Biopolymer-Based Minimal Formulations Boost Viability and Metabolic Functionality of Probiotics *Lactobacillus rhamnosus* GG through Gastrointestinal Passage

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Supporting Information

**ABSTRACT:** The delivery of probiotic microorganisms as food additives via oral administration is a straightforward strategy to improve the intestinal microbiota. To protect probiotics from the harsh environments in the stomach and small intestine, it is necessary to formulate them in biocompatible carriers, which finally release them in the ileum and colon without losing their viability and functions. Despite major progresses in various polymer-based formulations, many of them are highly heterogeneous and too large in size and hence often "felt" by the tongue. In this study, we established a new formulation for probiotics *Lactobacillus rhamnosus* GG (LGG) and systematically correlated the physicochemical properties of formulations with the functions of probiotics after the delivery to different gastrointestinal compartments. By reducing the stirring speed by 1 order of magnitude during the emulsification of polyalginate in the presence of xanthan gum, we fabricated microparticles with a size well below the limit of human oral sensory systems. To improve the chemical stability, we deposited chitosan and polyalginate layers on particle surfaces and found that the deposition of a 20 nm-thick layer is already sufficient to perfectly sustain the viability of all LGG. Compared to free LGG, the colony-forming units of LGG in these formulations were by factors of 10⁷ larger in stomach fluid and 10⁴ larger in small intestine fluid. The metabolic functionality of LGG in polymer formulations was assessed by measuring the amount of lactate produced by LGG in a human gastrointestinal simulator, showing 5 orders of magnitude larger values compared to free LGG. The obtained results have demonstrated that the minimal formulation of LGG established here boosts not only the viability but also the metabolic functionality of probiotics throughout oral uptake, passage through the gastrointestinal tract, and delivery to the ileum and colon.

**INTRODUCTION**

The human colonic lumen contains a large number of bacteria, amounting to 1 trillion per g of stool, that live in a symbiotic relationship with their host. Dysregulations of the natural intestinal microbiota can lead to severe, potentially fatal disorders like inflammatory bowel diseases such as Crohn’s disease and ulcerative colitis.¹,² A healthy intestinal microbiota can be supported by an uptake of probiotic microorganisms, whose metabolic activity supports the natural gastrointestinal microbiota.³ The beneficial effects of probiotics are (1) inhibition of binding and growth of pathogens either by blocking their binding sites or by secretion of antimicrobial substances like lactic acid, (2) improvement of the intestinal epithelial barrier, and (3) modulation of the host immune response by sustaining the homeostasis of intestinal microbiota.³⁻⁵ It is known that colon epithelial cells are protected from physical abrasion and the attack of pathogenic bacteria by a mucus layer consisting of mucin proteins, whose surface is covered by phospholipid layers.⁶,⁷ However, once the homeostasis of gastrointestinal microbiota is broken, pathogenic bacteria start producing phospholipase that degrades phospholipids. As a consequence, the mucus layer can no longer hold its barrier function. According to the International Scientific Association for Probiotics and Prebiotics, the maximum benefit is obtained by a minimum probiotics amount of 10⁹ colony-forming units (CFU) administrated per day.⁸ Probiotics have been used widely as food additives in a large scale since the 1980s, and the most commercially available probiotics are *Lactobacillus* or *Bifidobacterium*.⁹

One of the major problems in the delivery of probiotics via oral administration is that a substantial amount of the bacteria cannot survive under harsh environments in the upper
gastrointestinal tract, such a slow pH in the stomach, and the exposure to bile acid and pancreatin in the small intestine. A promising strategy is the formulation of probiotics in polymer capsules that can protect them in the stomach and small intestine and eventually release them in the ileum and colon. For food applications, the choice of biodegradable polymers approved by the US Food and Drug Administration (FDA) is desirable. Moreover, it is favored to keep the size of polymer particles below 80 μm in order that a consumer does not "feel" the particles by the tongue.

To date, a number of techniques have been developed for the microencapsulation of probiotics, such as spray-drying, freeze-drying, extrusion, and emulsification. Among those, the emulsification method is one of the most gentle techniques to minimize the damage to microorganisms. The most common matrix used for the encapsulation of probiotics is polyalginate (PA) cross-linked by divalent cations like Mg²⁺ or Ca²⁺. Polyalginate is an FDA-approved, linear biopolymer extracted from the cell wall of marine brown algae, consisting of disaccharide units based on l-guluronic acid and d-mannuronic acid.

However, previous studies have shown that polyalginate microcapsules are not stable in gastrointestinal fluids. A possible strategy to improve the stability of polyalginate particles is to deposit additional polymer layers. For example, the layer-by-layer deposition of cationic and anionic polyelectrolytes has been used for the stable encapsulation of enzymes, antigens, and living cells. Previous studies have suggested that the deposition of one layer of chitosan on polyalginate capsules increases the survival rate of Bifidobacterium and Lactobacillus by a factor of 100–1000 under gastric conditions compared to polyalginate microcapsules.

However, the size of particles used in these studies was 1 order of magnitude larger than the sensory limit of the human tongue, or even not controlled. Moreover, they failed to address key questions such as: (a) what is the minimum and thus the most effective number of layers for stable formulations, (b) whether these polymer capsules could release probiotics in the target organ (colon), and (c) whether polymer capsules interfere with the functionality of probiotics.

In this study, we have systematically established the minimum formulation of probiotics for optimal delivery via oral administration. As the probiotics, we selected Lactobacillus rhamnosus strain GG (LGG) from human intestine was purchased from ATCC (Hoersholm, Denmark), and polyglycolic polycaproic acid (PGPR) was from DANISCO GmbH (Niebuell, Germany). Polyalginate from brown algae (MW ≈ 90 kDa), xanthan gum from Xanthomonas campestris (MW ≈ 2.5–3.3 MDa), chitosan (MW ≈ 50–190 kDa), pepsin from porcine gastric mucosa, pancreatin from porcine pancreas, de Man, Rogosa, and Sharpe (MRS) broth, and starch were purchased from Sigma-Aldrich (Neu-Ulm, Germany). Anaerogen bags and formaldehyde were purchased from Thermo Fisher Scientific GmbH (Duisburg, Germany). The pluri strainier 10 μm was purchased from HiSS Diagnostic GmbH (Freiburg, Germany). Au sensors for quartz crystal microbalance with dissipation (QCM-D) were purchased from LOT-Quantum Design GmbH (Darmstadt, Deutschland). Throughout the study, double deionized water (Milli-Q, Merck Chemicals, Darmstadt, Germany) with a specific resistivity >18 MΩ cm was used. Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (Neu-Ulm, Germany) and used without any further purification. Human gastrointestinal liquid simulator, L-SHIME, was purchased from ProDigest (Zwijnaarde, Belgium), and bile extract from Difco (Livonia).

LGG Culture. Prior to the encapsulation, LGG were cultured for 18 h at 37 °C in MRS broth, harvested by centrifugation at 3500g for 15 min (Heraeus multifuge 3L-R, Thermo Fisher, Gmbh, Duisburg, Germany), and washed three times in phosphate-buffered saline (pH 7.4). For quantitative determination of CFU, 100 μL of a serial diluted bacteria suspension was plated on MRS broth agar dishes, and the number of colonies was determined by the plate count method.

Encapsulation of LGG in Polyalginate (PA⁺) Core. LGG were encapsulated in polyalginate (PA⁺) microparticles by emulsification. In brief, a 10 mL portion of LGG suspension (3.7 × 10^8 CFU/mL) containing 1 wt % polyalginate and 0.3 wt % xanthan gum was added dropwise to 40 mL of sunflower oil containing 1% (v/v) PGPR under constant stirring at 300 rpm. After 15 min, an aqueous solution of CaCl₂ (100 mM, 40 mL) was added and stirred for 30 min for gelation. PA⁺ microparticles were collected by centrifugation (pluri strain filter with a pore size of 10 μm) and rinsed five times with water.

Layer-by-Layer Coating of PA⁻ Cores. To improve the chemical stability, PA⁻ cores were further coated by the layer-by-layer deposition of polyelectrolytes.

Scanning Electron Microscopy (SEM). The surface morphology of microparticles was investigated using SEM (Leo 1530 scanning electron microscope, Carl Zeiss, Oberkochen, Germany) using an acceleration voltage of 5 kV. Prior to the SEM observation, the samples were immobilized on poly-l-lysine-coated glass coverslips and fixed with 3% (v/v) formaldehyde in water. Water was removed by rinsing the specimen with aqueous solutions of increasing ethanol concentrations. Subsequently, the samples were dried by critical point drying (Leica EM CPD030, Wetzlar, Germany) and sputter-coated with a 15 nm Au layer using a Bal-Tec Med 020 high vacuum coating system (Belenex BV, the Netherlands).

ζ-Potential Measurements. The ζ-potential of microparticles was measured utilizing a Malvern Zetasizer Nano ZS (Malvern, U.K.). The reported ζ-potential value corresponds to an average of six consecutive measurements.
Quartz Crystal Microbalance with Dissipation (QCM-D). The formation of multilayers was monitored by a QCM-D E4 (Q-Sense, Gothenburg, Sweden) using Au-coated AT-cut quartz crystals (LOT-Quantum design GmbH, Darmstadt, Germany).\(^{33,34}\) In brief, a 2 mL portion of PA\(^{-}\) solution (1 wt %) was applied to form the first layer. After reaching equilibrium, unbound polymers were removed by rinsing with water. Subsequently, gelation was induced by injection of 100 mM CaCl\(_2\) solution. For the layer-by-layer deposition, Ch\(^{+}\) and PA\(^{-}\) solutions (0.1 wt %) were applied alternately, followed by an intensive rinsing with water until the resonant frequency reached a constant value. The signals from fifth, seventh, and ninth overtones were analyzed using Q-Tools software from the manufacturer. Since the dissipation exceeded the critical level \(D = 2 \times 10^{-6}\),\(^{35}\) the obtained results were analyzed applying the Voigt model.

Viability of LGG in Simulated Gastrointestinal Fluids. Gastric fluid was simulated by 3 g/L pepsin and 85 mM NaCl (pH = 2.0) and small intestine fluid by 1 g/L pancreatin, 1 g/L bile salt, and 85 mM NaCl (pH = 6.5).\(^{34}\) Free or encapsulated LGG were incubated at 37 °C for 2 h under gentle shaking (100 rpm). The viability of LGG was assessed by counting CFU. The initial LGG concentration in capsules for 2 h under gentle shaking (100 rpm).

LGG Viability, Release, and Functionality of LGG in Gastrointestinal Simulator. As a realistic simulator of human intestinal environments, we employed the SHIME system.\(^{5-9}\) Prior to the experiments, the initial concentrations of free as well as encapsulated LGG were determined by an Accuri C6 flow cytometer (BD, San Jose, CA) (Scheme 1). As presented in Scheme 2, (i) free LGG; (ii) LGG encapsulated in PA\(^{-}\) cores, or (iii) LGG encapsulated in C3 particles first entered the container mimicking (1) stomach environments containing 1 g/L arabinogalactan, 2 g/L pectin, 1 g/L xylan, 4 g/L starch, 0.4 g/L glucose, 3 g/L yeast extract, 1 g/L pepton, 4 g/L mucin, and 0.5 g/L cysteine at pH = 4.8. After the incubation for 30 min, the sample was incubated at pH = 4.0, 3.5, 3.0, and 2.5 for 15 min at each step. Finally, pH was reduced to pH = 2.0 and incubated for further 30 min. (2) To mimic the small intestine, a simulated pancreatic juice (0.9 g/L pancreatin, 0.6 g/L bile extract, and 12.5 g/L NaHCO\(_3\)) was added, and the sample was incubated at pH = 6.5 for 3 h. After every step, a 1.2 mL portion sample was withdrawn, and the viability of LGG in the solution was assessed by flow cytometry.\(^{39}\) (3) As the ileum environment is anaerobic, the sample was flushed with N\(_2\). A diluted (1/100) fecal sample was added to simulate the ileum environment, incubated for 3 h. (4) Finally, the sample was incubated for another 18 h in colon environments in the presence of an additional fecal sample. The metabolic function of the released LGG was quantified by determining the lactic acid concentration in 1 mL samples withdrawn from each step by using a 930 Compact IC Flex ion-exchange chromatograph, equipped with a conductivity detector (Metrohm, Herisau, Switzerland).

Scheme 2. Viability and Functionality of LGG in Human Gastrointestinal Simulator

Characterization of Polymer Microparticles Encapsulating LGG. Figure 1A represents a typical bright field image of PA\(^{-}\) cores encapsulating LGG. As shown in the inset, the size distribution of PA\(^{-}\) microparticles obtained from \(N > 600\) can be well fitted with the Weibull function,\(^{40}\) yielding the mean diameter of 45 μm and the full width at half maximum (FWHM) of 33 μm. The particles are much smaller and more uniform in size compared to those fabricated in previous studies using the extrusion method.\(^{24,25}\) A previous single-blind test trial study has shown that soft and round particles, such as latex beads, were not perceived as gritty up to the diameter of 80 μm, whereas for hard and angular particles, such as garnet, the grittiness is evident already with the particle size of 10–20 μm.\(^{11}\) The fact that the size of our PA\(^{-}\) cores is well below this sensory limit of human suggests that they are potentially suited as food additives. The preparation of small, uniform microparticles can be achieved by emulsification.\(^{12}\) However, the preparation of particles smaller than 100 μm requires a high shear stress (or fast stirring), which could damage probiotic bacteria.\(^{11}\) To minimize the potential damage to LGG, we added xanthan gum that exhibits a shear-induced thinning and a fast recovery upon the removal of shear,\(^{41}\) which can be explained by a partial alignment of linear polymer chains.\(^{42}\) This enabled us to fabricate small, uniform microparticles at a low stirring speed (300 rpm), which is 1 order of magnitude smaller than that for the conventional high-pressure homogenization technique.

RESULTS AND DISCUSSION

Characterization of Polymer Microparticles Encapsulating LGG. Figure 1A represents a typical bright field image of PA\(^{-}\) cores encapsulating LGG. As shown in the inset, the size distribution of PA\(^{-}\) microparticles obtained from \(N > 600\) can be well fitted with the Weibull function,\(^{40}\) yielding the mean diameter of 45 μm and the full width at half maximum (FWHM) of 33 μm. The particles are much smaller and more uniform in size compared to those fabricated in previous studies using the extrusion method.\(^{24,25}\) A previous single-blind test trial study has shown that soft and round particles, such as latex beads, were not perceived as gritty up to the diameter of 80 μm, whereas for hard and angular particles, such as garnet, the grittiness is evident already with the particle size of 10–20 μm.\(^{11}\) The fact that the size of our PA\(^{-}\) cores is well below this sensory limit of human suggests that they are potentially suited as food additives. The preparation of small, uniform microparticles can be achieved by emulsification.\(^{12}\) However, the preparation of particles smaller than 100 μm requires a high shear stress (or fast stirring), which could damage probiotic bacteria.\(^{11}\) To minimize the potential damage to LGG, we added xanthan gum that exhibits a shear-induced thinning and a fast recovery upon the removal of shear,\(^{41}\) which can be explained by a partial alignment of linear polymer chains.\(^{42}\) This enabled us to fabricate small, uniform microparticles at a low stirring speed (300 rpm), which is 1 order of magnitude smaller than that for the conventional high-pressure homogenization technique.
As a number of studies have demonstrated, the polyelectrolyte complexes.19 Figure 2A represents a typical bright image of PA− cores encapsulating LGG. (C) SEM image with a higher magnification, implying LGG are trapped by polymer meshworks. (D) Degradation of PA− cores in gastric fluids (pH = 3.0): (D1) prior to the exposure, (D2) t = 30 min, (D3) t = 45 min, and (D4) t = 90 min.

Figure 1. (A) Bright field image of PA− cores encapsulating LGG. The size distribution is shown in the inset. The fitting with the Weibull function yields a mean diameter of 45 μm and FWHM of 33 μm. (B) Scanning electron microscopy (SEM) image of a PA− core. Yellow arrows indicate LGG. (C) SEM image of a C3 particle. Compared to the corresponding image of a PA− core (Figure 1B), the deposition of additional polyelectrolyte layers seemed to alter the texture of particle surfaces significantly. After the deposition, individual LGG could hardly be identified. Actually, the image with a higher magnification (Figure 2D) indicated that LGG are covered by the “folded” layers of polyelectrolytes.

Surface Coating with Polyelectrolyte Multilayers. To increase the chemical stability of microparticles as well as to sustain the viability of encapsulated LGG in gastric condition, we deposited several layers of polyelectrolytes on the PA− core. As a number of studies have demonstrated, the polyelectrolyte multilayers prepared by layer-by-layer deposition are highly stable owing to the formation of polycation–polyanion complexes.19 Figure 2A represents a typical bright field image of PA− cores encapsulating LGG, which are additionally coated with Ch+/PA−/Ch− layers (C3). The size distribution (inset) suggested that the deposition of C3 layers resulted in a decrease in the mean particle diameter from 45 to 35 μm. Interestingly, the particle size was the smallest after deposition of the first Ch− layer, implying that the PA− core was compacted due to the binding of oppositely charged Ch− (Supporting information, Figure S2). Figure 2B shows the fluorescence image taken at the same position as Figure 2A, confirming that fluorescein isothiocyanate (FITC)-labeled Ch+ was uniformly deposited on all the particles. Figure 2C represents the SEM image of a C3 particle. Compared to the corresponding image of a PA− core (Figure 1B), the deposition of additional polyelectrolyte layers seemed to alter the texture of particle surfaces significantly. After the deposition, individual LGG could hardly be identified. Actually, the image with a higher magnification (Figure 2D) indicated that LGG are covered by the “folded” layers of polyelectrolytes.

Layer-by-Layer Deposition of Polyelectrolytes. The successive deposition of polyelectrolyte multilayers was monitored by the change in ζ-potential (Figure 3). The PA− cores showed a negative value, ζ ≈ −30 mV. The incubation with a Ch− solution shifted the surface potential to a positive value, ζ ≈ +6 mV. The inversion of the sign of ζ-potentials was observed after each incubation cycle, confirming the alternate deposition of polycation (Ch+) and polyanion (PA−).

The kinetics of polyelectrolyte multilayer formation was monitored on planar surfaces by using QCM-D. As presented in Figure S3, the model of the PA− core was fabricated by the physisorption of PA− on the Au substrate, followed by the incubation with 100 mM CaCl2. The data were analyzed using the Voigt model, assuming the density of polymer layers to be 1400 kg/m3.43 Table 1 summarizes the calculated viscosity, shear modulus, and the thickness of each layer. The viscosity and shear modulus of the physisorbed PA− layer distinctly increased when CaCl2 was added, which corresponds to the cross-linking of PA− by divalent Ca2+. Moreover, the
thickness of the PA− layer decreased from 22.6 to 13.0 nm, suggesting the loss of hydrating water. The increase in the thickness after the incubation with Ch+ solution was rather small (3.5 nm), but the increase in viscosity was pronounced. This finding can be interpreted in terms of the compaction of the first PA− layer by the deposition of oppositely charged Ch+, which seems consistent with the decrease in particle size (Figure 2A). The monotonic increase in the film thickness caused by each incubation step confirmed the layer-by-layer deposition of oppositely charged polyelectrolytes.

**LGG Viability in Gastric and Intestinal Fluids.** In the next step, we evaluated the protective capacity of the different formulations in the upper gastrointestinal tract (stomach and small intestine). Figure 4 shows the number of CFU normalized by the initial value (CFU₀) after the exposure to the fluids mimicking gastric and small intestinal environments. In the case of encapsulated LGG, the particles were dissolved in 100 mM phosphate buffer (pH ≈ 7.8) following the previously reported protocols and subjected to CFU counting. As presented in Figure 4A, the normalized CFU count of free LGG (blue) in gastric fluid (pepsin, NaCl, pH = 2.0) was extremely low, CFU/CFU₀/free,stomach ∼ 10⁻⁷, implying that almost all LGG died after 2 h. Once LGG were encapsulated in the PA− core, a clear increase in the viability by 4 orders of magnitude was observed, CFU/CFU₀/free,stomach ∼ 10⁻³. However, this CFU level still remains low, suggesting that only 0.1% of the LGG were viable. The successive deposition of polyelectrolyte layers improved the viability of LGG significantly. C3 particles realized the maintenance of viability for almost all LGG, CFU/CFU₀/free,intestine ∼ 1. The obtained results have clearly demonstrated that the deposition of three additional polyelectrolyte layers onto PA− cores (C3) is sufficient to maintain the viability of all LGG during the passage through the stomach, which takes about 2 h.

The results obtained here suggested that the encapsulation of LGG followed by the deposition of additional three polyelectrolyte layers significantly increases the LGG survival by a factor of more than 10⁶ in stomach fluid and 10⁵ in small intestine fluid. It should be noted that the deposition of more than three polyelectrolyte layers did not affect the LGG viability (Supporting Information, Figure S4), implying that C3 is the minimum-necessary formulation for the maintenance of LGG viability under both stomach and small intestine conditions. This finding is remarkable if one considers that the total thickness of Ch+/PA−/Ch− layers is less than 20 nm. The significant improvement of the protection capability of PA− cores by the deposition of Ch+/PA−/Ch− layers can be attributed to (i) the tightening of polymer networks by the compaction of PA− core particles by the decrease in the amount of hydrating water (Figure 2A, Table 1) as well as (ii) the coating of individual LGG with polyelectrolytes (Figure 2D).

**Viability, Release, and Functionality of LGG in Human Gastrointestinal Simulator.** In the next step, we used the human gastrointestinal simulator not only to assess the protective capacity of our formulations but also to quantify the metabolic function of LGG during the passage through the entire gastrointestinal tract in the presence of nutrients (starch and other carbohydrates) and other bacteria (Scheme 2).

Figure 5 shows the number of living LGG released into the solution during the passage through the environments simulating the (A) stomach and (B) small intestine. First of all, it should be noted that we fluorescently labeled the "released LGG" in the solution and determined the number of
living LGG using flow cytometry, \( N_{FL} \). Namely, we can detect only the released LGG but not those encapsulated in the particles. Therefore, this assay is completely different from the previously described CFU assays (Figure 2), where all LGG were released by the dissolution of microparticles prior to CFU counting.

As presented in Figure 5, the fraction of living, free LGG (blue) decreased to \( N_{FL}/N_0(free,1h) \approx 10^{-1} \), implying that about 90% of free LGG already died in the initial phase of gastric condition (pH = 4.8, 1 h). The number of living LGG did not change by the successive exposure of free LGG to more acidic environments, pH = 3.0 (0.5 h) and pH = 2.0 (0.5 h), mimicking the later stage of gastric conditions. It is notable that the fraction of living LGG after 2 h, \( N_{FL}/N_0(free,2h) \approx 10^{-1} \), is much higher than the CFU of free LGG exposed to pH = 2.0 for 2 h, CFU/CFU(free,stomach) \approx 10^{-1} \) (Figure 4A, blue). These two findings can be explained by the presence of starch and other carbohydrates in the medium, helping the survival of LGG.46 Once free LGG were transferred to the environment simulating the small intestine for 3 h, a further decrease in living LGG was observed, \( N_{FL}/N_0(free,2+3h) \approx 10^{-2} \).

When PA\(^+\) cores encapsulating LGG (orange) were incubated in stomach fluid at pH = 4.8 for 1 h, no living LGG could be detected in the solution (\( N_{FL}/N_0(PA^+,1h) \approx 0 \)). However, this does not mean that all LGG died. In contrast, this result suggests that LGG were still encapsulated and thus not released into the solution. In fact, we found that the number of living LGG was close to 100% after incubating the LGG-loaded particles at pH = 3.0 for 0.5 h, \( N_{FL}/N_0(PA^+,1.5h) \approx 1 \). This finding suggests the release of LGG due to the dissolution of PA\(^+\) cores at pH = 3.0, which is in good agreement with the results presented in Figure 1D. No change in \( N_{FL}/N_0 \) was detected even after further incubation of the same sample at pH = 2.0 for 0.5 h (\( N_{FL}/N_0(PA^+,2h) \approx 1 \)). When this sample was incubated with the small intestine fluid (Figure 5B), the number of living LGG decreased approximately by 1 order of magnitude, \( N_{FL}/N_0(PA^+,2+3h) \approx 10^{-3} \). This decrease seemed to be in good agreement with free LGG.

Most remarkably, in the case of C3 particles (green), we found no fluorescence signals even after 2 h in the whole stomach phase, \( N_{FL}/N_0(C3,2h) \approx 0 \). As mentioned above, the absence of fluorescent LGG does not mean the death of all LGG but rather suggests the stable protection of all LGG in C3 particles throughout the entire stomach phase. Actually, LGG were released from C3 particles after the incubation with small intestine fluid for 1.5 h, which can be attributed to the deprotonation of Ch\(^+\) near pH = 6.5.47 It is notable that the number of living LGG was close to the initial level \( N_{FL}/N_0(PA^-,2+1.5h) \approx 1 \), and the \( N_{FL}/N_0 \) value significantly increased after incubation of the sample for another 1.5 h (\( N_{FL}/N_0(PA^-,4h) \approx 5 \)). These findings suggest two possible scenarios: (i) all LGG were released within the first 1.5 h of the small intestine phase and proliferate in the solution, or (ii) LGG proliferate in the microparticles and they were slowly released over time. The latter scenario is more plausible since there was no proliferation of released LGG observed for free LGG and LGG in PA\(^+\) cores. The obtained results suggest that C3 particles do not only protect LGG under the gastric conditions but also allow for the proliferation of LGG. It should be noted that the LGG viability in the lower gastrointestinal tract (ileum and colon) could not be assessed by flow cytometry due to the contamination of the simulated ileum and colon compartments by bacteria from fecal samples (Scheme 2).

To assess the metabolic function of LGG delivered to the ileum and colon, we determined the concentration of lactate produced by LGG in the (A) stomach, (B) small intestine, (C) ileum, and (D) colon phases in the simulator (Figure 6). The concentration of lactate was normalized by the initial concentration of LGG. As shown in Figure 6A, free LGG (blue) exhibited a poor lactate production capability already under stomach condition, \( 10^{-1} \) [ng/10\(^3\) × LGG]. The passage through the following phases led to a monotonic decrease in lactate production. LGG encapsulated in PA\(^+\) cores (orange)}
exhibited a distinctly improved function compared to free LGG. For example, the lactate production increased by a factor of $5 \times 10^2$ under stomach conditions. The enhancement of the metabolic activity of LGG was more pronounced when the sample goes to the later stages: $10^3$ times in the small intestine (Figure 6B), $5 \times 10^3$ times in the ileum (Figure 6C), and $10^4$ times in the colon (Figure 6D). When LGG were encapsulated in C3 particles (green), no lactate production was detected under the stomach condition. This seems understandable since no LGG were released (Figure 5A). C3 particles showed the release of viable LGG in small intestine conditions. Intriguingly, further deposition of polyelectrolyte layers did not result in any increase in the LGG viability, implying that the formulation of LGG in C3 particles boosts the metabolic function of LGG in the small intestine (Figure 6B), ileum (Figure 6C), and colon (Figure 6D) by a factor of $(2-3) \times 10^3$ times.

CONCLUSIONS

In this study, we optimized a new carrier that can deliver probiotics L. rhamnosus GG (LGG) to the ileum and colon via oral administration with no loss of viability and metabolic functionality. Since most of the probiotic bacteria cannot survive under harsh conditions in the stomach and small intestine, we encapsulated LGG into microparticles of polylginate (PA<sup>−</sup>) gels by means of emulsification. The use of a shear-thinning biopolymer (xanthan gum) and the replacement of centrifugation by filtration enabled us to fabricate highly uniform PA<sup>−</sup> particles (diameter: 45 μm) smaller than the limit of human oral sensory systems with the minimum shear stress to LGG (Figure 1A–C). As PA<sup>−</sup> gels are not stable under gastric condition (Figures 1D, S1), we deposited additional layers of chitosan (Ch<sup>+</sup>) and PA<sup>−</sup> layer-by-layer. As shown in Figure 2A, the deposition of three additional layers (Ch<sup>+</sup>/PA<sup>−</sup>/Ch<sup>+</sup>) on PA<sup>−</sup> cores, named as C3 particles, exhibited a slight decrease in the particle size (diameter: 35 μm), which can be attributed to the compaction of PA<sup>−</sup> cores by the deposition of a cationic polymer (Ch<sup>+</sup>). The formation of polyelectrolyte multilayers was quantitatively verified by fluorescence microscopy (Figure 2B), SEM (Figure 2C,D), and ζ-potential measurements (Figure 3). It is remarkable that C3, corresponding to the polyelectrolyte layer thickness of about 20 nm (Table 1), is already sufficient to maintain the viability of all LGG in fluids simulating gastric and small intestine environments. As presented in Figure 4A, compared to free LGG, the colony-forming unit of LGG in C3 particles was $6-7$ orders of magnitude larger in the gastric fluid containing pepsin and NaCl (pH = 2.0), compared to free, unprotected LGG. In small intestine fluid, the CFU of LGG in C3 particles was $10^4$ times larger than that of free LGG. Intriguingly, further deposition of polyelectrolyte layers did not result in any increase in the LGG viability, implying that the coating of C3 is the minimum-necessary formulation to protect LGG in the upper gastrointestinal tract. In addition to the protection against chemical degradations, we utilized a human gastrointestinal simulator with flow cytometry and demonstrated the release of viable LGG in small intestine conditions. In the final stage, we quantified the metabolic function of LGG by measuring the amount of lactate produced by LGG in a human gastrointestinal simulator. The lactate produced in the small intestine, ileum, and colon phases was by a factor of $(2-3) \times 10^3$ times larger compared to free LGG, respectively. Therefore, we have concluded that the minimal formulation of LGG (C3 particles) coated with ultrathin polyelectrolyte layer (thickness ≈ 20 nm) boosts not only the viability by $4-7$ orders of magnitude but also their metabolic functionality by $5$ orders of magnitude throughout oral uptake, passage through the gastrointestinal tract, and delivery to the ileum and colon.

ASSOCIATED CONTENT

Supporting Information

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Degradation of PA<sup>−</sup> microparticles; size distribution and FWHM of different layers; formation of the PA<sup>−</sup>/Ch<sup>+</sup>/PA<sup>−</sup>/Ch<sup>+</sup>/PA<sup>−</sup> multilayer films; viability of free and encapsulated LGG (PDF).

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Notes

The authors declare no competing financial interest.

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