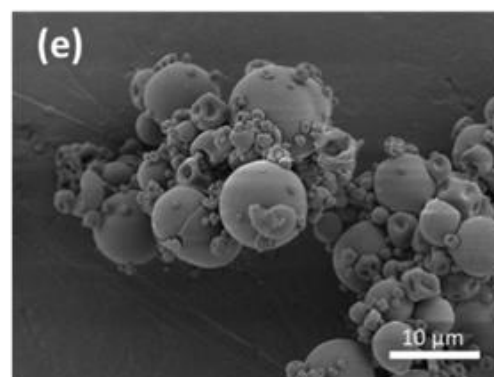
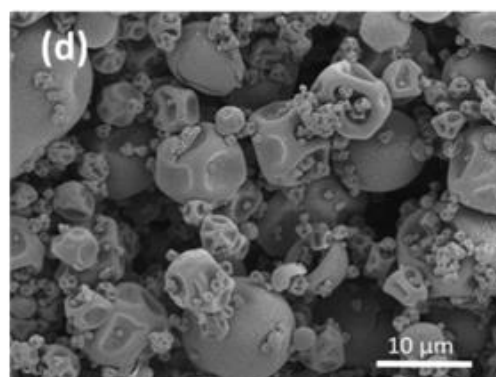
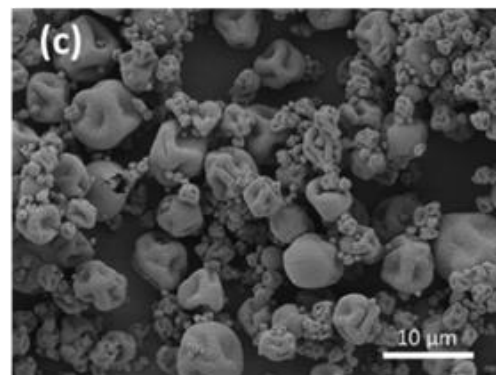
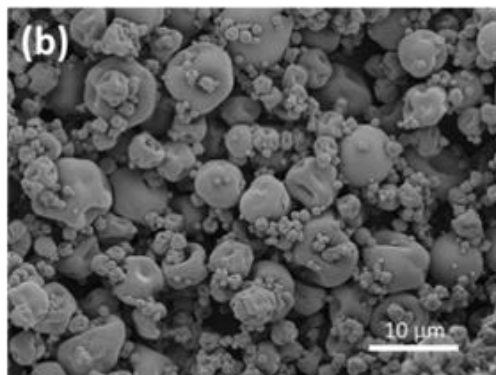
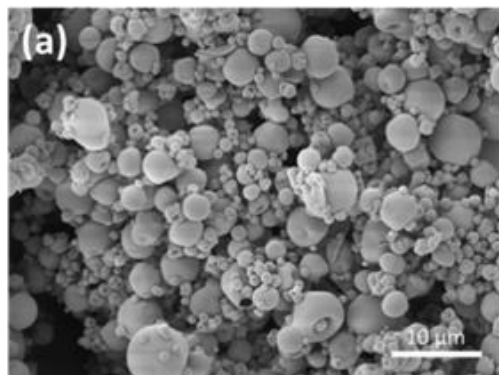
Fig. 8. SEM image of spray-dried MF3 microparticles after 2h incubation in gastric fluid.

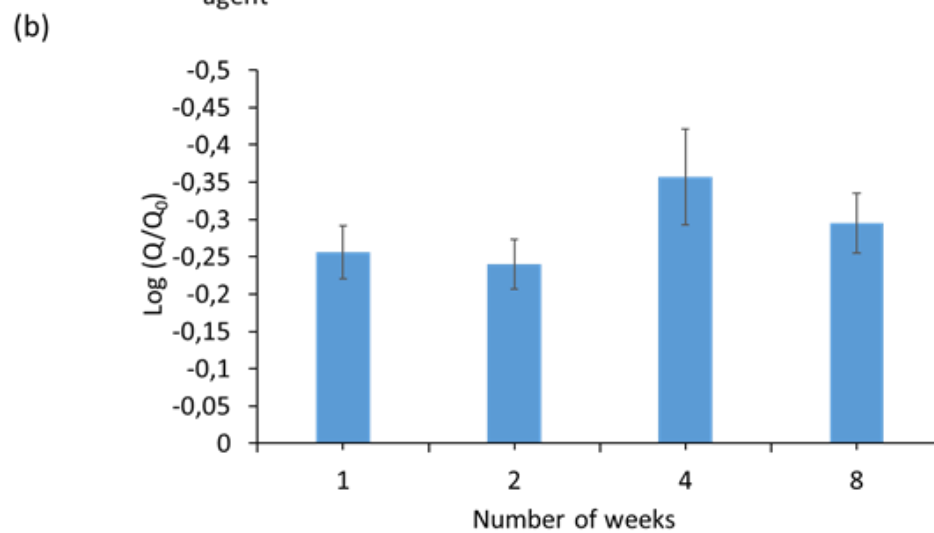
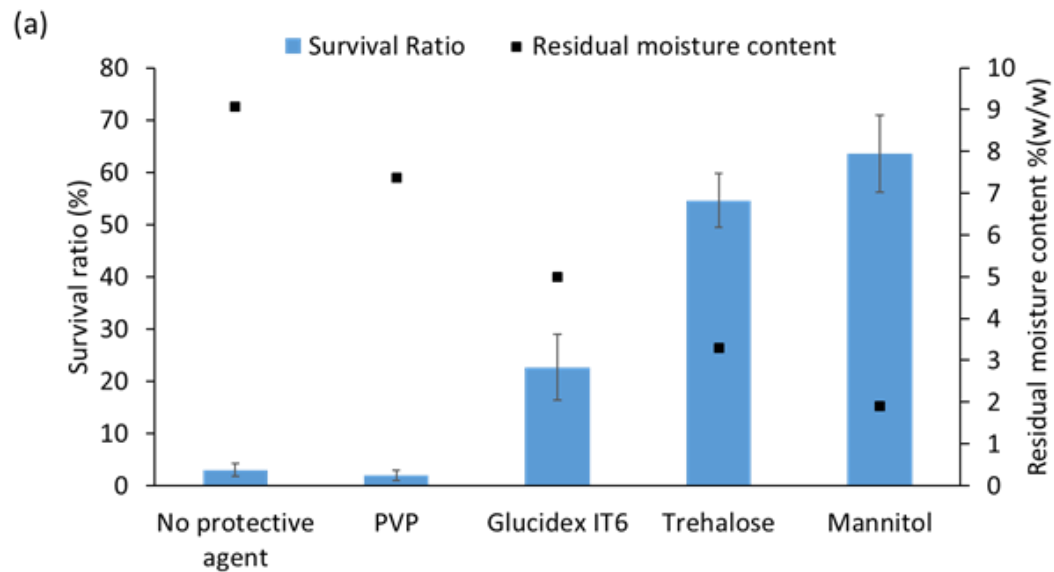




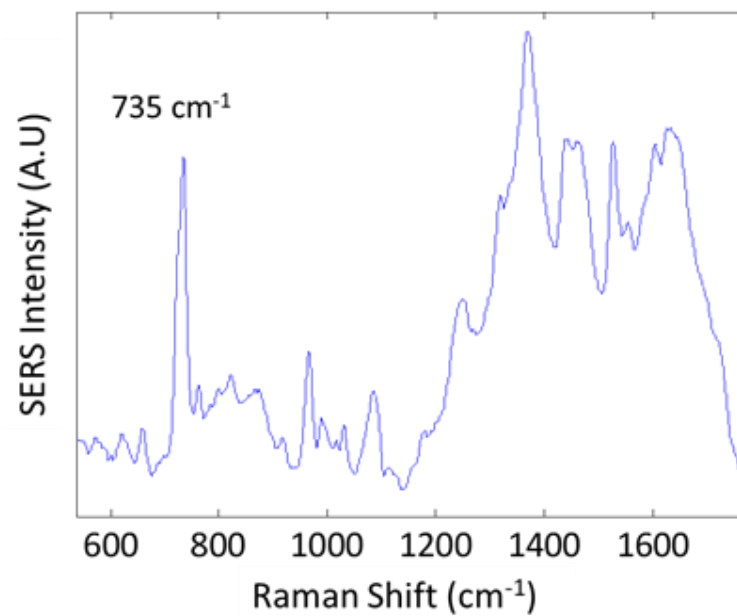
Eudragit® S100
Eudragit® S100 in PBS solution
Eudragit® S100/Mannitol MPs
Eudragit® S100/Mannitol MPs after acid incubation
Mannitol



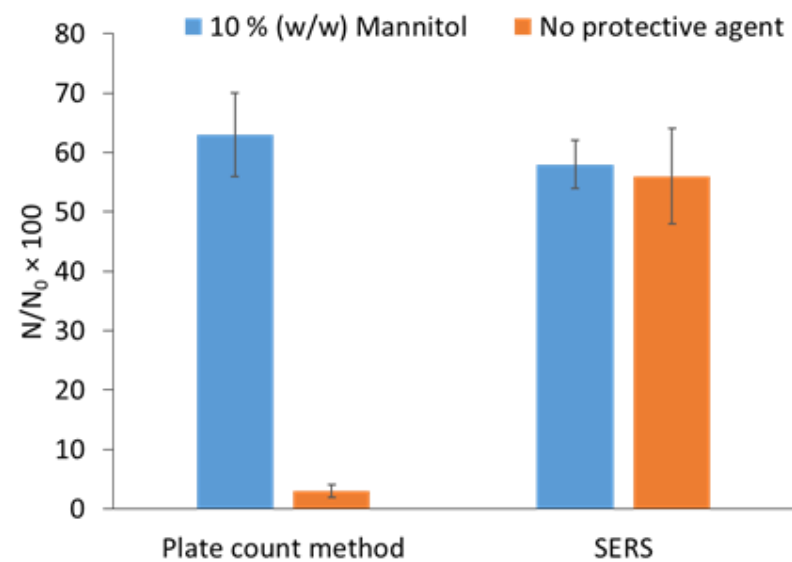


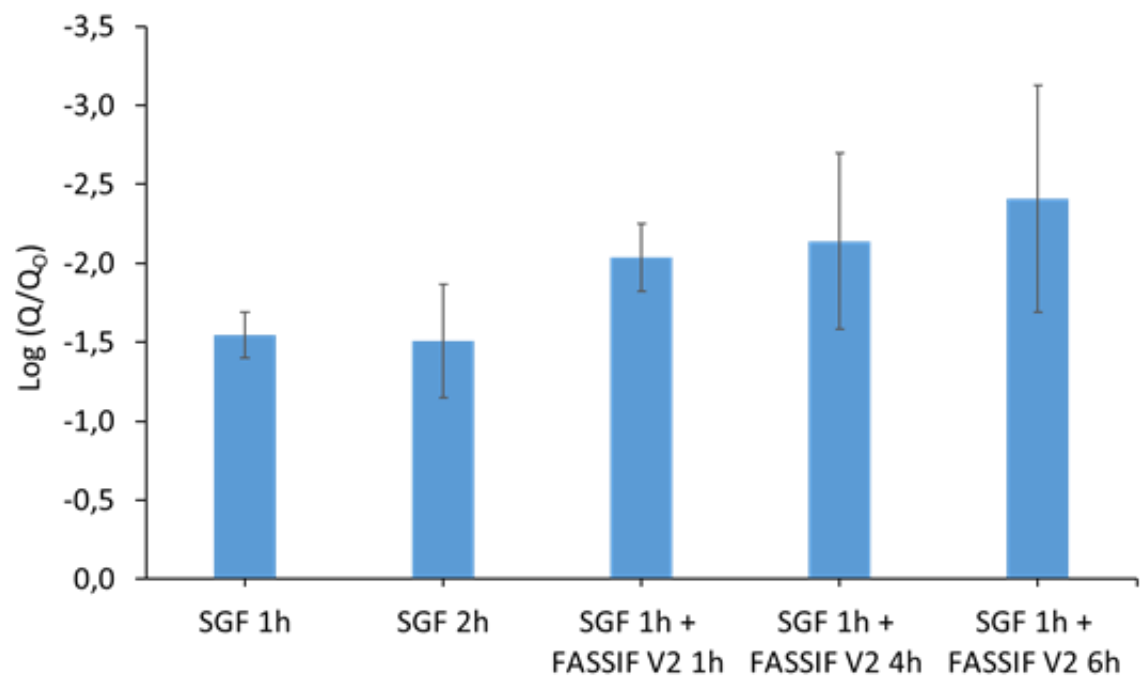


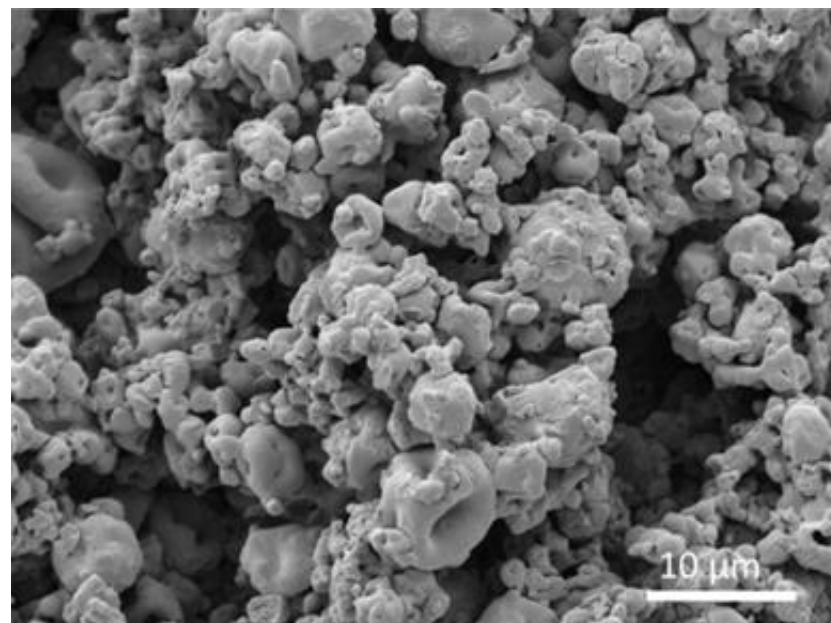
(a)



(b)







Tables

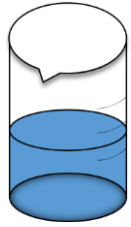
Table 1 Compositions of feed solutions.

Feed solution	Obtained Microparticles	Enteric polymer	Protective agent
F1	MF1		-
F2	MF2	Eudragit®	PVP 5% (w/v)
F3	MF3	S100 4.1%	Mannitol 10% (w/v)
F4	MF4	(w/v)	Trehalose 10% (w/v)
F5	MF5		Glucidex IT6 10% (w/v)

Table 2 Size and solid yield as a function of the protective agent used in the formulation.

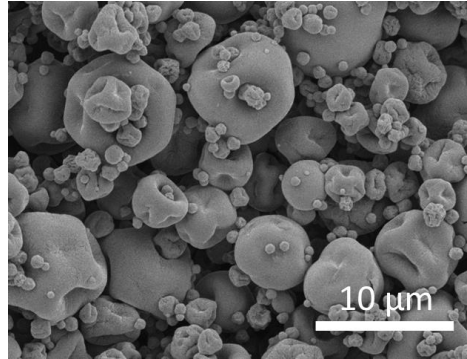
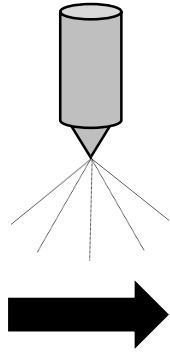
Protective agent	d ₁₀	d ₅₀	d ₉₀	Solid Yield (%)	Span
-	1.6	4.4	9.8	54±5	1.9
PVP	2.0	6.3	13.7	30±6	1.9
Mannitol	3.1	7.4	15.0	59±3	1.6
Trehalose	1.9	5.2	10.6	50±3	1.7
Glucidex IT6	2.2	7.3	16.3	54±4	1.9

Formulation



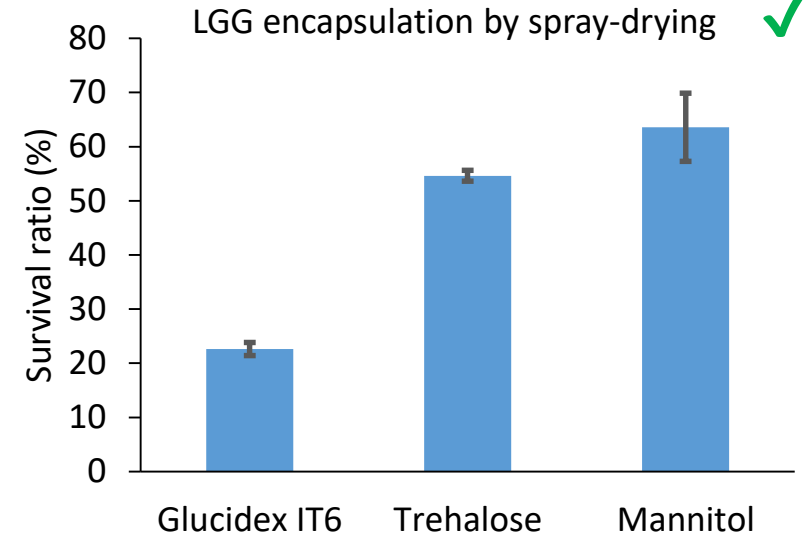
Aqueous solution :
 - *Lactobacillus rhamnosus*
 GG (LGG)
 - Eudragit® S100
 - Carbohydrates

Spray-drying



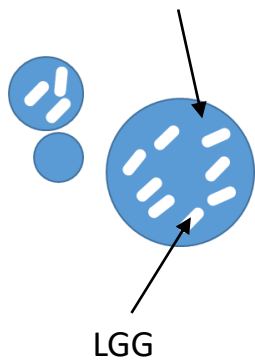
Spray-dried Eudragit® S100 MPs

Cell survival after process > 50 %



Protection in simulated gastro-intestinal (SGI) conditions

Eudragit® S100 /
 Carbohydrates



SGI
 conditions

