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Comparison of protection and release behavior of different capsule polymer combinations based on *L. acidophilus* survivability and function and caffeine release

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ABSTRACT

Oral administration of active pharmaceutical ingredients, nutraceuticals, enzymes or probiotics requires an appropriate delivery system for optimal bioactivity and absorption. The harsh conditions during the gastrointestinal transit can degrade the administered products, hampering their efficacy. Enteric or delayed-release pharmaceutical formulations may help overcome these issues. In a Simulator of Human Intestinal Microbial Ecosystem model (SHIME) and using caffeine as a marker for release kinetics and *L. acidophilus* survivability as an indicator for protection, we compared the performance of ten capsule configurations, single or DUOCAP® combinations. The function of *L. acidophilus* and its impact on the gut microbiota was further tested in three selected capsule types, combinations of DRcaps® capsule in DRcaps® capsule (DR-in-DR) and DRcaps® capsule in Vcaps® capsule (DR-in-VC) and single Vcaps® Plus capsule under colonic conditions. We found that under stomach and small intestine conditions, DR-in-DR and DR-in-VC led to the best performance both under fed and fasted conditions based on the slow caffeine release and the highest *L. acidophilus* survivability. The Vcaps® Plus capsule however, led to the quickest caffeine and probiotic release. When DR-in-DR, DR-in-VC and single Vcaps® Plus capsules were tested through the whole gastrointestinal tract, including under colonic conditions, caffeine release was found to be slower in capsules containing DRcaps® capsules compared to the single Vcaps® capsules. In addition, colonic survival of *L. acidophilus* was significantly increased under fasted conditions in DR-in-DR or DR-in-VC formulation compared to Vcaps® Plus capsule. To assess the impact of these formulations on the microbial function, acetate, butyrate and propionate as well as ammonia were measured. *L. acidophilus* released from DR-in-DR or DR-in-VC induced a significant increase in butyrate and a decrease in ammonia, suggesting a proliferation of butyrate-producing bacteria and reduction in ammonia-producing bacteria. These data suggest that *L. acidophilus* included in DR-in-DR or DR-in-VC reaching the colon is viable and functional, potentially contributing to changes in colonic microbiota composition and diversity.

1. Introduction

Oral route is the most preferred for active pharmaceutical ingredients, nutraceuticals or probiotic administration due to its convenience, potential controlled release, and patient compliance (Sosnik, 2014; Homayun et al., 2019). Despite these advantages, many challenges are associated with oral administration (Vinarov et al., 2021), such as specific patient populations, regional differences in the gastrointestinal tract, interaction with food, advanced and innovative formulations, and *in vitro* and *in silico* tools relevant for exploring product performance, including active pharmaceuticals or nutraceuticals and probiotics.

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In the upper gastrointestinal tract (GIT), orally administered drugs or nutraceuticals and probiotics are prone to degradation because of the harsh acidic conditions in the stomach and the gastric enzymes (i.e., pepsin). In the duodenum, pancreatic enzymes (i.e., lipase, trypsin, amylase, peptidases) and bile salts can significantly affect the stability of these ingredients, particularly probiotic viability. During the fasted or fed conditions, different transit times, pH profiles, and enzymatic levels have been described, requiring adjustments of the oral entity dosage forms for better efficacy and performance (Vinarov et al., 2021).

Therefore, immediate-release formulations should be avoided when pH-sensitive products are delivered orally. For example, probiotics which are live microorganisms, confer a health benefit to the host only when administered in adequate levels (Hill et al., 2014) and may have lower performance when the strain viability is reduced during the GIT transit because of a low pH for example (Marzorati et al., 2015; Dianawati et al., 2016). Nutritional supplements, like flavonoids, carotenoids, hydroxycinnamoyl acid or vitamin C, can also be highly degraded (80–91%) during gastrointestinal digestion, while bioactives like proteins and peptides can be damaged by the action of pepsin and trypsin degradation, thus significantly reducing their activity (Bao et al., 2019).

Different strategies, including tablet coating or bioactive encapsulation, have been developed to provide an adequate delivery systems for acid-sensitive products (Varum et al., 2020a, 2020b). Tablets have the disadvantages of low compressibility, slow dissolution or bitter taste (Al-Tabakha, 2010). In addition, during the early stages of drug development, the limited amount of drug availability can impede the development of a coated pellet or tablet formulation (Cole et al., 2002). Therefore, certain capsule polymers, like cellulose derivatives or acrylic/methacrylic acid derivatives may offer a better solid dosage form and also provide the possibility to deliver liquids or semi-solid formulations to the small or large intestine (Cole et al., 2002; Barbosa et al., 2019). Thus, capsule technology has made a significant progress in the last years, offering economically convenient alternatives for drug and nutraceutical formulation as well as functionality for targeted entity release.

To achieve controlled release and optimal performance or product bioactivity, modification of capsule polymers or capsule-in-capsule (DUOCAP®) technology has been developed. In addition to gelatine, more recently developed polymers such as hydroxyl propyl methylcellulose (HPMC) have been proven to be suitable for manufacturing of capsules with different characteristics. For example, Vcaps® and Vcaps® Plus capsules are vegetarian alternatives with an immediate release and similar performance than gelatine capsules. Vcaps® capsules are composed of HPMC and gellan gum as gelling agent to enable the melt to gel at room temperature (Sherry et al., 2010), whereas Vcaps® Plus capsules are composed only of HPMC and the manufacturing process is based on thermal gelling process using a hot-dip method (Ku et al., 2011). The combination of HPMC and gellan gum in DRcaps® capsule has been shown to provide a delayed-release in the small intestine (Smith et al., 2010; Hashem et al., 2011; Das and Giri, 2020; Venema et al., 2020). DUOCAP® capsule is another technology which can improve the performance of bioactives. It is a patented delivery system that further extends the time of capsule disintegration by incorporating a smaller prefilled capsule into a larger liquid or solid-filled capsule. This configuration also allows the incorporation of multiple ingredients and dual release products (Venema et al., 2020).

Both polymer modification and capsule combination solutions have been assessed in the gastrointestinal track in both human clinical trials and *in vitro* models. These models have been developed as a potential first step in the screening with the advantages of being economically-affordable, reproducible, time-efficient, parameter-controlled, and a useful tool for initial screenings of dosage forms within the different compartments of the gastrointestinal tract (Vardakou et al., 2011; Peanparkdee et al., 2018; Brodkorb et al., 2019). Improvements over the previously developed static gastrointestinal digestion models have been recently proposed. Such models include the simulation of the transient nature of gastric secretions and gradual acidification in the gastric phase (Mulet-Cabero et al., 2020).

In this research, the performance of DRcaps®, Vcaps® or Vcaps® Plus capsules individually or in DUOCAP® capsule combinations has been assessed by caffeine release and probiotic survival of *Lactobacillus acidophilus*, in an improved semi-dynamic *in vitro* model of the upper GIT. Further, the viability and the function of a probiotic strain was assessed in a simulated human colonic microbial ecosystem for three selected capsule configurations. *Lactobacillus acidophilus* has been chosen as a prototype as it is widely used in the probiotic market and known for its susceptibility to gastric acid degradation (Dodoo et al., 2017).

2. Materials and methods

All the reagents used in this study were provided by Sigma (Overijse, Belgium) unless otherwise stated.

2.1. Composition of capsule systems

Seven types of DUOCAP® systems and three single capsules were evaluated in this study (Table 1). The configuration of the DUOCAP® capsule technology was a combination of outer capsules (size #00) and inner capsules (size #3) as follows: Vcaps® Plus capsule in DRcaps® capsule (referred to as VCP-in-DR in the manuscript), Vcaps® capsule in DRcaps® capsule (VC-in-DR), DRcaps® capsule in DRcaps® capsule (DR-in-DR), Vcaps® capsule in Vcaps® capsule (VC-in-VC), Vcaps® Plus capsule in Vcaps® capsule (VCP-in-VC), DRcaps® capsule in Vcaps® capsule (DR-in-VC), DRcaps® capsule in Gelatine capsule (DR-in-HG). Glycerol was used as a filling for the outer capsule, except in Gelatine/DRcaps®, in which fish oil and silica were used. Single capsules tested were DRcaps®, Vcaps® or Vcaps® Plus capsules. Capsules were filled with caffeine (50 mg/capsule) as a marker for release and a probiotic strain (*L. acidophilus* ATCC-43121, LGC Standards) at a concentration of 2x10^10 CFU/capsule as indicated in Table 1. The capsules were supplied by (Capsules & Health Ingredients, Lonza Inc., USA).

2.2. Upper gastrointestinal tract simulation under fed or fasted conditions

The upper GIT simulation was performed in two sequential double-jacketed reactors simulating the stomach and small intestine digestion conditions. The temperature was maintained at 37 °C and continuous magnetic stirring (300 rpm) was applied during the experiments. Capsules were maintained in the stomach and small intestinal reactors with specially designed sinkers for capsule dissolution studies (ProSense, Oosterhout, The Netherlands). To mimic fed (i.e., consumption of the product during or immediately after a meal) and fasted (i.e., consumption of the product before a meal) conditions, the pH profile, enzyme levels and retention times were adjusted (Supplementary Fig. 1). Under fasted conditions, the stomach digestion was simulated with a 45 min incubation in a gastric fluid (76 mL, pH 2) containing KCl 0.66 g/L, NaCl 3.63 g/L and mucin 3.95 g/L, 0.4 mL of lecithin (Carl Roth GmbH + Co. KG, Germany) (3.4 g/L) and 3.6 mL pepsin (Chem Lab, Zedelgem, Belgium) (10 g/L). Continuous pH control was performed by a Senseline pH meter F410 (ProSense, Oosterhout, The Netherlands) and an automatic pump dosage of HCl (0.5 M) or NaOH (0.5 M) to keep the pH constant at 2. After the stomach incubation, the gastric digestion volume was measured and adjusted to 100 mL with MilliQ water. Capsule sinkers and gastric fluids were transferred to the small intestine reactors and 35.2 mL pancreatic juice ($NaHCO_3$ 2.6 g/L, Oxgall 4.8 g/L and pancreatin 1.9 g/L), 2.15 mL trypsin (10 g/L) and 2.7 mL chymotrypsin (10 g/L) were added. The small intestine pH was gradually increased from 2 to 6.5 and maintained at this pH over a 27 min period, simulating the duodenal incubation. This phase was followed by a stepwise pH increase (0.1 pH units every 7 min) to 7.5 within a 63 min period, mimicking the jejunal environment. Finally, the pH remained constant at 7.5 during a 90 min ileal incubation. The pH increase was achieved by

Table 1
Capsule configurations.

Capsule	Configuration	Outer Capsule	Outer Capsule Fill	Inner Capsule	Probiotic Strain
VCP-in-DR	DUOCAP® system	Size #00 DRcaps® capsule	Glycerol	Size #3 Vcaps® Plus capsule	*L. acidophilus* LA-14
VC-in-DR	DUOCAP® system	Size #00 DRcaps® capsule	Glycerol	Size #3 Vcaps® capsule	*L. acidophilus* LA-14
DR-in-DR	DUOCAP® system	Size #00 DRcaps® capsule	Glycerol	Size #3 DRcaps® capsule	*L. acidophilus* LA-14
VC-in-VC	DUOCAP® system	Size #00 Vcaps® capsule	Glycerol	Size #3 Vcaps® capsule	*L. acidophilus* LA-14
VCP-in-VC	DUOCAP® system	Size #00 Vcaps® capsule	Glycerol	Size #3 Vcaps® Plus capsule	*L. acidophilus* LA-14
DR-in-VC	DUOCAP® system	Size #00 Vcaps® capsule	Glycerol	Size #3 DRcaps® capsule	
DR-in-HG	DUOCAP® system	Size #00 Gelatine capsule	Fish Oil 18/12 + Silica	Size #3 Banded DRcaps® capsule	*L. acidophilus* LA-14
DRcaps	Single	Size #00 DRcaps® capsule	–	–	*L. acidophilus* LA-14
Vcaps	Single	Size #00 Vcaps® capsule	–	–	*L. acidophilus* LA-14
Vcaps Plus Capsule	Single	Size #00 Vcaps®Plus capsule	–	–	*L. acidophilus* LA-14

the addition of $NaHCO_3$ (8.4 g/L) at 60, 90 and 120 min, mimicking the dilution of the intestinal contents (Riethorst et al., 2018). Under fed conditions, testing was carried out in similar way than the fasted conditions with the following modifications. The stomach digestion was simulated with a 120 min incubation in a solution of 76 mL of gastric juice containing the SHIME® nutritional medium (PDNM001B 20.53 g/L, ProDigest, Ghent, Belgium), NaCl (3.63 g/L), KCl (0.65 g/L), 0.4 mL lecithin (13.5 g/L) and 3.6 mL pepsin (40 g/L) at pH 4.6. During the fed-stomach digestion, a sigmoidal decrease of the pH from 4.6 to 2 was obtained by a controlled pump of HCl (0.5 M) at established time points. After the stomach incubation, a small intestinal phase was performed as described before, but with different compositions of pancreatic juice ($NaHCO_3$ 7.7 g/L, oxgall 15 g/L and pancreatin 10 g/L), 2.15 mL trypsin (10 g/L), 2.7 mL chymotrypsin (10 g/L). The pH increase was achieved by adding $NaHCO_3$ (4.8 g/L) at 60, 90 and 120 min. A blank control without capsules, caffeine or *L. acidophilus* was included in all the assays as a background media for the caffeine HPLC analysis. The negative control consisted of naked *L. acidophilus* and caffeine. All the assays were performed in triplicate.

2.3. Whole gastrointestinal tract simulation and colonic fermentation

Following the upper GIT incubations under fed and fasted conditions, as described above, a colonic incubation was simulated by addition of 160 mL fresh colonic anaerobic medium [KH_2PO_4 (6.6 g/L), K_2HPO_4 (20.5 g/L), NaCl (5 g/L), yeast extract (2 g/L), peptone (2 g/L), glucose (1 g/L), starch (2 g/L), mucin (1 g/L), L-cysteine HCl (0.5 g/L), Tween® 80 (2 mL)], 40 mL of anaerobic PBS [K_2HPO_4 (8.8 g/L), KH_2PO_4 (6.4 g/L), NaCl (8.5 g/L) and L-cysteine HCl (0.5 g/L)]. A fixed pH interval between 6.5 and 5.8 was implemented and automatically adjusted by adding HCl (0.5 M) or NaOH (0.5 M). Next, a fecal inoculum derived from a healthy donor (male, 32 y) was used to inoculate the colonic incubation, as previously described (Van den Abbeele et al., 2018a,b; Ghyselinck et al., 2021). Briefly, a mixture of 1:10 (w/v) of fecal sample and anaerobic phosphate buffer (K_2HPO_4 8.8 g/L; KH_2PO_4 6.8 g/L; sodium thioglycolate 0.1 g/L; sodium dithionite 0.015 g/L) was homogenized for 10 min (BagMixer 400, Interscience, Louvain-La-Neuve, Belgium). After centrifugation (2 min, 500g) (Centrifuge 5417C, Eppendorf, VWR, Belgium), large particles were removed and the fecal inocula was added to the different reactors at 20% (v/v) to the upper GIT digestion fluids. Colonic incubations were performed under anaerobic conditions at 37 °C, and 90 rpm agitation during 24 h (MaxQ 4000 Benchtop Orbital Shaker, Thermo Fisher Scientific, Belgium).

2.4. Caffeine release quantification

Caffeine was quantified by HPLC-UV/Vis (Hitachi Chromaster HPLC-DAD, Hitachi High-Tech Corporation, Japan) using an isocratic separation method (25 %methanol:75 %water) on a Kinetex® C18 LC column (serial number 00D-4601-E0; 5 μm,100 Å, LC Column 100 × 4.6 mm, solid support of Core-shell Silica) (Phenomenex, Belgium). The column temperature was kept controlled at 25 ± 0.1 °C. The retention time of caffeine was 3.18 ± 0.2 min and the total run time 7 min. The injection volume was 10 μL and the UV/Vis detector was operated at 272 nm. Quantification of caffeine was performed using external standards (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). Prior to injection in the column, the samples were centrifuged for 15 min at 5000 g. Subsequently, the supernatant was filtered through a 0.2 μm filter into HPLC vials. Caffeine analysis was performed on gastric samples at 15, 30 and 45 min (fed and fasted) and 60, 90 and 120 min (fed). Small intestinal samples were collected at 30, 60, 90, 120, 150 and 180 min. Colonic samples were obtained at 1, 2 and 24 h of incubation.

2.5. L. acidophilus survival by PMA-based qPCR

Bacterial survival was tested by propidium monoazide (PMA) based qPCR. For this procedure 1:1 (v/v) dilution of sample in anaerobic phosphate buffer was mixed with 1.25 μL PMAxx™ dye (20 mM) (VWR International Europe, Leuven, Belgium). Samples were incubated 5 min in constant shaking (500 rpm) in the dark and centrifuged at max. speed (18,327 g) for 30 sec. Subsequently, the samples were placed in the PhAST blue PhotActivation System (GenIUL, Barcelona, Spain), a LED-active Blue system (GenIUL, Barcelona, Spain), for 15 min and centrifuged 10 min at 13,000g. The supernatant was immediately removed, and DNA was isolated as described before (Boon et al., 2003) with modifications described in (Duysburgh et al., 2019). The qPCRs were performed with specific primers for *Lactobacillus acidophilus* [L.acid_F (5′-GAAAGAGCCCAAACCAAGTGATT-3′) and L. acid_R (5′- CTTCCCA-GATAATTCAACTATCGC-3′)] (Haarman and Knol, 2006), using a QuantStudio 5 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) with the program conditions previously described in (Van den Abbeele et al., 2018a,b). *L. acidophilus* survival was tested at the end of the stomach incubations (45 min for fasted condition and 120 min for fed condition), at 60, 120 and 180 min of the small intestinal digestion and at 1, 2 and 24 h of colonic fermentation.

2.6. L. acidophilus cultivability

In samples obtained during the entire gastrointestinal tract passage, *L. acidophilus* cultivability was tested through MRS agar plating. Samples were collected at the end of the gastric (45 min for fasted and 120 min for fed) and small intestinal phase (180 min) and a ten-fold dilution series in anaerobic phosphate-buffered saline were plated in MRS agar plates. Plates were incubated aerobically at 37 °C for at least 48 h. The number of colony-forming units (CFU) is reported as average log (CFU) ± SEM (n = 3).

2.7. Evaluation of the L. acidophilus function and the metabolic activity of gut microbiota under colonic conditions.

During 24 h of colonic incubation, samples at time point 0, 1, 2 and 24 h were obtained for microbial activity assessment. The pH

measurements were performed using a Senseline pH meter F410 (ProSense, Oosterhout, The Netherlands). Short chain fatty acids (SCFA) (acetate, propionate, and butyrate) and branched chain fatty acids (BCFA) (isobutyrate, isovalerate, and isocaproate) were determined by gas chromatography as previously described (Ghyselinck et al., 2020). Lactate production was assessed with a kit (R-Biopharm, Darmstadt, Germany), according to the manufacturer's instructions.

2.8. Statistical methods

Results are presented of the mean and standard error of the mean (SEM) from triplicates. Two-way ANOVA tests including time and different conditions were applied, with t-Tukey test for multiple comparisons. Significant statistical differences were set as a $p < 0.05$. Analysis were performed using GraphPad Prism software, version 9.0 (GraphPad Software, CA, USA). The detailed comparison between capsules is presented in the supplementary Tables S2–S4.

3. Results

3.1. Characterization of capsule release behavior during the upper gastrointestinal tract passage under fed or fasted conditions

In the first part of the study, 10 capsule configurations were subjected to passage in upper GIT simulation under fasted and fed conditions (Fig. S1). Caffeine was used as an active marker to evaluate the capsules dissolution at different time points during gastric and small intestinal digestion-like environment. During the fasted condition (Fig. 1 A B) and after 15 min of gastric digestion, there was a release of caffeine from the Vcaps® Plus capsules (19.7 ± 1.3 mg) and in to lesser extend for Vcaps® capsules (0.7 ± 0.3 mg) and DRcaps® capsules (0.2 ± 0.04 mg). After 30 min of incubation, the free caffeine for Vcaps® Plus capsules increased rapidly (40.8 ± 2.6 mg), and in to less extend for Vcaps® capsules (5.7 ± 1.4 mg), DRcaps® capsules (0.7 ± 0.1 mg), VCP-in-VC (0.5 ± 0.1 mg), VCP-in-DR (0.1 ± 0.003 mg) and DR-in-HG (0.1 ± 0.02 mg). At the end of the stomach incubation (45 min), Vcaps® Plus capsules had the highest caffeine release (41.9 ± 2.8 mg), showing a complete dissolution of the capsule. Other capsules displayed a partial caffeine release, with values of 11.3 ± 2.2 mg, 2.9 ± 1.5 mg and 1.5 ± 0.3 mg in Vcaps® capsules, VCP-in-VC and DRcaps® capsules, respectively. Finally, the lowest values of caffeine release (0.1–0.2 mg), indicating a highest capsule integrity, was found for DR-in-HG, DR-in-VC, VCP-in-DR, VC-in-VC, DR-in-DR and VC-in-DR.

At the end of the duodenal incubation (Fig. 1 C D), there was a significant increase in caffeine release for Vcaps® capsules (36.4 ± 3.9 mg) and VCP-in-VC (27.0 ± 9.8 mg) and a small but steady release for DRcaps® capsules (6.5 ± 1.5 mg), VC-in-VC (5.8 ± 1.4 mg), VC-in-DR (3.8 ± 0.6 mg), DR-in-HG (1.7 ± 0.5 mg), DR-in-VC (1.0 ± 0.1 mg) and VCP-in-DR (0.5 ± 0.2 mg). DR-in-DR remained intact (0.2 ± 0.1 mg), while the caffeine from the Vcaps® Plus capsules was already released during the stomach incubation. After 60 min of the small intestine incubation, there was a significant increase in caffeine release for VC-in-VC (42.6 ± 4.8 mg) and VCP-in-VC (36.5 ± 3.0 mg), indicating a complete dissolution of the capsules in the middle of jejunal phase. Vcaps® capsules were also completely disintegrated (41.8 ± 2.3 mg). Slow but steady release continued for DR-in-HG (18.5 ± 13 mg), VC-in-DR (14.3 ± 1.2 mg), DRcaps® capsules (12.7 ± 1.7 mg), DR-in-VC (3.8 ± 0.1 mg) and VCP-in-DR (2.5 ± 0.9 mg). The first release of caffeine was detected for DR-in-DR (0.9 ± 0.2 mg) under the jejunal incubation conditions. At the start of the ileal phase, after 90 min of small intestinal incubation, there was a significant increase in caffeine release for VC-in-DR (38.2 ± 4.6 mg) and DR-in-HG (31.1 ± 9.8 mg), and slower release for DRcaps® capsules (20.1 ± 3.4 mg), VCP-in-DR (8.8 ± 3.8 mg), DR-in-VC (8.8 ± 0.1 mg) and DR-in-DR (3.01 ± 0.7 mg). Further in the ileal phase, after 120 min of small intestinal incubation, VC-in-DR and DR-in-HG were completely dissolved. A significant increase in caffeine release was observed for the VCP-in-DR (31.6 ± 6.6 mg). The other capsules, i.e., DRcaps® capsules (26.2 ± 4.7 mg), DR-in-VC (21.4 ± 0.6 mg) and DR-in-DR (7 ± 1.7 mg), still showed high integrity, with lower and continuous caffeine release, until the end of the small intestinal phase. Partially dissolved capsules at the end of the incubation were DR-in-DR (24.2 ± 6.8 mg), while DR-in-VC (46.3 ± 4.2 mg), VCP-in-DR (41.5 ±

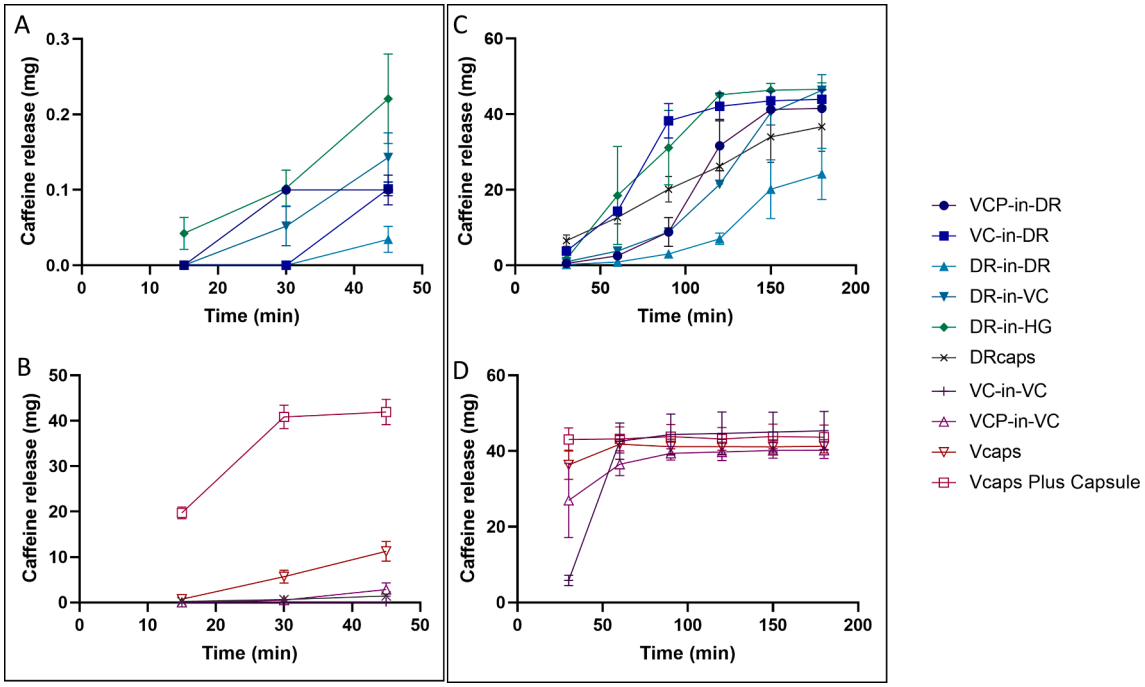

Fig. 1. Effect of capsule configuration on caffeine release during the stomach (left panel) and small intestinal (right panel) simulated digestion in fasted conditions. Dots represent caffeine release in gastric digestion media (A-B) or in the small intestinal digestion media (C-D) at different time points (mean ± SEM, n = 3). Significant differences between different capsule configurations and control are presented in supplementary Table S2.

0.4 mg) and DRcaps® capsules (36.7 ± 6.5 mg) were completely dissolved.

During the fed incubation (Fig. 2 A. B.), and after 15 min of stomach digestion, caffeine was detected in Vcaps Plus capsules (19.5 ± 7.6 mg) and Vcaps® capsules (2.4 ± 1.4 mg), while after 30 min of gastric digestion, only a small caffeine release (0.1–0.9 mg) occurred for VCP-in-VC, DRcaps® capsules, VCP-in-DR, VC-in-VC, DR-in-VC and DR-in-HG. An increase in caffeine release was observed for Vcaps® capsules (32.2 ± 3.3 mg) and Vcaps® Plus capsules (35.3 ± 2.5 mg). After 45 min, the Vcaps® Plus capsules were dissolved. The Vcaps® capsules released 35.4 ± 2.3 mg caffeine. Other capsules showed a slow but steady release: VCP-in-VC (1.3 ± 0.7 mg), DRcaps® capsules (1.3 ± 0.1 mg), VCP-in-DR (0.5 ± 0.1 mg), VC-in-VC (0.4 ± 0.1 mg), DR-in-VC (0.2 ± 0.01 mg) and DR-in-HG (0.2 ± 0.1 mg), with VC-in-DR and DR-in-DR showing a first sign of caffeine release (0.1 ± 0.003 mg). In the mid-stomach incubation (60 min), the Vcaps capsule is completely dissolved. Slow but steady release continued for the following capsules: VC-in-VC (5.2 ± 2.3 mg), VCP-in-VC (4.3 ± 1.0 mg), VCP-in-DR (1.9 ± 0.1 mg), DRcaps® capsules (2.3 ± 0.4 mg), VC-in-DR (0.5 ± 0.1 mg), DR-in-HG (0.5 ± 0.2 mg), DR-in-VC (0.4 ± 0.1 mg) and DR-in-DR (0.3 ± 0.2 mg). After 90 min of the stomach incubation, a significant increase in caffeine release occurred for VC-in-VC (39.8 ± 0.1 mg), indicating a complete dissolution of the capsule, and in a lesser extend for VCP-in-VC (20.1 ± 2.1 mg) and VCP-in-DR (11.7 ± 3.5 mg). Slow but steady release continued for DRcaps® capsules(5.6 ± 1.1 mg), VC-in-DR (2.8 ± 0.6 mg), DR-in-HG (2.1 ± 0.8 mg), DR-in-VC (1.7 ± 0.5 mg) and DR-in- DR (0.2 ± 0.01 mg). At the end of the stomach incubation, the VCP-in-VC capsules were completely dissolved. The other capsules were partially dissolved: VCP-in-DR (20.6 ± 3.6 mg), DRcaps® capsules (9.5 ± 2.1 mg), VC-in-DR (6.2 ± 1.0 mg), DR-in-HG (5.8 ± 1.5 mg), DR-in-VC (4.7 ± 1.4 mg) and DR-in-DR (0.7 ± 0.04 mg).

The small intestinal incubation (Fig. 2 C D) started with four completely dissolved capsules: Vcaps® Plus capsules, VC-in-VC, VCP-in-VC and VCP-in-DR. After the duodenal incubation, the VCP-in-DR capsules were also completed dissolved (41.4 ± 0.7 mg). The slow caffeine release continued in the small intestine for DR-in-HG (23.7 ± 7.3 mg), DR-in-VC (22.4 ± 4.8 mg), DRcaps® capsules (14.5 ± 3.1 mg), VC-in-DR (12.3 ± 1.9 mg) and DR-in-DR (5.5 ± 0.1 mg). In the jejunal phase (60 min of small intestinal digestion), caffeine release of DR-in-HG (37.1 ± 5.0 mg) and DR-in-VC (35.7 ± 5.9 mg) was complete, while DRcaps® capsules (18.8 ± 3.1 mg), VC-in-DR (17.7 ± 3.9 mg) and DR-in-DR (6.3 ± 3.8 mg) showed higher integrity and lower caffeine release to the digestion media. In the ileal phase (90 min of small intestine digestion), DR-in-HG and DR-in-VC were completely dissolved. There was a significant increase in caffeine release of the VC-in-DR (37.4 ± 7.8 mg) at the start of the ileal phase, indicating a complete dissolution of the capsule. DRcaps® capsules (22.8 ± 3.1 mg) and DR-in-DR (15.8 ± 3.2 mg) continued having a slow caffeine release until 120 min of small intestine incubation, when all the caffeine contained in DR-in-DR was present in the digestion fluids (44.5 ± 2.0 mg), indicating a complete disintegration of the capsule. Only the DRcaps® capsules continued their slow and steady caffeine release throughout the whole incubation until a final release of 32.7 ± 2.5 mg of caffeine at the end of the small intestinal incubation.

3.2. *Protection of L. acidophilus by DR-in-DR and VC-in-DR during the stomach and small intestinal-like environment digestion promote probiotic survival at colonic level*

In the second part of the study, three capsule configurations (DR-in-DR, VC-in-DR and Vcaps® Plus capsule) were selected based on their delayed release in the first part of the testing, to evaluate their behavior during the full gastro-intestinal tract under fasted or fed conditions. *L. acidophilus* survival and its modulatory effect in a colonic ecosystem were further tested. Vcaps® Plus capsule was selected as a control for immediate release. DR-in-DR was selected as this was the DUOCAP® system with the slowest caffeine release in the upper GI sections in fasted and fed conditions. The third capsule, VC-in-DR, was selected as the second slowest caffeine release delayed DUOCAP® system in fed conditions. As previously observed, under both fed and fasted conditions, caffeine release was significantly faster for Vcaps® Plus capsules than for the dual configurations (Fig. 3 A C), indicating a disintegration of the capsule before arriving to the colonic environment. At the end of the small intestine incubation time, DR-in-DR were partially dissolved, with a complete capsule dissolution after one hour of colonic incubation. At the end of the stomach incubation, and under both fed and fasted states, PMA-DNA copies *L. acidophilus* (Fig. 3 B D) were similar for both VC-in-DR (log 5.2 ± 0.1 copies/mL) and DR-in-DR (log 5.0 ± 0.2 copies/mL), while higher PMA-DNA copies were detected for Vcaps® Plus capsules (log 7.94 copies/mL), likely due to higher release of the probiotic strain into the digestion fluid. However, after 60 min of small intestinal incubation, this number was reduced to log 6.2 ± 0.3 copies/mL, while for

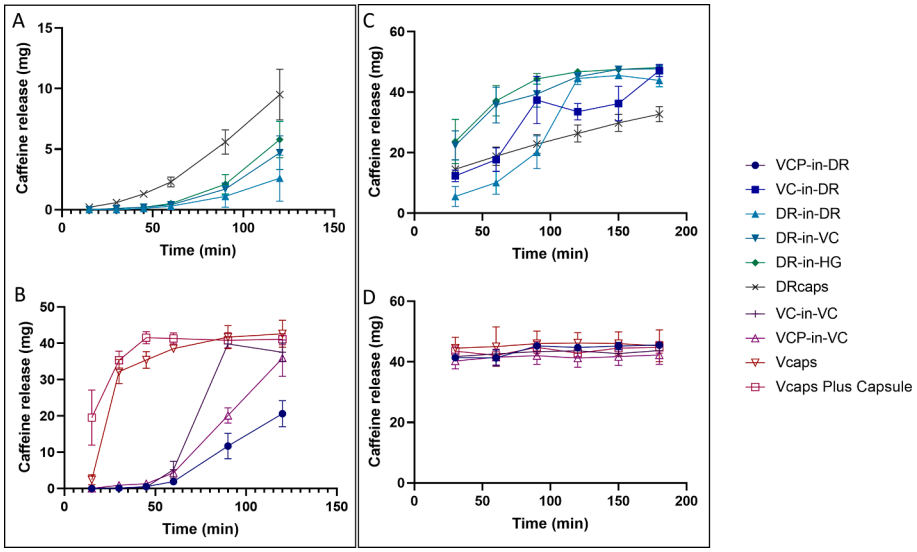

Fig. 2. Effect of capsule configuration on caffeine release during the stomach (left panel) and small intestinal (right panel) simulated digestion in fed conditions. Dots represent caffeine release in gastric digestion media (A-B) or in the small intestinal digestion media (C-D) at different time points (mean ± SEM, n = 3). Significant differences between different capsule configurations and control are presented in supplementary table S3.

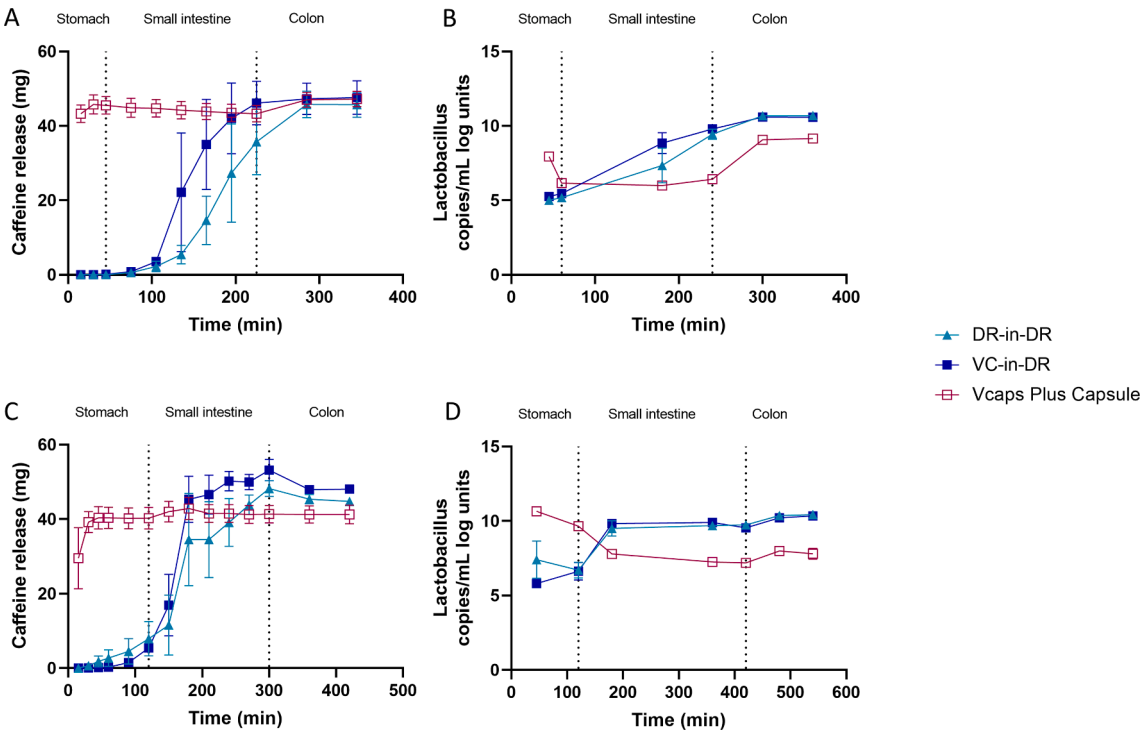

Fig. 3. Effect of capsule configuration on caffeine release and probiotic survival. (A, C) Time-course of caffeine release during the gastric, small intestine and colonic digestion in fasted (A) and fed (C) conditions. Dots represent caffeine content in the corresponding digestion or fermentation media at selected time points (mean ± SEM, n = 3). (B, D) Time-course of Lactobacillus acidophilus survival in the gastric, small intestine and colonic digestion if fasted (B) and fed (D) conditions. Dots represent copies/mL in log units of PMA treated samples in the corresponding digestion or fermentation media at selected time points (mean ± SEM, n = 3). Significant differences between the capsule configurations and control are presented in supplementary table S4.

the other capsules, PMA-DNA copies remained within similar values. After 120 min of small intestinal incubation, the PMA-DNA copies *L. acidophilus* were log 8.8 ± 0.7 copies/mL for VC-in-DR and log 7.4 ± 1.2 copies/mL for DR-in-DR, indicating a high survival of the strain until the end of the small intestinal conditions.

L. acidophilus survival based on its growth on agar plates after gastric and intestinal passage is presented for both fasted and fed conditions (Fig. 4). *L. acidophilus* from DR-in-DR and VC-in-DR showed a significantly higher growth than when it is included in Vcaps® Plus capsules under fasted conditions in the small intestinal environment (Fig. 4 A), while in the fed state, the difference was observed in the stomach phase after 120 min, with slower colony forming units (CFU) in DR-in-DR and VC-in-DR than in Vcaps® Plus capsules (Fig. 4 B). This is likely caused by a higher release of capsule contents from Vcaps® Plus capsules on stomach medium.

In the simulated colon environment, the probiotic administration's effect on the microbial activity via the three capsules was assessed by measuring the levels of SCFA at different time points (Fig. 5). In general, less effect was observed under fasted than fed conditions. Butyrate was the most affected metabolite. Under fasted conditions, significant difference was observed between VC-in-DR and DR-in-DR in one hand and Vcaps® Plus capsules in the other hand (Fig. 5). Specifically, butyrate was increased when *L. acidophilus* was included in DR-in-DR (6.0 ± 0.3 mM) or VC-in-DR (5.6 ± 0.3 mM) compared to the control Vcaps® Plus capsules (3.4 ± 0.1 mM). Ammonium levels slightly increased with Vcaps® Plus capsules (156.1 ± 6.1 mg/L) compared to VC-in-DR (143.8 ± 1.7 mg/L) while BCFA showed the opposite trend, with a significant decrease with Vcaps® Plus capsules (0.3 ± 0.01 mM) compared to DR-in-DR and VC-in-DR (0.48–0.5 mM). Under fed condition, pH decrease, a general marker for microbial activity was higher with DR-in-DR and VC-in-DR (−0.6 ± 0.01 Δ24-0 h), while lactate levels were significantly increased in both dual configurations (1.3–2.8 mM). Acetate and

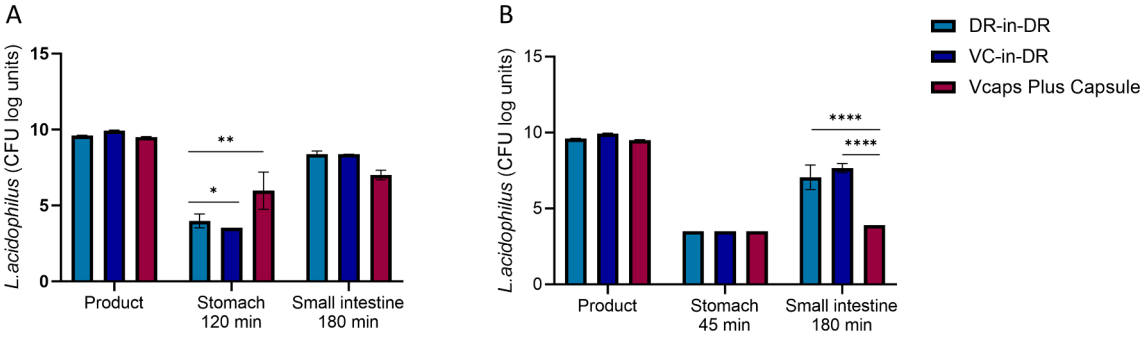

Fig. 4. Effect of capsule configuration on cultivability of L. acidophilus strain after the stomach and small intestinal simulated digestion in fasted (A) and fed (B) conditions. Bars represent CFU in log units (mean ± SEM, n = 3) obtained by plate counting from the stomach and small intestinal digestion fluids exposed to different capsule configurations. Product refers to the maximum L. acidophilus CFUs inoculated in the different capsules. Significant differences are marked with asterisks ($p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***, $p < 0.0001$ ****).

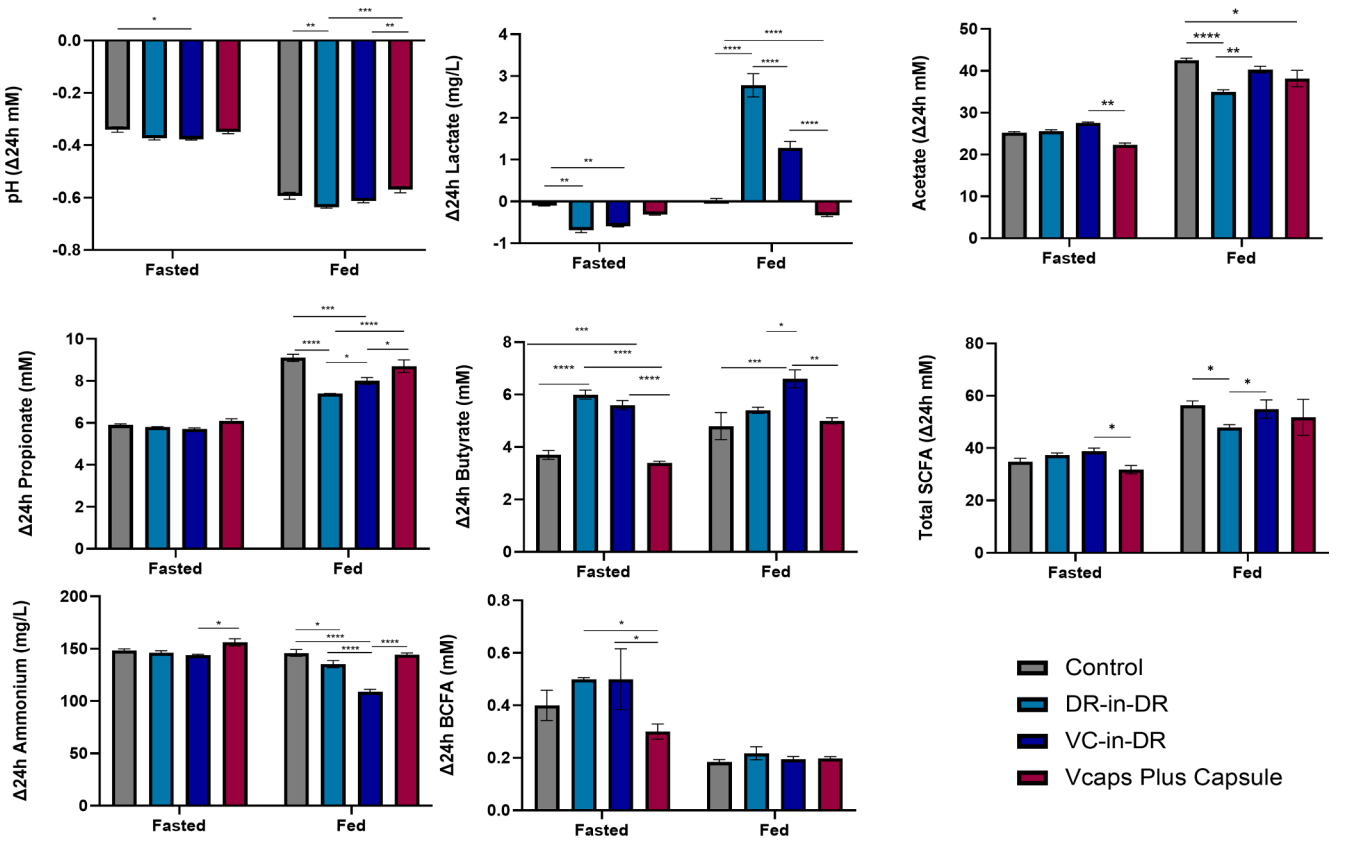

Fig. 5. Effect of probiotic administration through different capsules on microbial activity modulation in a simulated colonic environment. Bars represent the relative increase of different metabolites between time 0 and 24 h ($\Delta_{24h\text{-}0h}$) (mean ± SEM, n = 3) for pH, lactate, acetate, propionate, butyrate, total short chain fatty acids, ammonium and branched fatty acids.

propionate were reduced in DR-in-DR (acetate = 35.0 ± 0.8 mM; propionate = 7.4 ± 0.01) compared to the fed conditions (acetate = 38.2–42.5 mM; propionate = 8–9.1 mM). Contrarily, the highest butyrate levels were detected in VC-in-DR reactors (6.6 ± 0.3 mM), and the opposite effect was observed for ammonium (108.8 ± 4.2 mg/L). There were no significant differences in branched chain fatty acid production between the different capsules under fed conditions.

4. Discussion

Targeted delivery of pharmaceutically active compounds, nutritional supplements or probiotics is essential for providing the product performance and probiotic survivability and its function, including colonization (Yoha et al., 2021).

The most common capsule material has been gelatine due to its accessibility, low price, non-toxicity, solubility in biological fluids at body temperature, and gelation characteristics (Majee et al., 2017). However, some disadvantages have been described for gelatine such as reactivity towards aldehyde groups, sugars, metal ions, plasticizers, or preservatives. In addition, moisture changes due to high environmental humidity, dependent temperature release, and animal (porcine, bovine) origin are all disadvantages of gelatine (Majee et al., 2017). HPMC can overcome these limitations of the gelatine-based capsules, as it is a non-animal-based material, has low cross-reactivity with excipients, is stable in a wide range of temperatures and moisture conditions and has a proven safety record for human consumption (Al-Tabakha, 2010).

The aim of this research was to evaluate the release and disintegration characteristics of different HPMC-based capsule combinations as DUOCAP® capsule technology, using caffeine and probiotic survival as markers. SHIME model has been used to simulate the full length gastrointestinal tract conditions. We found that combinations which included DRcaps® capsules showed delayed caffeine release in the stomach and the small intestine under both fed and fasted conditions, and confered a significant increase in probiotic viability and performance at the colonic level.

The nature and the concentration of the gelling agent dictate the release behavior. Our research showed that at the end of the fasted and fed gastric environment, caffeine release was complete in single the Vcaps® capsule while its release was low with DRcaps® capsule. Vcaps® and DRcaps® capsules are both manufactured from HPMC, with gelling agent (gellan gum) incorporated in DRcaps® capsules as compared to Vcaps® capsules (Stegemann et al., 2018; Bucci et al., 2019). Gellan insolubility at pH lower than 4 and changes in HPMC films physical properties with gelation, increased resistance to the mechanical stress during the gastric passage (Yamamoto and Cunha, 2007; Ku et al., 2010; Grimm et al., 2019) and may be responsible of the delayed release behavior of DRcaps® capsules. It has been reported elsewhere that the HPMC capsules containing carrageenan as a gelling agent showed a fast disintegration profile *in vivo* under fasted conditions (complete release after 7–9 min), similar to gelatine capsules (Tuleu et al., 2007). In addition, gelling additives are also required for capsule shell HPMC manufacturing, because of the lower mechanical strength of the cellulosic film. Carrageenan and potassium chloride have been proven effective in HPMC gelation, while gellan gum combined with ethylene diamine tetraacetic acid (EDTA) or sodium citrate have been used in HPMC capsule production (Majee et al., 2017).

In the small intestinal phase, the highest delayed caffeine release was observed for DR-in-DR under fasted conditions and for DRcaps® capsules under fed conditions, both not achieving, however, release of all the caffeine even at the end of the small intestine. This observation suggests that DR-in-DR can be used for colonic-targeted delivery beyond the small intestine, possible to the colon where it can be useful in

delivering viable probiotics at their site of action, as demonstrated by the *L. acidophilus* viability and function. Probiotic viability along with storage or administration are important factors of its efficacy (Govender et al., 2014; Dodoo et al., 2017). Thus, orally administered probiotics, delivered alive and in the right dose is a requisite for their performance (Han et al., 2021).

The caffeine release from DRcaps® capsules followed a linear trend ($R^2 > 0.9$) under both fed and fasted conditions, suggesting a steady-state delivery sustained in time, which may also be beneficial for probiotic engraftment in the gut. The change in the SCFA profile, suggest that other bacteria from the microbiota are affected by the introduction of the exogeneous *L. acidophilus*, indicating that this target delivery to the colon enabled modulation of the microbiome composition. In particular, the observed increased in lactic acid suggest conization by *L. acidophilus.* A viable "colonizer" microorganism in a sufficient mass, introduced in a complex ecosystem, can compete with other commensals thus modulating the diversity of the microbiome (Walter et al., 2018). This process is known as the propagule pressure hypothesis, where successful invasions require a sufficient number of individuals to enter the ecosystem, which relates to the cell numbers (or dose) of the treatment and frequency with which they are applied (Catford et al., 2009). Probiotic strains are not easily engrafting in the human gut ecosystem, due to the resilience of pre-established niches of commensal microorganisms (Walter et al., 2018). However, under dysbiotic conditions following antibiotic intake for example, the potential benefit of probiotic microorganisms to colonize and restore gut homeostasis may be improved by a targeted colonic delivery using DUOCAP® formulations. Indeed, previous research *in vivo* showed that DRcaps® capsule-based DUOCAP® systems were resistant to low pH gastric environment under fasted conditions (Grimm et al., 2019). The same authors reported high interindividual variability in gastric emptying time, which can significantly affect disintegration times and product release. Despite *in vivo* conditions that may differ from *in vitro* tests due to the complex nature of the gastrointestinal processes and the inter-individual variability, different *in vitro* models simulating the gastrointestinal digestion have been developed to mimic the human physiology under fasted and fed conditions (Li et al., 2020; Mulet-Cabero et al., 2020). Physiological gastric and intestinal pH and bile salts concentrations undergo gradual changes during the digestion processes (Mudie et al., 2010; Amara et al., 2019), which were reproduced in this research by steady addition of acid and digestive fluids, improving the previously developed static settings (Brodkorb et al., 2019). Including duodenal, jejunal and ileal phases, with different pH, retention times, and bile salts concentrations, brought the *in vitro* systems closer the gastrointestinal digestion in humans.

Changes in caffeine release were accompanied by differences in viability of *L. acidophilus*, especially under fasted conditions. To further assess the function of *L. acidophilus* at its site of action, we evaluated if these changes in probiotic viability had an effect on gut microbial modulation under colonic conditions. Gastrointestinal digestion was continued with a simulated colonic fermentation for three selected capsules. Detection of viable *L. acidophilus* in the colonic environment was significantly higher when administered in DR-in-DR or VC-in-DR. Vcaps® Plus capsule was used as a negative control, as suggested by lactic acid decrease. In addition, DR-in-DR and VC-in-DR also affected the microbial colonic function, suggesting a potential modulation of its composition and diversity, based on the resulting decrease in acetate and propionate and increase in butyrate. Protection of *L. acidophilus* may have induced higher acidification of colonic media and lactate production, potentially by providing lactate as a substrate to other bacteria in the microbiota (cross-feeding interactions). It has been previously described that probiotic *Lactobacillus* spp. can ferment non-digestible fibers to enable lactate production, used subsequently as a substrate by butyrate-producing bacteria (Duncan et al., 2004; Belenguer et al., 2007; Belenguer et al., 2011). Butyrate is a microbial metabolite with a key role in maintaining gut homeostasis, including immunoregulation, gut motility and epithelial barrier function (Hiippala et al., 2018).

Low stomach pH and high bile acid concentrations are the major factors in reducing probiotic viability (Sahadeva et al., 2011; Millette et al., 2013). Thus delayed-release delivery systems such as DRcaps® capsules or VC-in-DR, targeting colonic delivery, may improve probiotic performance in modulating gut microbial function and potentially its diversity and composition, as observed in this study *in vitro*, leading to various health benefits. On the other hand, the fast caffeine release from Vcaps® Plus capsules may suggest that this formulation can be used for targeted gastric release.

5. Conclusion

Using an improved SHIME model to simulate the GIT conditions and caffeine and *L. acidophilus*, viability as markers for capsule release and disintegration, we showed that DR-in-DR and VC-in-DR formulations led to the slowest release profile and therefore can be used to target delivery to the colonic environment, the main site of action for probiotics. Vcaps® Plus showed the fasted release profile, and can be used for ingredients intended to be released immediately. The other capsules showed an intermediate release profiles, making them good candidates for delivery of ingredient at different sites of the GI tract. Our data suggest controlled release of orally administered ingredients can optimize their doses, stability and overall performance.

CRediT authorship contribution statement

Massimo Marzorati: Supervision. **Marta Calatayud:** Writing – original draft. **Chloë Rotsaert:** Investigation. **Michiel Van Mele:** Investigation. **Cindy Duysburgh:** Investigation. **Shane Durkee:** Funding acquisition, Supervision. **Tyler White:** Data curation, Supervision. **Kelli Fowler:** Investigation. **Vincent Jannin:** Writing – review & editing. **Aouatef Bellamine:** Conceptualization, Writing – review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: At the time of the study, Shane Durkee, Tyler White, Kelli Fowler, Vincent Jannin and Aouatef Bellamine were employees of Lonza manufacturing and selling capsules used in this study.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpharm.2021.120977.

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