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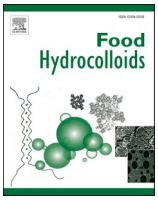

Encapsulation of *Lactobacillus plantarum* in casein-chitosan microparticles facilitates the arrival to the colon and develops an immunomodulatory effect

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ABSTRACT

The current work describes the capability of casein-chitosan microparticles to encapsulate *Lactobacillus plantarum* (CECT 220 and WCFS1 strains) and evaluates their ability to target the distal areas of the gut and to stimulate the immune system. Microparticles were prepared by complex coacervation, between sodium caseinate and chitosan in an aqueous suspension of the bacteria, and dried by spray-drying. In order to increase the survival rate of the loaded bacteria, microparticles were cross-linked with one of the following cross-linkers: tripolyphosphate, calcium salts or vanillin.

Overall, microparticles displayed a mean size of about 7.5 μm with a bacteria loading of about 11 Log CFU/g, when cross-linked with vanillin (MP-LP-V). For conventional microparticles, the payload was 10.12 Log CFU/g. The storage stability study at 25 °C/60% RH, MP-LP-V offered the highest degree of protection without significant modification of the payload in 260 days. Compared with control (aqueous suspension of bacteria), MP-LP-V also displayed a significantly higher degree of protection against probiotic inactivation in simulated gastric and intestinal fluids. *In vivo* results evidenced that microparticles, orally administered to rats, were able to reach the distal ileum and colon in about 4 h post-administration. Additionally, the effect of the daily administration of 10^7 CFU/mouse of MP-LP-V, for 3 weeks, induced an immunomodulatory effect characterized by an important enhancement of Th1 and Th17 responses. In conclusion, these microparticles seem to be a promising strategy for increasing survival and efficacy of probiotics, allowing the formulation of cost-effective and more stable and effective probiotic-based nutraceuticals.

1. Introduction

The gut microbiota in healthy adult individuals is relatively constant and contains diverse beneficial bacterial populations, including *Lactobacillus* and *Bifidobacterium* species, which play an important role on the health of the host (Bibbò et al., 2016). Moreover, in the healthy gut, the intestinal microbiota prevents the adherence and gut colonization by pathogenic bacteria (Hills et al., 2019; Kim et al., 2019). Consequently, an imbalance of normal gut microbiota (dysbiosis) can contribute to the development of a variety of health complications, including inflammatory bowel disease and irritable bowel syndrome (Khan et al., 2019; Simon, Călinoiu, Mitrea, & Vodnar, 2021), as well as to worsen particular manifestations of some diseases such as obesity, type 2 diabetes, and hypersensitivity disorders (Hills et al., 2019; Kim et al., 2019). The

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main factors affecting the microbiota composition and bacterial diversity includes diet, lifestyle, infections, antibiotic treatments or geographical provenance (Tandon et al., 2018). These evidences led to a recommendation, in 2001, from a joint FAO and WHO expert to exploit the therapeutic and prophylactic potential of beneficial microorganisms to prevent the risks associated to dysbiosis (Hill et al., 2014).

The most popular microorganisms currently employed as probiotics include lactic acid bacteria (LAB) and bifidobacterial but other types of bacteria (e.g., *Escherichia coli* Nissle 1917 or *Enterococcus faecalis*) and certain yeasts (e.g., *Saccharomyces boulardii*) are also employed (Wieërs et al., 2020). In order to ensure health-relevant effects, it is generally accepted that products including probiotics should have a minimum concentration of about 10^6 CFU/mL or per gram and that around 10^8–10^9 CFU of probiotic microorganisms per day should be consumed (Binda et al., 2020).

Among LAB, *Lactobacillus plantarum* (Lp) is widely employed for dairy, meat, and vegetable industrial fermentation (Liu, Liong, & Tsai, 2018) and has been proven to be able to colonize human intestinal mucosa and, thus, be part of the human microbiota of healthy individuals (Sonnenburg, Chen, & Gordon, 2006). Moreover, Lp is recognise as safe for humans and, from a regulatory point of view, has both a "Generally Recognized As Safe" (GRAS) and a "Qualified Presumption of Safety" (QPS) status from FDA and EFSA, respectively (Behera, Ray, & Zdolec, 2018; Wei, Chuantao, Arbab, Lai-Yu, & Wenyi, 2021). Several studies have proven the applicability of various strains of Lp as probiotic by improving the gastrointestinal barrier function and by preventing the overgrowth of pathogenic bacteria (Paolillo, Carratelli, Sorrentino, Mazzola, & Rizzo, 2009; Wang et al., 2019).

Additionally, Lp would induce an immunomodulation of the immune response of the gut-associated lymphoid tissue and epithelial cells (Santarmaki et al., 2017). Thus, *L. plantarum* Lp91 has shown a strong immunoregulatory capability in a murine colitis model induced by TNBS (Duary, Bhausaheb, Batish, & Grover, 2012). Another interesting example of this immunomodulatory effect has been described for Lp WCFS1. In healthy individuals, oral supplementation with this strain stimulated antigen presentation and memory immune responses (de Vos et al., 2017).

However, the delivery of probiotic bacteria (particularly Lp) to the large intestine and the preservation of their efficacy are still important challenges for the development of effective functional food products. Thus, Lp is very sensitive to industrial processing, transport and storage conditions that markedly limit its use in food products (Parlindungan, Dekiwadia, May, & Jones, 2019; Mahmoud, Abdallah, El-Shafei, Tawfik, & El-Sayed, 2020). Another important factor determining the beneficial effect of probiotics would be their ability to resist and survive the passage through the gut, irrespective of gastric pH conditions as well as the presence of bile salts and digestive enzymes (Hlaing et al., 2020), in their "journey" to the ileum and colon where they should colonize the mucosa. One alternative to improve the viability during both processing and transit to the distal areas of the intestine might be their encapsulation in biodegradable microparticles. Thus, in the last years, a vast number of developments using food-grade polymers as material for microencapsulation of Lp and other probiotics have been proposed, including the use of alginates (Jiang et al., 2013; Mahmoud et al., 2020), gums (Paula et al., 2019), celluloses (Li, Liu, Tian, Luo, & Liu, 2019), soy proteins (González-Ferrero, Irache, & González-Navarro, 2018), or fructooligosaccharide and whey protein (Rajam, Kumar, Prabhasankar, & Anandharamakrishnan, 2015). However, information about the capability of microparticles to both carry the loaded bacteria to the site of colonization and promote their beneficial *in vivo* effect is scarce.

The aim of this work was to study and evaluate the potential of casein-chitosan microparticles to encapsulate *L. plantarum* (strain CECT 220 and WCFS1). The work describes the increased viability of the microencapsulated bacteria during processing and storage, as well as their ability to target the distal areas of the gut and to stimulate the immune system *in vivo*.

2. Material and methods

2.1. Materials

Sodium caseinate was provided by Anvisa (Madrid, España) and chitosan (deacetilation degree 90.2%, MW 105 kDa) was from Guinama (Valencia, España). Mannitol, pancreatin, pepsine, vanillin, rhodamine B isothiocyanate (RBITC), PBS tablets, and tripolyphosphate (TPP) were purchased from Sigma-Aldrich (St Louis, USA). MRS broth and Buffer Peptone Water (BPW) broth, sodium hydroxide, calcium chloride and calcium acetate were acquired from Merck KGaA (Darmstadt, Germany). Lumogen® F Red was from BASF (Ludwigshafen, Germany), 4',6-diamidino-2-phenylindole (DAPI) from Biotium Inc (Fremont, USA), and O.C.T.™ Compound Tissue-Tek® from Sakura Finetek Europe BV (Alphen aan der Rijn, The Netherlands). Ethanol was provided by Panreac (Barcelona, Spain). All other chemicals were of analytical grade.

Lactobacillus plantarum CECT 220 was purchased from the Colección Española de Cultivos Tipo (Valencia, Spain), and *Lactobacillus plantarum* WCFS1 was from the American Type Culture Collection (ATCC BAA-793, Virginia, USA).

2.2. Production of probiotics

Commercial freeze-dried samples of Lp (both, Lp CECT 220 and Lp WCFS1) were revitalized in a MRS broth at 37 °C for 12 h in microaerobic conditions. This preculture was used to inoculate flasks containing 500 mL MRS broth and incubated for 24 h until reaching the early stationary growth phase.

The final working suspensions was prepared by centrifugation of 2 L MRS broth culture at 6,000×*g* for 20 min. Supernatants were eliminated and the pellets washed with sucrose 2% w/v two-times before final dispersion in a 2% (w/v) sucrose solution. The bacterial population was counted by plating decimal dilutions using 0.1% BPW on MRS agar. The mean count of final suspensions was 5×10^{11} CFU/mL.

In order to evaluate the viability during storage, aliquots of Lp CECT 220, in 2% w/v sucrose, were lyophilized in a LyoAlfa freeze-drier (Telstar, Terrassa, Spain).

2.3. Fluorescence-labeling of probiotic bacteria

Bacteria were labeled with a solution of RBITC in phosphate buffer (1 mg/mL; pH 7.4). Then, 0.1 mL of this solution was added to 10 mL of *L. plantarum* (5×10^{11} CFU/mL) in 2% sucrose and the mixture was maintained under agitation at 20 °C for 10 min. Then, the bacterial suspension was centrifuged at 6,000×*g* for 20 min. The pellet was washed twice with sucrose 2% w/v and finally dispersed in the sucrose solution.

2.4. Preparation of probiotic-loaded microparticles

The microencapsulation of probiotic bacteria was performed by complex coacervation by promoting the ionic interaction between sodium caseinate and chitosan. Eventually, these microparticles were cross-linked with different agents, including vanillin, TPP or calcium salts. Finally, microparticles were dried by spray-drying.

More specifically, for the preparation of microparticles, 1.5 mL of a bacterial suspension (about 5×10^{11} CFU/mL) was added to 25 mL of an aqueous solution of sodium caseinate (10 mg/mL). After 5 min of incubation under magnetic stirring, 10 mL of an aqueous solution of chitosan (1.6 mg/mL; pH 5.5–6) were poured under agitation at room temperature. Finally, after 5 min of incubation, 100 mg mannitol were dissolved and the resulting mixture was dried by spray-drying in a Büchi B-290 Mini Spray-Dryer. The main parameters of the drying process were as follows: air inlet temperature, 85 °C; air outlet temperature, 43 °C; sample pumping rate, 3.5 mL/min; air pressure, 6 bar and air flow

rate of 600 L/h. The resulting microparticles were identified as MP-LP.

For the cross-linkage of the microparticles with vanillin, 0.5 mL of an aqueous solution of vanillin (5 mg/mL) were added to the sodium caseinate solution and, after 15 min of incubation, bacteria and chitosan was incorporated as described above. For the cross-linkage of microparticles with either TPP (0.8 mL of an aqueous solution 1 mg/mL) or calcium salts (2 mL calcium acetate 2% w/v and 2 mL calcium chloride 2% w/v in water), the reagents were incorporated in the just formed microparticles. Then, the mannitol was added and the microparticles dried by spray-drying as described above. These cross-linked microparticles were named as follows: MP-LP-V (when cross-linked with vanillin), MP-LP-TPP (when cross-linked with TPP) and MP-LP-Ca (when cross-linked with calcium salts).

Microparticles containing fluorescently labeled bacteria were prepared in the same way as aforementioned. Finally, control microparticles (MP) were prepared as described above but in the absence of probiotic bacteria.

2.5. Physico-chemical characterization

2.5.1. Size, moisture content and morphology analysis

The mean size of the microparticles, measured as the volume mean diameter (D [4,3]) in micrometers (μm), was determined in a Mastersizer S laser sizer equipment (Malvern Instruments, UK). All the measurements were performed at RT after dispersion in water of the dried samples.

The moisture contents of the powder materials obtained from the Spray-drying were measured in terms of the losses on drying using a MOC-120H moisture balance (Shimadzu Scientific Instruments, Nettetal, Germany). Drying was conducted at 105 °C.

The morphology and shape of probiotic-loaded microparticles were evaluated by scanning electron microscopy. Microparticles dispersed in an aqueous medium were mounted on double-faced adhesive tape on metal stubs, coated with gold to a thickness of 16 nm (Emitech K550; Quorum Technologies, UK). Observations were performed by scanning electron microscopy (Zeiss DSM 940 A, Germany) with a digital imaging capture system (Point Electronic GmBH, Germany). Morphology analysis was additionally performed by optical and fluorescent microscopy (Olympus CH40 apparatus).

2.5.2. Quantification of bacteria loaded in microparticles and determination of bacterial death cycles

The viability of microencapsulated bacteria was evaluated after dispersion of an accurately weighed amount of microparticles in 1 mL NaOH (0.1 mM; pH 10) (González-Ferrero et al., 2018). Then, dilutions of these samples were performed in 0.1% BPW broth (w/v). These dilutions were seeded in MRS plates, incubated for 48 h at 37 °C under anaerobic conditions. The colony were counted and the payload of microparticles was expressed as the quotient between the counts of viable bacteria (expressed as log) and the mass of powder containing the microparticles.

2.6. Evaluation of stability of probiotic bacteria during storage

Probiotic-loaded microparticles and control samples (a fresh aqueous suspension in 2% sucrose and freeze-dried bacteria) were stored in 50 mL sterilized glass containers hermetically sealed in a climate chamber (Memmert GmbH, HPP108, Schwabach, Germany) at 25 °C and 60% relative humidity (RH). At different times, selected vials were opened, and the counts of viable bacteria were calculated as described above.

The stability was evaluated by representing the relative viability (the quotient between the total viable bacteria at a particular storage time and the number of viable cells at the beginning of storage, expressed as logarithm).

2.7. Viability of encapsulated probiotic bacteria in simulated gastrointestinal media

The gastrointestinal resistance of microencapsulated probiotic was evaluated in simulated gastric (SGF) and simulated intestinal (SIF) fluids. SGF, with an adjusted pH of 2.5 ± 0.1, was prepared by dissolving 2 g NaCl, 7 mL HCl and 3.3 g pepsin in enough distilled water to obtain a final volume of 1 L. For SIF, 6.8 g potassium phosphate monobasic was first dissolved in 250 mL distilled water. In parallel, 77 mL 0.2 N NaOH and 500 mL distilled water containing 10 g pancreatin were prepared. The SIF solution was obtained by mixing the different components and the pH was adjusted to pH 6.8 ± 0.1, before adding distilled water to obtain a volume of 1 L.

For the experiment, an accurately weighed amount of microparticles were dispersed in 990 μL SGF. At different times, the samples were centrifuged and the number of viable bacteria was calculated as described above. In order to simulate the *in vivo* conditions, microparticles containing probiotics were also dispersed in SGF for 2 h. Then, these samples were centrifuged at 6,000×*g* for 10 min and the supernatants removed before dispersion in SIF. Again, at predetermined times, the samples were centrifuged and the number of viable bacteria was calculated. The experiments were performed in triplicate.

As controls, both lyophilized and aqueous suspensions of bacteria in sucrose 2% (w/v) were employed.

2.8. Radiolabeling of microparticles

Probiotic-loaded casein-chitosan microparticles cross-linked with vanillin (MP-LP-V) were radiolabeled with technetium-99 m by reduction with tin chloride following a previously described protocol (Sánchez-Martínez et al., 2013). Briefly, 40 μL of a 0.02 mg/mL tin chloride dihydrate solution in water at pH 4 (adjusted with HCl 0.1 N) were mixed with 0.5 mg microparticulated samples. Then, 200 μL (370 MBq) of [^{99m}Tc]TcO_4Na eluate from a ^{99}Mo/^{99m}Tc generator (Ultratechneflow™, Mallinkrodt, The Netherlands) were added to the mixture. For *in vivo* studies, 40 μL of the radiolabeled microparticle suspension (^{99m}Tc-microparticles; ≈150 MBq) were mixed with 1.75 mg of unlabeled formulation.

2.9. Biodistribution studies in rats

2.9.1. Gastro-intestinal transit studies with ^{99m}Tc-microparticles

These studies were carried out in female Wistar rats weighing 250 g. All the procedures were performed following a protocol previously approved by the Ethics Committee of the University of Navarra in line with the European legislation on animal experiments (protocol 066–16).

Animals were slightly anaesthetised with 2% isoflurane gas for administration of ^{99m}Tc-microparticles, dispersed in 1 mL water, by oral gavage, and then quickly awakened. Each animal received one single dose of radiolabeled microparticles (150 MBq; 0.8–1.0 mg ^{99m}Tc-microparticles) that were completed with up to 10 mg with unlabeled microparticles. The total estimated dose of microencapsulated bacteria was calculated to be 1×10^8 CFU/mL.

Single photon emission computed tomography (SPECT) scans were acquired in a U-SPECT6/E-class (MILabs) using a UHR-RM-1 mm multipinhole collimator. At different times post administration (1-, 4- and 8-h), rats were placed prone on the scanner bed under continuous anesthesia with isoflurane (2% in 100% O_2 gas) to acquire a whole body scan in list mode format over 15 min. Following the SPECT acquisition, CT scans were performed to obtain anatomical information using a tube setting of 55 kV and 0.33 mA. The SPECT images were reconstructed using the ^{99m}Tc photopeak centered at 140 keV with a 20% energy window width and using a calibration factor to obtain the activity information (MBq/mL). Finally, attenuation correction was applied using the CT attenuation map. Studies were visualized using PMOD software (PMOD Technologies Ltd., Adliswil, Switzerland).

2.9.2. Distribution studies in the gut mucosa with fluorescently labeled probiotic bacteria

The fate of probiotics in the gastrointestinal tract of Wistar rats was visualized by fluorescence microscopy after oral administration to laboratory animals (approved protocol by the Ethics Committee: 045–18). For this purpose, fluorescently labeled Lp WCFS1 (free or encapsulated in casein-based microparticles) were dispersed in 1 mL water and orally administered as single of dose of 1×10^8 CFU/mL to rats. For free bacteria, a dose of 3.3×10^{11}/mL was also assayed. Four hours after administration, animals were sacrificed and, for each animal, the whole gastrointestinal tract was removed. Portions of 1 cm [from the stomach, proximal (duodenum), medium (jejunum), and distal (ileum) small intestine, and cecum] were collected, cleaned and stored in the tissue proceeding medium OCT™ before freezing at −80 °C. From these samples, 5-μm sections obtained on a cryostat were attached to glass slides, treated with formaldehyde and DAPI, before visualization in a fluorescence microscope (Axioimager M1, Zeiss, Oberkochen, Germany) with a coupled camera (Axiocam ICc3, Zeiss, Oberkochen, Germany) and fluorescent source (HBO 100, Zeiss, Oberkochen, Germany). The images were captured with the software ZEN (Zeiss, Oberkochen, Germany).

2.10. Immunomodulation potential of encapsulated probiotic

Experiments were performed in compliance with the regulation of the Ethics Committee of the University of Navarra in line with the European legislation on animal experiments (approved protocol 011/13).

CD-1 male mice (20 ± 1 g) purchased from Envigo (Barcelona, Spain) were divided into four randomized groups of 6 animals each. The first group received orally 0.1 mL PBS per day for 21 days (control). The second group of animals was orally treated with a daily aqueous suspension of Lp CECT 220 in 2% sucrose at a dose of 1.0×10^7 CFU/mouse (free LP). The third group of animals received daily the physical mixture between Lp CECT 220 in 2% sucrose (10^7 CFU/mouse) and empty microparticles cross-linked with vanillin (100 μg/mouse). Finally, the fourth group was daily treated with the previously described formulation of LP CECT 220 incorporated into microparticles cross-linked with vanillin (1.0×10^7 CFU/mouse). The total duration of the treatments was 21 days.

On day 22, blood samples were taken and, subsequently, the animals were sacrificed, and the spleens removed, whose cells were isolated in RPMI 1640 medium. The erythrocytes were lysed and the splenocytes concentration was adjusted in complete RPMI medium. On replicas of 100 μL of the cell suspension, Lp CECT 220 was added as a stimulus (10:1 ratio with respect to splenocytes). After 48 h of incubation at 37 °C, 5% CO_2, the cell suspensions were centrifuged and the supernatants containing the released cytokines were kept at −80 °C until use. Cytokine levels were determined by flow cytometer (Attune® Acoustic Focusing Cytometer) using the "BD cytometric bead array Th1/Th2/Th17 CBA" kit (BD, USA). Moreover, cells were labeled with the corresponding specific monoclonal antibodies conjugated with a fluorochrome: CD4 (FITC anti-mouse CD4, Biolegend), CD8 (PE anti-mouse CD8, Biolegend), and levels were determined by flow cytometer (Attune® Acoustic Focusing Cytometer).

2.11. Statistical analysis

The physico-chemical characteristics of nanoparticles as well as the *in vitro* studies were compared using the Student's *t*-test. For *in vivo* studies, comparisons and statistical analyses were performed using a one-way ANOVA test followed by a Tukey-Kramer multiple comparisons test. In all cases, $p < 0.05$ was considered as a statistically significant difference. All calculations were performed using GraphPad Prism v6 (GraphPad Software, San Diego, USA) and the curves were plotted with the Origin 8 software (OriginLab Corp, Northampton, USA).

Table 1
Mean particle size and payload of viable bacteria in the casein-chitosan microparticles. MP: empty microparticles; MP-LP: Lp-loaded casein-chitosan microparticles; MP-LP-V and MP-LP-V2: Lp-loaded microparticles cross-linked with vanillin; MP-LP-TPP: Lp-loaded microparticles cross-linked with TPP; MP-LP-Ca: Lp-loaded microparticles cross-linked with calcium salts. Data expressed as mean ± SD ($n > 3$). *$p < 0.05$ compared to MP-LP.

	Strain *L. plantarum*	Particle size (μm)	Lp loading (Log CFU/g)
MP	–	6.5 ± 1.4	–
MP-LP	Lp CECT 220	7.6 ± 0.7	10.12 ± 0.5
MP-LP-V	Lp CECT 220	7.3 ± 0.5	10.98 ± 0.3 *
MP-LP-V2	Lp WCFS1	7.1 ± 0.4	9.89 ± 0.3
MP-LP-TPP	Lp CECT 220	7.1 ± 0.4	10.34 ± 0.2
MP-LP-Ca	Lp CECT 220	7.4 ± 0.5	10.23 ± 0.3

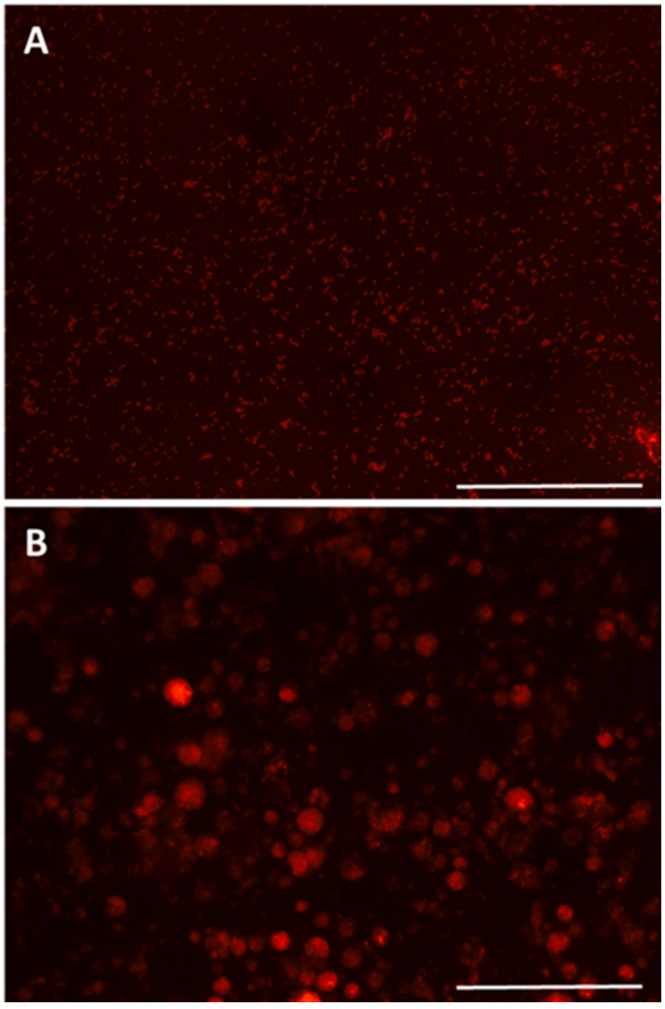

Fig. 1. Optical fluorescence microscopy images of Lp CECT 220 fluorescence-labeled with RBITC in an aqueous suspension (A) or microencapsulated in casein-chitosan microparticles (B). The bar represents 100 μm.

3. Results and discussion

3.1. Characterization of microparticles

Microparticles were prepared by complex coacervation, after promoting the interaction between sodium caseinate and chitosan in an aqueous medium containing Lp. The resulting probiotic-loaded microparticles, after the addition of mannitol, were dried in a spray-drying apparatus. Mannitol was used as drying agent and to facilitate the

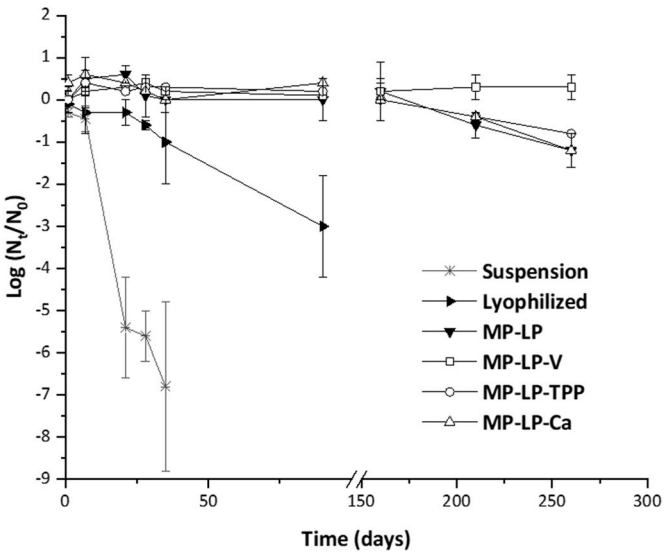

Fig. 2. Survival of Lp CECT 220 under controlled conditions (25 °C/60% RH) over time. Suspension: fresh aqueous suspension in 2% sucrose; Lyophilized: non-encapsulated freeze-dried bacteria; MP-LP: casein-chitosan microparticles; MP-LP-V: microparticles cross-linked with vanillin; MP-LP-TPP: microparticles cross-linked with TPP; MP-LP-Ca: microparticles cross-linked with calcium salts. Data expressed as mean ± SD (n = 3).

redispersion of microparticles in aqueous media. In all cases, the resulting powder presented a moisture content lower than 4%. For MP-LP (probiotic-loaded microparticles) the mean size was close to 7.5 μm (Table 1), slightly higher than empty ones (MP; about 6.5 μm, $p < 0.05$). Likely, Lp cells were efficiently encapsulated in casein-chitosan microparticles (Fig. 1) with a Lp loading of about 10 Log CFU/g.

In order to evaluate the effect of cross-linkage on the survival of loaded bacteria during the preparative process and storage, as well as on the gut conditions, three different cross-linking agents were studied: tripolyphosphate (TPP), calcium salts and vanillin.

TPP ($Na_5P_3O_{10}$) is one of the most employed anionic cross-linkers that may produce inter and intramolecular linkages between TPP phosphates and protonated amino groups in chitosan, producing a network that enhances the mechanical resistance and stability of chitosan-based devices (Helbling et al., 2018; Timilsena, Akanbi, Khalid, Adhikari, & Barrow, 2019).

Similarly, calcium-dependent cross-linking is mediated by the establishment of ionic bonds between a calcium ion and two neighboured negatively charged functional groups of sodium caseinate, facilitating the preparation of micro- and nanoparticles from this protein (Lai, Renneberg, & Mak, 2016; Penalva et al., 2014). Lastly, vanillin, one of the most popular flavouring agents, has been proposed as a natural cross-linker to prepare chitosan-based hydrogels, microparticles and films (Zou, Li, & Li, 2015; Arya, Rookes, Cahill, & Lenka, 2021). The aldehyde moiety of vanillin would react with an amino group of one chitosan molecule through Schiff-base reaction and its hydroxyl moiety would interact (via hydrogen bond) with the hydroxyl or the amino groups in another chitosan molecule (Peng et al., 2010; Xu et al., 2018). Although the ability of vanillin to crosslink protein-based devices has

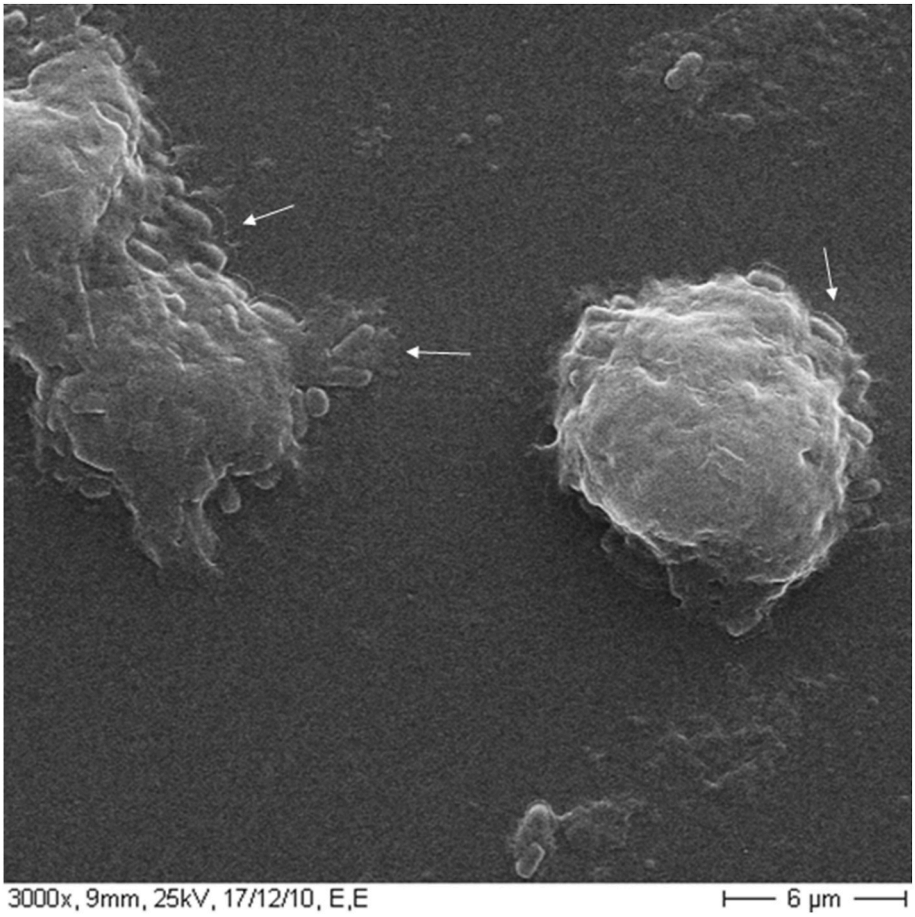

Fig. 3. SEM microphotograph showing Lp CECT 220-loaded microparticles (MP-LP-V), in the process of degradation after 2 h of incubation in SGF and 1 h in SIF. The arrows indicate the presence of bacteria released from the microparticles.

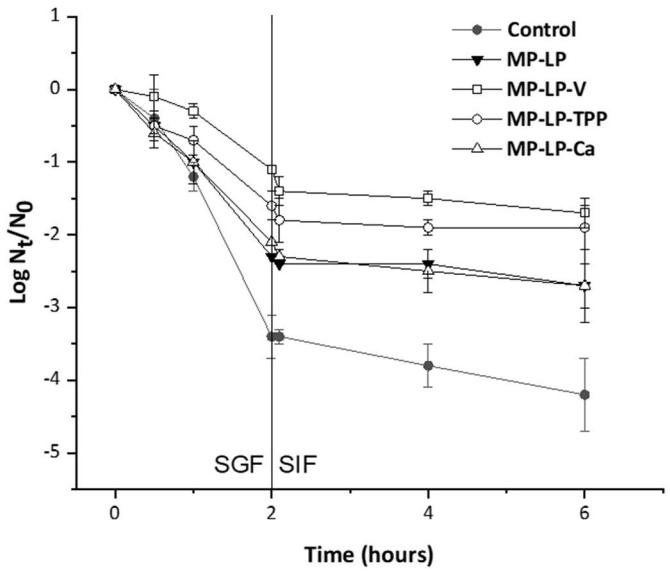

Fig. 4. *In vitro* gastrointestinal resistance of Lp CECT 220. Suspension: fresh aqueous suspension in 2% sucrose; MP-LP: casein-chitosan microparticles; MP-LP-V: microparticles cross-linked with vanillin; MP-LP-TPP: microparticles cross-linked with TPP; MP-LP-Ca: microparticles cross-linked with calcium salts. Data expressed as mean ± SD (n = 3).

not been described in the literature yet, the fact that molecules with aldehyde groups in their structure can establish covalent bonds with primary amino groups (as can be found in proteins), via formation of a Schiff base adduct (Metz et al., 2004), suggests that vanillin would act as cross-linker of chitosan and caseinate.

In this study, the cross-linking process, regardless of the agent used for this purpose, did not modify the average size of the resulting microparticles (Table 1). Overall, the Lp loading increased when microparticles were cross-linked. For Lp CECT220, the amount of viable microencapsulated cells was calculated to be between 1.3×10^{10} and 9.6×10^{10} CFU/g with slightly higher counts for vanillin cross-linked microparticles than for those treated with TPP o calcium salts ($p < 0.05$). For Lp WCFS1, the payload in microparticles cross-linked with vanillin was calculated to be 0.8×10^{10} CFU/g (Table 1).

3.2. Storage stability study

Microencapsulation has been proposed as an adequate technique to improve the viability of probiotics (including Lp) during storage and extend their useful lifetime (O'Riordan, Andrews, Buckle, & Conway, 2001; Oliveira et al., 2007; Albadran, Chatzifragkou, Khutoryanskiy, & Charalampopoulos, 2015). In this context, the possibility of storage at room temperatures, rather than freezing or low temperature conditions, is preferable for minimizing costs of handling and transportation.

The viability of Lp CECT 220, after microencapsulation in casein-chitosan microparticles and packed in glass containers hermetically sealed, was evaluated at 25 °C and 60% RH (Fig. 2). As controls, a freshly prepared aqueous suspension in 2% sucrose and the freeze-dried form of this microorganism were employed. All the microparticle-based formulations provided a significant increase in the survival of Lp CECT 220, when compared with controls. Thus, after 90 days of storage, both the suspension of bacteria and the lyophilized product showed a loss of

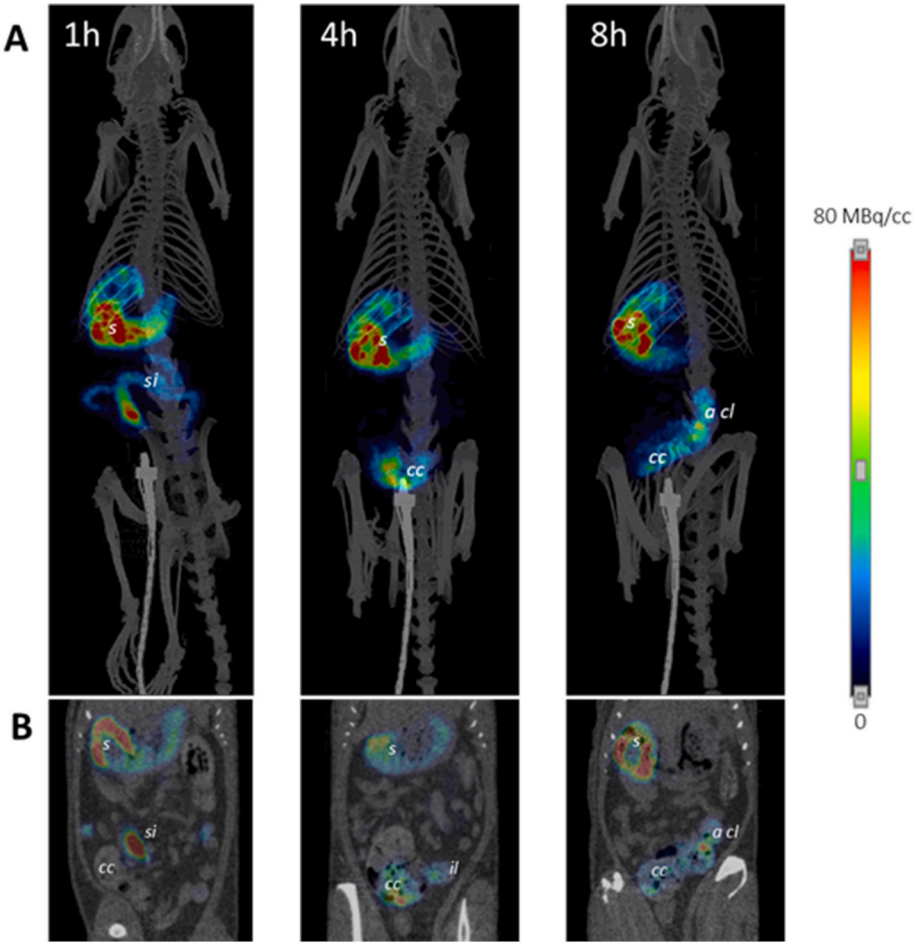

Fig. 5. Gastro-intestinal transit of Lp WCFS1-loaded ^{99m}Tc-labeled microparticles (MP-LP-V2) after oral administration to rats. A: Volume rendered fused SPECT-CT images from a representative animal at different times post-administration (1, 4 and 8 h). B: Representative longitudinal slices of the abdominal area of the rat. The fusion of SPECT image with the CT allow to precisely locate the microparticles along the different parts of the intestine. In order to compare the images correctly, the detected activity was corrected taking into account the radioactive decay of ^{99m}Tc. s: stomach; si: small intestine; cc: cecum; a cl: ascending colon.

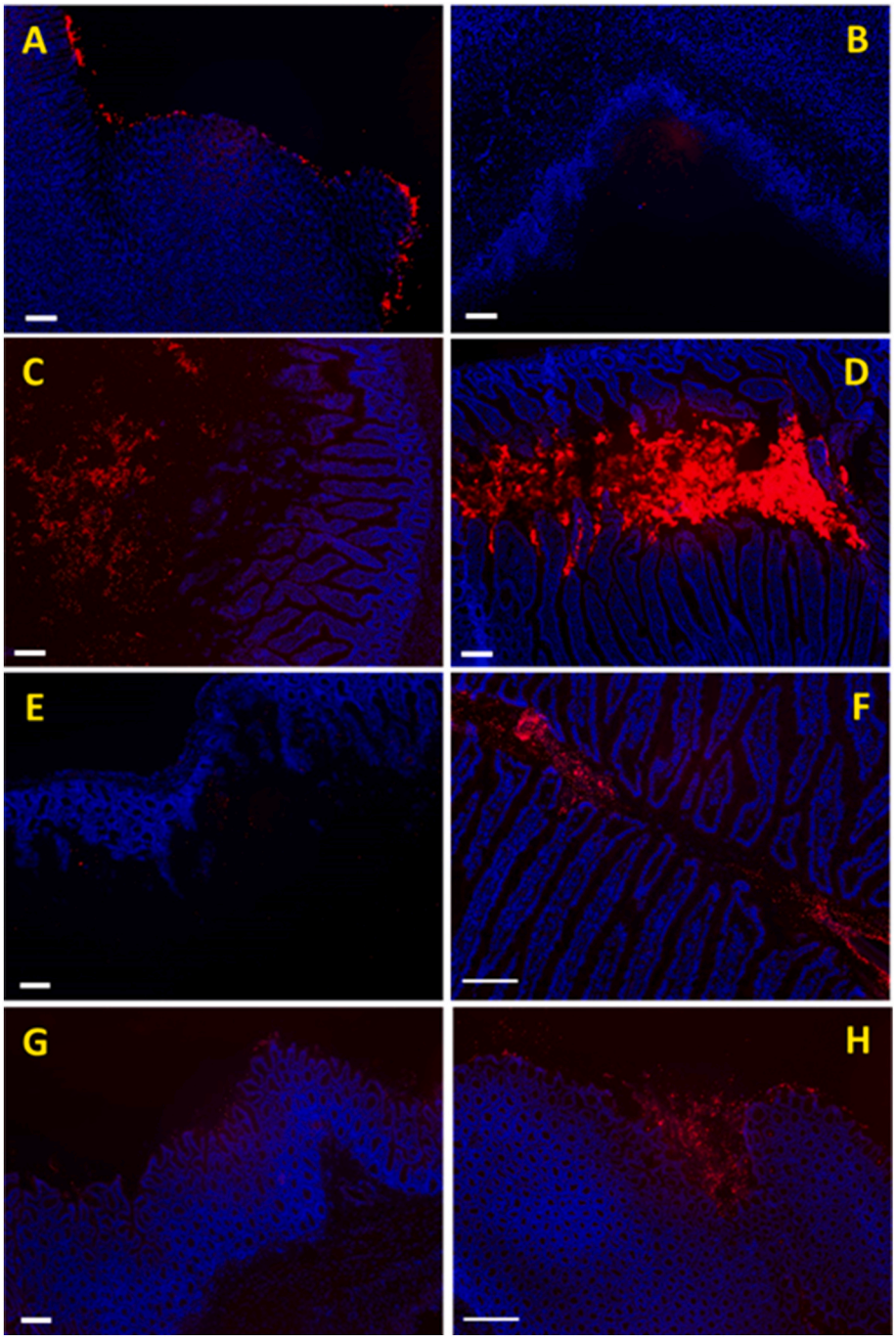

Fig. 6. Fluorescence microscopic visualization of fluorescently labeled Lp WCFS1 formulated as an aqueous suspension (A, C, D, E, G) or encapsulated in casein-chitosan microparticles cross-linked with vanillin (B, F, H). Longitudinal sections of different parts of the gut of rats 4 h post-administration. A and B: stomach; C and D: proximal region of the small intestine; E and F: distal region of the small intestine; G and H: cecum. The length of the bar represents 100 μm.

viability greater than 3 Log. This loss of viability in the lyophilized samples was found to be of the same order as that previously described in the literature (Dhewa, Pant, & Mishra, 2014). In contrast, the microencapsulated bacteria did not show substantial changes in viability for at least 6 months of storage. At the end of the experiment (approx. 8 months), the formulation offering the greatest protection against inactivation of the encapsulated bacteria was MP-LP-V that presented a bacterial load similar to that defined at the beginning of the study. For the other microparticle formulations, the viability loss was approximately of 1 Log in 260 days of storage at 25 °C.

3.3. *In vitro gastrointestinal resistance study*

Another important challenge associated to the oral administration of Lp (and other probiotics) is a relatively low tolerance to resist the harsh conditions encountered in the gastrointestinal tract; particularly, their high susceptibility to acidic pH conditions (Cook, Tzortzis, Charalampopoulos, & Khutoryanskiy, 2012; Hlaing et al., 2020). Thus, one important aspect in the development of successful probiotic delivery system should be the capability of such system to offer a significant degree of protection facilitating the survival of encapsulated microorganisms in the gut environment long enough to reach the intestinal surfaces where they colonize and provide health benefits.

In this context, our strategy consisted of developing microcapsules able to resist acidic pH conditions and slowly degrade under pH conditions close to the neutrality, in which their content should be released. Fig. 3 shows a SEM image of Lp-loaded microparticles when incubated in SGF (pH 2.5) for 2 h and, then, in SIF (pH 6.8) for 1 h. Under acidic pH conditions, no signs of erosion were found (data not shown). However, when samples were incubated under neutral pH conditions, microparticles displayed obvious signs of erosion and some released bacteria were observed.

The *in vitro* gastrointestinal resistance study was performed in simulated gastric and intestinal fluids (Fig. 4). After 2 h of incubation in SGF, the number of viable bacteria in the control formulation (aqueous suspension in 2% sucrose) was reduced in more than 3.5 log. On the contrary, for the different formulations of microparticles tested, the number of viable bacteria was significantly higher than for the control (reduction between 1.2 and 2.2 Log; $p < 0.01$). This reduction was slightly higher for MP-LP and MP-LP-Ca formulations than for the TPP- or vanillin-crosslinked microparticles. Under SIF conditions, the viability of the microencapsulated bacteria did not vary significantly; which contrasts with the viability reduction (more moderate than under SGF conditions) observed in the control formulation. Again, the cross-linking of the microparticles with either vanillin or TPP treatments offered a significantly higher protection against bacterial inactivation than cross-linkage with calcium salts ($p < 0.05$).

3.4. Biodistribution studies in rats

In order to study the ability of casein-chitosan microparticles to reach the distal areas of the small intestine and colon, where the loaded bacteria should be released to facilitate its implantation, two complementary studies were carried out. First, the gastrointestinal transit of microparticles, containing Lp WCFS1, radiolabeled with ^{99m}Tc was evaluated. Second, the microscopic distribution of fluorescently labeled Lp WCFS1 encapsulated in microparticles, was studied. In all cases, microparticles and bacteria were orally administered by gavage after dispersion in water.

Fig. 5 shows the gastro-intestinal transit of MP-LP-V2 (Lp WCFS1-loaded microparticles cross-linked with vanillin), radiolabeled with ^{99m}Tc, after oral administration. During the overall experiment, a high intensity of radioactivity was observed in the stomach of animals. This observation may be explained by the release of a fraction of the radioactive tag bound to the surface of microparticles inside the stomach and/or by the high binding affinity of pertechnetate for the stomach mucosa (Kiratli, Aksoy, Bozkurt, & Orhan, 2009). In spite of this aspect, casein-chitosan microparticles moved rapidly along the gastrointestinal tract. Thus, after 4 h post-administration, radiolabeled microparticles were found in the ileum and the cecum of the animals. Eight hours post-administration, radioactivity signals were also observed in the ascending colon.

Fig. 6 shows fluorescence microscopy images of gut samples from animals treated with fluorescently labeled bacteria formulated as aqueous suspension or encapsulated in casein-chitosan microparticles (MP-LP-V2) at a dose of 1×10^8 CFU/mL. The biodistribution of Lp, when administered as aqueous suspension, appeared to be different to that observed for the microencapsulated formulation. Thus, 4 h post-administration, the presence of bacteria seemed to be restricted to the surface of the stomach mucosa (Fig. 6A) and the lumen of the proximal area of the small intestine (Fig. 6C). In contrast, in the distal areas of the intestine and the cecum, no relevant presence of fluorescent signal was observed (Fig. 6E and G). A similar biodistribution was found with a more concentrated Lp WCFS1 aqueous suspension (3.3×10^{11} CFU/mL); although, in this case, bacteria appeared as large aggregates in the proximal region of the small intestine (Fig. 6D). On the contrary, the microencapsulation of bacteria offered a completely different biodistribution within the gut. As a result, 4 h post-administration, fluorescent signals were only observed in the distal areas of the small intestine (ileum, Fig. 6F) and cecum (Fig. 6H). Conversely, no evidence of the presence of bacteria in the stomach (Fig. 6B) and duodenum of the animals were found. All these findings suggest that casein-chitosan microparticles may be an adequate strategy to promote the arrival of the loaded probiotics to the colon.

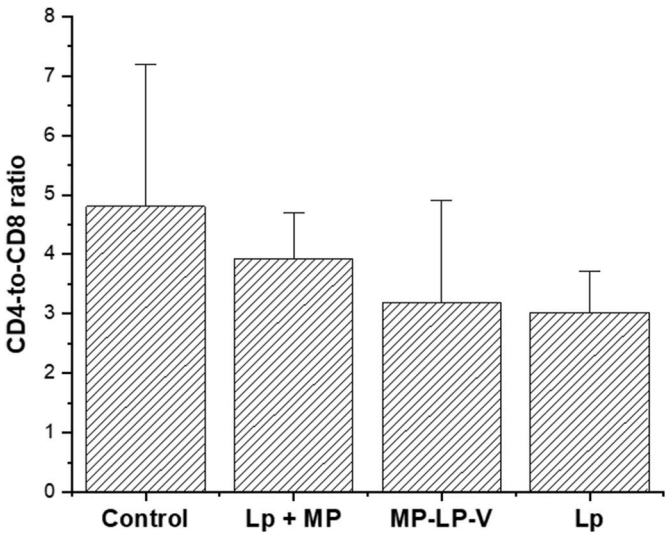

Fig. 7. Effect of a daily administration of Lp CECT 220 in CD1 mice on the splenic CD4-to-CD8 ratio (daily dose of 1.0×10^7 CFU/g for 3 weeks). LP free (aqueous suspension of Lp); MP-LP-V (Lp CECT 220 microencapsulated in casein-chitosan microparticles cross-linked with vanillin); Lp + MP (physical mixture of Lp CECT 220 and empty casein-based microparticles cross-linked with vanillin). The results are shown as the mean levels for triplicate determination ± SD.

3.5. Immunization study

Fig. 7 shows the effect of the daily administration of 10^7 CFU/mouse (for 3 weeks) of Lp CECT 220 (either free, as physical mixture with empty microparticles or microencapsulated) on the CD4-to-CD8 ratio in mice. All the treatments displayed a slight decrease of this ratio, compared with control values; although no statistically significant differences were found. The microencapsulation of Lp in microparticles did not affect the ability of the bacteria to alter the CD4+/CD8+ ratio.

The production of cytokines by splenocytes restimulated with Lp CECT 220 *in vitro* was determined at day 22 (Fig. 8). Results indicated that the oral administration of Lp (free or microencapsulated in MP-LP-V) induced an increase in the release of Th1 and Th17 cytokines (particularly IFN-γ, IL-2 and IL-6 and IL-17, respectively). These findings appear to be in line with previous studies in mice describing the bias toward a Th1 response by different Lp strains (Ren, Wang, Liu, Shen, & Yu, 2019; Smelt et al., 2012; Wiese et al., 2012). However, in our study, the cytokine profile was different for Lp and MP-Lp-V groups. For the Lp group, the re-stimulation of splenocytes mainly promoted the secretion of IL-6; whereas, for the MP-Lp-V group, the re-stimulation basically produced IFN-γ. This different behaviour for Lp (free or microencapsulated) is clearly expressed when representing the IFN-γ-to-IL6 ratio as a function of the treatment orally administered (Fig. 8H). IL-6 is considered the main pro-inflammatory cytokine and, thus, is recognized as a biomarker and potential target to control inflammatory diseases (Gabay, 2006; Ridker & Rane, 2021). Moreover, IL-6 promotes Th2 differentiation and interferes with IFN-g signalling and the development of Th1 cells (Diehl et al., 2000). Thus, the favoured modulation of the Th1 and Th17 immune response rather than the Th2 response by administration of microencapsulated Lp may be beneficial for treating mucosal infections, allergies, or cancer (Marques et al., 2021; Nguyen et al., 2021).

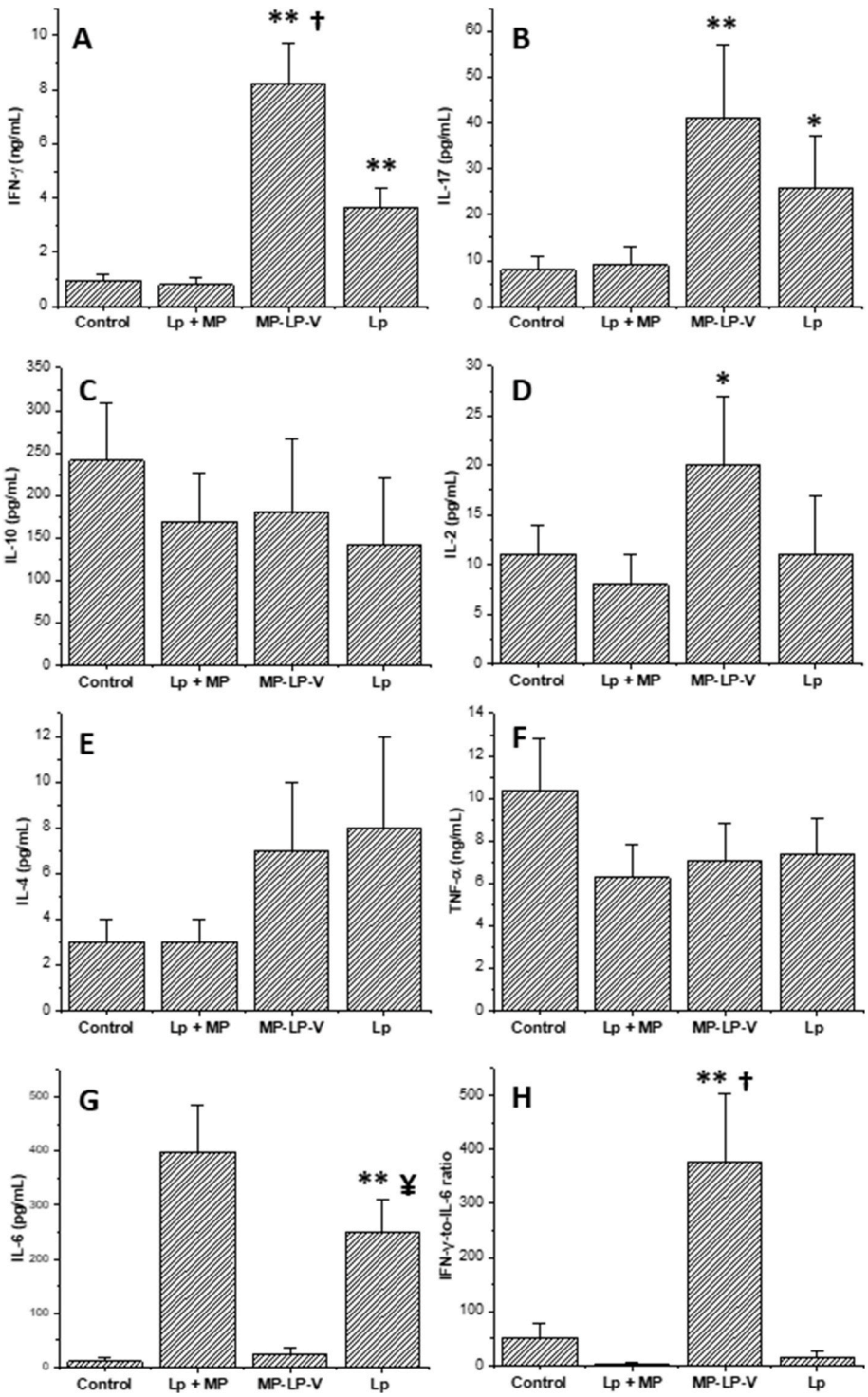

Fig. 8. Splenic cytokine profile after treatment of CD1 mice with Lp CECT 220 (daily dose of 1.0×10^7 CFU/g during 3 weeks). LP free (aqueous suspension of Lp); MP-LP-V (Lp CECT 220 microencapsulated in casein-chitosan microparticles cross-linked with vanillin); Lp + MP (physical mixture of Lp CECT 220 and empty casein-based microparticles cross-linked with vanillin). Production of cytokines by splenocytes restimulated with Lp *in vitro* was determined at day 22. The results are shown as the mean levels for triplicate determination ± SD (*$p < 0.05$ for treated mice (Lp or MP-LP-V groups) vs. control; **$p < 0.01$ for treated mice (Lp or MP-LP-V groups) vs. control; †$p < 0.01$ for MP-LP-V vs. Lp; ¥ $p < 0.01$ for Lp vs. MP-LP-V).

4. Conclusions

In conclusion, the microencapsulation of *L. plantarum* into casein-chitosan microparticles significantly improved the viability of the probiotic during storage, particularly when the resulting microparticles were cross-linked with vanillin (MP-LP-V formulation). Moreover, MP-LP-V also conferred the highest protection when incubated in simulated gastric and intestinal fluids. Further, the biodistribution studies in rat evidenced that MP-LP-V displayed an important ability to target the distal small intestine and colon areas where, after release of the loaded probiotic, facilitate its implantation and the generation of beneficial effects. Thus, microencapsulated Lp CECT 220, after oral administration, induced an immunomodulatory effect characterized by an important enhancement of Th1 and Th17 responses. Hence, the study findings suggest that these microparticles might be a promising strategy for increasing survival and efficacy of probiotics, allowing the formulation of cost-effective and more stable and effective probiotic-based nutraceuticals useful for treating mucosal infections, allergies, or other diseases where a Th1 and/or Th17 response would be beneficial.

Author statement

Rebeca Peñalva: conceptualization, methodology, data curation, investigation, software, visualization, formal analysis, writing - original draft.

Ana Luisa Martínez-López: conceptualization, methodology, data curation, investigation, formal analysis.

Carlos Gamazo: conceptualization, methodology, writing – review & editing, supervision.

Carlos J. González-Navarro: conceptualization, writing – review & editing, resources.

Carolina González-Ferrero: methodology, data curation, writing – review & editing.

Raquel Virto-Resano: methodology, data curation, resources.

Ana Brotons-Canto: methodology, data curation, resources.

Ana Isabel Vitas: methodology, formal analysis.

Maria Collantes: conceptualization, methodology, formal analysis, writing – review & editing.

Ivan Peñuelas: conceptualization, methodology, writing – review & editing, supervision.

Juan M. Irache: conceptualization, resources, writing – review & editing, funding acquisition, supervision, project administration.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Ana Brotons-Canto reports a relationship with Nucaps Nanotechnology SL that includes: employment.

Data availability

Data will be made available on request.

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References

Albadran, H. A., Chatzifragkou, A., Khutoryanskiy, V. V., & Charalampopoulos, D. (2015). Stability of probiotic *Lactobacillus plantarum* in dry microcapsules under accelerated storage conditions. *Food Research International, 74*, 208–216. https://doi.org/10.1016/j.foodres.2015.05.016

Arya, S., Rookes, J. E., Cahill, D. M., & Lenka, S. K. (2021). Vanillin: A review on the therapeutic prospects of a popular flavouring molecule. *Adv Tradit Med, 21*, 1–17. https://doi.org/10.1007/s13596-020-00531-w

Behera, S. S., Ray, R. C., & Zdolec, N. (2018). *Lactobacillus plantarum* with functional properties: An approach to increase safety and shelf-life of fermented foods. *BioMed Research International, 2018*, Article 9361614. https://doi.org/10.1155/2018/9361614

Bibbò, S., Ianiro, G., Giorgio, V., Scaldaferri, F., Masucci, L., Gasbarrini, A., et al. (2016). The role of diet on gut microbiota composition. *European Review for Medical and Pharmacological Sciences, 20*, 4742–4749.

Binda, S., Hill, C., Johansen, E., Obis, D., Pot, B., Sanders, M. E., et al. (2020). Criteria to qualify microorganisms as "probiotic" in foods and dietary supplements. *Frontiers in Microbiology, 11*, 1662. https://doi.org/10.3389/fmicb.2020.01662

Cook, M. T., Tzortzis, G., Charalampopoulos, D., & Khutoryanskiy, V. V. (2012). Microencapsulation of probiotics for gastrointestinal delivery. *Journal of Controlled Release, 162*, 56–67. https://doi.org/10.1016/j.jconrel.2012.06.003

Dhewa, T., Pant, S., & Mishra, V. (2014). Development of freeze dried synbiotic formulation using a probiotic strain of *Lactobacillus plantarum*. *Journal of Food Science & Technology, 51*, 83–89. https://doi.org/10.1007/s13197-011-0457-2

Diehl, S., Anguita, J., Hoffmeyer, A., Zapton, T., Ihle, J. N., Fikrig, E., et al. (2000). Inhibition of Th1 differentiation by IL-6 is mediated by SOCS1. *Immunity, 13*, 805–815. https://doi.org/10.1016/s1074-7613(00)00078-9

Duary, R. K., Bhausaheb, M. A., Batish, V. K., & Grover, S. (2012). Anti-inflammatory and immunomodulatory efficacy of indigenous probiotic *Lactobacillus plantarum* Lp91 in colitis mouse model. *Molecular Biology Reports, 39*, 4765–4775. https://doi.org/10.1007/s11033-011-1269-1

Gabay, C. (2006). Interleukin-6 and chronic inflammation. *Arthritis Research and Therapy, 8*, S3. https://doi.org/10.1186/ar1917

González-Ferrero, C., Irache, J. M., & González-Navarro, C. J. (2018). Soybean protein-based microparticles for oral delivery of probiotics with improved stability during storage and gut resistance. *Food Chemistry, 239*, 879–888. https://doi.org/10.1016/j.foodchem.2017.07.022

Helbling, I. M., Busatto, C. A., Fioramonti, S. A., Pesoa, J. I., Santiago, L., Estenoz, D. A., et al. (2018). Preparation of TPP-crosslinked chitosan microparticles by spray drying for the controlled delivery of progesterone intended for estrus synchronization in cattle. *Pharmaceutical Research, 35*, 66. https://doi.org/10.1007/s11095-018-2363-z

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., et al. (2014). Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nature Reviews Gastroenterology & Hepatology, 11*, 506–514. https://doi.org/10.1038/nrgastro.2014.66.E

Hills, R. D., Jr., Pontefract, B. A., Mishcon, H. R., Black, C. A., Sutton, S. C., & Theberge, C. R. (2019). Gut microbiome: Profound implications for diet and disease. *Nutrients, 11*, 1613. https://doi.org/10.3390/nu11071613

Hlaing, S. P., Kim, J., Lee, J., Kwak, D., Kim, H., & Yoo, J. W. (2020). Enhanced viability of probiotics against gastric acid by one-step coating process with poly-L-lysine: In vitro and in vivo evaluation. *Pharmaceutics, 12*, 662. https://doi.org/10.3390/pharmaceutics12070662

Jiang, T., Kim, Y. K., Singh, B., Kang, S. K., Choi, Y. J., & Cho, C. S. (2013). Effect of microencapsulation of *Lactobacillus plantarum* 25 into alginate/chitosan/alginate microcapsules on viability and cytokine induction. *Journal of Nanoscience and Nanotechnology, 13*, 5291–5295. https://doi.org/10.1166/jnn.2013.7042

Khan, I., Ullah, N., Zha, L., Bai, Y., Khan, A., Zhao, T., et al. (2019). Alteration of gut microbiota in inflammatory bowel disease (IBD): Cause or consequence? IBD treatment targeting the gut microbiome. *Pathogens, 8*, 126. https://doi.org/10.3390/pathogens8030126

Kim, S. K., Guevarra, R. B., Kim, Y. T., Kwon, J., Kim, H., Cho, J. H., et al. (2019). Role of probiotics in human gut microbiome-associated diseases. *Journal of Microbiology and Biotechnology, 29*, 1335–1340. https://doi.org/10.4014/jmb.1906.06064

Kiratli, P. O., Aksoy, T., Bozkurt, M. F., & Orhan, D. (2009). Detection of ectopic gastric mucosa using 99mTc pertechnetate: Review of the literature. *Annals of Nuclear Medicine, 23*, 97–105. https://doi.org/10.1007/s12149-008-0204-6

Lai, K. K., Renneberg, R., & Mak, W. C. (2016). High efficiency single-step biomaterial-based microparticles fabrication via template-directed supramolecular coordination chemistry. *Green Chemistry, 18*, 1715–1723. https://doi.org/10.1039/C5GC02424B

Li, W., Liu, L., Tian, H., Luo, X., & Liu, S. (2019). Encapsulation of *Lactobacillus plantarum* in cellulose based microgel with controlled release behavior and increased long-term storage stability. *Carbohydrate Polymers, 223*, Article 115065. https://doi.org/10.1016/j.carbpol.2019.115065

Liu, Y. W., Liong, M. T., & Tsai, Y. C. (2018). New perspectives of *Lactobacillus plantarum* as a probiotic: The gut-heart-brain axis. *Journal of Microbiology, 56*, 601–613. https://doi.org/10.1007/s12275-018-8079-2

Mahmoud, M., Abdallah, N. A., El-Shafei, K., Tawfik, N. F., & El-Sayed, H. S. (2020). Survivability of alginate-microencapsulated *Lactobacillus plantarum* during storage, simulated food processing and gastrointestinal conditions. *Heliyon, 6*, Article e03541. https://doi.org/10.1016/j.heliyon.2020.e03541

Marques, H. S., de Brito, B. B., da Silva, F. A. F., Santos, M. L. C., de Souza, J. C. B., Correia, T. M. L., et al. (2021). Relationship between Th17 immune response and cancer. *World Journal of Clinical Oncology, 12*, 845–867. https://doi.org/10.5306/wjco.v12.i10.845

Metz, B., Kersten, G. F., Hoogerhout, P., Brugghe, H. F., Timmermans, H. A., de Jong, A., et al. (2004). Identification of formaldehyde-induced modifications in proteins: Reactions with model peptides. *Journal of Biological Chemistry, 279*, 6235–6243. https://doi.org/10.1074/jbc.M310752200

Nguyen, N., Guleed, S., Olsen, A. W., Follmann, F., Christensen, J. P., & Dietrich, J. (2021). Th1/Th17 T cell tissue-resident immunity increases protection, but is not required in a vaccine strategy against genital infection with *Chlamydia trachomatis*. *Frontiers in Immunology, 12*, Article 790463. https://doi.org/10.3389/fimmu.2021.790463

Oliveira, A. C., Moretti, T. S., Boschini, C., Baliero, J. C. C., Freitas, O., & Favaro-Trindade, C. S. (2007). Stability of microencapsulated *B. lactis* (BI 01) and *L. acidophilus* (LAC 4) by complex coacervation followed by spray drying. *Journal of Microencapsulation, 24*, 673–681. https://doi.org/10.1080/02652040701532908

O'Riordan, K., Andrews, D., Buckle, K., & Conway, P. (2001). Evaluation of microencapsulation of a *Bifidobacterium* strain with starch as an approach to prolonging viability during storage. *Journal of Applied Microbiology, 91*, 1059–1066. https://doi.org/10.1046/j.1365-2672.2001.01472.x

Paolillo, R., Carratelli, C. R., Sorrentino, S., Mazzola, N., & Rizzo, A. (2009). Immunomodulatory effects of *Lactobacillus plantarum* on human colon cancer cells. *International Immunopharmacology, 9*, 1265–1271. https://doi.org/10.1016/j.intimp.2009.07.008

Parlindungan, E., Dekiwadia, C., May, B. K., & Jones, O. A. H. (2019). Nutrient stress as a means to enhance the storage stability of spray dried microencapsulated *Lactobacillus plantarum* B21. *Lebensmittel-Wissenschaft und -Technologie, 111*, 204–210. https://doi.org/10.1016/j.lwt.2019.05.010

Paula, D. A., Martins, E. M. F., Costa, N. A., de Oliveira, P. M., de Oliveira, E. B., & Ramos, A. M. (2019). Use of gelatin and gum Arabic for microencapsulation of probiotic cells from *Lactobacillus plantarum* by a dual process combining double emulsification followed by complex coacervation. *International Journal of Biological Macromolecules, 133*, 722–731. https://doi.org/10.1016/j.ijbiomac.2019.04.110

Penalva, R., Esparza, I., Agüeros, M., Gonzalez-Navarro, C. J., Gonzalez-Ferrero, C., & Irache, J. M. (2014). Casein nanoparticles as carriers for the oral delivery of folic acid. *Food Hydrocolloids, 44*, 399–406. https://doi.org/10.1016/j.foodhyd.2014.10.004

Peng, H., Xiong, H., Li, J., Xie, M., Liu, Y., Bai, C., et al. (2010). Vanillin cross-linked chitosan microspheres for controlled release of resveratrol. *Food Chemistry, 121*, 23–28. https://doi.org/10.1016/j.foodchem.2009.11.085

Rajam, R., Kumar, S. B., Prabhasankar, P., & Anandharamakrishnan, C. (2015). Microencapsulation of *Lactobacillus plantarum* MTCC 5422 in fructooligosaccharide and whey protein wall systems and its impact on noodle quality. *Journal of Food Science & Technology, 52*, 4029–4041. https://doi.org/10.1007/s13197-014-1506-4

Ren, D., Wang, D., Liu, H., Shen, M., & Yu, H. (2019). Two strains of probiotic *Lactobacillus* enhance immune response and promote naive T cell polarization to Th1. *Food and Agricultural Immunology, 30*, 281–295. https://doi.org/10.1080/09540105.2019.1579785

Ridker, P. M., & Rane, M. (2021). Interleukin-6 signaling and anti-interleukin-6 therapeutics in cardiovascular disease. *Circulation Research, 128*, 1728–1746. https://doi.org/10.1161/CIRCRESAHA.121.319077

Sánchez-Martínez, M., da Costa Martins, R., Quincoces, G., Gamazo, C., Caicedo, C., Irache, J. M., et al. (2013). Radiolabeling and biodistribution studies of polymeric nanoparticles as adjuvants for ocular vaccination against brucellosis. *Revista Española de Medicina Nuclear e Imagen Molecular, 32*(2), 92–97. https://doi.org/10.1016/j.remn.2012.11.005

Santarmaki, V., Kourkoutas, Y., Zoumpopoulou, G., Mavrogonatou, E., Kiourtzidis, M., Chorianopoulos, N., et al. (2017). Survival, intestinal mucosa adhesion, and immunomodulatory potential of *Lactobacillus plantarum* strains. *Current Microbiology, 74*, 1061–1067. https://doi.org/10.1007/s00284-017-1285-z

Simon, E., Călinoiu, L. F., Mitrea, L., & Vodnar, D. C. (2021). Probiotics, prebiotics, and synbiotics: Implications and beneficial effects against irritable bowel syndrome. *Nutrients, 13*, 2112. https://doi.org/10.3390/nu13062112

Smelt, M. J., de Haan, B. J., Bron, P. A., van Swam, I., Meijerink, M., Wells, J. M., et al. (2012). *L. plantarum, L. salivarius*, and *L. lactis* attenuate Th2 responses and increase Treg frequencies in healthy mice in a strain dependent manner. *PLoS One, 7*, Article e47244. https://doi.org/10.1371/journal.pone.0047244

Sonnenburg, J. L., Chen, C. T. L., & Gordon, J. I. (2006). Genomic and metabolic studies of the impact of probiotics on a model gut symbiont and host. *PLoS Biology, 4*, e413. https://doi.org/10.1371/journal.pbio.0040413

Tandon, D., Haque, M. M., Saravanan, R., Shaikh, S., Sriram, P., Dubey, A. K., et al. (2018). A snapshot of gut microbiota of an adult urban population from Western region of India. *PLoS One, 13*, Article e0195643. https://doi.org/10.1371/journal.pone.0195643

Timilsena, Y. P., Akanbi, T. O., Khalid, N., Adhikari, B., & Barrow, C. J. (2019). Complex coacervation: Principles, mechanisms and applications in microencapsulation. *International Journal of Biological Macromolecules, 121*, 1276–1286. https://doi.org/10.1016/j.ijbiomac.2018.10.144

de Vos, P., Mujagic, Z., de Haan, B. J., Siezen, R. J., Bron, P. A., Meijerink, M., et al. (2017). *Lactobacillus plantarum* strains can enhance human mucosal and systemic immunity and prevent non-steroidal anti-inflammatory drug induced reduction in T regulatory cells. *Frontiers in Immunology, 8*, 1000. https://doi.org/10.3389/fimmu.2017.01000

Wang, T., Teng, K., Liu, Y., Shi, W., Zhang, J., Dong, E., et al. (2019). *Lactobacillus plantarum* PFM 105 promotes intestinal development through modulation of gut microbiota in weaning piglets. *Frontiers in Microbiology, 10*, 90. https://doi.org/10.3389/fmicb.2019.00090

Wei, Z., Chuantao, P., Arbab, S. H., Lai-Yu, K., & Wenyi, Z. (2021). Meta-Analysis: Randomized trials of *Lactobacillus plantarum* on immune regulation over the last decades. *Frontiers in Immunology, 12*, Article 643420. https://doi.org/10.3389/fimmu.2021.643420

Wieërs, G., Belkhir, L., Enaud, R., Leclercq, S., Philippart de Foy, J. M., Dequenne, I., et al. (2020). How probiotics affect the microbiota. *Frontiers in Cellular and Infection Microbiology, 9*, 454. https://doi.org/10.3389/fcimb.2019.00454

Wiese, M., Eljaszewicz, A., Andryszczyk, M., Gronek, S., Gackowska, L., Kubiszewska, I., et al. (2012). Immunomodulatory effects of *Lactobacillous plantarum* and *Helicobacter pylori* CagA$^+$ on the expression of selected superficial molecules on monocyte and lymphocyte and the synthesis of cytokines in whole blood culture. *Journal of Physiology & Pharmacology, 63*, 217–224.

Xu, C., Zhan, W., Tang, X., Mo, F., Fu, L., & Lin, B. (2018). Self-healing chitosan/vanillin hydrogels based on Schiff-base bond/hydrogen bond hybrid linkages. *Polymer Testing, 66*, 155–163. https://doi.org/10.1016/j.polymertesting.2018.01.016

Zou, Q., Li, J., & Li, Y. (2015). Preparation and characterization of vanillin-crosslinked chitosan therapeutic bioactive microcarriers. *International Journal of Biological Macromolecules, 79*, 736–747. https://doi.org/10.1016/j.ijbiomac.2015.05.037