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► **To cite this version:**

Abhik Mallick, Fabienne Quilès, Gregory Francius, Jennifer Burgain, Claire Gauzelin-Gaiani, et al.. An easy and robust method of preparation of capsules for delivering probiotic bacteria by a 3D bioprinting. *Food Hydrocolloids for Health*, 2022, 2, pp.100088. 10.1016/j.fhfh.2022.100088 . hal-03795936

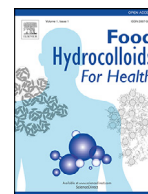
HAL Id: hal-03795936

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Submitted on 5 Oct 2022

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An easy and robust method of preparation of capsules for delivering probiotic bacteria by a 3D bioprinting

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ARTICLE INFO

Keywords:

Probiotics
3D bioprinting
Survival in the gastrointestinal fluids
Industrial applications

ABSTRACT

Probiotic bacteria such as *Lactobacillus rhamnosus* GG (LGG) play an important role in maintaining a healthy microbiota environment. Oral administration is the only way to deliver probiotic bacteria to the small intestine. Due to the harsh environment of the gastrointestinal tract, only a small proportion of probiotic bacteria can reach the target site, limiting their beneficial effects. Although many encapsulation techniques exist, the loss of bacterial cell viability, high cost, and low payload remain a major problem. In this work, we present a state-of-the-art microextrusion technique for encapsulating probiotic bacteria using a 3D bio-printer. Using a composite of sodium alginate, gelatin gel and LGG as bioink, we printed encapsulated LGG bacteria that were stable for 7 days. The production of the encapsulated bacteria was a very fast and simple process. We printed 120 probiotic capsules, each containing 10^8 CFU/ml, within 30 min. On microscopic examination of the LGG capsules, the encapsulated LGG bacteria were evenly distributed in the alginate-gelatin hydrogel. The bacteria in the capsules were viable and alive for 7 days at 4 °C and survived the harsh gastrointestinal fluid environment *in vitro*. This bioprinting method for encapsulating probiotic bacteria for their specific delivery in the gut could become a revolutionary method for industrial applications.

Introduction

Since Döderlein warned about the microbiota in the regulation of human health one century ago, many studies have confirmed that not only diseases such as obesity and diabetes, but also autoimmune pathologies, inflammatory disorders and allergies could result from microbiota alteration (Belkaid & Hand, 2014; Döderlein, 2012; Yap & Mariño, 2018). Thus, for a healthy and functional microbiota environment, the intake of probiotic bacteria is essential, and the metabolic activity of the probiotic bacteria is responsible for the improvement of the natural gastrointestinal microbiota (Yan et al., 2007). According to the World Health Organization/ Food and Agricultural Organization (WHO/FAO), probiotic bacteria are defined as living microorganisms, which provides health benefit to its host, when taken in an adequate amount (Food & Agricultural Organization, of the United Nations & World Health Organization, 2002). They also exert a variety of beneficial effects such as (a) positive changes in the microbiota composition, (b) competitive ex-

clusion of pathogenic microorganism by either blocking their binding sites or secreting antimicrobial substances like lactic acid, (c) regulation of the epithelial barrier function, (d) modulation of immune responses, and (e) interactions with the gut-brain barrier (Babenco, Bubnov, Lazarenko & Spivak, 2018; Farzi, Fröhlich & Holzer, 2018; Yap & Mariño, 2018). *Lactobacillus rhamnosus* GG (LGG) is one of the best clinically documented and most commercialized probiotic microorganism, which confers health benefits ranging from gastro-intestinal health to immune modulatory effects (Belkaid & Hand, 2014; Gismondo, Drago & Lombardi, 1999; Gorbach, 2000).

According to the International Scientific Association of probiotics and prebiotics, the minimum number of probiotics administered to achieve the maximal health benefit is 10^7 – 10^8 colony-forming units (CFU) (Hill, Guarner & Reid, 2014).

For the specific targeted delivery of probiotics to the small intestine, the available delivery mode is the oral administration (Gismondo et al., 1999; Gorbach, 2000). Unfortunately, a large proportion of bacteria cannot survive the harsh environment of the upper gastrointestinal tract due

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to the combination of acidic medium in the stomach, and the presence of bile salts and proteolytic enzymes in the small intestine (Marteau & Shanahan, 2003; Ohashi & Ushida, 2009). Thus, the gastro-intestinal tract environment that prevents LGG to reach the intestine surface and act as a recovery agent limits the efficiency of the probiotic activity (Marteau & Shanahan, 2003).

In this context, many methodologies in food industries have been developed to encapsulate bacteria in order to maintain the probiotic activity from oral administration until reaching their site of action (Broeckx, Vandenheuvel, Claes, Lebeer & Kiekens, 2016; Burgain, Gaiani, Linder & Scher, 2011; Huang, Vignolles & Chen, 2017; Krasaekoopt, Bhandari & Deeth, 2003; Schuck, Dolivet, Mejean, Hervé & Jeantet, 2013). Given that they should stay alive and functional, one of the major issues concerning the formulation of probiotics is their loss in viability after processing or during storage (Gomand et al., 2019). Many factors, such as temperature, oxygen level, moisture level, pressure and formulation have an influence on the viability of probiotics and thus their health-promoting activity (Kim, Muhammad, Jhun & Yoo, 2016). Many technologies have been developed to encapsulate bacterial cells with well-designed strategies to protect the bacteria during processing steps (Haffner et al., 2016). Freeze-drying is the most commonly used method to preserve bacteria. However, the high-energy costs of the technique are a major drawback and the need for cheaper and efficient technologies is undeniable. Moreover, cellular damages to bacterial cells can also be caused (M A, U FP & K, 2015). Other technologies have been developed at lab scale but difficulties are encountered for their scaling up. The alternative technologies are emulsification, spray chilling, electro spraying, vibrating nozzle, fluidized-bed and spray-drying (Haffner, van de Wiele & Pasc, 2017). These encapsulation methodologies succeeded in protecting bacteria by designing matrices formulated for specific bacterial strain. However, the bacterial loading efficiency and the bacterial location control still remains a challenging task (Guerin, Petit & Burgain, 2017). Moreover, as far as the food industries are concerned, the most promising encapsulation methodologies do not meet the ease in implementation required by industries (Burgain et al., 2011; Krasaekoopt et al., 2003).

Thus, to tackle the inconsistency and inefficiency of the known encapsulation technologies for the specific targeting of probiotic bacteria to the small intestine via oral administration, we have used a meticulous encapsulation methodology using a 3D bioprinting technology. Bioprinting is defined as the simultaneous deposition of biomaterials, living cells and growth factors in layer-by-layer pattern to fabricate living tissues or organs (Murphy & Atala, 2014). The main advantage of bioprinting is to print complex geometric porous structures in a controlled manner. Usually, different types of hydrogels and cells are used for printing. There are mainly three different methods of bioprinting: extrusion based bioprinting, inkjet based bioprinting and laser assisted bioprinting (Mota, Camarero-Espinosa, Baker, Wieringa & Moroni, 2020). Micro-extrusion based bioprinting is a highly developed and widely used bioprinting technique due to the low expenditure, possibility of printing homogenous and heterogeneous structures with uniform cellular distribution, and precise integrity in the 3D printed layers with high cell viability (Mota et al., 2020). Many hydrogels are used as encapsulating matrices also called bio-ink for extrusion technique of bioprinting. Sodium alginate and gelatin are the most widely used bio-ink due to their biocompatibility, excellent viscoelasticity, ability to adhere cells and help it to survive, ease of gelation and the fast-crosslinking rate (Bociaga, Bartniak, Grabarczyk & Przybyszewska, 2019). Thus, for 3D bioprinting, there are three basic parameters, which has to be maintained properly: (a) concentration of the components in the bio-ink, (b) printing temperature and pressure and (c) holding time (Bociaga et al., 2019).

Bioprinting with mammalian cells and hydrogel is a widely used approach that is applied in various research areas such as tissue engineering and regenerative medicine, transplantation and clinics, cancer research and drug screening, and high-throughput testing, thus having a

revolutionary impact on the medical field (Sneddon, 1965). However, there are very few reports on 3D bioprinting of bacterial cells. Evita et al. reported the formation of a bacterial biofilm using a 3D bioprinter for antimicrobial resistance testing, and Manuel et al. have developed 3D printing methods that allow the additive fabrication of complex living 3D architectures from bacteria-laden hydrogels with complete localization and concentration control of bacteria (Gavara & Chadwick, 2012; Yunda & Quilès, 2019). Recently, Anna et al. developed a microbial bioink produced by genetically engineered microbial cells programmed to perform hierarchical bottom-up self-assembly of protein monomers into nanofibers and thus nanofiber networks comprising extrudable hydrogels (ESPACE, 2021). While there have been some reports of 3D bioprinting of bacterial cells, the preservation of the health-promoting aspects of bacteria through encapsulation techniques using 3D bioprinting has never been investigated.

In this work, a simple and easy technique of encapsulating probiotic bacteria for oral administration via micro-extrusion technique by 3D bioprinting is developed. To the best of our knowledge, this is the first report on preparation of capsules with a probiotic bacterium (*Lactobacillus rhamnosus* GG hereafter called LGG) using a 3D bioprinter. The features of the LGG capsules were then characterized by standard microbiology methods, confocal microscopy, scanning electron microscopy (SEM), atomic force microscopy (AFM), and infrared spectroscopy. LGG bacteria and the matrix encapsulated probiotics growth and viability were monitored for 0, 2 and 7 days-old LGG capsules, which was in accordance to the minimum amount referred by the International Scientific Association of probiotics and prebiotics (Hill et al., 2014). Eventually, LGG capsules were exposed to gastrointestinal fluids in an *in vitro* digestion setup to check for their behavior when reaching the intestinal target.

Materials and methods

Chemicals and nutritive medium

Sodium alginate, gelatin, sodium citrate, calcium chloride, sodium chloride, sodium taurodeoxycholate (NaTDC) and porcine pancreatic extract, PPE (P7545: 8 × USP specifications activity) were purchased from Sigma Aldrich. Phosphate buffer saline (PBS), Man-Rogosa-Sharpe medium (MRS, ref. 288,130) and agar powder (ref. A1296) were supplied by Difco and Sigma-Aldrich, respectively. Cell proliferation reagent WST 1 and LIVE/DEAD® *BacLight*TM kit were purchased from Roche and ThermoScientific respectively. Ultra-pure water (18.2 MΩ.cm) was used for preparation of all media (Elga, Purelab Option-Q). Rabbit gastric extract (RGE15) was provided by Lipolytech, Marseille, France. The extrusion based bioprinter BIO XTM was purchased from CELLINK (CELLINK bioprinting AB, Sweden).

1 Bacterial strain, culture conditions and CFU counting

S. Lebeer (University of Antwerp, Belgium) kindly provided *Lactobacillus rhamnosus* GG (LGG, ATCC 53,103). *Lactobacillus rhamnosus* was cultured in MRS medium under static condition at 37 °C without special caution to avoid oxygen. For monitoring the bacterial growth, the optical density at 600 nm (OD₆₀₀) of the bacterial suspensions was measured using a cell density meter (model 40, Fisher Scientific, Illkirch, France). Briefly, sub-cultures of LGG in MRS (~15 ml) were grown for 24 h in 15 mL closed centrifuge tubes (Greiner Bio-One, ref 188,271) from one UFC grown on MRS agar at 37 °C. They were used to start cultures in the same conditions with fresh MRS (OD₆₀₀ = 0.05 ± 0.01). After 16 h of growth, the centrifugation tubes containing the culture were centrifuged at 3000 g for 10 min at 4 °C. After removal of the supernatant, 15 ml of fresh sterile MRS was added in the tube, and was ready for further experiments.

CFU counting of LGG suspensions was performed by serial dilution of the bacterial suspensions in sterile PBS solution. 100 µl of the diluted

suspensions were spread on MRS agar plates in triplicates. The CFU were counted after 48 h at 37 °C.

1 Preparation of gelatin-sodium alginate hydrogel composite

1.5 g of gelatin was taken in a sterile 10 ml vial and dissolved in 6 ml MRS. It was kept for stirring at 37 °C for 2 h by a magnetic stirrer. After 2 h, when the gelatin was totally dissolved, 0.2 g of alginate was added with 3 mL of sterile MRS. It was kept for stirring at 37 °C overnight. Next day, the alginate-gelatin solution at 37 °C was poured in a cartridge and allowed to cool at room temperature (at 23 °C) for 15 min to form the hydrogel. Then the bioprinting was performed with the following parameters: Cartridge temperature 29.6 °C, Pressure 100 atm, Speed 10 m/s and Print bed temperature 8 °C. The obtained gels were then cross-linked with 0.5 ml 1.1% CaCl₂ solution and stored at 4 °C in MRS after discarding the CaCl₂ solution.

1 Preparation of LGG capsules

1.5 g of gelatin was dissolved in 6 ml MRS at sterile conditions and kept for stirring at 37 °C for 2 h by a magnetic stirrer. After 2 h, when the gelatin is totally dissolved; 0.2 g of alginate was added with 3 mL of MRS. It was kept for stirring at 37 °C overnight. Next day, 1 ml of LGG suspension in MRS was added to the alginate-gelatin solution at 37 °C and gently shaken with hand for 5 to 10 min and poured in a cartridge and allowed it to cool at room temperature (at around 23 °C) for 15 min until it forms a gel. Then the bioprinting was performed with the following parameters: Cartridge temperature of 29.6 °C, Pressure of 100 atm, Printing Speed of 10 m/s and Print bed temperature 8 °C and 22-Gauge needles. The capsules were then physically crosslinked with 0.5 ml 1.1% CaCl₂ solution and stored either in 1 mL sterile MRS, water, PBS or sodium citrate after discarding the CaCl₂ solution. The stability of the prepared LGG capsules was studied after 7 days in these media at 23 °C, 37 °C or 4 °C.

1 Characterization of the LGG capsules:

a) Optical microscopy

Each LGG capsule stored at 4 °C was observed under an optical microscope (Carl Zeiss microscopy) after day 0, day 2 and day 7.

a) Scanning electron spectroscopy

Each LGG capsule was washed three times with PBS and then fixed with 0.5 ml of 4% formaldehyde for 15 min. The LGG capsules were washed again three times with PBS and kept for drying in the air for 24 h and then investigated using a Quanta 600 FEG (FEI) scanning electron microscope (SEM).

a) Imaging and nanomechanical properties by Atomic Force Microscopy (AFM)

Imaging and nanoindentation measurements were performed using a MFP3D-BIO instrument (Asylum Research Technology, Oxford Instruments Company, Wiesbaden, Germany) respectively. Gel morphology was obtained by AFM operating in air with contact mode using silicon nitride cantilevers purchased from Bruker (PeakForce HIRS-SSB, Bruker France SAS, Palaiseau, France) with a spring constant of about 0.01 nN.nm⁻¹. All images were recorded with a resolution of 512 × 512 pixels for a scan rate of 0.5 Hz and a maximum applied force of 0.3 nN.

Nanomechanical properties of gels before and after bacterial embedding were obtained by nanoindentation technique using silicon nitride cantilevers purchased from Bruker (MLCT, Bruker France SAS, Palaiseau, France) with a spring constant of about 0.01 nN.nm⁻¹ at indentation rate of 1 μm.s⁻¹. The nanoindentation technique was operated in PBS medium by recording at least 5 Force-Volume Images at different locations of the gel surface. Each FVI consisted of a grid of 32 × 32 force curves. Gel stiffness (Young modulus) *E* was evaluated by analysing the force-indentation curves within the framework of the

Sneddon model. (EEER, 2021; Kandemir, Vollmer, Jakubovics & Chen, 2018) In this model, the Young modulus is related to the applied force according to the equation given below:

$$F = \frac{2 E \cdot \tan(\alpha)}{\pi (1 - \nu^2)} R^{1/2} \delta^2 \cdot f_{\text{BECC}} \quad (2)$$

where δ is the indentation depth, ν the Poisson coefficient, α the semi-top angle of the conical tip and f_{BECC} is the bottom effect cone correction function. All FVI were analysed using an automatic Matlab algorithm detailed elsewhere, and average Young moduli values given in this work were derived from all the force curves.

a) ATR-FTIR spectroscopy

ATR-FTIR spectra were recorded on a Bruker Tensor 27 spectrometer equipped with a KBr beam splitter and a DTGS detector. Spectra recording and data processing were performed using the Bruker OPUS 7.5 software. The resolution of the single beam spectra was 4 cm⁻¹. Two hundred scans were collected per spectrum corresponding to a 2-minutes accumulation time. All interferograms were Fourier processed using the Mertz phase correction and a Blackman-Harris three-term apodization function. A nine-reflection diamond ATR accessory (DurasamplIR™, SensIR Technologies, incidence angle: 45°) was used for acquiring spectra. No ATR correction was performed. ATR-FTIR spectra are shown with an absorbance scale corresponding to $\log(R_{\text{reference}}/R_{\text{sample}})$, where *R* is the internal reflectance of the device. The contribution of water vapor was removed when necessary. All spectra were baseline corrected at 1800 and 900 cm⁻¹. Measurements were performed at 22 ± 1 °C in an air-conditioned room. The bioprinted capsules were either analyzed after a few hours (day 0), or after storage at 4 °C during 2 days (day 2) or 7 days (day 7). Before the infrared measurements, the capsules were dissolved in an aqueous solution of Na citrate (1.5% w/v) at 37 °C during 30 min. Then, the as obtained suspension was centrifuged at 3000 rpm during 5 min. 100 μl of the bacterial suspension was laid on the ATR crystal. The spectral background was removed by recording the spectrum of the supernatant free of bacteria (obtained by centrifugation).

a) Rheological properties

The rheology of the 3D printed hydrogels without LGG was studied with a strain-imposed ARES Rheometer from TA instruments. All the rheological experiments were carried out with fresh 3D printed gels. The gels were placed in a parallel plate rotor-stator geometry and were submitted to oscillatory tests. First, the linear viscoelastic domain (LVE) of the material was determined through amplitude sweep tests performed at $\omega = 1 \text{ rad.s}^{-1}$. A deformation amplitude of $\gamma = 10^{-3}$ was chosen within the LVE domain. The storage and loss modulus *G'* and *G''* were measured at this amplitude and for $\omega = 1 \text{ rad.s}^{-1}$. The measurements were repeated for three different samples.

1 Behavior of the bacteria in the capsules

a) Plate count

Each probiotic capsule was dissolved in 0.5 ml of 1.5% sodium citrate and 0.5 ml MRS and kept at 37 °C for 30 min. Then 0.1 mL of the dissolved capsule was diluted to 1 mL by MRS solution. Ten-fold dilutions were made until 10⁹. Then 0.1 mL was added to the MRS agar plates in triplicates and kept it for 48 h at 37 °C in the incubator. The bacterial colonies were counted after 48 h.

a) Confocal microscopy

The bacteria shape and the integrity of the bacterial membrane were assessed using the BacLight™ stain kit. With this kit, bacteria with intact membranes exhibit green fluorescence (Syto 9), while bacteria with damaged membranes show red fluorescence (propidium iodide, PI). Each LGG capsule was washed three times with 0.85% NaCl and

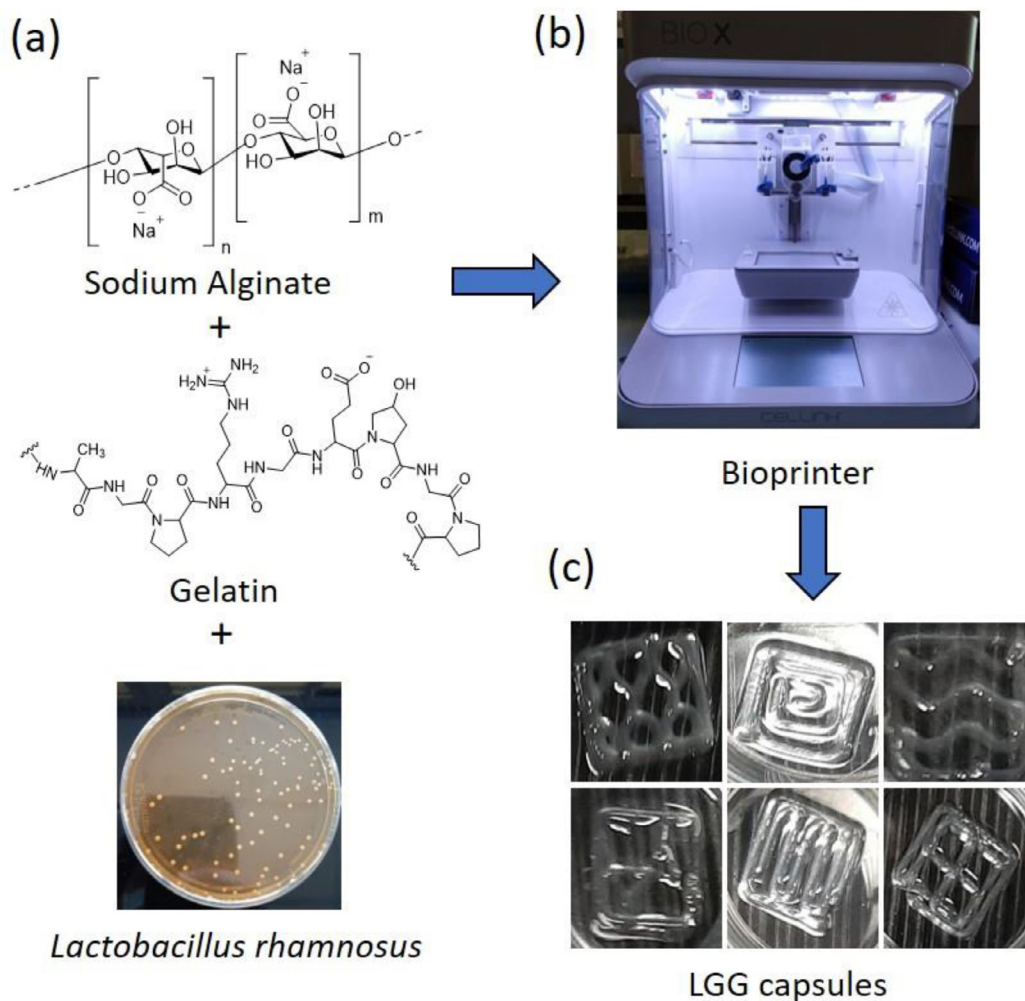


Fig. 1. Preparation of LGG capsules by 3D bioprinter using alginate-gelatin polymer and *Lactobacillus rhamnosus* GG. (a) Chemical structure of alginate and Gelatin and LGG grown on MRS agar, (b) Picture of the BIO-X (Celink) bioprinter used, (c) different shapes of LGG capsules from the 3D bioprinter visualized by optical microscopy.

then stained with 3 μL BacLight™ solution (1.5 μL of SYTO 9 and 1.5 μL of PI) in 1 mL, 500 μL and 200 μL of 0.85% NaCl for 30 min at 23 °C in the dark. After 30 min, the LGG capsules were then washed 5 times with 0.85% NaCl (to remove excess dyes), put on a coverslip and finally placed on a glass slide in mounting oil. Confocal laser scanning microscopy (CLSM) images were taken using a Leica TCS SP5-X-AOBS confocal laser scanning microscope (Leica Microsystems CMS GmbH, Mannheim, Germany) equipped with WLL lasers. The objective lens used was a HCX PL APO CS 100 \times 1.40 (oil immersion). The imaging was done with the appropriate filters listed below:

The images were processed and exported using LAS X software, version 3.4.2.18368. The measurements were performed at day 0, day 2 and day 7 for LGG capsules kept at 4 °C.

a) WST 1 assay

WST 1 assay is a method of colorimetric detection of the enzymatic activity in microbial metabolism by which bacterial viability can be monitored. WST 1 is a colorimetric indicator producing a water-soluble formazan dye upon receiving electrons from the viable bacterial cell through an electron mediator. Monitoring the color intensity of WST formazan dye helps us in determining bacterial cell viability ($\lambda_{\text{max}}=460 \text{ nm}$). Each LGG capsule was washed three times with PBS

and dissolved in 1 ml of 1.5% sodium citrate at 37 °C for 30 min. LGG capsules were also treated with methanol for 30 min as a reference for dead bacteria and capsules were also made without LGG as a negative control. 100 μL of the dissolved capsules were plated in a 96 well plate and 10 μL of WST solution was added in each well and kept at 37 °C for 2 h. The absorbance was measured at 450 nm using a microplate reader. The assay was performed at day 0, day 2 and day 7 keeping the capsules at 4 °C.

1 wo-step static *in vitro* digestion procedure:

- a) Gastric step: The LGG capsules, free probiotic bacteria and the non-encapsulated capsules (alginate gelatin composite separately) with the probiotic bacteria were first resuspended in the buffer. The initial volume of the buffer taken was 15 mL containing 10 mM MES, pH 5.5, 150 mM NaCl and 1.4 mM CaCl_2 . At $t = 0 \text{ min}$, 3 mL of a freshly prepared gastric extract solution RGE at 100 μg gastric lipase/mL (equivalent to 25 mg RGE15 in 3 mL buffer) were added to the reaction vessel to obtain a final concentration of 17 $\mu\text{g}/\text{ml}$ of gastric lipase (20 U/mL) and the pH was kept constant at 5.5 during 30 min, via an automated titration of FFAs with 0.1 M NaOH using a pH-stat device. Agitation was kept at 1 speed.
- b) Duodenal step: At $t = 30 \text{ min}$, 11 ml of pancreatic extract /bile salts (NaTDC) solution were added to the mixture and the pH

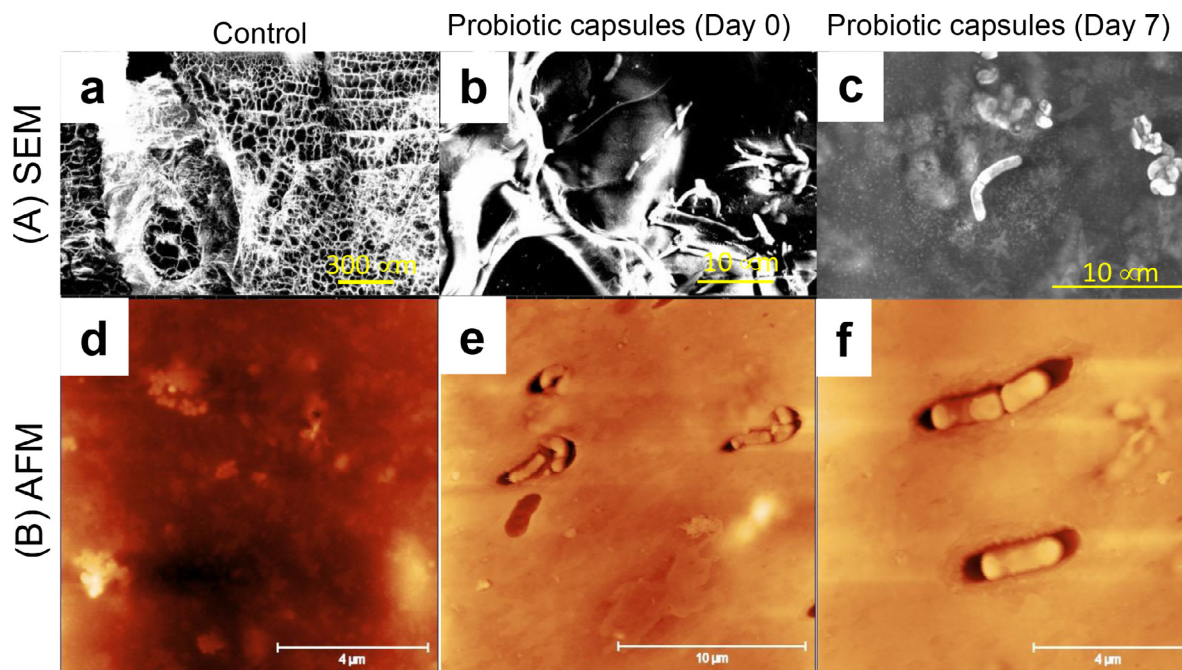


Fig. 2. Microscopic images of capsules without bacteria (left panel, control) and LGG capsules at day 0 (middle panel) and day 7 (right pane). (A) SEM images and (B) AFM images.

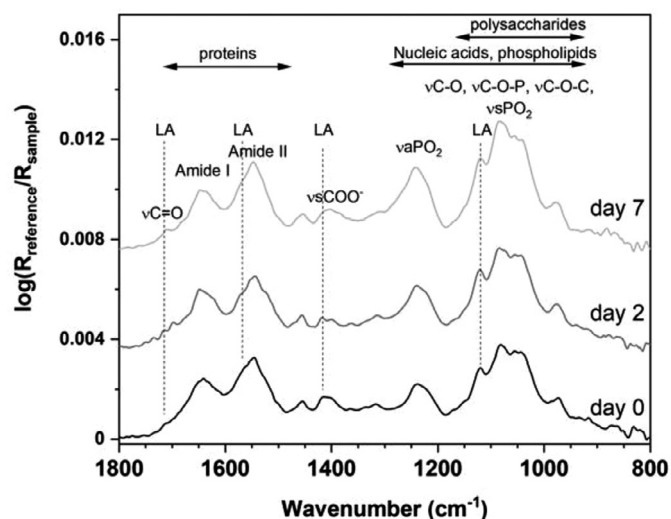


Fig. 3. Infrared spectra in ATR mode in aqueous medium of LGG after dissolution of the LGG capsules just prepared (day 0) and after storage at 4 °C during 2 (day 2) and 7 (day 7) days in MRS medium. Spectra are normalized to one in region 1604 – 949 cm^{-1} to allow comparison. Offsets are used for clarity. Main assignments are provided. Key: ν , stretching; a, antisymmetric; s symmetric, LA, lactic acid or lactate.

was kept at 6.25 for 60 more min. After adding the pancreatic extract/bile salt solution to the reaction vessel (675 mg porcine pancreatic extract, PPE at 1% and 57 mg NaTDC), the final pancreatic lipase concentration was 250 $\mu\text{g}/\text{mL}$ (2000 U/ml), the bile salt concentration was 4 mM, and the gastric phase was diluted 1.7-fold. Agitation was kept at 3 speed.

At $t = 90$ min, the medium was harvested and centrifuged at 3000 rpm, 4 °C for 10 min and the pellet was used for plate counting experiment.

Results and discussion

3D-bioprinting of the LGG capsules and their stability

Due to the lack of consistency and limitations of the encapsulation techniques used for the oral administration of probiotic bacteria, we aimed to develop, a controlled bacteria encapsulation process for further industrial implementation.

For that, we have used a micro-extrusion technique with the help of a 3D-bioprinter. In this method, two biocompatible and bio functional polymers; i.e. alginate and gelatin in a fixed ratio (2% Alginate and 15% Gelatin) were mixed with LGG and led to LGG embedded bioprinted structures called LGG capsules (Supp. Info S1).

Alginate and gelatin were chosen as the matrix for encapsulation because of their cytological compatibility and ease of gelation when used in the proper ratio. We optimized the exact ratio of alginate and gelatin by preparing different concentrations of alginate and gelatin for its use as a bio ink. After several trials, the ratio 2:15 (% /%) was considered as the best concentration for hydrogel formation. The main reason for choosing this concentration, is that below this value, the alginate gelatin composite was runny liquid and very difficult to bio print.

The solubility of the LGG capsules at different temperatures and in different aqueous media was assessed. The capsules were stable at 4 °C, 23 °C and 37 °C (Figure S2). The capsules were also stable in water and PBS but on addition of sodium citrate at 37 °C for 30 min, they were dissolved easily (Figure S3). Around 120 capsules were prepared within half an hour. Therefore, this procedure could be very useful for industrial applications.

Characterization of LGG capsules by microscopic and spectroscopic techniques

Alginate-gelatin matrix (with and without probiotic) were first visualized by light microscope in brightfield images on both day 0 and day 7 of storage at 4 °C in MRS medium (Figure S4 a-c). As shown in figure S4 2b and S4 2c, when the bacteria were incorporated within the poly-

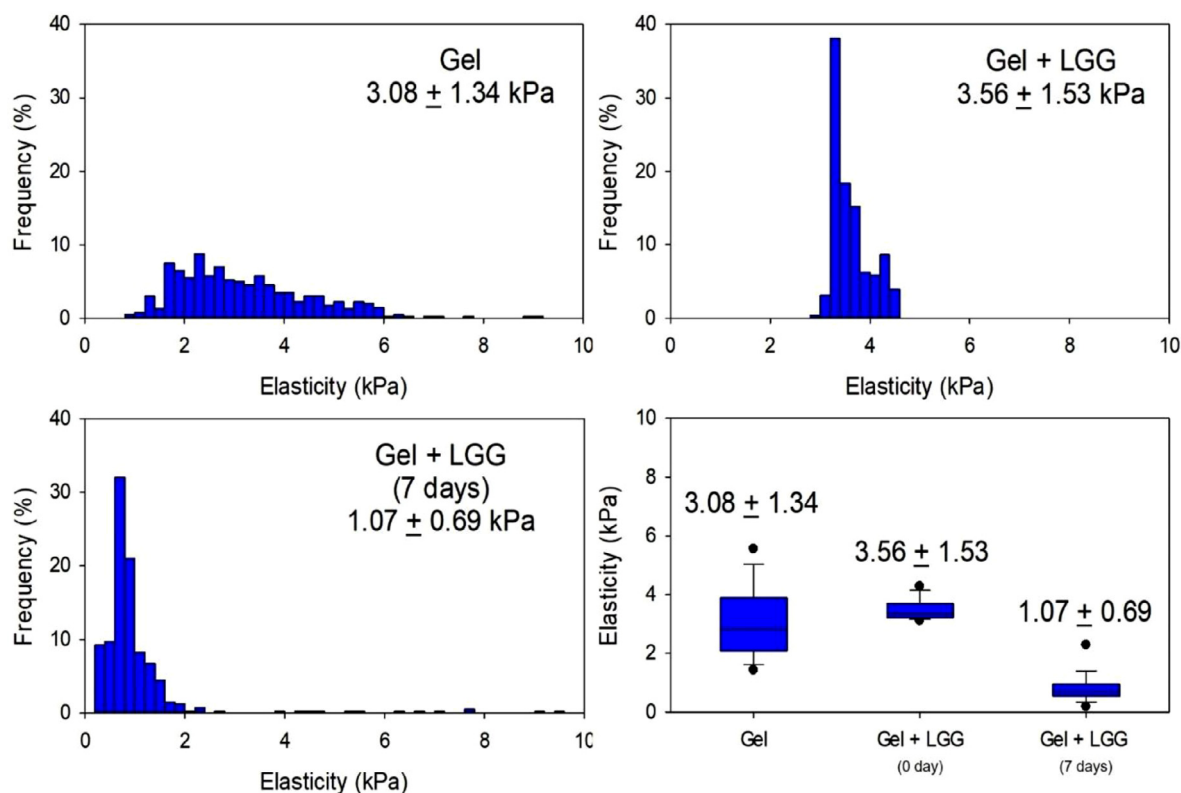


Fig. 4. Statistic distribution of elastic modulus of (a) freshly prepared alginate-gelatin hydrogel, (b) freshly prepared alginate-gelatin hydrogel and probiotic bacteria and (c) alginate-gelatin hydrogel and probiotic bacteria kept at 4 C for 7 days. (d) Evolution of the elastic modulus of alginate-gelatin matrix after the addition of bacteria on the 0th day and after 7 days (statistical analysis of gel stiffness measured by AFM are presented in Table S1 in the Supp. Info.).

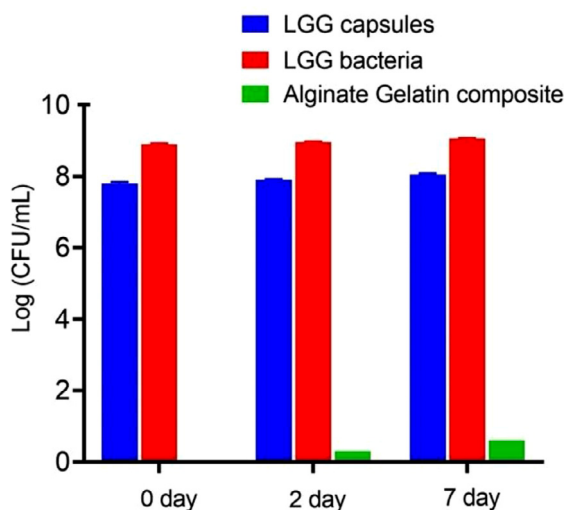


Fig. 5. Evaluation of bacterial cell viability. (a) CFU counting of LGG free in MRS, in capsules, CFU counting in capsules without LGG as a control experiment at day 0, day 2 and day 7.

mer matrix, black features were seen confirming the good dispersion of the LGG cells within the 3D-bioprinted film. The non-harmfulness of the bio-printing process was assessed first by the LGG morphological characterization by combining SEM (Fig. 2a-c) and AFM (Fig. 2d-f). As illustrated in Fig. 2b-c(SEM) and 2e-f (AFM), the LGG were embedded in the alginate-gelatin polymer and have kept their morphology up to 7 days. No bacteria were present in the control, which consisted only of alginate-gelatin gel, as described in Fig. 2a and 2d. Thus, we could confirm from the microscopic data that the LGG strains were completely and

homogeneously encapsulated in the alginate-gelatin composite while keeping their initial shape.

To compare the physiological state of LGG cells as a function of the time spent in the alginate-gelatin capsule, ATR-FTIR spectra were recorded few hours after encapsulation, on day 2 and on day 7 (Fig. 3, fingerprint region). The spectra were typical of LGG bacteria and were previously assigned³³. They showed major bands associated with proteins (mainly amide I and II bands), nucleic acids (regions around 1240 and 1085 cm^{-1}), polysaccharides (region 1150 - 900 cm^{-1}) and lactic acid/lactate (bands at 1712, 1574, 1417 and 1121 cm^{-1}). The general characteristics changed only slightly, suggesting that the biochemical composition of LGG cells remained similar during 7 days of storage. *L. rhamnosus* grows slowly³⁴, which is consistent with the minor differences in spectral fingerprints. The ratios of the integrated intensities of the band regions assigned to proteins (amide II), {nucleic acids + phospholipids} (sPO2) and {nucleic acids + phospholipids + polysaccharides} (PS), were calculated from day 0 to day 7 (Figure S5). Interestingly, the ratio of the integrated intensities of the bands amide II / vsPO₂ and amide II / PS bands decreased from day 0 to day 7, indicating metabolic activity of the bacteria. Nucleic acids, some lactic acid and polysaccharide-based compounds were synthesized. Thus, these data indicated that the bacteria were safely stored in the capsules and the metabolic activity was maintained.

Mechanical properties of the alginate gelatin composite and the LGG capsules by atomic force microscopy and rheological studies

A storage modulus of $G' = 1075 \pm 64$ Pa and a loss modulus of $G'' = 262 \pm 22$ Pa were measured for the 3D printed hydrogels swelled in MRS, which indicates the good robustness of the gel used as a matrix. It is worth mentioning that the gel with and without LGG was easily

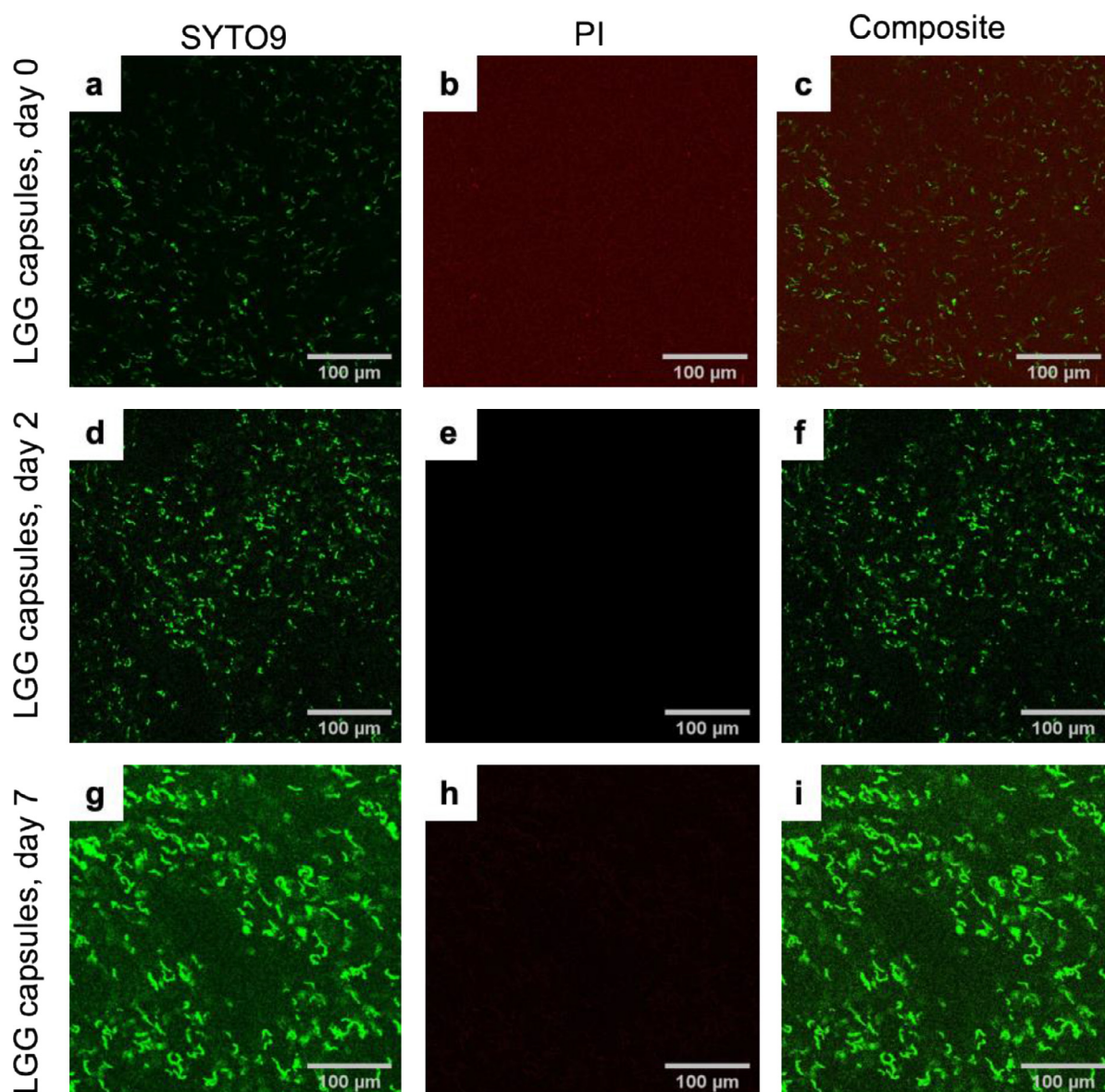


Fig. 6. Confocal images of day 0, day 2 and day 7 LGG capsules after *BacLight*TM staining after day 0, day 2 and day 7.

extruded through a 22-gauge needle, demonstrating its shear-thinning properties when we will consider the in-vivo injection of the gel.

Statistical analysis of gel stiffness measured by AFM shows important variations when the gel was loaded with bacteria and after 7 days of storage, as shown in Fig. 4 and Table SI-1. Indeed, as shown in Fig. 4, the gel stiffness slightly increased from 3.1 to 3.6 kPa after loading with LGG bacteria. After 7 days, a mechanical breakdown was observed with a decrease from 3.6 to 1.1 kPa. This mechanical weakening can be attributed to gel degradation due to bacterial activity (nutrients and gelatin consumption). Despite this mechanical weakening, no significant morphological damage to the gel was detected by AFM imaging, as shown in Fig. 4. Thus, it is quite obvious that the freshly prepared alginate-gelatin hydrogel is very soft and the LGG bacteria can easily survive in it³⁵.

Studies of the LGG capsules

To evaluate the integrity of the bacterial cells and viability of the LGG after encapsulation in the alginate-gelatin matrix, three standard techniques were used: (a) CFU counting (Fig. 5), (b) *BacLight*TM staining

and confocal microscopy imaging (Fig. 6), and (c) WST 1 assay (Fig. 7)³⁶.

Though, CFU counting is considered as an essential quantitative tool in clinical microbiology laboratories. For convenience, the results were reported as CFU/mL for liquids. According to the scientific community, the minimum recommendation of probiotics in any food item is 6 log CFU/mL and 7 log CFU/mL at the targeted point of delivery. The log CFU/mL was 7.8 at day 0 and slightly increased to 7.9 and 8, at day 2 and day 7 respectively, for LGG capsules stored at 4 °C in this work. We also performed the CFU counting with the free LGG bacteria, which was around 8.8 log CFU/mL on the 0th day and 8.9 log CFU/mL and 9 log CFU/mL on 2nd day and 7th day respectively. The log CFU/mL of the alginate-gelatin capsule without the probiotic bacteria were of course found to be approximately 0 at all the time points. Thus, from the above results, Fig. 5, it was clear that our LGG capsules were viable and in accordance to the recommended probiotic content in the food product.

The membrane integrity the bacterial cells inside the matrix was assessed by *BacLight*TM. All the LGG bacteria inside the capsules emitted green fluorescence when they were stored at 4 °C either for day 0, day

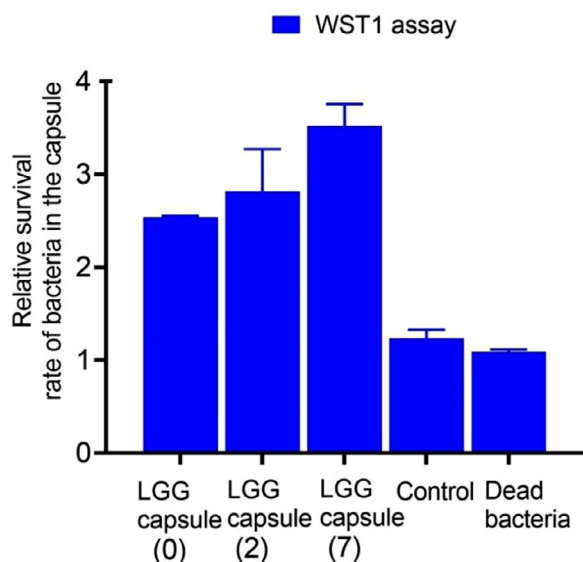


Fig. 7. Evaluation of bacterial cell viability: WST 1 assay was performed with LGG capsules (day 0, day 2 and day 7), control (alginate gelatin capsule) and LGG capsules containing dead bacteria.

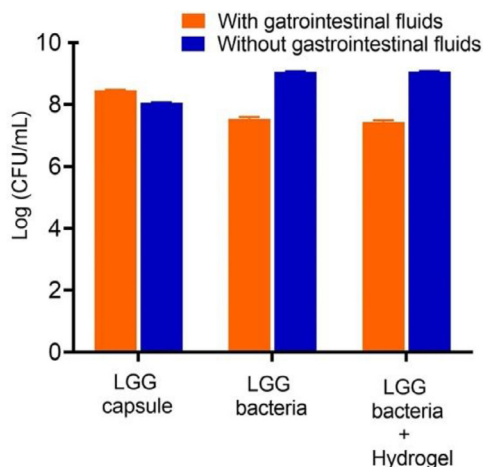


Fig. 8. Colony counting assay performed after passing the LGG capsules, free probiotic bacteria and the non-encapsulated capsules with the free probiotic bacteria through the simulated *in vitro* digestion experimental setup containing gastrointestinal fluids (gastric enzymes and pancreatic enzymes/bile salts solution). For comparison, a control was kept without using gastrointestinal fluids.

2 and day 7, showing that they did have no compromised membranes (Fig. 6a-i).

For LGG capsules, the absorbance at $\lambda_{\max}=460$ nm was found to be increased by 2.7, 2.8 and 3.3-fold with respect to that of the only media after the addition of the WST 1 reagent day 0, day 2 and day 7 respectively. The absorbance at $\lambda_{\max}=460$ nm of the control (alginate gelatin capsules without the probiotic bacteria) and the capsules with dead bacteria (LGG capsules treated with methanol) was almost equal to that of the media. From this data, Fig. 7, there is a clear indication that the probiotic bacteria in the capsules remained alive on day 0, day 2 and day 7.

Study of the probiotic capsules stability in an *in vitro* model mimicking the gastro-intestinal environment

Finally, the major aim of this work was to ensure the preservation of probiotic bacteria until it reaches the target site, *i.e.*, the intestine, by using this encapsulation technique with the 3D bio-printer. For this

Table 2

	SYTO 9	PI
Excitation/Emission (in nm)	480/500	490/635
Standard set filters	FITC	Texas Red

purpose, an experimental *in-vitro* model was used to mimic the gastro-intestinal digestion. The LGG capsules, the free probiotic bacteria and the non-encapsulated capsules to which the probiotic bacteria were added separately were subjected to a solution of gastric enzymes and pancreatic enzyme/bile salts in a simulated *in-vitro* gastro-intestinal digestion experiment at 37 °C, and the bacteria obtained at the end of the experiment were counted after culture on MRS agar (CFU/mL, Fig. 6). The log CFU/mL for LGG capsules after passing through the mimetic fluids of the gastrointestinal tract was 8.1, compared to 7.9 without the *in-vitro* digestion step. For the free bacteria and the non-encapsulated capsules with the probiotic bacteria, the log CFU/mL after passing the gastrointestinal fluids was 7.4 and 7.5, respectively. Without gastrointestinal fluids, the log CFU/mL was 9.1 and 9.3, indicating a 30-fold decrease (Fig. 8). Thus, from the above data in Fig. 5, it can be confirmed that the probiotic bacteria encapsulated in the gelatin-alginate matrix were protected from the harsh conditions such as low pH in the stomach and could resist bile acid and pancreatin in the small intestine, while the free probiotic or the non-encapsulated capsules associated to the probiotic bacteria (but separately) couldn't survive in substantial amount while reaching the targeting site.

Conclusion

Encapsulation of probiotic bacteria remains a big challenge for the food industries. Thus, we have developed a novel strategy of encapsulating probiotic bacteria by 3D bioprinter using sodium alginate-gelatin as an encapsulating material. We printed about 120 LGG capsules within 30 min each containing 8 log CFU/mL of LGG. The bacteria inside the LGG capsules were uniformly distributed in the gelatin alginate matrix. LGG inside the capsules were viable. The probiotic bacteria inside the capsule also have endured the harsh gastrointestinal fluidic condition prepared in an *in vitro* digestion set up. Thus, this bioprinting methodology will open up the possibility of fast and easy preparation of encapsulated bacteria, which will bring about huge change in the food industries.

Fig. 1, Table 2

SUPPORTING INFORMATION:

The Supporting Information is available free of charge on the ACS Publications website.

The shape versatility of the 3D bioprinted capsules can be easily visualized by optical

Microscopy (Figure S1).

LGG capsules are stable up to 7 days at different temperatures 23 °C, 4 °C and 37 °C (Figure S2).

The LGG capsules are not soluble neither in water or PBS, but they are in 1.5% sodium citrate at 37 °C (Figure S3).

The bacteria are uniformly dispersed in the gel after the 3D bioprinting (Figure S4).

The bacteria viability in the Alginate-gelatin matrix (with and without probiotic) was confirmed by ATR FT-IR measurement (Figure S5)

The statistical analysis of gel stiffness showed the important variations when the gel was loaded with bacteria and confirmed that the 3D printed hydrogels swelled in MRS indicating indicates the good robustness of the gel used as a matrix after 7 days of storage. (Table S1)

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by the French PIA project “Lorraine Université d’Excellence” reference ANR-15-IDEX-04-LUE.

AM and HA thank the CC3M for the SEM and optical microscopy analysis.

The CLSM was performed at the UMS2008 Imaging Core Facility from Lorraine University, <https://umsibslor.univ-lorraine.fr/en/facility/imaging>.

FQ, GF thank the Spectroscopy and Microscopy Service Facility (SMI) of LCPME (Université de Lorraine-CNRS – <http://www.lcpme.cnrs-nancy.fr>) where the ATR-FTIR and AFM experiments were performed.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.fhfh.2022.100088](https://doi.org/10.1016/j.fhfh.2022.100088).

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