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Dry alginate beads for fecal microbiota transplantation: From model strains to fecal samples

Adèle Rakotonirina [a,b], Tatiana Galperine [c,d], Maxime Audry [e], Marie Kroemer [e], Aurélie Baliff [c], Laurent Carrez [e], Farshid Sadeghipour [a,b,e], Jacques Schrenzel [f], Benoît Guery [c], Eric Allémann [a,b,*]

[a] School of Pharmaceutical Sciences, University of Geneva, 1211 Geneva, Switzerland
[b] Institute of Pharmaceutical Sciences of Western Switzerland, University of Geneva, 1211 Geneva, Switzerland
[c] Infectious Diseases Service, Department of Medicine, University Hospital and University of Lausanne, 1011 & 1015 Lausanne, Switzerland
[d] French Group of Faecal Microbiota Transplantation, Paris, France
[e] Service of Pharmacy, Lausanne University Hospital, 1011 Lausanne, Switzerland
[f] Genomic Research Lab, Service of Infectious Diseases, Geneva University Hospitals and University of Geneva, 1211 Geneva, Switzerland

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ABSTRACT

Clostridioides difficile infection (CDI) is a critical nosocomial infection with more than 124,000 cases per year in Europe and a mortality rate of 15–17 %. The standard of care (SoC) is antibiotic treatment. Unfortunately, the relapse rate is high (~35 %) and SoC is significantly less effective against recurrent infection (rCDI). Fecal microbiota transplantation (FMT) is a recommended treatment against rCDI from the second recurrence episode and has an efficacy of 90 %. The formulation of diluted donor stool deserves innovation because its actual administration routes deserve optimization (naso-duodenal/jejunal tubes, colonoscopy, enema or several voluminous oral capsules).

Encapsulation of model bacteria strains in gel beads were first investigated. Then, the encapsulation method was applied to diluted stools.

Robust spherical gel beads were obtained. The mean particle size was around 2 mm. A high loading of viable microorganisms was obtained for model strains and fecal samples. For plate-counting, values ranged from 10^{15} to 10^{17} CFU/g for single and mixed model strains, and 10^{6} to 10^{8} CFU/g for fecal samples. This corresponded to a viability of 30 % to 60 % as assessed by flow cytometry.

This novel formulation is promising as the technology is applicable to both model strains and bacteria contained in the gut microbiota.

1. Introduction

The intestinal microbiome is well-known for its contribution to the host's health: food digestion, immunity and neural development are a few examples (Berg et al., 2020; Biedermann and Rogler, 2015). This homeostasis can be disrupted by environmental factors such as lifestyle, diet, physical activity, infections, but primarily by drugs such as antibiotics. This disruption is called dysbiosis and can be characterized by an imbalance of the microbiota function and composition, notably a modification of its diversity but also specific changes to certain phyla and strains (Biedermann and Rogler, 2015; Hooks Katarzyna and O'Malley Maureen, 2017; Kaiko and Stappenbeck, 2014; Rakotonirina et al., 2022). Recent research highlighted the relationship between dysbiosis and the pathophysiology of several diseases, such as *Clostridioides difficile* infection (CDI), inflammatory bowel disease, irritable bowel syndrome, obesity, among others (Hooks Katarzyna and O'Malley Maureen, 2017; Kaiko and Stappenbeck, 2014; Thursby and Juge, 2017).

Abbreviations: 2xYT, Tryptone and Yeast 2x; BMI, Body mass index; CDI, Clostridioides difficile infection; CFU, Colony forming unit; CHUV, University Hospital of Lausanne; FMT, Fecal microbiota transplantation; MRS, de Man Rogosa & Sharpe; OTU, Operational taxonomic unit; PBS, Phosphate buffer saline; PI, Propidium iodide; rCDI, Recurrent Clostridioides difficile infection; SEM, Scanning electron microscopy; SoC, Standard of care; VBNC, Viable but non-culturable.

* Corresponding author at: Centre Médical Universitaire, Rue Michel-Servet 1, 1211 Genève 4, Switzerland.
E-mail address: eric.allemann@unige.ch (E. Allémann).

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CDI is one of the most important nosocomial infections, with more than 124,000 cases annually in Europe and a mortality rate of 15–17 % (Gupta and Ananthakrishnan, 2021). Symptoms are caused by exotoxins secreted by *C. difficile* and are mainly abundant diarrhea (Bristol 6–7, three or more liquid stools lost in 24 h) but could amount to hemodynamic instability, peritonitis, colonic ileus, colon distension, colonic wall thickening, pseudomembranous colitis, or even toxic megacolon (Guery et al., 2019). The standard of care (SoC) is antibiotic treatment using fidaxomicin or vancomycin (Johnson et al., 2021; van Prehn et al., 2021). Unfortunately, the relapse rate is high (~35 %), and the SoC is significantly less effective against recurrent infection (rCDI) following the second episode. As CDI is associated with a gut dysbiosis, treatments to modulate the microbiota have shown promise to treat rCDI (Cammarota et al., 2014).

Fecal microbiota transplantation (FMT) is a well-recommended treatment against rCDI due to its high efficacy of 80–95 % compared to 30 % with anti-CDI antibiotics alone (Johnson et al., 2021; Quraishi et al., 2017; van Prehn et al., 2021). The treatment consists in transferring a fecal material from a healthy donor's stool to a patient suffering from a pathology linked to an alteration of the intestinal microbiota to exert therapeutic effects (Johnson et al., 2021; van Prehn et al., 2021). The exact mode of action of FMT is still unknown but FMT could enable reconstitution of the microbiota and the suppression of *C. difficile* by commensal strains using competition mechanisms and secretion of specific protective metabolites, among others. The current ways of delivering donor stool are naso-duodenal/jejunal tubes, colonoscopy, enema or 30–40 voluminous oral capsules (size 00 with an overall closed length of 23.3 mm and an external diameter of 8.5 mm for the cap, and an internal volume of 0.91 mL). The number of capsules to be taken depends on the FMT center's protocol, but a single treatment is corresponding to a total solid volume of approximatively 36 mL (Kao et al., 2017; Youngster et al., 2016). This undoubtedly deserves innovation because these routes of administration are either invasive when requiring tube placement and colonoscopy, or the ingestion of large capsules would potentially prove to be difficult to swallow. In addition, the storage at −80 °C enabling long-term conservation is binding. Usage of freeze-dried stool emerged to address these issues and improve the modalities of administration, but more clinical data are needed to prove treatment efficacy (Reigadas et al., 2019; Staley et al., 2017; Tian et al., 2015; Youngster and Gerding, 2017; Zain et al., 2022; Hecker et al., 2016). In a previous review article, we extensively discussed FMT formulation issues and proposed that live bacteria particulate formulation techniques could be used to enhance the handling, storage, and administration of FMT treatment (Rakotonirina et al., 2022).

Here, we present the microencapsulation of live bacteria using sodium alginate. Alginate is a biocompatible polysaccharide isolated from brown algae composed of (1 → 4) linked β-D-mannuronic acid (M) and α-L-3 guluronic acid (G) residues. In the presence of multivalent cations, here Ca^{2+}, G residues' conformation is reorganized into an "egg-box" structure and increases the gel's strength. First, the method was developed for single strains, then it was applied to a complex mixture of several microorganisms, and finally, fecal samples from healthy donors were formulated.

2. Methods

Bacterial strains were kindly provided by the Infectious Diseases Service, Department of Medicine of the University Hospital of Geneva (HUG). All reagents were purchased from Sigma-Aldrich, ThermoFisher Scientific and Becton Dickinson, Switzerland.

All strains were cultured aerobically at 35 °C, 140 rpm, 95 % relative humidity in their respective medium. *Escherichia coli* was inoculated and cultured in Tryptone and Yeast 2x (2xYT) medium for 3 h, *Enterococcus faecalis* and *Lactobacillus paracasei* were inoculated and cultured in de Man, Rogosa & Sharpe (MRS) broth for 24 h.

All products and glassware were sterilized by autoclaving at 121 °C for 20 min prior use.

2.1. Encapsulation protocol for model strains

The microencapsulation protocol based on the extrusion of alginate droplets was adapted from Sheu and Marshall (1993) and Krasaekoopt and Watcharapoka (2014).

The inoculated bacteria were harvested and concentrated by centrifugation at 4000 rpm, 4 °C for 10 min. In the case of single strains, the pellet of bacteria cells was rinsed with milliQ water and resuspended in 1 mL PBS. Each strain's optical density measured at 670 nm was adjusted to 1.0 prior formulation. This OD value was chosen because of the growth rate of the strains and the high proportion of live cells observed with flow cytometry at this value. For mixed strains, each individual strain's optical density measured at 670 nm was adjusted to 1.0 and they were consequently mixed at a 1:1:1 ratio prior concentration. Then, 100 µL of concentrated bacteria suspension was mixed with 900 µL of a polymer solution containing 2.5 % sodium alginate, 10 % trehalose and 5 mM sodium pyruvate. This polymer solution was sterilized by heat-treatment at 80 °C for 15 min prior including the bacteria to avoid undesirable growth of microorganisms. The resulting bacterial suspension was then transferred in a 1 mL sterile syringe and extruded through a 27G needle in 20 mL of 0.10 M calcium chloride solution under magnetic stirring at 350 rpm. The produced particles were then left to harden in the cross-linking solution for 20 min. Then, the obtained particles were harvested by filtration (folded paper filter, grade 1289, diameter 125 mm, 84 g/m^2, Munktell Ahlstrom) and rinsed twice with milliQ water to remove calcium chloride excess. Each sample was then resuspended in 1 mL of 10 % trehalose solution, transferred to a flat-bottom sealable glass vial and snap-frozen at −78 °C using dry ice in ethanol. Then, all samples were lyophilized overnight at −85 °C and 8–13 Pa (Christ Alpha 2–4 LD Plus, Kühner AG, Switzerland). Controls consisted in a non-encapsulated bacterial cells suspension that was also put in a glass vial, snap-frozen, then lyophilized.

2.2. Donor selection

Fecal samples were obtained from healthy donors at the University Hospital of Lausanne (CHUV). This project is registered at the Ethical Committee of the Canton of Vaud under the ID 2022–01216. The CHUV FMT Centre (Infectious diseases Service) recruits all the volunteers. The screening of the donors is based on international recommendations (Cammarota et al., 2019; Keller et al., 2021; Sokol et al., 2016). The outpatient clinic of the infectious diseases department of the CHUV performs donor selection by nurses and physicians trained and dedicated to FMT. The selection procedure is standardized and similar for all FMT products manufactured by the CHUV Pharmacy. Donors are volunteers and healthy adults unrelated to the patients (universal donors). During the on-site evaluation, volunteers eligible for the first screening visit provide informed written consent that covers the selection process, stool donations, participation to a registry and a biobank, and the use of their biological samples for research.

The general profile of the suitable volunteers is:

- Adult volunteer greater than 18 years old less than 50 years old
- Volunteer in usually good health
- Asymptomatic volunteer
- Body mass index (BMI) of more than 18 kg/m^2 and less than 28 kg/m^2
- No significant medical (personal or family) or surgical history (except for past resolved traumatic injury or routine surgery e.g., wisdom teeth extraction, appendectomy…)
- No medication(s) with a risk of altering the intestinal microbiota (antibiotics…)
- No risky travel within 12 months
- No infectious risk factors

- No risk factors to alter the fecal microbiota
- With regular intestinal transit (no constipation or diarrhea)
- With no abnormality in the biological screening
- With no abnormality in physical examination
- Vaccinated against SARS-CoV-2 according to current recommendations
- Omnivore, pescatarian or flexitarian (no vegetarian or vegan diet)
- Bristol scale (2–5)

We selected 4 different donors, 2 of whom donated several times. Each sample was labeled as follows "Donor ID – Donation Number". The characteristics of each donor are presented in Table 1.

2.3. Encapsulation protocol for fecal samples

Purified stool samples were prepared following an in-house protocol consisting in diluting and homogenizing 100 g of stool in 600 mL of 0.9 % NaCl. The obtained slurry was filtered thrice through gauze compresses to remove the undigested food residues. The filtered suspension was then centrifuged at 4000 rpm, 4 °C for 20 min. The supernatant was discarded, and the pellet was resuspended in 4 mL of 10 % trehalose solution. Two pellets were used for each donation. The encapsulation process was done within 8 h after the donation.

The obtained suspension was further diluted 4-fold in a 10 % trehalose solution and 20 mL of the resulting suspension were mixed with 1.5 % alginate and 5 mM sodium pyruvate for microencapsulation. The stool and polymer suspension were transferred in several sterile syringes of 5 mL and extruded through a 21G needle in 150 mL of 0.10 M calcium chloride solution under magnetic stirring at 350 rpm. The produced particles were then left to harden in the cross-linking solution for 20 min. Next, the obtained particles were harvested by filtration and rinsed twice with milliQ water to remove calcium chloride excess. The particles were divided into several flat-bottom sealable glass vials, each one containing 2 mL of 10 % trehalose solution, and snap-frozen. Then, all samples were lyophilized overnight at −85 °C and 8–13 Pa (Christ Alpha 2–4 LD Plus, Kühner AG, Switzerland). Controls consisted in non-encapsulated purified stools that were put in a glass vial, snap-frozen, then lyophilized.

2.4. Particles characterization

The size and morphology of the particles were assessed on a binocular optical microscope (Wild M3Z, Wild + Leitz SA, Switzerland). For each batch, at least 10 particles were sampled and observed. Micrographs were then taken with an Infinity CCD camera (Infinity 2-1C, Gloor Instruments, Switzerland) and processed on ImageJ (version 1.52a, National Institute of Health, USA).

Scanning electron microscopy (SEM) (Jeol JSM7001F, Jeol Europe BV) was used to assess alginate particles' morphological aspects and inner structure. Samples were prepared by placing lyophilized particles on an adhesive carbon disc (Science Services GmbH, Germany). Particles were then desiccated overnight and sputter-coated with gold prior to observation (Leica EM SCD 500, Leica, Austria). SEM micrographs were taken at different magnifications with an accelerating voltage of 5 kV.

2.5. Viability assessment

Particles were dissolved in 1 mL of 3 % sodium citrate on a rotator (Heidolph Reax 2, Heidolph Instruments GmbH & Co. KG, Germany) in triplicates. About 200 mg of fresh particles and 2 mg of lyophilized particles were used for model strains. About 80 mg of fresh particles and 10 mg of lyophilized particles were used for fecal samples. Samples were then serially diluted to obtain an accountable number of colonies and plated either onto 2xYT or MRS agar for model strains and Columbia agar supplemented with 5 % horse blood for fecal samples. Model strains were cultured in aerobic conditions at 37 °C during 24 h for *E. coli,* and 48 h for *E. faecalis* and *L. paracasei*. Fecal samples were separately cultured in aerobic and anaerobic (5 % H_2 (5.0), 20 % CO_2 (4.5), 75 % N_2 (5.0)) conditions at 37 °C for 48 h.

In parallel, the viability of bacteria was also evaluated by flow cytometry (BD LSRFortessa™ 2, Becton Dickinson, USA; Gallios, Beckman Coulter, USA for model strains; and Attune™ NxT, Thermo Fischer Scientific, Switzerland for fecal samples) using the LIVE/DEAD™ BacLight™ Bacterial Viability Kit (Invitrogen, Thermo Fischer Scientific, Switzerland) for model strains and Draq7™ (Invitrogen, Thermo Fischer Scientific, Switzerland) instead of PI for fecal samples. Briefly, the content of dissolved particles was diluted as indicated by the kit manufacturer, stained with 1.5 µL of an equal-parts mixture of SYTO™ 9 and PI or Draq7™, incubated for 15 min in the dark at room temperature, and finally analyzed on the flow cytometer. Counting tubes were also used for fecal samples (BD TruCount™ Absolute Counting Tubes, Becton Dickinson, Switzerland). Data were processed and analyzed on FlowJo (version 10.7.1, Becton Dickinson & Company) or Kaluza Analysis (version 2.1, Beckman Coulter, USA).

2.6. Statistical analyses

Data were plotted and statistically analyzed on GraphPad Prism (version 9.5.0.730, GraphPad Software LLC). Results are presented as mean ± SD. Data normality was assessed by confronting several tests: Anderson-Darling, D'Agostino-Pearson, Shapiro-Wilk and Kolmogorov-Smirnov normality tests. For normally distributed data, a two-way ANOVA with post-hoc Tukey's multiple comparisons test was applied. For non-normally distributed data, a non-parametric Mann-Whitney test with False Discovery Rate multiple comparisons was used.

3. Results and discussion

3.1. Formulation optimization

Alginate beads are formed upon cross-linking of guluronic acid chains with Ca^{2+} ions. The morphology and integrity of those particles depend on several parameters, such as the polymer concentration, the cross-linking solution concentration, the needle diameter, the distance between the needle and the solution, the flow rate of injection, and the stirring rate of the solution (Chan et al., 2009; Sheu and Marshall, 1993). The particles need to be sturdy enough to entrap the bacteria while enabling the release of live bacterial cells in the intestine, which is related to the concentration of alginate and Ca^{2+} (Petraitytė and Šipailienė, 2019). A higher concentration of both alginate and Ca^{2+} will

Table 1
Selected donors' profile.

	Donation date	Age	Sex	Origins	Diet	Bristol score	BMI
Donor 1-1	21.10.2021	41	Male	Western Europe	Flexitarian	type 4	20.7
Donor 1-2	04.11.2021					type 4	
Donor 2	25.11.2021	24	Female	Western Europe	Pescatarian	type 4	18.1
Donor 3	20.01.2022	24	Female	Western Europe	Omnivore	type 5	23.4
Donor 4-1	22.02.2022	23	Female	Western Europe	Omnivore	type 4	20.1
Donor 4-2	03.03.2022					type 3	

produce sturdier beads, but as the viscosity of alginate increases rapidly as a function of its concentration, care should be taken so that the mixture is extruded through the syringe. Indeed, to obtain smaller and spherical particles, a thinner needle and a higher dropping distance are needed (Chan et al., 2009).

As shown in Fig. 1, the appearance and integrity of alginate beads depends on the previously mentioned parameters. After several steps of optimization, we indeed found that increasing the concentration of alginate from 1.0 % to 4.0 % and calcium chloride from 0.05 M to 0.10 M produced sturdier beads. It is however important to keep calcium chloride to an isoosmotic concentration to preserve the microorganisms' viability, so its concentration should not be indefinitely increased. Likewise, we confirmed that the injectability of alginate decreased as its concentration increased, because we were unable to extrude 4.0 % alginate. Consequently, the most favorable balance between calcium chloride and alginate concentration must be determined first to obtain optimal particles. We also observed that using a thinner needle produced smaller particles when we compared a 27G needle to 30G. However, a thinner needle also affects the injectability of alginate so one should take care in constantly adjusting their experimental setup to different samples. This principle was applied throughout this study because of the high diversity of samples. The experimental setup was carefully fine-tuned to each sample type, from single model strains to their mixture, and finally purified stools. In fact, purified stools were more viscous than cultured bacteria, so less concentrated alginate and a larger needle were needed to transpose the process developed for model strains.

Sterilization of the polymer mixture should also be taken into consideration to avoid undesirable sample contamination. The integrity of alginate is known to be sensitive to heat. Indeed, sterilization by autoclaving generates a depolymerization of sodium alginate (Daigle and Cotty, 1997). This results in the decrease of both the viscosity of alginate and the strength of calcium alginate particles. Alternative sterilization processes, such as ɣ-irradiation and ethylene oxide, were also previously tested and resulted in the same phenomenon (Lee et al., 2003; Leo et al., 1990). The degree of depolymerization proved to be dependent on the heat temperature or irradiation energy, and the sterilization time. As shown in Fig. 1, we confirmed that autoclaved alginate cannot produce spherical and sturdy particles. Taking these observations into consideration, heat treatment was then tested in this project as less heat and a shorter exposure time are needed (Pitino et al., 2020). This enabled the production of well-shaped alginate beads. The morphology of the produced alginate beads during the optimization process and the experimental setup with the selected parameter values are shown in Fig. 1.

The following section presents the morphology and size characterization of the bacteria-loaded alginate beads.

3.2. Particles characterization

Freshly produced particles were spherical and slightly decreased in size after lyophilization (Fig. 2). Lyophilized particles containing a mixture of model strains and fecal samples had a mean size of 2.1 ± 0.2

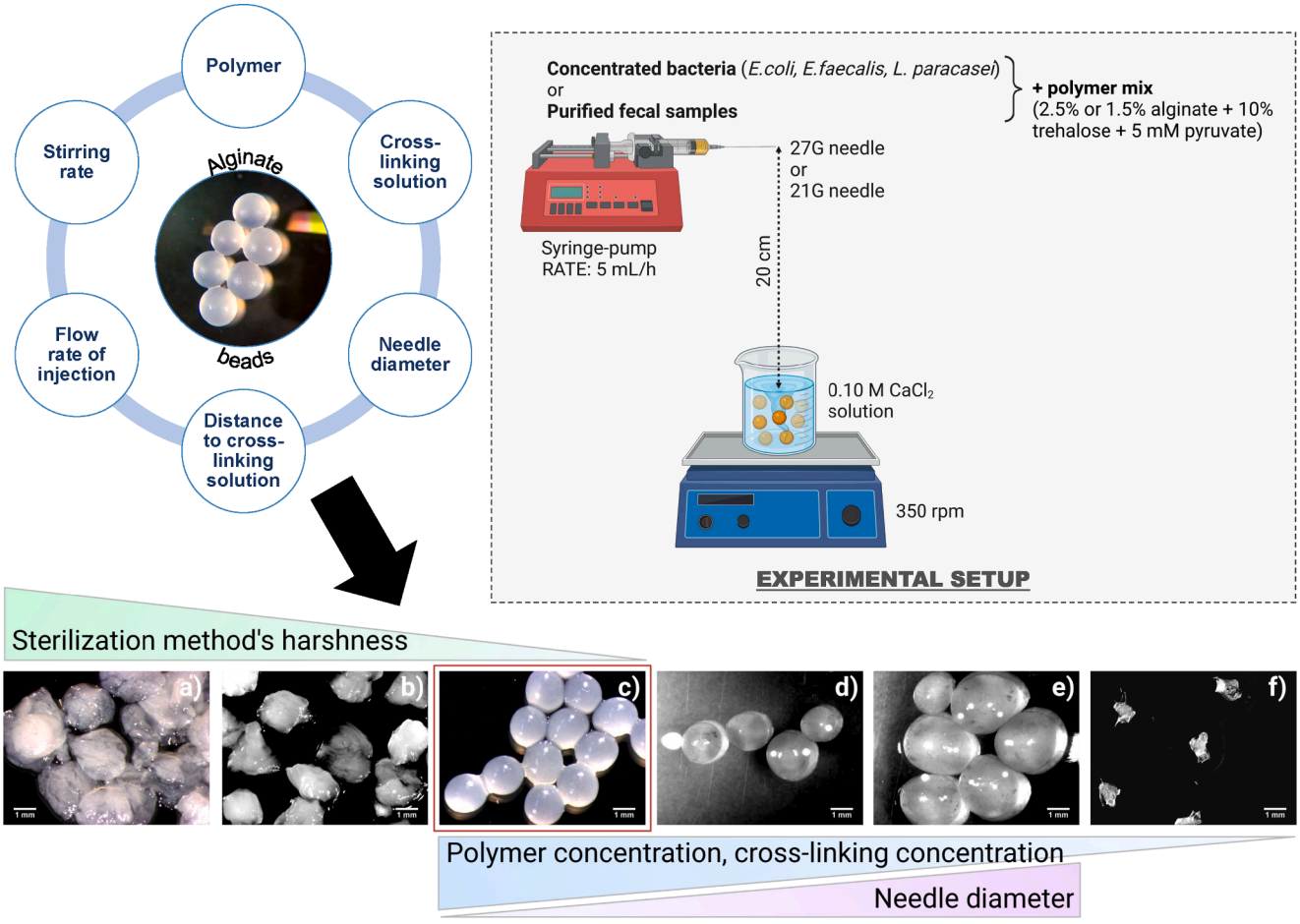

Fig. 1. Experimental setup used to produce alginate beads (gray box) and its optimization process. The optimization process was done on unloaded particles. Scale bar = 1 mm. a and b) particles made of autoclaved alginate, c) particles made using the current experimental setup and heat-treated alginate at 80 °C during 15 min, d) particles made of 3.0 % alginate and extruded through a 30G needle, e) particles made of 3.0 % alginate and extruded through a 27G needle, f) particles made of 1.0 % alginate. The concentration and sterilization method of alginate were significant parameters to consider to produce sturdy particles.

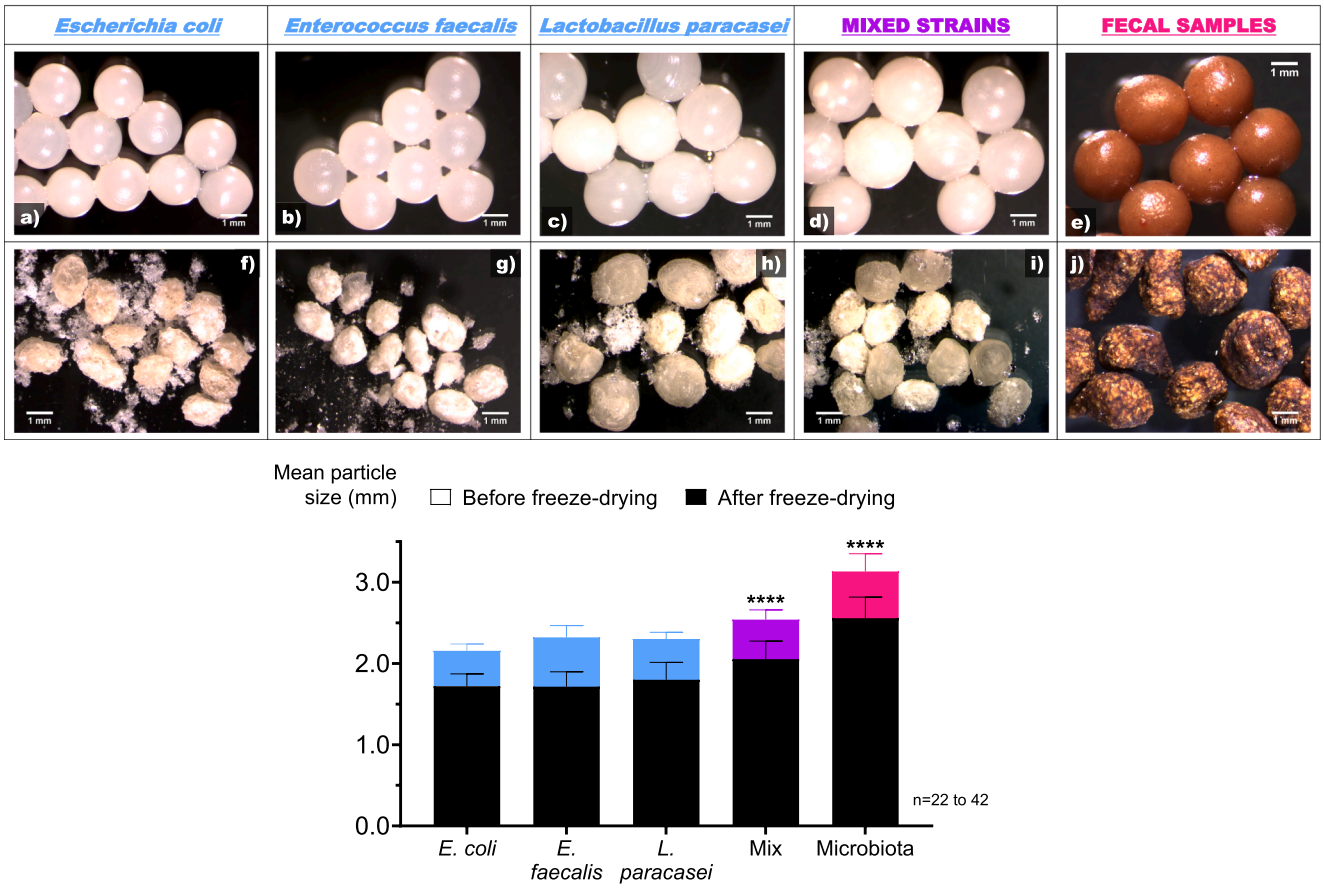

Fig. 2. Particles characterization and size. Scale bar = 1 mm. Freshly produced particles are represented on the first line (a to e), and their appearance after lyophilization is presented on the second line (f to j).

mm (n = 36) and 2.6 ± 0.3 mm (n = 30), respectively. For single model strains, a mean size of 1.72 ± 0.15 mm (n = 22), 1.71 ± 0.18 mm (n = 38), 1.80 ± 0.22 mm (n = 42) was obtained for *E. coli*, *E. faecalis*, and *L. paracasei*, respectively. As mentioned previously, the viscosity of the bacterial suspension increased with the sample complexity. As such, the larger size of the particle when using mixed strains compared to single strains was due to using a 27G instead of 30G, and using a 21G needle instead of 27G when using fecal samples compared to mixed strains, respectively. This confirmed that using a larger needle led to the increase of the size of the obtained beads (Chan et al., 2009). The corresponding micrographs are presented in Fig. 2.

According to Fig. 2, the morphology and size of alginate beads was reproducible from one batch to another, and the size was mostly influenced by the size of the needle.

SEM micrographs of lyophilized alginate particles showed the importance of using flash-freezing to maintain their internal structure compared to regular freezing at −80 °C (see Fig. 3). Indeed, regularly frozen particles presented a collapsed surface and a hollow core, whereas flash-frozen particles were less porous. As presented in Fig. 3j, pores containing bacteria are consistently present throughout the particles' core. These pores were formed due to the water surrounding the bacteria that was sublimated during the lyophilization process (Hansen et al., 2002). Additionally, these pores were not observed for regularly frozen particles because of the collapsed internal structure as shown in Fig. 3e. Preliminary experiments also showed that the two freezing methods maintained the viability of bacteria after lyophilization, and that there was no significant difference between both methods (data not shown). Similar observations were also made in previous studies by Hansen et al. (2002) and Qi et al. (2020). The same phenomenon was observed for fecal samples. However, particles formulated with fecal sample were more compact than particles with model strains, and pores were less present. This was due to the more complex fecal matrix compared to pure bacteria cultures.

3.3. Viability assessment of model strains

As previously mentioned, the concentration of Ca^{2+} influences the strength of the gelification process of alginate particles. To dissolve alginate particles and release their bacteria, these cations should be displaced. To do so, Na^{+} is used and as Ca^{2+} ions are replaced, the "egg-box" model loosens, and the particles dissolve (Guo and Kaletunç, 2016). Phosphate buffer is often used, but as the dissolution rate proved to be slow, trisodium citrate was used instead, as suggested by Murujew et al. (2021). Indeed, they investigated several sodium salts to determine the most suitable dissolution medium. They compared sodium chloride, sodium carbonate and sodium citrate and determined that sodium citrate was the best candidate. This could be due to the higher amount of Na^{+} ions available in sodium citrate to displace Ca^{2+} ions.

The viability of the encapsulated model strains after their dissolution by sodium citrate is presented in Fig. 4. A very high loading of viable microorganisms was obtained for single and mixed model strains. For plate-counting, particle loading ranged from 10^{14} to 10^{20} CFU/g of product and viability after lyophilization ranged from 10^{14} to 10^{17} CFU/g of product. This corresponded to a viability of 16 % to 82 % as assessed by flow cytometry. On Fig. 4, the original suspensions' bacterial count is quasi-similar to the encapsulated mixed strains count. The decrease in viability was observed after the freeze-drying process with a 2-log reduction for alginate particles and 5-log reduction for controls. This shows that the decrease of viability was more due to the freeze-drying process than the encapsulation method.

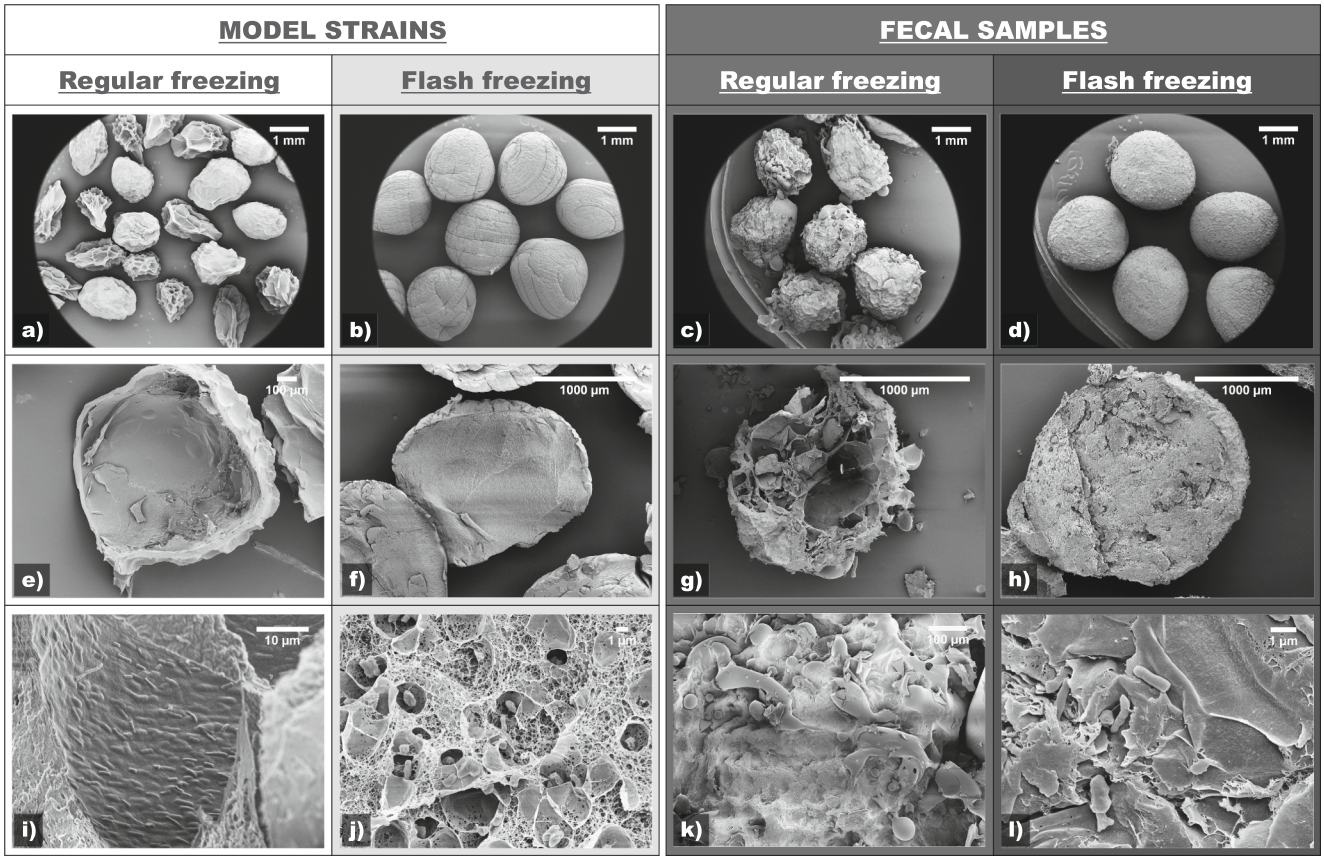

Fig. 3. SEM micrographs of alginate particles containing a mixture of *E. coli*, *E. faecalis* and *L. paracasei* (model strains), or a fecal transplant (fecal samples). Regular freezing corresponds to freezing at −80 °C for several hours, while flash freezing corresponds to snap-freezing with dry ice in ethanol at −78 °C. Several magnifications were considered to display the morphology of alginate beads: numerous particles at a small magnification (first line, a to d), single particle section at a medium magnification (second line, e to h), and details of bacterial distribution at a high magnification (i to l). The particles frozen at −80 °C were more collapsed and hollower than snap-frozen particles.

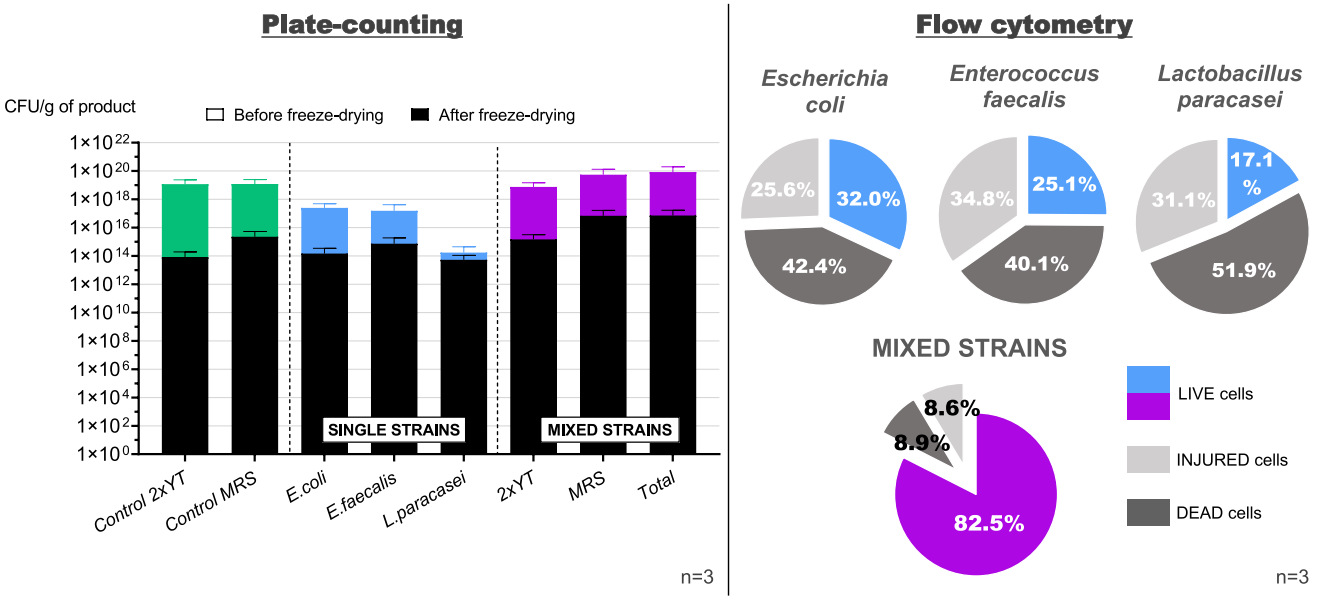

Fig. 4. Viability assessment of encapsulated single and mixed model strains: fresh and lyophilized particles for plate-counting and lyophilized particles for flow cytometry. Controls correspond to free bacteria cells: *E. coli* for 2xYT, and *E. faecalis* and *L. paracasei* for MRS. A high loading up to 10^{20} CFU/g of dry particles was obtained and viability was maintained after lyophilization.

Lyophilization is a process where water is frozen and eliminated through sublimation under vacuum. Depending on the freezing process and the use of cryoprotectants, cells can face extreme conditions and their viability can be affected. The use of trehalose and pyruvate in the formulation provides protection against the osmotic shock during freezing and oxidative conditions (Bircher et al., 2018). Bacterial viability varies from one strain to another due to their intrinsic resistance to freezing and lyophilization processes. In our study, the percentage of live *L. paracasei* cells is 17.1 % compared to 32.0 % and 25.1 % for *E. coli* and *E. faecalis*, respectively. The difference of bacterial viability is often associated with their physiological characteristics such as cell membrane, optimal pH, presence of biofilm or growth state (Xu et al., 2022). Indeed, *E. coli* is a Gram-negative bacteria, and possesses an additional protective outer membrane layer compared to gram-positive *E. faecalis* and *L. paracasei*. It has also been reported that *L. paracasei* is very sensitive to cold stress, which could explain the loss of viability after lyophilization (Wang et al., 2005). Additionally, Bellali et al. (2020) evaluated the viability of *E. coli* and *Akkermansia muciniphila* isolated from stools and observed a difference in viability between these strains after lyophilization and during storage, which is in agreement with our findings. In our study, the mixture of *E. coli, E. faecalis* and *L. paracasei* showed a promising viability with nearly 82 % of live cells in lyophilized particles. Bacteria are known to communicate among themselves via quorum sensing and shift their dynamics to ensure their survival. Overall, the viability results of model strains particles showed that the encapsulation method does not massively affect the bacterial viability and that the use of protectants can maintain this viability after lyophilization. Additionally, a mixture of microorganisms appeared to be beneficial for maintaining a higher viability compared to single strains, which was encouraging for the encapsulation of purified stool.

3.4. Viability assessment of fecal samples

The active component of FMT is still unknown. Some argue that live bacteria are needed to recolonize the gut, but some studies suggested that metabolites or purified *Firmicutes* spores are sufficient to treat rCDI (Brunse et al., 2021; Feuerstadt et al., 2022; Ott et al., 2017). In this study, encapsulation of the whole fecal filtrate was considered so that the content of the novel formulation would be similar to the currently used FMT oral capsules. All actual processes in hospital pharmacy are done aerobically. Oral FMT capsules produced in this way exhibit a very good clinical efficacy at treating rCDI and some studies have shown that anaerobic bacteria are not necessarily essential to the treatment's efficacy (Papanicolas et al., 2019; Hirotaka et al., 2018; Mendolia, 2020). In addition, very few papers evaluated the viability and the count of bacteria in the microbiota, so this study aims to bridge that gap (Ben-Amor et al., 2005; Chu et al., 2017; Papanicolas et al., 2019; Vandeputte et al., 2017). The viability of encapsulated bacteria contained in purified stool is presented in Fig. 5. Plate-counting showed a viability from 10^6 to 10^8 CFU/g. Similar results were obtained by Rougé et al. (2010). However, the microbiota is estimated to 10^{14} bacteria cells (Simon and Gorbach, 1984). In fact, most of the gut microbes are non-culturable. Ito et al. (2019) evaluated the ability of 26 rich non-selective and selective culture media to grow strains from a fecal sample. The combination of these media enabled to culture 61 % of the operational taxonomic units (OTUs) in the original fecal sample. These results indicate that culturing bacteria is not the optimal way to determine the viability of gut microbes. It is important to take viable but non-culturable (VBNC) bacteria into account. Several culture-independent methods were evaluated in the literature, such as viability quantitative polymerase chain reaction v-qPCR, DNase I protection assay, or flow cytometry (Fleischmann et al., 2021; Hammes et al., 2011; Li et al., 2014; Roussel et al., 2018). Flow cytometry was chosen in the present study.

Flow cytometry is a fast and robust method to evaluate the viability of bacterial cells. Indeed, the ability to use various fluorescent probes enables to investigate the cell viability in a complementary way to plate-counting (Van Nevel et al., 2017). The LIVE/DEAD™ viability kit from Invitrogen is often used to evaluate bacterial cells' viability by evaluating the cell membrane integrity and was used in this study (Berney et al., 2007). The obtained count of viable cells by flow cytometry was 10^{14} cells/g of product, which is far superior to the plate-counting results. This could account for the VBNC cells and is close to the estimated count of gut microbes.

These results showed the potential of the novel formulation to encapsulate and maintain the gut microbes' viability. In addition, viability was found to be donor and donation dependent. As mentioned in the previous section, the optimization of the extrusion method was crucial when we encapsulated purified stool compared to model strains. The optimal experimental setup with the right alginate concentration, needle size and release method was developed through Donors 1 and 2. Indeed, as shown in the flow cytometry count on Fig. 5, the higher loading was obtained from Donor 3 after several optimization steps.

As previously mentioned, the active portion of FMT is unknown. Live bacteria could have an effect, but bile acids and metabolites should also be considered. As such, further studies linking the bacterial viability to FMT treatment efficacy are needed and the use of compact novel formulations could enable such endeavors. Indeed, the total volume of FMT treatment using 30 size 00 capsules is 27.3 mL, corresponding to 23.0 g. On the other hand, using our formulation would require far less volume. The matching volume and weight of particles needed for an equivalent treatment is 8.7 mL, corresponding to 1.9 g. To determine these values, we analyzed how many bacterial cells were present in one FMT capsule using flow cytometry and extrapolated how many bacteria were contained in one oral FMT capsules treatment (see Table 2). No research to date has discussed this value, which reinforces the relevance and importance of our study. Compared to previous works involving similar types of microbeads, Krasaekoopt and Watcharapoka (2014) showed an initial cell count of 8.3–10.8 log CFU/mL before encapsulation and this cell count was reduced by 0.1–2.6 logs after encapsulation. Similar observations were done by Hansen et al. (2002), with an initial bacterial count of 8 logs CFU/mL and 1 log reduction after encapsulation. These authors did not freeze-dry their particles after encapsulation. Additionally, none of these studies has evaluated the viability of a whole microbiota. As such, our encapsulation method is performing better, as we have no reduction of CFU after encapsulation, the number of bacteria loaded in our particles is significantly higher, we propose a dry formulation and we encapsulated a whole microbiota.

In all, alginate beads allowed to keep a very high amount of live bacteria in a dry and compact formulation. By encapsulating the fecal microbiota contained in purified stool, we were able to reduce the volume needed to perform oral FMT treatment. In addition, dry alginate particles can be coated using gastro-resistant polymers such as cellulose acetate phthalate or polymethacrylate-based copolymers to further protect the encapsulated fecal microbiota and enable its precise modified-release in any part of the gastro-intestinal tract (Ramos et al., 2018; Salawi, 2022). Finally, having small particles of purified stool could enhance storage conditions and could ease treatment administration for the patients by mixing 1.9 g of particles to their food or drink. Further studies are needed to assess the efficacy of alginate beads and to evaluate the link between bacterial viability and FMT treatment's outcome.

4. Conclusion

Alginate particles containing a high number of viable microorganisms were obtained. Reproducible and robust batches were obtained in terms of particles' appearance and size. The viability of encapsulated bacteria was successfully maintained after lyophilization by using cryoprotectants. This novel formulation shows promise as the technology was applicable to single model strains, mixed model strains, and fecal material contained in purified stool. The use of plate-counting and flow cytometry to evaluate the viability of gut microbes proved to be a

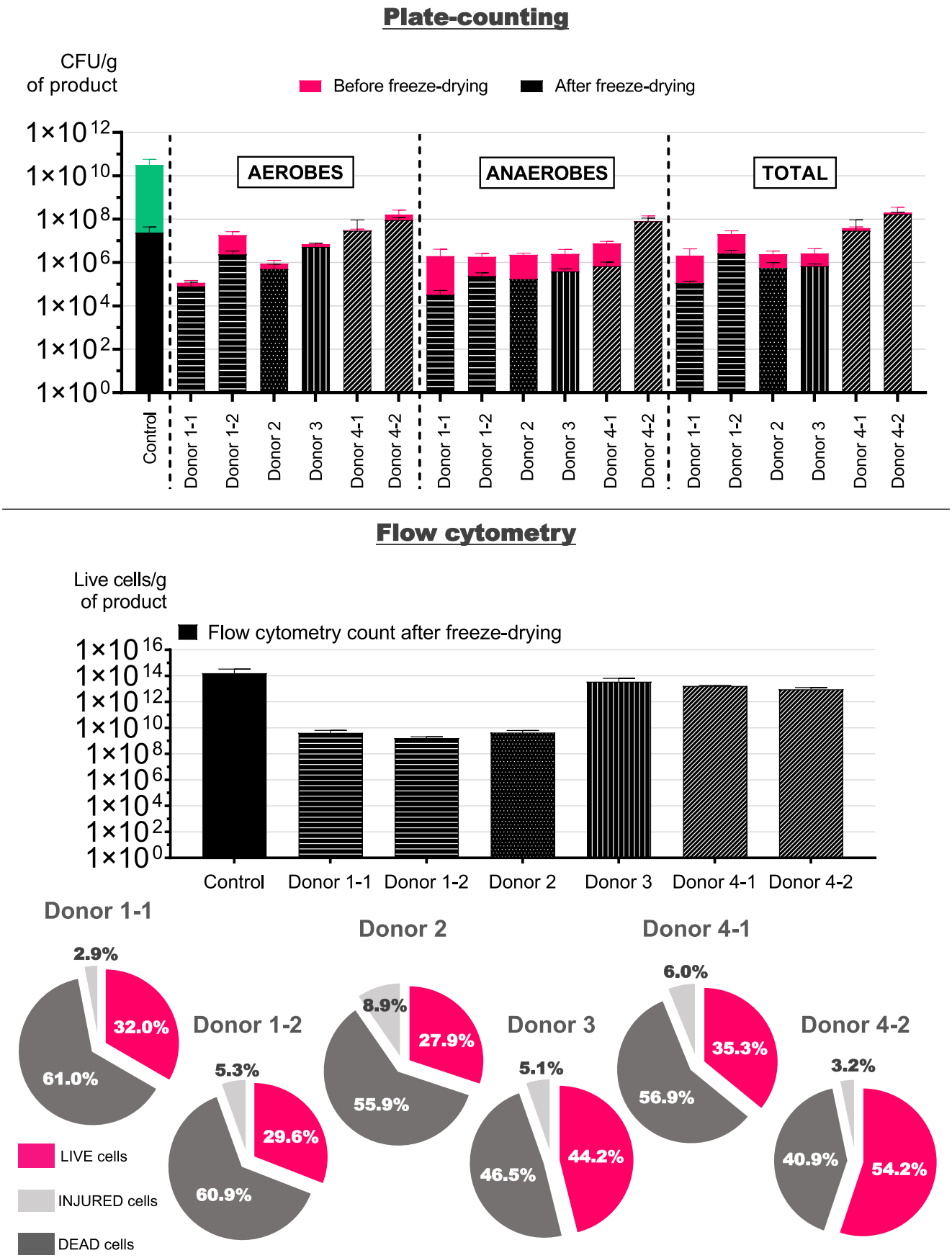

Fig. 5. Viability assessment of encapsulated purified stool: fresh and lyophilized particles for plate-counting and lyophilized particles for flow cytometry (n=3).

Table 2
Quantity equivalence between current oral FMT capsule treatment and alginate beads determined by flow cytometry. Results are presented as mean ± SD.

Sample	Bacteria cells/capsule	Bacteria cells/treatment	Bacteria cells/g	Volume per unit (mL)	Weight per unit (g)	Treatment volume (mL)	Treatment weight (g)
FMT capsules	$3.3 \times 10^{13} \pm 2.7 \times 10^{13}$(n = 3)	$9.9 \times 10^{14} \pm 8.2 \times 10^{14}$(n = 3)		0.910	0.768	27.3	23.0
Alginate beads			$5.2 \times 10^{14} \pm 5.5 \times 10^{14}$(n = 5)	0.008	0.002	8.7	1.9

good selection of complementary methods. We managed to determine the amount of bacterial cells contained in one oral FMT capsules treatment and found that using our formulation would need far less volume. The characteristics of an efficient donation are yet to be determined and further studies are needed to link the bacterial viability to treatment efficacy. The use of novel compact FMT formulations could help bridge that gap, as our study suggests.

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CRediT authorship contribution statement

Adèle Rakotonirina: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Visualization. **Tatiana Galperine:** Conceptualization, Resources, Writing – review & editing, Validation. **Maxime Audry:** Resources. **Marie Kroemer:** Resources, Writing – review & editing. **Aurélie Baliff:** Resources. **Laurent Carrez:** Writing – review & editing. **Farshid Sadeghipour:** Writing – review & editing. **Jacques Schrenzel:** Resources, Writing – review & editing. **Benoît Guery:** Writing – review & editing, Validation. **Eric Allémann:** Conceptualization, Supervision, Validation, Resources, Funding acquisition.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: co-author is a member of the editorial board of IJP - Eric Allémann.

Data availability

Data will be made available on request.

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