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Research paper

Development of a time-dependent oral colon delivery system of anaerobic *Odoribacter splanchnicus* for bacteriotherapy

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A R T I C L E I N F O

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A B S T R A C T

Odoribacter (O.) splanchnicus is an anaerobic member of the human intestinal microbiota. Its decrease in abundance has been associated with inflammatory bowel disease (IBD), non-alcoholic fatty liver, and cystic fibrosis. Considering the anti-inflammatory properties of *O. splanchnicus* and its possible use for IBD, intestinal isolate *O. splanchnicus* 57 was here formulated for oral colonic release based on a time-dependent strategy. Freeze-drying protocol was determined to ensure *O. splanchnicus* 57 viability during the process. Disintegrating tablets, containing the freeze-dried *O. splanchnicus* 57, were manufactured by direct compression and coated by powder-layering technique with hydroxypropyl methylcellulose (Methocel™ E50) in a tangential-spray fluid bed. Eudragit® L was then applied by spray-coating in a top-spray fluid bed. Double-coated tablets were tested for release, showing gastric resistance properties and, as desired, lag phases of reproducible duration prior to release in phosphate buffer pH 6.8. The cell viability and anti-inflammatory activity of the strain were assessed after the main manufacturing steps. While freeze-drying did not affect bacterial viability, the tableting and coating processes were more stressful. Nonetheless, *O. splanchnicus* 57 cells survived manufacturing and the final formulations had 10^6-10^7 CFU/g of viable cells. The strain kept its anti-inflammatory properties after tableting and coating, reducing *Escherichia coli* lipopolysaccharide-induced interleukin-8 cytokine release from HT-29 cells. Overall, *O. splanchnicus* 57 strain was formulated successfully for oral colon delivery, opening new ways to formulate pure cultures of single anaerobic strains or mixtures for oral delivery.

1. Introduction

Odoribacter (O.) splanchnicus is a gram-negative, mesophilic, anaerobic member of the human intestinal microbiota and short-chain fatty acid (SCFA) producer, belonging to the order Bacteroidales within the family 'Porphyromonadaceae' [1,2]. It was formerly named *Bacteroides splanchnicus* due to the similarities with the members of the *Bacteroides* genus, but it was reclassified in 2008 by Hardham *et al.* to a new genus, *Odoribacter*, based on its biochemical, morphological, and molecular phylogenetic characteristics [3]. Although it shares a significant number of characteristics with the genus *Bacteroides*, several biochemical features differ from other *Bacteroides* species [4]. Regardless of being classified as a potential opportunistic pathogen when found in extra-intestinal locations, its beneficial properties and commensal role derived from SCFAs production have weighted *O. splanchnicus* to be considered a key component of the gut microbiota [5,6]. Its beneficial functions include promoting healing during colitis, modulating regulatory T-cell responses, and contributing to overall gut health [7].

O. splanchnicus is considered to establish a beneficial interaction with the human host, and a decrease in its abundance is related to a wide range of pathologies, such as inflammatory bowel disease (IBD), non-alcoholic fatty liver (NAFL), and cystic fibrosis [8–10]. A decrease in SCFA levels in the gut together with a lower presence of their dominant bacterial producers were related to active IBD compared with healthy

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controls [11]. As an SCFA-producing organism, *O. splanchnicus* contributes to the production of butyrate, which is absorbed and metabolized by the epithelium leading to the consumption of (local) O_2 and stabilization of the hypoxia-inducible factor [11]. A drop in butyrate levels and an increase in O_2 levels in the gut epithelium may favour facultatively anaerobic, pro-inflammatory species such as *Escherichia coli* and *Salmonella* spp. [12]. Also, butyrate reduces gut hyperpermeability by increasing expression of tight-junction proteins, thereby decreasing inflammation and endotoxemia derived from leaky gut syndrome [6].

O. splanchnicus has been described to be part of the immune-reactive bacterial core strains that are transferred during fecal microbiota transplantation (FMT) from donor to recipient [5]. The study identified *O. splanchnicus* as species that correlated with the clinical response of ulcerative colitis (UC) patients to FMT therapy. Furthermore, in UC mice model, *O. splanchnicus* shaped mucosal immunity, increased Foxp3$^+$/ROR γ t$^+$ regulatory T cells, and induced interleukin 10 (IL-10) [5].

Given the anti-inflammatory properties of *O. splanchnicus* and its possible therapeutic use for IBD, colon delivery would be highly beneficial to protect the bacteria from the hostile upper gastrointestinal environment [13]. Nowadays, encapsulation of anaerobic bacteria is the most used technique to formulate anaerobic bacteria, such as *Bifidobacterium* spp. for oral delivery [14]. The challenge is ensuring that probiotic bacteria reach the colon in viable, active form after being exposed to a wide range of pH (from pH 1–3 in the stomach and increasing above pH 7 in the intestine), enzymes, metabolites, and presence of food or drugs. Some bacteria like *Bifidobacterium* spp. can resist the acidic environment of the stomach and other harsh upper gastrointestinal conditions [15], however, most of the anaerobic species that are considered as next-generation probiotics cannot survive transit through the upper gastrointestinal tract, due to the lack of cell protective systems [16]. Therefore, new oral formulation technologies need to be employed to deliver these bacteria to the desired site with the maximum number of active cells [17].

The colonic region is a highly favourable environment for anaerobic bacteria, and it harbours the densest population of the gut microbiome, especially anaerobes [18]. The delivery of viable anaerobic bacteria to the colon by oral route is a challenge since there is a need to protect the bacterial cells from O_2 during manufacture, storage, and from the hostile conditions of the upper intestinal tract.

Several formulation strategies have been proposed for colon delivery purposes, including exploitation of small intestinal transit time [19]. Swellable/erodible hydrophilic polymer layers have been applied when a time-controlled delivery is sought [20]. Particularly, a coated system was developed having a low-viscosity hydroxypropyl methylcellulose (HPMC) coating to delay the onset of release from a drug-containing core [21]. Additionally, an outer enteric coating was needed to protect the system from aqueous fluids during unpredictable residence in the stomach, so that time-based colon delivery could be achieved. Eudragit® L-30 D55 was used to this end. Spray-coating was mainly employed to apply the HPMC layer to tablets and capsules. However, it was recently reported that powder-layering, allowing powdered drugs to be layered onto inert seeds, may involve lower working temperatures and shorter processing times, which would presumably be advantageous to preserve the viability of bacteria [22].

Based on these premises, the present work aimed to formulate a suitable oral system of pure culture of *O. splanchnicus* 57 for bacteriotherapy according to a time-dependent colonic delivery strategy. The strain used in this study was previously isolated from a healthy fecal donor, thoroughly characterized, and whole genome sequenced [1]. The use of a novel coating technique, powder-layering, was explored to apply the functional HPMC coating to reduce exposure of the bacteria to possibly harmful operating conditions. The *in vitro* release performance of the resulting delivery system was studied through assay of an analytical tracer, purposely incorporated in the tablet core along with the bacterial powder. The bacterial viability was assessed after the main steps involved in the manufacturing process. Furthermore, the anti-inflammatory properties of *O. splanchnicus* 57 were evaluated before and after formulation development.

2. Materials and methods

2.1. *O. splanchnicus* strain, human enterocyte cell line, and their growth conditions

O. splanchnicus 57 strain was isolated from a healthy fecal donor, purified, and characterized in our previous study [1]. Frozen stocks were made in skim milk (Tammer BioLab, FI) and kept at −80 °C for further cultivation. The strain was grown in Gifu Anaerobic Medium (GAM, Nissui Pharmaceutical, JP) at 37 °C in the anaerobic chamber (Don Whitley A85 anaerobic workstation, UK; 10% H_2, 10% CO_2, and 80% N_2) for 2–3 days.

The HT-29 human enterocyte cell line (ACC 299) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, DE). The cells were grown at 37 °C, under an oxic atmosphere with 5% of CO_2. Cells were passaged every 3–4 days when 80% of confluence was reached by using TryplExpress (Lonza, US-MD) to detach the cells. HT-29 cells were cultivated in McCoy 5A (Lonza) medium supplemented with 100 U/ml Penicillin-Streptomycin (PEST; Lonza) and 10% of heat-inactivated fetal bovine serum (FBS, Gibco, US-MA).

2.2. Freeze-drying of bacterial cells

O. splanchnicus 57 cultures were grown by plating frozen stock bacteria on GAM agar as described above. Afterwards, single colonies were inoculated into 4 ml of GAM broth and cultivated under the same conditions. After 48 h of cultivation, bacterial cells were harvested by centrifugation (4,000 rpm, 15 min, +4°C), washed with phosphate-buffered saline (PBS), and suspended into cryoprotectant media.

Two cryoprotectant solutions were tested. Medium 1 is described below and medium 2, which was not finally used in the formulation, is described in the supplementary material. Cryoprotectant solution was based on peptone-buffered water (Neogen, US-MI), skim-milk (Tammer BioLab, FI; 5% w/v), maltodextrin (Sigma-Aldrich, US-MO; 5% w/v), glycerol (Sigma-Aldrich; 5% v/v) and L-cysteine hydrochloride monohydrate (Sigma-Aldrich; 0.6 g/L), acting as an oxygen scavenger. Stock solutions of the different components of the cryoprotectant solution were first sterilized by autoclaving or filter sterilization and then combined to reach the final concentrations. 1 ml of bacterial suspension in cryoprotectant medium was aliquoted to Greiner Bio-one 2-vent tubes for freeze-drying. The vials were frozen overnight at −80 °C and freeze-dried the day after in a CoolSafe 110–4 Pro freeze-drier (Labogene, DK) at −105 °C and under 0.05 Pa for 24 h.

2.3. Manufacturing of the colon delivery system

Convex cylindrical tablets (250 mg) for immediate release containing the freeze-dried powder (40% w/w), microcrystalline cellulose (MCC, Avicel® pH 102, FMC Co., IT; 50.5% w/w), paracetamol (Mallinckrodt™, UK; 4% w/w) as a release marker, sodium starch glycolate (Explotab® CLV, JRS Rettenmaier Italia, IT; 4.5% w/w), colloidal silica (Giusto Faravelli S.p.A, IT; 0.5% w/w) and magnesium stearate (Carlo Erba reagents, IT; 0.5% w/w) were manufactured by mixing in a mortar and direct compression (rotary tablet press AM-8S, Officine Ronchi, IT, equipped with concave punches of 8 mm in diameter and 8 mm in curvature radius) under a compaction force of 8 kN.

The tablets were then coated by powder-layering in a tangential-spray fluid bed (GPCG1.1, Glatt, DE) with hydroxypropyl methylcellulose (HPMC, Methocel™ E50, Colorcon Ltd, UK) up to a nominal value of 40% weight gain after sealing with a 5% w/w Methocel™ E50 solution that was also used as the powder-layering binder [22]. The process parameters were: 1.2 mm nozzle port size, 0.8 bar atomizing air

pressure, 55–65 m^3/h drying air volume, 50 °C inlet air temperature, 31–36 °C product temperature, 9–10 g/min/kg spray rate. Eudragit® L-30 D55 (Evonik Degussa Italia, IT), in aqueous dispersion (17 % w/w as a solid) with triethyl citrate (TEC, Honeywell International Inc, US-NC; 10 % w/w on dry polymer), glyceryl monostearate (GMS, Gattefossé SA, FR; 5% w/w on dry polymer) and polysorbate 80 (Tween® 80; ACEF S.p. A., IT; 6% w/w on dry polymer) was finally applied onto HPMC-coated tablets using a top-spray fluid bed (Mini-Glatt, Glatt, DE) up to nominal 10% weight gain. The process parameters were: 0.5 mm nozzle port size, 1.0 bar atomizing air pressure, 40–45 m^3/h drying air volume, 35 °C inlet air temperature, 30–33 °C product temperature, 65–70 g/min/kg spray rate. Curing was performed after coating with Eudragit® L-30 D55. The chosen conditions were 35 °C for 2 h.

All intermediates and final double-coated tablets were stored in vacuum-sealed plastic bags at −10 °C.

2.4. *In vitro release test*

The resulting double-coated tablets were tested in triplicate for release using a tracer drug, paracetamol. The release test was performed in an adapted USP 43 disintegration apparatus (Sotax DT3, Sotax S.r.l., IT; 31 cycles/min) to avoid impact on data reliability of the adhesion of swollen HPMC layer to the vessels [21,22]. The release medium (37 ± 0.5 °C) was firstly 0.1 N hydrochloric acid solution (Merk, DE), for 2 h, and then phosphate buffer (PB) pH 6.8 (VWR Int. S.r.l., IT). A volume of 300 ml was used for both fluids to set up a testing procedure that could apply to bacterial cell count in the study follow-up. Sink conditions were ensured throughout the entire duration of the test. At predetermined time points, fluid samples were automatically collected. Paracetamol released was assayed spectrophotometrically at $\lambda = 248$ nm (Lambda 35, PerkinElmer® Italia, IT), and *in vitro* lag time, expressed as the time to 10% release ($t_{10\%}$), was calculated by linear interpolation of the data before and after 10% of the drug was released in phosphate buffer pH 6.8.

2.5. *Bacterial viability test*

The bacterial viability was assessed by viable cell count method plating on GAM and/or Fastidious Anaerobe Agar (FAA, Tammer Bio-Lab, FI) agar using sterilized PBS for the serial dilutions and incubating the plates in an anaerobic chamber or jars with anaerobic gas generator (Oxoid™ AnaeroGen™, ThermoFisher Scientific, US-MA) at 37 °C for 72 h.

For the viable cell count method, a volume of 100 μl of cell suspension dilution (10^3, 10^4, 10^5) was plated onto 90 mm petri agar plates. After incubation, each viable cell in the appropriately diluted sample forms a visible colony, which were counted. Based on the dilution and the number of colony-forming units (CFUs), an estimate of viable cells was obtained.

Freeze-dried (FD) powders were kept at room temperature, +4 °C, −20 °C, or −80 °C, and the viability was tested in triplicates before FD, after FD, 1 month, and 3 months after FD. Additionally, one replicate of FD powders stored at −10 °C was tested after FD and 3 months after FD. The bacterial viability in all manufacturing intermediates and the final double-coated tablets was assessed as described above for FD powders. The solid formulations were previously vortexed in sterilized PBS until mechanical disintegration and dispersion.

2.6. *Assay for anti-inflammatory activity on enterocytes*

Active culture of *O. splanchnicus* strain 57, freeze-dried powders, bacteria-loaded double-coated tablets, and the respective placebos, wherein the bacteria load was replaced with MCC, were tested for their capacity to attenuate *Escherichia coli* lipopolysaccharide (LPS, Sigma, US) induced interleukin-8 (IL-8) release in the HT-29 cell line. The assay was described in detail previously [23]. Briefly, HT-29 cells were seeded onto a Corning™ Falcon™ 96-well cell culture-treated, flat-bottom microplate (ThermoFisher Scientific, US-MA), 12.500 cells per well, and grown in Gibco™ McCoy 5A medium (ThermoFisher Scientific) with supplements under 95% air–5% of CO_2 atmosphere at 37 °C for 8 days. The HT-29 growth medium was changed after 3–4 days and the day before the assay.

McCoy 5A medium was used to wash *O. splanchnicus* 57 cells after harvest from the culture media and to disperse the FD powder and the double-coated tablets. Bacteria-loaded and placebo double-coated tablets were suspended 1:10 (v/v) and vortexed together with glass beads to ensure mechanical disintegration and detachment of bacteria from the components of the drug core. Supernatants collected after glass bead vortexing were further used. The freshly cultured strain 57 as well as strain 57 grown from FD powder and double-coated tablets were adjusted to OD_{600nm} 0.25 (Novaspec II, Pharmacia Biotech, SE) in McCoy 5A medium. 8-day-old HT-29 cells were co-incubated with 100 μl of the corresponding bacterial suspensions for 1 h under the cell culturing conditions followed by removing of bacterial suspensions, placebo, and controls from the monolayer and addition of McCoy 5A medium supplemented with 1 ng/ml of *E. coli* LPS. Only McCoy 5A medium supplemented with FBS 10% was used for the negative background controls. After incubating the plate for 4 h in the same conditions as before, IL-8 levels were measured from the supernatants by performing an ELISA assay (BD OptEIA™ Set, BD Biosciences, US-NJ) according to the kit manufacturerś instructions.

2.7. *Statistical analysis*

The statistical analyses were performed with GraphPad Prism 9 software. The differences between two groups were calculated by an unpaired, two-tailed *t*-test, and p-values < 0.05 were considered significant. The equality of variances was checked by carrying out F-test.

3. Results

3.1. *Effect of freeze-drying and storage on O. splanchnicus viability*

The viability of freeze-dried bacterial cells is the basis for the formulation as biotherapeutic bacteria need to be delivered alive and in active form at the desired release location. Thus, it is essential to track the bacterial viability at each step to proceed with further formulation steps. The first step in formulating a dry bacterial product is freeze-drying, which is a stressful procedure for bacterial cells, and viability assessment during this crucial first step as well as during the following storage is basis for the successful formulation.

We first tested two cryoprotectant media for freeze-drying of *O. splanchnicus* 57 and selected medium 1, which gave better results in bacterial viability after freeze-drying (see supplementary material), for further formulation studies. The final cryoprotectant medium for *O. splanchnicus* 57 contained peptone-buffered water, skim milk, maltodextrin, glycerol, and l-cysteine.

To address the impact of storage temperature on the survival of freeze-dried *O. splanchnicus* 57, we evaluated the viability of freeze-dried cell powders by comparing multiple storage temperatures; room temperature, +4 °C, −20 °C and −80 °C (Fig. 1**A**). Freeze-drying of *O. splanchnicus* 57 lowered viable bacterial cells slightly, but not statistically significantly. On the one hand, storage for 1 and 3 months at the lowest temperatures of −20 °C and −80 °C did not decrease bacterial viable counts statistically significantly. On the other hand, higher storage temperatures of +4 °C and room temperature lead to a significant decrease in cell survival after 1 and 3 months.

Furthermore, we addressed the viability of freeze-dried *O. splanchnicus* 57 at −10 °C and found that, although viable cell counts decreased after 3 months of storage, the viable cell count was still above 10^7 CFU/ml (Fig. 1**B**).

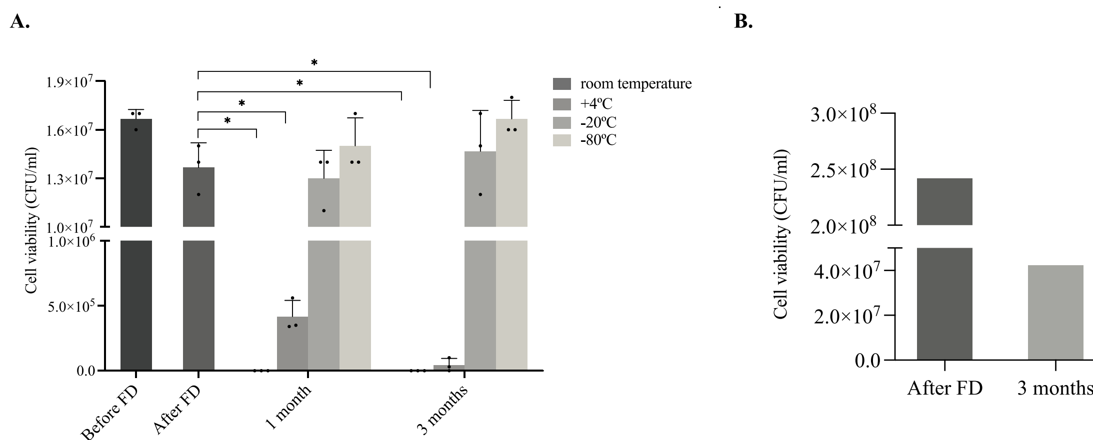

Fig. 1. Cell viability after freeze-drying and storage of *O. splanchnicus* 57 strain **(A)** at room temperature, +4 °C, −20 °C, and −80 °C and **(B)** at −10 °C. Columns indicate **(A)** the CFU/ml present in three replicates compared to after freeze-drying and suspension into the original volume and **(B)** the CFU/ml present in one replicate. An asterisk indicates a statistically significant difference (* = $p < 0.05$) in reduction of viable counts of *O. splanchnicus* 57 during storage.

3.2. Formulation and manufacturing of the oral delivery system

For every manufacturing step and outcome formulation, we assessed the viability of *O. splanchnicus* 57 cells by plate counts, and the results showed viable counts of cells ranging from 10^6 to 10^7 CFU/g of freeze-dried powder. The tablets were manufactured from freeze-dried powders that were kept at −10 °C, which was not optimal but still allowed good bacterial cell survival (Fig. 1**B). First, we tested the effect of mixing *O. splanchnicus* 57 powders with the tablet core excipients, which did not affect the viable counts significantly (Fig. 2**A**). Next, a comparison between the successive tableting and mixing was done, and the differences were found to be non-significant (Fig. 2**A**), but statistically significant when comparing freeze-dried powder and tableting from another batch (Fig. 2**B**). Thus, the applied compaction force to manufacture the core matrix decreased the viable counts in the tablets, but overall freeze-dried *O. splanchnicus* 57 cells tolerated the process well. After tableting, the viable counts were still above 10^6 CFU/g.

Next, we tracked bacterial viability also during the following formulation steps (Fig. 2**B**), where two coating processes, for application of HMPC and gastric-resistant layers (GR), respectively, were carried out. When analysing the manufacturing step-by-step, statistically significant drops in viability were observed when the freeze-dried powders were tableted but not when the tablets were coated with HPMC by powder-layering and with Eudragit® L-30 D55 by spray-coating. The viable counts of *O. splanchnicus* 57 remained above 10^6 CFU/g (Fig. 2**B**). The bacterial viable counts in the coated tablets did not decrease after 1 week of storage in vacuum-sealed bags at −10 °C (Fig. 2**B**).

3.3. Release test

Double-coated tablets were evaluated in triplicate for *in vitro* performance first in 0.1 N HCl and then in phosphate buffer pH 6.8 (Fig. 3). While in contact with the acidic medium, they showed gastric resistance properties since no drug release or signs of disintegration were observed. When the medium was changed, the tablets lost their gastric-resistant layer and HPMC got into contact with the aqueous fluid. Therefore, the polymer started to swell delaying the onset of release of the tracer drug. Reproducible lag times with an average duration of 55 min were

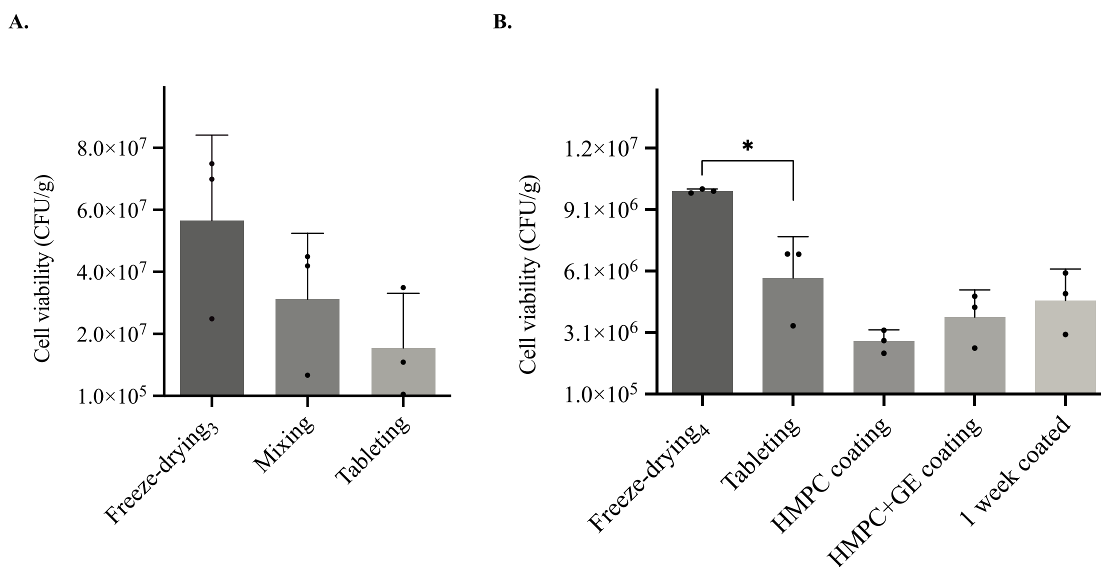

Fig. 2. Cell viability after mixing and tableting of *O. splanchnicus* strain 57 **(A)** and during the different processing steps until double-coated tablets and after 1 week of storage in airtight bags at −10 °C **(B)**. Bars indicate the standard deviations from three replicates. An asterisk indicates a statistically significant difference (* = $p < 0.05$) in reduction of viable counts of *O. splanchnicus* 57 between the processing steps.

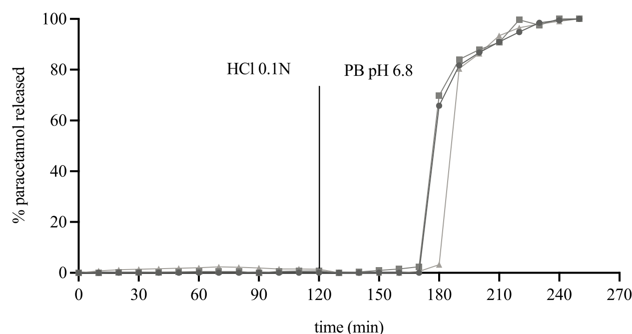

Fig. 3. Release of paracetamol in HCl 0.1 N and PB pH 6.8 from the double-coated tablets containing *O. splanchnicus* strain 57. Tablets containing freeze-dried *O. splanchnicus 57* powder, MCC, paracetamol, sodium starch glycolate, colloidal silica, and magnesium stearate, were firstly coated with HPMC and then with Eudragit® L.

achieved. After release was started, coinciding with the breakup of the tablets, 80% of the drug was released within 20 min. Subsequently, the release of paracetamol was slowed down possibly because of collapse of the coating structure observed during the test.

3.4. Anti-inflammatory capacity of O. splanchnicus after formulation

The capacity of *O. splanchnicus* 57 strain to attenuate *E. coli* LPS-induced inflammatory interleukin IL-8 release from HT-29 cells was tested for active cultures, freeze-dried powders, and double-coated tablets. The assay was carried out with the already established protocol [1]. HT-29 cells pre-incubated with *O. splanchnicus* 57 cells that were sub-cultivated from the freeze-dried powders or the double-coated tablets, showed a drop in IL-8 release of 70 % and 59 %, respectively, as compared to the LPS control without prior exposure to the bacterium (Fig. 4**A**). The result was comparable to that obtained with *O. splanchnicus* 57 cultivated from the regular −80 °C stock (Fig. 4**A**). Thus, the capability of *O. splanchnicus* 57 to attenuate the release of pro-inflammatory IL-8 from enterocytes after *E. coli* LPS induction was not compromised by freeze-drying, tableting, powder-layering, or spray-coating.

Next, we addressed the anti-inflammatory effect of *O. splanchnicus* 57 double-coated tablets as compared to placebo ones (Fig. 4**B**). Overall, data from three experiments showed that *O. splanchnicus* 57 bacteria from double-coated tablets had an anti-inflammatory effect by reducing IL-8 cytokine release from HT-29 cells, which were induced with *E. coli* LPS. Placebo double-coated tablets did not statistically significantly attenuate the release of IL-8 cytokine, except for experiment 2 where they also reduced IL-8 release, but statistically significantly less than *O. splanchnicus* 57 double-coated tablets. Thus, the *O. splanchnicus* 57 tablets exerted anti-inflammatory action also directly without the retrieval of bacterial viability and growth.

4. Discussion

In this study, we formulated double-coated tablets containing freeze-dried *O. splanchnicus* 57 powders for oral colon delivery. The system had a tablet core of freeze-dried powders from a pure culture of *O. splanchnicus* 57 coated by an inner layer of HPMC and an outer layer of Eudragit® L. Preservation and formulation of *O. splanchnicus* for site-selective release pave the way to treat inflammation in the colon epithelium by targeted delivery of bacteria that have anti-inflammatory properties.

Freeze-drying technique is widely implemented to preserve bacterial strains that are used in diverse probiotic formulations. During the process, a protective media is needed to support bacterial cells from the stressful procedures of freezing or freeze-drying [24]. First, we evaluated two cryoprotectant media for *O. splanchnicus* 57 cells and continued with the better one to achieve stable survival and high viable counts of cells after freeze-drying. The composition of the cryoprotectant medium was designed based on the requirements of anaerobic bacteria for *O. splanchnicus* 57 strain. Optimally, selection of cryoprotectants is strain-specific, especially when freeze-drying is applied to strict anaerobic human gut microbes [25,26]. Our protectant medium was based on a mixture of skim milk (5%), maltodextrin (5%), and glycerol (5%) in peptone-buffered water. Skim milk interacts with the lipid bilayer structural proteins of bacterial cells forming biofilms and stabilizing cell membranes [27]. Polysaccharide maltodextrin was chosen amongst other disaccharide polyols or polysaccharides as it has been commonly used as a probiotic cryoprotectant [28]. Glycerol has proved to be a good supplement for freeze-drying butyrate-producing bacteria [29], enhancing the recovery of the cells by disrupting the hydrogen bonding between water molecules, and blocking the formation of ice crystals [30]. L-cysteine enhances energy production of anaerobic bacteria by reducing the oxidation–reduction potential and improves in that way cell growth and expression of their functional key genes encoding substrate conversion and energy production [31].

O. splanchnicus 57 cells did not suffer statistically significant viability

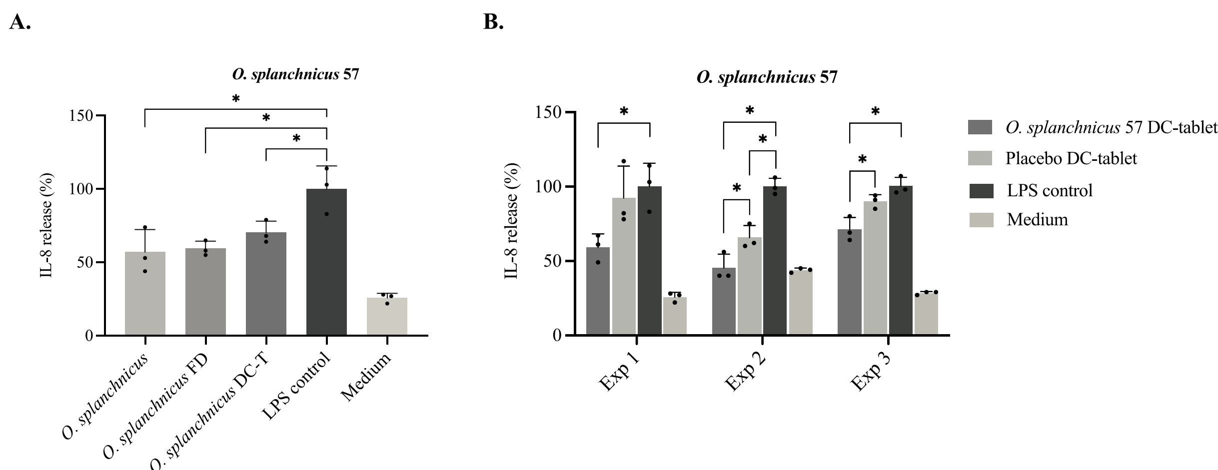

Fig. 4. Attenuation of *E. coli* LPS induced IL-8 release (%) from the human HT-29 cell line exposed to *O. splanchnicus* 57 from **(A)** *O. splanchnicus* 57 cells cultivated from stock culture (control), sub-cultivated from freeze-dried (FD) powder, and double-coated tablets (DC-T); **(B)** *O. splanchnicus* 57 and *placebo* supernatants from double-coated tablets. Bars indicate the standard deviations from three replicates. An asterisk indicates a statistically significant difference (* = $p < 0.05$) in reduction of IL-8 production by HT-29 cell line as compared to the control, or between the *O. splanchnicus* 57 and *placebo* double-coated tablets.

loss when undergoing the freeze-drying process, and subsequently stored for 1 or 3 months at either −20 °C or −80 °C. On the contrary, viable cell counts of *O. splanchnicus* 57 freeze-dried powder were significantly decreased when stored at +4 °C or room temperature. Our results are in line with previous studies showing that the recovery rate of anaerobic bacteria after freeze-drying declines as the storage temperature increases [32]. Major factors influencing the survival of bacterial cells, notably anaerobic strains, include exposure to oxygen and humidity, and nutrient lack [33].

To ensure the delivery of freeze-dried *O. splanchnicus* 57 cells to the colon and to protect the cells from degradation by the hostile upper gastrointestinal tract, freeze-dried bacterial powder was tableted for oral dosing and subsequently double coated. The manufactured tablet consisted of a mix of freeze-dried *O. splanchnicus* 57 strain and excipients. The tablet core was coated by an inner layer of HPMC to delay the onset of disintegration throughout the intestine, according to the time-dependent colonic release strategy, and an outer coating of Eudragit® L to protect the delivery system during gastric residence.

In the core formulation, MCC was chosen since it was shown to remarkably improve the flow properties of the powder mixture and the relevant compaction ability compared to other diluents, such as the broadly used lactose monohydrate. Sodium starch glycolate (Explotab® CLV), a commonly employed super-disintegrant, was used to promote the disintegration of the dosage form by more effectively counteracting the possible matrix effect associated with the relatively high percentage amount of freeze-dried component. As this also tended to impair flowability during processing, due to marked hygroscopicity characteristics, colloidal silica (Aerosil® 200) was needed as a glidant. Magnesium stearate was finally added as a lubricant to the pre-mixed tablet components and subjected to a shorter mixing to avoid a negative impact on disintegration of the tablet core.

We addressed the effect of mixing and tableting on *O. splanchnicus* 57 cell survival and observed that mixing of the excipients did not show any significant effect. However, tableting appeared a more stressful step, decreasing the viable count by approximately one logarithmic unit. Survival rates in tableting can depend on the used excipients or mechanical stress from compression forces, lowering microorganism viability possibly due to an increase in temperature induced by inter-particle and particle-die wall frictions. However, when anaerobic probiotic tablets need to be formulated, direct compression might protect the oxygen-sensitive strains when stored [34,35].

As previously described, the tablet cores used in our study, containing freeze-dried powders of *O. splanchnicus* 57, underwent a sealing phase followed by powder-layering in a tangential-spray fluid bed with a low-viscosity swellable hydrophilic polymer, Methocel™ E50 [22]. The main goal of coating was to obtain oral dosage forms with a lag phase before the bacteria are released, to achieve a time-dependent colon delivery through swelling and erosion of the functional HPMC layer when in contact with the aqueous intestinal fluid [19–22]. Powder-layering, only recently introduced as a coating technique, was purposely selected for this process given the highly sensitive nature of the bacteria conveyed in the tablet cores. Indeed, it allowed lower operating temperatures and, above all, smaller water volumes to be employed *vs.* spray-coating. On the other hand, the extent of mechanical stress was by far reduced as compared with compression-coating. Moreover, although the latter technique would have involved fully dry processing, it is known to bring about difficulties in achieving reproducible and fine-tuned lag times followed by prompt and quantitative release [36]. The coating process parameters were attentively set up in order to make their impact on the bacteria as mild as possible. Particularly, an adequate balance of the binder spray and powder addition rates was sought, which would enable powder adhesion while avoiding excessive wetting of the tablets and stickiness of the cores. After coating the tablets with HPMC, *O. splanchnicus* 57 cell viability was stable (10^6 CFU/g), and the viable counts remained unchanged when applying an enteric coating, also after storage for 1 week at −10 °C. This may indicate that the HPMC coating would serve as a protective barrier during the subsequent spray-coating process with Eudragit® L. Given the early stage of the work, the usual probiotic dose ranges, and particularly the critical oxygen and humidity sensitivity characteristics of *O. splanchnicus*, the overall results could be considered successful pointing out the interesting potential of the chosen coating technique in this specific area and the suitability of the formulation design applied. However, more research needs to be conducted to increase the number of viable cells at the end of all the manufacturing steps.

To evaluate the release performance of the colon delivery system proposed, paracetamol was included as a component of the tablet core, together with the bacterial freeze-dried powder, for use as an analytical tracer given its well-known solubility and solid-state properties as well as ease of UV detection [37–39]. In addition, a preliminary literature survey allowed any detrimental influence on bacteria to be ruled out, which is an aspect that also needs to be taken into account when dealing with microorganisms. The release started on average after 55 min of immersion in PB pH 6.8, and over 80% release was achieved in 20 min. This would confirm that, despite a porous structure associated with the nature of the coating technique employed, the applied HPMC layer would be effective in preventing drug release over a lag phase of programmable duration. The subsequent decrease in the release rate may have been due to a collapse of the coating structure that was noticed during the test, roughly coinciding with the final phases of release. Because of the high percentage of freeze-dried bacterial powder, the effect of core disintegration may have been less effective in aiding complete disruption of the coating.

The *O. splanchnicus* strain 57 used in this study has previously shown anti-inflammatory properties in the human enterocyte model [1] and we assessed whether these properties were maintained during the manufacturing process of the double-coated tablets for colonic release. Anti-inflammatory tests were performed based on its attenuation capacity of cytokine IL-8 release from HT-29 human cell line upon *E. coli* LPS induction. *O. splanchnicus* 57 cells attenuated the IL-8 release from enterocytes induced by *E. coli* LPS when the bacteria were recultivated from freeze-dried powders or double-coated tablets. Thus, the strain kept its anti-inflammatory action after re-growth from freeze-dried formulation. Double-coated tablets conveying *O. splanchnicus* 57 also exerted an anti-inflammatory effect in the enterocyte model. Our results imply that the effector molecules of *O. splanchnicus* 57 were active in the described formulation and could exert anti-inflammatory action even before the bacterium retrieved its growth.

5. Conclusions

An oral colon delivery system was designed for targeted release of anaerobic *O. splanchnicus* 57, a potentially therapeutic bacterium. Tableting and double coating of the freeze-dried *O. splanchnicus* 57 cells were attempted and viable cell counts, release tests, and bacterial anti-inflammatory assays were performed. The results showed the survival of *O. splanchnicus* 57 and a time-controlled release of the delivery system contents. *O. splanchnicus* 57 cells that had undergone the formulation steps were capable of attenuating *E. coli* LPS-induced IL-8 release from HT-29 cells, *i.e.,* exerted anti-inflammatory action. Our study suggests that it is possible to include in oral double-coated tablets anaerobic bacteria and deliver them alive and in active form after a lag phase, which should help avoid degradation by hostile upper gastrointestinal tract conditions and allow selective release into the colon. Therefore, this opens new avenues to formulate pure cultures of single anaerobic strains or mixtures for oral delivery to become the so-called live biotherapeutic products.

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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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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References

[1] K. Hiippala, G. Barreto, C. Burrello, A. Diaz-Basabe, M. Suutarinen, V. Kainulainen, J.R. Bowers, D. Lemmer, D.M. Engelthaler, K.K. Eklund, F. Facciotti, R. Satokari, Novel *Odoribacter splanchnicus* Strain and Its Outer Membrane Vesicles Exert Immunoregulatory Effects in vitro, Front Microbiol. 11 (2020) 1–14, https://doi.org/10.3389/fmicb.2020.575455.

[2] F. Nagai, M. Morotomi, Y. Watanabe, H. Sakon, R. Tanaka, Alistipes indistinctus sp. nov. and Odoribacter laneus sp. nov., common members of the human intestinal microbiota isolated from faeces, Int J Syst Evol Microbiol. 60 (2010) 1296–1302, https://doi.org/10.1099/ijs.0.014571-0.

[3] J.M. Hardham, K.W. King, K. Dreier, J. Wong, C. Strietzel, R.R. Eversole, C. Sfintescu, R.T. Evans, Transfer of Bacteroides splanchnicus to Odoribacter gen. nov. as *Odoribacter splanchnicus* comb. nov., and description of Odoribacter denticanis sp. nov., isolated from the crevicular spaces of canine periodontitis patients, Int J Syst Evol Microbiol. 58 (2008) 103–109, https://doi.org/10.1099/ijs.0.63458-0.

[4] M. Göker, S. Gronow, A. Zeytun, M. Nolan, S. Lucas, A. Lapidus, N. Hammon, S. Deshpande, J.F. Cheng, S. Pitluck, K. Liolios, I. Pagani, N. Ivanova, K. Mavromatis, G. Ovchinikova, A. Pati, R. Tapia, C. Han, L. Goodwin, A. Chen, K. Palaniappan, M. Land, L. Hauser, C.D. Jeffries, E.M. Brambilla, M. Rohde, J. C. Detter, T. Woyke, J. Bristow, V. Markowitz, P. Hugenholtz, J.A. Eisen, N. C. Kyrpides, H.P. Klenk, Complete genome sequence of *odoribacter splanchnicus* type strain (1651/6 T), Stand Genomic Sci. 4 (2011) 200–209, https://doi.org/10.4056/sigs.1714269.

[5] S.F. Lima, L. Gogokhia, M. Viladomiu, L. Chou, G. Putzel, W.B. Jin, S. Pires, C.J. Guo, Y. Gerardin, C. v. Crawford, V. Jacob, E. Scherl, S.E. Brown, J. Hambor, R.S. Longman, Transferable Immunoglobulin A–Coated *Odoribacter splanchnicus* in Responders to Fecal Microbiota Transplantation for Ulcerative Colitis Limits Colonic Inflammation, Gastroenterology. 162 (2022) 166–178. https://doi.org/10.1053/j.gastro.2021.09.061.

[6] H. Zafar, M.H. Saier, Gut Bacteroides species in health and disease, Gut Microbes. 13 (2021) 1–20, https://doi.org/10.1080/19490976.2020.1848158.

[7] J. Wang, J. Qie, D. Zhu, X. Zhang, Q. Zhang, Y. Xu, Y. Wang, K. Mi, Y. Pei, Y. Liu, G. Ji, X. Liu, The landscape in the gut microbiome of long-lived families reveals new insights on longevity and aging–relevant neural and immune function, Gut Microbes. 14 (2022), https://doi.org/10.1080/19490976.2022.2107288.

[8] X.C. Morgan, T.L. Tickle, H. Sokol, D. Gevers, K.L. Devaney, D. v. Ward, J.A. Reyes, S.A. Shah, N. LeLeiko, S.B. Snapper, A. Bousvaros, J. Korzenik, B.E. Sands, R.J. Xavier, C. Huttenhower, Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment., Genome Biol. 13 (2012) R79. https://doi.org/10.1186/gb-2012-13-9-r79.

[9] F. Li, G. Sun, Z. Wang, W. Wu, H. Guo, L. Peng, L. Wu, X. Guo, Y. Yang, Characteristics of fecal microbiota in non-alcoholic fatty liver disease patients, Sci China Life Sci. 61 (2018) 770–778, https://doi.org/10.1007/s11427-017-9303-9.

[10] D.G. Burke, F. Fouhy, M.J. Harrison, M.C. Rea, P.D. Cotter, O. O'Sullivan, C. Stanton, C. Hill, F. Shanahan, B.J. Plant, R.P. Ross, The altered gut microbiota in adults with cystic fibrosis, BMC Microbiol. 17 (2017) 1–11, https://doi.org/10.1186/s12866-017-0968-8.

[11] D.P. Venegas, M.K. De La Fuente, G. Landskron, M.J. González, R. Quera, G. Dijkstra, H.J.M. Harmsen, K.N. Faber, M.A. Hermoso, Short chain fatty acids (SCFAs)mediated gut epithelial and immune regulation and its relevance for inflammatory bowel diseases, Front Immunol. 10 (2019), https://doi.org/10.3389/fimmu.2019.00277.

[12] M.Y. Zeng, N. Inohara, G. Nuñez, Mechanisms of inflammation-driven bacterial dysbiosis in the gut, Mucosal Immunol. 10 (2017) 18–26, https://doi.org/10.1038/mi.2016.75.

[13] H. el Enshasy, K. Malik, R.A. Malek, N.Z. Othman, E.A. Elsayed, M. Wadaan, Anaerobic probiotics: The key microbes for human health, Adv Biochem Eng Biotechnol. 156 (2016) 397–432, https://doi.org/10.1007/10_2015_5008.

[14] R. Rajam, P. Subramanian, Encapsulation of probiotics: past, present, and future, Beni Suef Univ J Basic Appl Sci. 11 (2022), https://doi.org/10.1186/s43088-022-00228-w.

[15] J. Mättö, H.L. Alakomi, A. Vaari, I. Virkajärvi, M. Saarela, Influence of processing conditions on Bifidobacterium animalis subsp. lactis functionality with a special focus on acid tolerance and factors affecting it, Int Dairy J. 16 (2006) 1029–1037, https://doi.org/10.1016/j.idairyj.2005.10.014.

[16] M. Naissinger da Silva, B.L. Tagliapietra, V. do A. Flores, N.S. Pereira dos Santos Richards, In vitro test to evaluate survival in the gastrointestinal tract of commercial probiotics, Curr Res Food Sci. 4 (2021) 320–325, https://doi.org/10.1016/j.crfs.2021.04.006.

[17] C. Lacroix, S. Yildirim, Fermentation technologies for the production of probiotics with high viability and functionality, Curr Opin Biotechnol. 18 (2007) 176–183, https://doi.org/10.1016/j.copbio.2007.02.002.

[18] E.M.M. Quigley, Gut bacteria in health and disease, Gastroenterol Hepatol (N Y). 9 (2013) 560–569. http://www.ncbi.nlm.nih.gov/pubmed/24729765.

[19] A. Gazzaniga, S. Moutaharrik, I. Filippin, A. Foppoli, L. Palugan, A. Maroni, M. Cerea, Time-based formulation strategies for colon drug delivery, Pharmaceutics 14 (2022) 2762, https://doi.org/10.3390/pharmaceutics14122762.

[20] A. Maroni, L. Zema, M. Cerea, A. Foppoli, L. Palugan, A. Gazzaniga, Erodible drug delivery systems for time-controlled release into the gastrointestinal tract, J. Drug Deliv. Sci. Technol. 32 (2016) 229–235, https://doi.org/10.1016/j.jddst.2015.10.001.

[21] A. Foppoli, A. Maroni, S. Moutaharrik, A. Melocchi, L. Zema, L. Palugan, M. Cerea, A. Gazzaniga, In vitro and human pharmacoscintigraphic evaluation of an oral 5-ASA delivery system for colonic release, Int. J. Pharm. 572 (2019), 118723.

[22] A. Foppoli, M. Cerea, L. Palugan, L. Zema, A. Melocchi, A. Maroni, A. Gazzaniga, Evaluation of powder-layering vs. spray-coating techniques in the manufacturing of a swellable/erodible pulsatile delivery system, Drug Dev Ind Pharm. 46 (2020) 1230–1237, https://doi.org/10.1080/03639045.2020.1788060.

[23] V. Kainulainen, Y. Tang, T. Spillmann, S. Kilpinen, J. Reunanen, P.E.J. Saris, R. Satokari, The canine isolate Lactobacillus acidophilus LAB20 adheres to intestinal epithelium and attenuates LPS-induced IL-8 secretion of enterocytes in vitro, BMC Microbiol. 15 (2015), https://doi.org/10.1186/s12866-014-0337-9.

[24] S. Bellali, J. Bou Khalil, A. Fontanini, D. Raoult, J.C. Lagier, A new protectant medium preserving bacterial viability after freeze drying, Microbiol Res. 236 (2020), https://doi.org/10.1016/j.micres.2020.126454.

[25] L. Bircher, A. Geirnaert, F. Hammes, C. Lacroix, C. Schwab, Effect of cryopreservation and lyophilization on viability and growth of strict anaerobic human gut microbes, Microb, Biotechnol. 11 (2018) 721–733, https://doi.org/10.1111/1751-7915.13265.

[26] K. Vorländer, I. Kampen, J.H. Finke, A. Kwade, Along the process chain to probiotic tablets: Evaluation of mechanical impacts on microbial viability, Pharmaceutics. 12 (2020), https://doi.org/10.3390/pharmaceutics12010066.

[27] Z. Zhang, Y. Xiang Yu, Y. Geng Wang, X. Xian Wei, M. Jie Liao, X. Jun Rong, J. Chen, Development of a new protocol for freeze-drying preservation of Pseudoalteromonas nigrifaciens and its protective effect on other marine bacteria, Electronic Journal of Biotechnology. 44 (2020) 1–5, https://doi.org/10.1016/j.ejbt.2019.12.006.

[28] S.O. Oluwatosin, S.L. Tai, M.A. Fagan-Endres, Sucrose, maltodextrin and inulin efficacy as cryoprotectant, preservative and prebiotic – towards a freeze dried Lactobacillus plantarum topical probiotic, Biotechnology Reports. 33 (2022), https://doi.org/10.1016/j.btre.2021.e00696.

[29] A. Biclot, G.R.B. Huys, R. Bacigalupe, K. D'hoe, D. Vandeputte, G. Falony, R. Y. Tito, J. Raes, Effect of cryopreservation medium conditions on growth and isolation of gut anaerobes from human faecal samples, Microbiome. 10 (2022), https://doi.org/10.1186/s40168-022-01267-2.

[30] C.M. Koh, Storage of bacteria and yeast, in: Methods Enzymol, Academic Press Inc., 2013: pp. 15–21. https://doi.org/10.1016/B978-0-12-420067-8.00002-7.

[31] Y. Wang, B. Xi, X. Jia, M. Li, X. Qi, P. Xu, Y. Zhao, M. Ye, Y. Hao, Characterization of hydrogen production and microbial community shifts in microbial electrolysis cells with L-cysteine, Science of the Total Environment. 760 (2021), https://doi.org/10.1016/j.scitotenv.2020.143353.

[32] O.F. Celik, D.J. O'Sullivan, Factors influencing the stability of freeze-dried stress-resilient and stress-sensitive strains of bifidobacteria, J Dairy Sci. 96 (2013) 3506–3516, https://doi.org/10.3168/jds.2012-6327.

[33] Z. Zhang, M. Gu, X. You, D.A. Sela, H. Xiao, D.J. McClements, Encapsulation of bifidobacterium in alginate microgels improves viability and targeted gut release, Food Hydrocoll. 116 (2021), 106634, https://doi.org/10.1016/j.foodhyd.2021.106634.

[34] R. Allouche, S. Dupont, A. Charriau, P. Gervais, L. Beney, O. Chambin, Optimized tableting for extremely oxygen-sensitive probiotics using direct compression, Int J Pharm. 538 (2018) 14–20, https://doi.org/10.1016/j.ijpharm.2018.01.010.

[35] S. Klayraung, H. Viernstein, S. Okonogi, Development of tablets containing probiotics: Effects of formulation and processing parameters on bacterial viability, Int J Pharm. 370 (2009) 54–60, https://doi.org/10.1016/j.ijpharm.2008.11.004.

[36] A. Foppoli, A. Maroni, L. Palugan, L. Zema, S. Moutaharrik, A. Melocchi, M. Cerea, A. Gazzaniga, Erodible coatings based on HPMC and cellulase for oral time-controlled release of drugs, Int. J. Pharm. 585 (2020), 119425, https://doi.org/10.1016/j.ijpharm.2020.119425.

[37] F. Giordano, A. Rossi, R. Bettini, A. Savioli, A. Gazzaniga, C.S. Novák, Thermal behavior of paracetamol-polymeric excipients mixtures, J. Therm. Anal. Calorim. 68 (2002) 575–590, https://doi.org/10.1023/A:1016004206043.

[38] S. Moutaharrik, A. Maroni, A. Melocchi, L. Zema, A. Foppoli, M. Cerea, L. Palugan, C. Neut, F. Siepmann, J. Siepmann, A. Gazzaniga, Oral colon delivery platform based on a novel combination approach: Design concept and preliminary evaluation, J. Drug Deliv. Sci. Technol. 66 (2021), 102919, https://doi.org/10.1016/j.jddst.2021.102919.

[39] S. Moutaharrik, A. Maroni, C. Neut, C. Dubuquoy, L. Dubuquoy, A. Foppoli, M. Cerea, L. Palugan, F. Siepmann, J. Siepmann, A. Gazzaniga, In vitro and in vivo evaluation of a pH-, microbiota- and time-based oral delivery platform for colonic release, Eur. J. Pharm. Biopharm. 183 (2023) 13–23.