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S. Moutaharrik[a], A. Maroni[a]*, C. Neut[b], C. Dubuquoy[b], L. Dubuquoy[b], A. Foppoli[a], M. Cerea[a], L. Palugan[a], F. Siepmann[c], J. Siepmann[c] and A. Gazzaniga[a]

[a]Università degli Studi di Milano, Dipartimento di Scienze Farmaceutiche (DISFARM), Sezione di Tecnologia e Legislazione Farmaceutiche "Maria Edvige Sangalli", 20133 Milan, Italy; saliha.moutaharrik@unimi.it; alessandra.maroni@unimi.it; anastasia.foppoli@unimi.it; matteo.cerea@unimi.it; luca.palugan@unimi.it; andrea.gazzaniga@unimi.it
[b]Univ. Lille, Inserm, CHU Lille, U1286 - INFINITE - Institute for Translational Research in Inflammation, F-59000 Lille, France; christel.neut@univ-lille.fr; carodubu@yahoo.fr; laurent.dubuquoy@inserm.fr;
[c]Univ. Lille, Inserm, CHU Lille, U1008, F-59000 Lille, France; florence.siepmann@univ-lille.fr; juergen.siepmann@univ-lille.fr.
*Corresponding author: alessandra.maroni@unimi.it

ABSTRACT

Several formulation strategies have been proposed for oral colon delivery, particularly for the therapy of inflammatory bowel disease (IBD). However, targeting the large intestine remains a challenging goal. The aim of this study was to develop and evaluate a novel type of drug delivery system, which is based on multiple drug release triggers for reliable performance. The system consists of: *(i)* a drug core, *(ii)* an *inner* swellable low-viscosity hydroxypropyl methylcellulose (HPMC) layer, and *(iii)* an *outer* film coating based on a Eudragit® S : high-methoxyl (HM) pectin (7:3 w/w) blend, optionally containing chitosan. Convex immediate release tablets (2 or 4 mm in diameter) containing paracetamol or 5-aminosalicylic acid (5-ASA) were coated in a fluid bed. The double-coated tablets exhibited pulsatile release profiles, when changing the release medium from 0.1 N HCl to phosphate buffer pH 7.4. Also, drug release was faster in simulated colonic fluid (SCF) in the presence of fecal bacteria from IBD patients compared to control culture medium from tablets with outer Eudragit® S : HM pectin : chitosan coatings. The latter systems showed promising results in the control of the progression of colitis and the alteration of the microbiota in a preliminary rat study.

KEYWORDS

Oral colon delivery; 5-aminosalicylic acid; combined time-, pH- and microbiota-dependent approach; hydroxypropyl methylcellulose; hydroxypropyl cellulose; Eudragit® S; pectin; chitosan; spray-coating.

1. INTRODUCTION

Over the last decades, numerous strategies have been proposed for achieving site-selective drug delivery to the colon in order to better treat local disorders, *e.g.* inflammatory bowel disease (IBD) and irritable bowel syndrome, and to enhance oral bioavailability of biotechnological drugs compared to gastric and/or small intestinal delivery. Such strategies are typically based on the exploitation of physiological features of the gastrointestinal tract (GIT) such as pH, microbiota, transit time and luminal pressure [1].

The pH-dependent formulation approach, covering the vast majority of commercially available products indicated for the treatment of IBD, leverages pH changes along the GIT [2]. In particular, typical gastroresistant polymers, such as Eudragit® S, Eudragit® L and Eudragit® FS, are applied as coating agents. Nevertheless, the dissolution pH threshold of such polymers is often exceeded in the small intestine, while pH values below the threshold are typical for the proximal colon [3]. As a result, these systems may go through premature release or release failure. In the former case, the drug is released before reaching the colonic region, while in the latter case no drug is released and the delivery unit may be voided intact. Such issues were clearly highlighted by γ-scintigraphy studies of pH-sensitive delivery systems, which have reported erratic behavior *in vivo* [4–6].

In order to reduce the risk of release failure, a new design concept based on a coating layer composed of Eudragit® S in admixture with a microbially-degradable polysaccharide, namely high amylose starch, was proposed [7,8]. The idea is that the polysaccharide undergoes selective degradation by the microbiota at the target site, inducing coating rupture/permeability increase in case the system is not exposed to pH values above the dissolution threshold for a sufficient time period. An additional layer was added beneath the Eudragit® S : high amylose starch coating, facilitating its dissolution [9]. This inner coating layer is composed of partially neutralized Eudragit® S, combined with a buffer agent. Once water penetrates into this layer, it rapidly dissolves and creates an internal environment of high buffer capacity. Hence, the outer Eudragit® S layer can dissolve from both sides (outside and inside). A high-dose 5-aminosalicylic acid (5-ASA) tablet based on this double-coating design (Opticore™) has been

granted marketing authorization for the treatment of mild to moderate ulcerative colitis and maintenance of remission also of Crohn's disease.

To address the risk of potential *premature* release of enteric coated dosage forms, the following strategy has been proposed: A "back-up" polymer layer can be added beneath the outer enteric barrier. If the latter becomes permeable too early, the back-up layer protects the drug reservoir from release, adding a time lapse [10]. For instance, an outer polymer coating based on Eudragit® S and high amylose starch was "backed-up" with an underneath swellable hydrophilic layer, consisting of hydroxypropyl methylcellulose (HPMC). Also, a hydroxypropyl cellulose (HPC) capsule shell manufactured by injection-molding has been used for this purpose. The newly proposed formulations leveraging the luminal pH, enzymatic activity of the microbiota as well as the intestinal transit time, were shown to release the drug in simulated colonic fluid. On the other hand, these withstood exposure to 0.1 N HCl medium and, in phosphate buffer pH 7.4, a lag phase was imparted by the hydrophilic back-up layer.

A variety of other naturally-occurring polysaccharides, such as pectin, guar gum and chitosan, has been investigated as colon targeting excipients due to their intrinsic degradability by the microbiota in the large bowel [11]. It should be pointed out that certain starches require a heat treatment in the presence of 1-butanol prior to their use, to strengthen their resistance to digestion by pancreatic enzymes and their susceptibility to selective microbial degradation [8]. Such a pre-treatment step is to be conducted under well-defined experimental conditions, which could be dependent on the natural sources of the polysaccharide [8,12].

Pectin, a D-galacturonic acid-rich hetero-polysaccharide found in plant cell walls, has been employed either as a matrix-forming or compression-coating agent [1,13,14]. Due to the inherent hydrophilicity and water solubility, it was often applied in admixture with insoluble polymers (ethyl cellulose, cellulose acetate, polymethacrylates) to avoid premature drug release before the dosage form reaches the colon. Alternatively, the formation of a polyelectrolyte complex with positively-charged polymers, such as Eudragit® RL and chitosan, was attempted in order to slow down its dissolution [15–19].

In the present work, pectin was investigated as a microbially-degradable pore-former dispersed in an enteric film aiming at colon targeting, with a hydrophilic swellable polymer layer beneath. Optionally,

chitosan was added to the outer layer. Paracetamol and 5-aminosalicylic acid (5-ASA) loaded tablets were used as cores. The systems were characterized *in vitro* and *in vivo*, in HLA-B27 transgenic rats - a validated rodent model for colitis. Pentasa® served as a commercialized reference product.

2. MATERIALS AND METHODS

2.1 MATERIALS

Paracetamol for direct compression (Rhodapap™ DC 90, Novacyl, Lyon, France), 5-aminosalicylic acid (5-ASA, Chemi Spa, Cinisello Balsamo, Italy), Pentasa® prolonged-release granules (Ferring SAS, Gentilly, France; batch number: T0977A), microcrystalline cellulose (Avicel® PH-101, FMC Corporation, San Colombano al Lambro, Italy), sodium starch glycolate (Explotab® CLV, JRS PHARMA, Castenedolo, Italy), vinylpyrrolidone-vinyl acetate copolymer (Kollidon® VA 64, BASF Italia Spa, Cesano Maderno, Italy), povidone (Kollidon® K30, BASF Italia Spa) and crospovidone (Kollidon® CLM, BASF Italia Spa), glyceryl dibehenate (Compritol® 888 ATO, Gattefossé SA, Saint-Priest, France), lactose (Pharmatose® DCL11, DMV International bv, Veghel, Netherlands), talc (Tradeco Group Srl, Pioltello, Italy), hydrophilic fumed silica (Aerosil® 200, Evonik Degussa Italia Spa, Pandino, Italy), magnesium stearate (Carlo Erba Reagents, Srl, Cornaredo, Italy), hydroxypropyl methylcellulose (HPMC, Methocel® E50, Colorcon, Dartford, Kent, United Kingdom), polyethylene glycol (PEG 400, Clariant SE, Sulzbach am Taunus, Germany), methacrylic acid-methyl methacrylate copolymer (1:2) (Eudragit® S, EuS, Evonik Degussa Italia Spa, Pandino, Italy), high-methoxyl pectin (pectin, pec, Aglupectin HS-RP, JRS Silvateam Ingredients Srl, Bergamo, Italy), chitosan (chit, 90 % degree of deacetylation, MADAR Co., Fordingbridge, United Kingdom), triethyl citrate (TEC, Honeywell International Inc, Charlotte, North Carolina, United States), glyceryl monostearate (GMS, Gattefossé SA, Saint-Priest, France), sodium hydroxide (VWR International Srl, Milano, Italy), polysorbate 80 (Tween® 80, ACEF Spa, Fiorenzuola d'Arda, Italy), ammonia solution 25 % v/v (Carlo Erba Reagents Srl, Cornaredo, Italy), acetic acid (Merck KGaA, Darmstadt, Germany), hydrochloric acid 37 % v/v (Merck KGaA, Darmstadt, Germany). Beef extract (Becton-Dickinson, LePont de Claix, France), yeast extract (Oxoid, Basingstoke, United Kingdom), tryptone (Becton-Dickinson, LePont de

Claix, France), sodium chloride (NaCl, Acros, Geel, Belgium), L-cysteine hydrochloride (Acros, Geel, Belgium). Columbia Blood Agar (Oxoid, Basingstoke, United Kingdom), Drigalski Agar (Oxoid, Basingstoke, United Kingdom), Wilkins-West Agar [20], Kligler Iron Agar (Oxoid, Basingstoke, United Kingdom), May-Grünwald (Eosin methylene blue) Q path® solution (VWR International SAS, Rosny-sous-Bois cedex, France) and Giemsa's azur eosin methylene blue solution (Merck KGaA, Darmstadt, Germany).

2.2 METHODS

2.2.1 Manufacturing and physical characterization of tablet cores

Immediate-release tablets containing paracetamol were prepared from paracetamol DC (80 %), Avicel® PH 101 (12.5 %), Explotab® CLV (4.5 %), Kollidon® VA 64 (2.0 %), Aerosil® 200 (0.5 %) and magnesium stearate (0.5 %) powders, blended in a Turbula mixer (Willy A. Bachofen AG, Muttenz 1, Switzerland; 12 + 3 min, 200 rpm). Tableting was performed by a rotary press (AM-8S, Officine Ronchi, Italy), equipped with concave punches (4 mm diameter, 4 mm curvature radius), resulting in tablets with a nominal weight of 40 mg.

Immediate-release minitablets containing 5-ASA were prepared as follows: Drug (200 g) and lactose (57.6 g) powders were blended with a 10 % w/v povidone aqueous solution (98.0 g) and kneaded in a mortar for 5 min. The wet mass was then forced through a 500 µm net sieve. The obtained granules were oven-dried at 40 °C overnight and sieve-calibrated (500 µm). Crospovidone (3.7 %), talc (1.5 %) and glyceryl behenate (1.5 %) were added to the granules (93.3 %) and mixed in a V-blender (Erweka GmbH, Langen, Germany). The mixture was tableted using a rotary machine, equipped with concave punches of 2 mm in diameter and 1.5 mm in curvature radius, resulting in minitablets with a nominal weight of 7 mg.

The paracetamol tablets and 5-ASA minitablets were characterized with respect to their weight (analytical balance BP211D Sartorius Italy Srl, Varedo, Italy; n=20), friability (friabilometer TA3R Erweka GmbH, Langen, Germany), crushing strength (crushing tester TBH30 Erweka GmbH, Langen, Germany; n=10), height & diameter (digital micrometer, Mitutoyo Italiana Srl, Lainate, Italy; n=20) and disintegration time (three-position disintegration apparatus DT3 Sotax Srl, Milano, Italy; n=6). The

weight, height, diameter, crushing strength (mean ± s.d.), friability and disintegration time were as follows: For paracetamol tablets - 40.75 ± 0.50 mg, 3.20 ± 0.05 mm, 4.01 ± 0.01 mm, 40.6 ± 9.1 N, < 1 %, < 5 min; for 5-ASA minitablets - 7.22 ± 1.31 mg, 2.29 ± 0.06 mm, 2.00 ± 0.01 mm, 10.00 ± 1.87 N, < 1 %, < 5 min, respectively.

2.2.2 Manufacturing and physical characterization of coated systems

The tablet cores were coated with an aqueous solution of HPMC (8 % w/w + 10 % PEG 400 on the dry polymer) up to different coating levels, corresponding to 70, 120 and 200 μm nominal thickness (HPMC$_{70}$, HPMC$_{120}$ and HPMC$_{200}$, respectively), in a tangential-spray fluid bed (Glatt GPCG 1.1, Glatt GmbH, Binzen, Germany) [21]. HPMC-coated tablet cores were then overcoated with a blend of Eudragit® S and pectin in a bench-top bottom-spray fluid bed apparatus (Mini-Glatt, Glatt GmbH, Binzen, Germany) up to 7 or 14 mg/cm^2 of polymethacrylate. Optionally, chitosan was added to form a polyelectrolyte complex with pectin. A 7:3 solid weight : weight ratio was maintained between Eudragit® S and the polysaccharide(s), irrespective of whether pectin alone or pectin with chitosan was used. TEC and GMS were added as plasticizing and anti-tacking agents, respectively. Eudragit® S was dispersed in distilled water at a concentration of 21.4 % w/w. 1 N ammonia solution was added dropwise up to theoretical 15 % neutralization of the polymethacrylate under magnetic stirring. After 1 h, TEC (70 % w/w based on dry Eudragit® S) and a fine 5 % w/w GMS (10 % based on dry Eudragit® S) dispersion were incorporated, and the blend was stirred for 1 h. The GMS dispersion was prepared by suspending the anti-tacking agent into an aqueous Tween® 80 (40 % w/w based on dry GMS) solution under vigorous mixing and heating to 75 °C for 15 min. When pectin was incorporated ”alone” in the polymethacrylate dispersion, it was dissolved in distilled water at 1.6 % w/w under magnetic stirring and heated to 60 °C. Alternatively, when pectin was used along with chitosan, it was first dissolved at 2 % w/w in 0.1 N HCl, followed by slowly adding a 2 % w/w chitosan solution in 0.1 N HCl under continuous magnetic stirring up to a pectin : chitosan weight : weight ratio of 5:1 [19]. The pH was then adjusted to approximately 6.0 with 1 N NaOH. Finally, the aqueous suspension of Eudragit® S, TEC and GMS was added dropwise to the pectin solution or pectin-chitosan suspension under magnetic

stirring to obtain the coating dispersions. The compositions of the coating formulations are summarized in Table I. The coating equipment and process parameters used are given in Table II.

Table I: Composition of the formulations used for the outer coating (amounts are indicated in % w/w)

Components	Eudragit® S : pectin coating	Eudragit® S : pectin : chitosan Coating
Eudragit® S	2.88	3.23
Pectin	1.24	1.15
Chitosan	-	0.23
Triethyl citrate	2.02	2.25
Glyceryl monostearate	0.29	0.32
Tween® 80	0.11	0.13
1 N NaOH	-	5.42
Distilled water	92.01	17.88
1 N Ammonia solution	1.44	1.61
0.1 N HCl	-	66.00

Table II: Coating equipment and process parameters

	HPMC inner coating	Eudragit® S : pectin outer coating	*Eudragit® S : pectin : chitosan* outer coating
Equipment	Tangential-spray fluid bed	Bottom-spray fluid bed	Bottom-spray fluid bed
Batch size (g)	1000	75	75
Nozzle pore size (mm)	1.2	0.5	0.5
Atomizing air pressure (bar)	2	1.0	1.0
Pattern air pressure (bar)	-	-	-
Drying air volume (m^3/h)	100	37 – 47	35 – 47
Inlet air temperature (°C)	59	40	40 – 43
Outlet air temperature (°C)	53	-	-
Product temperature (°C)	52	30 – 34	32 – 35
Spray rate (g/min/kg)	3 – 5	12 – 13	14 – 15
Post drying time (min)	30	10	5
Curing (h – °C)	-	*48 h – 40 °C*	*48 h – 40 °C*

At the end of the process, all units were oven-cured at 40 °C for 48 h.

The amount of coating material per unit area (mg/cm^2) was obtained from weight measurements (n=100) acquired before and after coating, calculating the surface of the tablets as follows:

$$\text{Tablet surface} = 4\pi(\mathrm{R}-\mathrm{r})\left(\mathrm{R}-\sqrt[2]{\mathrm{R}^2-\mathrm{r}^2}\right)+2\pi\mathrm{r}h_{cpr}$$

where R, r and h_{cpr} are the curvature radius, radius and height of the tablets. The thickness of the applied coatings was measured using a digital microscope (Dyno-Lite Pro AM-413T, AnMo Electronics Co., Hsinchu, Taiwan). Tablets were cut with a scalpel. The indicated coating thicknesses are mean values of 10 measurements performed in different regions in each of 3 cross-sectioned tablet). Cross-sections of tablets were also analyzed with a scanning electron microscope (SEM, VEGA-TS5136 XM, Tescan Analytics, BrnoKohoutovice, Czech Republic) after gold-sputtering in a plasma evaporator under argon flow (Sputter coater, Edwards, S150B, West Sussex, United Kingdom; 1 kV, 40 mA; 1 min). Photomicrographs were acquired at an accelerated voltage of 20 kV using a 100x magnification. SEM images were also used to randomly confirm coating thicknesses measured with the optical microscope.

2.2.3. In vitro characterization of coated tablets

Drug release from coated tablets (n=3) was measured using the USP 43 paddle apparatus (Dissolution System 2100B, Distek Strumenti & Misure Srl, Napoli, Italy; 100 rpm) in 800 mL 0.1 N HCl for 2 h, followed by phosphate buffer (PB) pH 7.4, at 37±0.5 °C. In the case of coated 5-ASA *mini*tablets, 7 units were placed in each vessel to achieve a total amount of 5-ASA equal to 37.5 mg, corresponding to the daily drug dose in the rat study. Fluid samples were automatically withdrawn at predetermined time points. The drug released over time

was assayed with a spectrophotometer (Lambda 35, PerkinElmer® Italia, Milano, Italy) at 248 and 302 nm in the case of paracetamol and 5-ASA, respectively.

Drug release was also studied in simulated colonic fluid (SCF). In this case, the coated tablets were pre-treated by exposure to 0.1 N HCl for 2 h, followed by PB pH 4.5 for another 2 h in a USP 43 dissolution paddle apparatus (100 rpm, 800 mL). Afterwards, the systems were transferred into 100 mL culture medium inoculated with fecal samples (1 g) collected from IBD patients [10,22]. For reasons of comparison, drug release was also measured in CM free of feces. CM was prepared by dissolving 1.5 g beef extract, 3 g yeast extract, 5 g tryptone, 2.5 g NaCl and 0.3 g L-cysteine hydrochloride in 1 L distilled water (pH 6.5±0.2) [23–25]. The resulting solution was sterilized in an autoclave, followed by incubation with the fecal samples under anaerobic conditions at 37±0.5 °C and horizontal shaking (50 rpm) for 20 h. Upon transfer of coated tablets into this release medium, 2 mL fluid samples were withdrawn at predetermined time points, centrifuged (13,000 rpm, 5 min), filtered (0.22 µm) and analyzed for their drug content by HPLC (ThermoFisher Scientific Ultimate 3000, Massachusetts, United States). In the case of *paracetamol*, a Gemini® 5 µm C18 110 Å, 150 × 4.6 mm column (Phenomenex, Le Pecq, France) was employed, and the mobile phase consisted of (A) water adjusted to pH 2 with orthophosphoric acid and (B) acetonitrile [10,26]. A gradient program was applied as follows: 0-10 min 5-20 % B; 10-11 min 20-5 % B. The flow rate was set at 1 mL/min, and the injection volume was 10 µL. Paracetamol was detected spectrophotometrically at 248 nm. In the case of *5-ASA*, a Gemini® 5 µm C18 110 Å, 100 × 4.6 mm column (Phenomenex, Le Pecq, France) was used. A blend of a 1 % w/v acetic acid solution and methanol (9:1 v/v ratio) was employed as the mobile phase. The flow rate and the injection volume were 0.9 ml/min and 10 µL, respectively. 5-ASA was detected spectrophotometrically at 302 nm [27].

2.2.4 In vivo evaluation of coated minitablets

5-ASA-loaded coated minitablets were evaluated in a rat colitis model. The study was conducted respecting all governmental guidelines (including n° 2010/63/UE; Décret 2013-118) and ethical rules (APAFIS#201809281814746). The rats came from two females (Cochin Hospital, Paris, France) carriers of various inflammatory pathologies, including ankylosing spondylitis and colitis, mated with Wild Type Fisher 344 male rats (Charles River Laboratoire France CRLF, Ecully, France) [28]. The treatment was started on 10 weeks old rats, *i.e.* 15 days before the onset of macroscopic lesions. The animals were divided into two groups (A and B), each group gathering 7 rats: Group A received the coated minitablets, while Group B served as a control, treated with Pentasa®. In both arms, the daily dose of 5-ASA was 37.5 mg. Feces were collected on Day 0 and Day 15. The rats were sacrificed after 15 days of treatment.

Bacteriological analyses were performed blindly. Fecal samples were diluted 1:10 and seeded by spreading 0.1 mL of the suspension onto Columbia Blood Agar and Drigalski Agar in Petri dishes. The Columbia Blood Agar plates were incubated at 37 °C for 7 days in anaerobiosis. The colonies were counted and identified by sub-culturing in Wilkins-West Agar and Wilkins-West fluid [20]. Identification of the bacteria was carried out by Matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF MS; Microflex®, Bruker Daltonics, Wissembourg, France): Samples were deposited in the form of spots onto a steel plate. Each spot was covered with an alpha-cyano-4-hydroxy-cinnamic acid matrix and allowed to dry before analysis. The generated spectra were processed using Biotyper 3.0 software (Bruker Daltonics) by comparison with the ones present in the database [29]. Spectral scores below 1.7 did not allow reliable identification, between 1.7

and 2.0 allowed identification to the genus level, and above 2 were associated with bacterial species. Petri dishes with Drigalski Agar, selective for Enterobacteriaceae, were incubated at 37° C for 24 h under aerobic conditions. Subsequently, enumeration, sub-culturing and identification of the different colonies were performed when necessary.

For histological analysis, colon and ileum samples were collected from the rats immediately after sacrifice and fixed in buffered formaldehyde overnight. Samples were then dehydrated, included in paraffin and stored at room temperature. Four micrometer sections were obtained with a RM2245 microtome (Leica microsystems SA, Nanterre Cedex, France) for histological staining (May-Grünwald-Giemsa). Briefly, slides were deparaffinized (2 successive baths of 5 min in xylene), rehydrated (4 successive baths of 5 min in ethanol 100°, ethanol 95°, distilled water and distilled water, respectively), incubated with May-Grünwald solution (20% in water, 15 min at 37 °C), followed by Giemsa (1% in water, 40 min at 37 °C). To highlight different shades of staining, a sequence of washing steps was carried out in acetic acid, acetone and 100° ethanol 1:1 (until transparency) and finally xylene twice for mounting. The specimens were observed under a light microscope (DM5500B, Leica, Bensheim, Germany). Histological lesions were blindly evaluated by two investigators according to a previously established score criterion (Table III) [30]. This scoring system rates the lesions on a scale from 0 to 6, based on the extent of inflammatory infiltrate, the presence of erosion, ulceration or necrosis, and the depth and surface extension of the lesions. The scores obtained with each formulation were then collected into 2 inflammation levels, *i.e.* low (<3) and medium to severe (≥3). For comparison, colonic and ileal specimens from a single untreated HLA-B27 transgenic rat were also collected and analyzed.

Table III: Scoring system for the analysis of histological lesions, adapted from [30]

Score	Description
0	Normal histology
1	Mucosal and/or submucosal inflammatory infiltrate with edema. Punctiform mucosal erosion often associated with capillary proliferation and/or architectural changes of the villi present on less than 1/3 of the specimen. Integrity of the mucous membrane
2	Same as score 1 but with abnormalities present on >1/3 of the specimen
3	Same as score 1 but with abnormalities present on >2/3 of the specimen
4	Significant inflammatory infiltrate with areas of loss of villi and areas of ulceration extending at least to the muscularis mucosae present on 1/3 of the specimen
5	Same as score 4 but with abnormalities present on >1/3 of the specimen
6	Same as score 4 but with abnormalities present on >2/3 of the specimen

3. RESULTS AND DISCUSSION

3.1 Manufacturing and *in vitro* evaluation of coated paracetamol-containing tablets

The drug delivery platform here presented is based on multiple mechanisms controlling release, leveraging physiological variables. A drug-containing core is coated with an inner swellable & soluble HPMC layer and an outer Eudragit® S-based coating. The latter also contained microbially-degradable polysaccharides of natural origin, namely pectin or pectin in combination with chitosan.

Paracetamol-containing tablets (4 mm in diameter) were successively coated with different amounts of an aqueous HPMC solution (containing also 10 % PEG 400 based on dry coating polymer) and an aqueous Eudragit® S : pectin formulation, optionally containing chitosan. Triethyl citrate, glyceryl monostearate, Tween® 80, NaOH, 1 N aqueous ammonia solution and 0.1 N HCl were added to the Eudragit® S : pectin formulations, as indicated in Table I. The coating equipment and processing parameters set up are listed in Table II. The obtained coating thicknesses are shown in Table IV. Batch codes refer to the coating polymers and nominal coating thickness in the case of the HPMC layer.

Table IV: Amounts of coating materials applied to paracetamol tablets and corresponding coating thicknesses

Batch code	HPMC inner layer		Eudragit® S/polysaccharides outer layer		
	Amount per unit area (mg/cm^2)	Thickness (µm)	Total amount applied (mg/cm^2)	Eudragit® S amount applied (mg/cm^2)	Thickness (µm)
$HPMC_0$-EuS/pec	-	-	30.7	13.5	205.2 ± 7.1
$HPMC_{70}$-EuS/pec	8.3	79.0 ± 3.7	30.6	13.4	219.1 ± 12.8
$HPMC_{120}$-EuS/pec	13.3	119.6 ± 3.8	29.7	13.1	213.0 ± 7.2
$HPMC_{200}$-EuS/pec	27.7	203.5 ± 6.7	35.5	15.6	234.3 ± 13.6
$HPMC_0$-EuS/pec-chit	-	-	33.3	14.7	210.7 ± 4.0
$HPMC_{70}$-EuS/pec-chit	8.3	79.0 ± 3.7	31.4	13.8	234.2 ± 8.6
$HPMC_{120}$-EuS/pec-chit	13.3	119.6 ± 3.8	32.4	14.3	222.8 ± 6.9
$HPMC_{200}$-EuS/pec-chit	27.7	203.5 ± 6.7	31.7	14.0	210.4 ± 6.1

Figure 1 shows cross-sections of paracetamol tablets coated with an inner HPMC layer and an outer Eudragit® S : pectin layer (1a), or an outer Eudragit® S : pectin : chitosan layer (1b). As it can be seen, the structures and thicknesses of all types of coatings were quite uniform, including the regions at the tablet edges. No evident structural differences between the Eudragit® S : pectin and the Eudragit® S : pectin : chitosan layers were observed (Figures 1a *vs.* 1b).

Drug release from these coated tablets upon exposure to 0.1 N HCl for 2 h, followed by phosphate buffer pH 7.4, is shown in Figure 2. The release profiles at the top are relevant to tablets coated with an inner HPMC layer and an outer Eudragit® S : pectin layer. The thickness of the HPMC coating was varied from 0 to 200 µm. Clearly, all tablet formulations provided enteric drug release: The amounts of paracetamol released under acidic conditions were very limited. Upon exposure to phosphate buffer pH 7.4, pulsatile drug release was observed, the lag time being strongly affected by the thickness of the inner HPMC layer. The thicker the HPMC layer, the longer the lag time was. Some drug was already released prior to the onset of the release pulse in all cases (Figure 2a). This can be explained by the penetration of water into the system, dissolving paracetamol in the core. Once dissolved, the drug would slowly diffuse out of the tablet. The coatings were not completely impermeable for water and the drug. The longer the lag time, the more important this “pre-pulse” drug release was. It has to be pointed out that once the pulse started, drug release was always rapid: steep release curves were observed, irrespective of the thickness of the inner HPMC layer.

The use of pectin in combination with chitosan was aimed at improving the performance of the outer coating. Chitosan, a linear polysaccharide of randomly distributed N-acetyl-D-glucosamine and D-glucosamine obtained by deacetylation of chitin, is widely utilized in

pharmaceutical formulations because of its safety, biocompatibility and versatility [31]. It has also been proposed for colon delivery, since it can be degraded by the microbiota [32]. Through amino groups, it can form polyelectrolyte complexes via ionic interactions with oppositely-charged polymers, such as pectin [15–18]. This property was exploited in the present study: The idea was to complex pectin with chitosan to slow down its dissolution and leaching from the outer coating. The pectin : chitosan weight : weight ratio was 5:1, the polymethacrylate : total polysaccharides (pectin + chitosan) ratio was kept constant at 7:3 [19].

As desired, better protection of the drug core was obtained when pectin was combined with chitosan (Figure 2b). At a coating level of 14 mg/cm^2 gastroresistance was obtained, and the onset of dissolution of the enteric film was delayed in pH 7.4 fluid. Importantly, "pre pulse" release of paracetamol was reduced and the lag time was prolonged upon chitosan addition, irrespective of the thickness of the inner HPMC layer. However, when the latter increased, the pre-pulse release phase became more evident (Figure 2a *vs.* 2b). Thus, thicknesses of the HPMC coating beyond 120 μm did not appear advantageous.

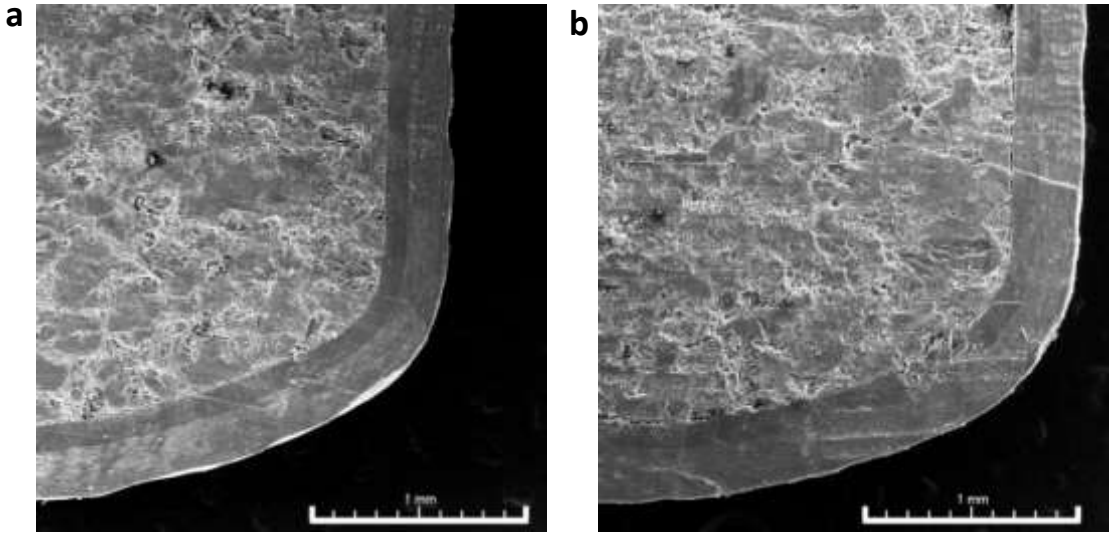

Figure 1: SEM photomicrographs of cross-sections of paracetamol tablets coated with an inner HPMC (120 µm) and an outer (a) Eudragit® S : pectin or (b) Eudragit® S : pectin : chitosan layer.

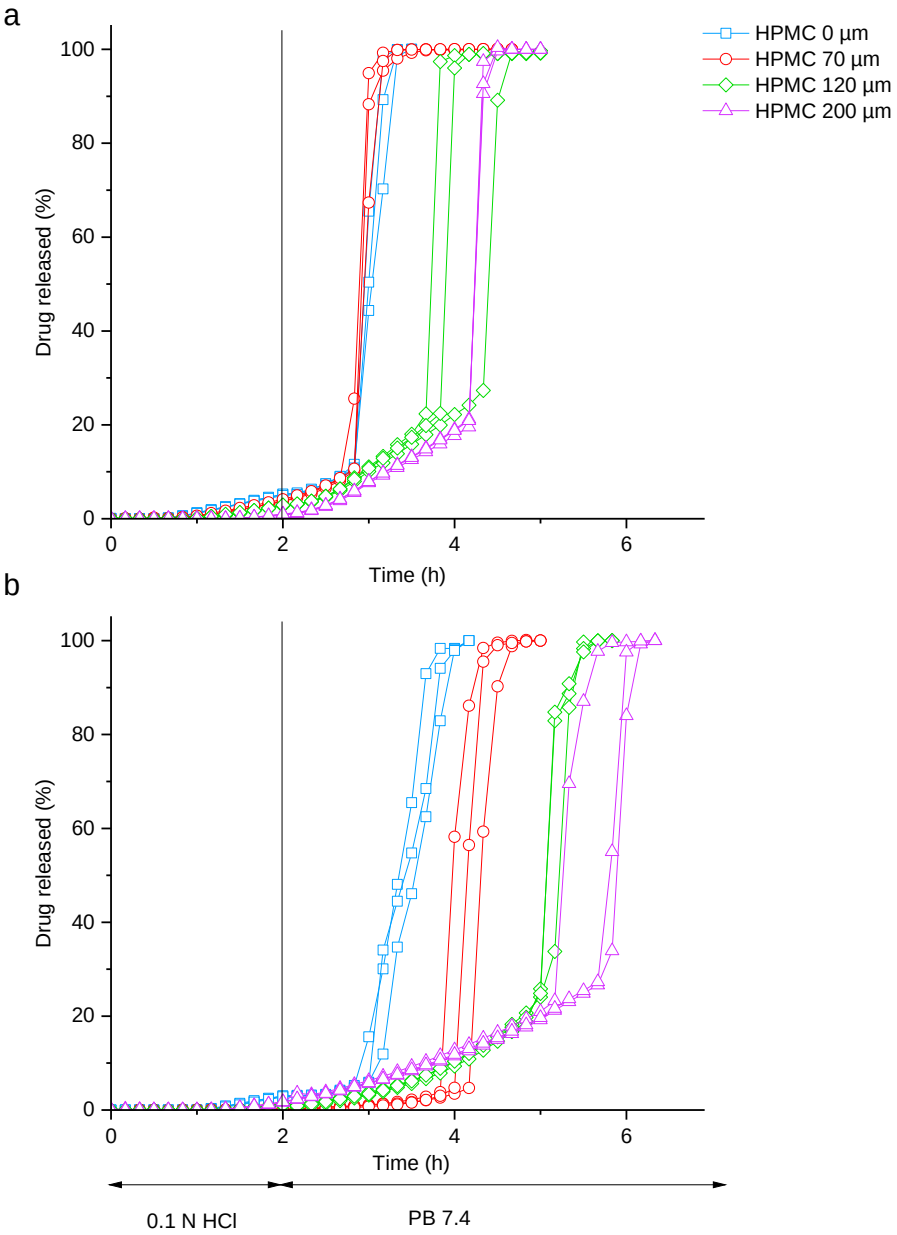

Figure 2: Paracetamol release from tablets coated with an inner HPMC and an outer (a) Eudragit® S : pectin or (b) Eudragit® S : pectin : chitosan layer, upon exposure to 0.1 N HCl and phosphate buffer (PB) pH 7.4. The thickness of the inner HPMC layer was varied as indicated in the legend.

To explore the possible role of the microbiota, release testing of the developed formulations was also carried out in simulated colonic fluid (SCF), prepared with culture medium (CM) upon inoculation with fecal samples from IBD patients [10,23]. The pH was set at 6.5, *i.e.* below the dissolution threshold of the enteric soluble polymer Eudragit® S, to evaluate whether microbial degradation of the polysaccharide may be effective in promoting colonic drug release in case a pH value of 7 is not reached and/or maintained for a sufficient time lapse *in vivo.* For reasons of comparison, also drug release in CM free of fecal samples was studied. Before transferring the dosage forms to the simulated colonic fluid, they were exposed to 0.1 N HCl for 2 h and phosphate buffer pH 4.5 for another 2 h, to simulate conditions encountered in the upper gastrointestinal transit.

As can be seen in Figure 3, drug release was limited (but not completely suppressed) upon exposure to 0.1 N HCl and phosphate buffer pH 4.5 (for the reasons discussed above). Importantly, paracetamol release was much faster upon subsequent exposure to simulated colonic fluid (containing fecal samples from IBD patients) compared to pure culture medium. This clearly indicates that the presence of pectin (or pectin : chitosan) in the outer coating fulfils its purpose: The back-up mechanism allows for the onset of drug release under colonic conditions and, even if the threshold value of pH 7 required for Eudragit® S dissolution is not reached, drug release sets on.

In 0.1 N HCl, phosphate buffer pH 4.5 and pure culture medium, where bacteria were not present, the HPMC layer of 70 µm was proved to more effectively protect the drug core than the thicker one (120 µm). This might be due to a possible dual role played by HPMC depending on the amount of polymer applied. Particularly, because of its water swelling nature, it may either defer penetration of the aqueous fluids into the system, or act as a rupturing agent accelerating breakup of the overlaid enteric coating through the outward

pressure exerted upon hydration. Accordingly, it could be hypothesized that, in the event the enteric coating is dissolved before the coated units have passed the ileo-cecal junction, the inner layer may further delay release to increase the chances of targeting the large intestine. On the other hand, swelling of the HPMC coating, along with bacterial degradation of the naturally-occurring polysaccharides, may promote breakup of the outer film when this is maintained until colon arrival. Such a rupturing effect might thus help rule out the issue of release failure.

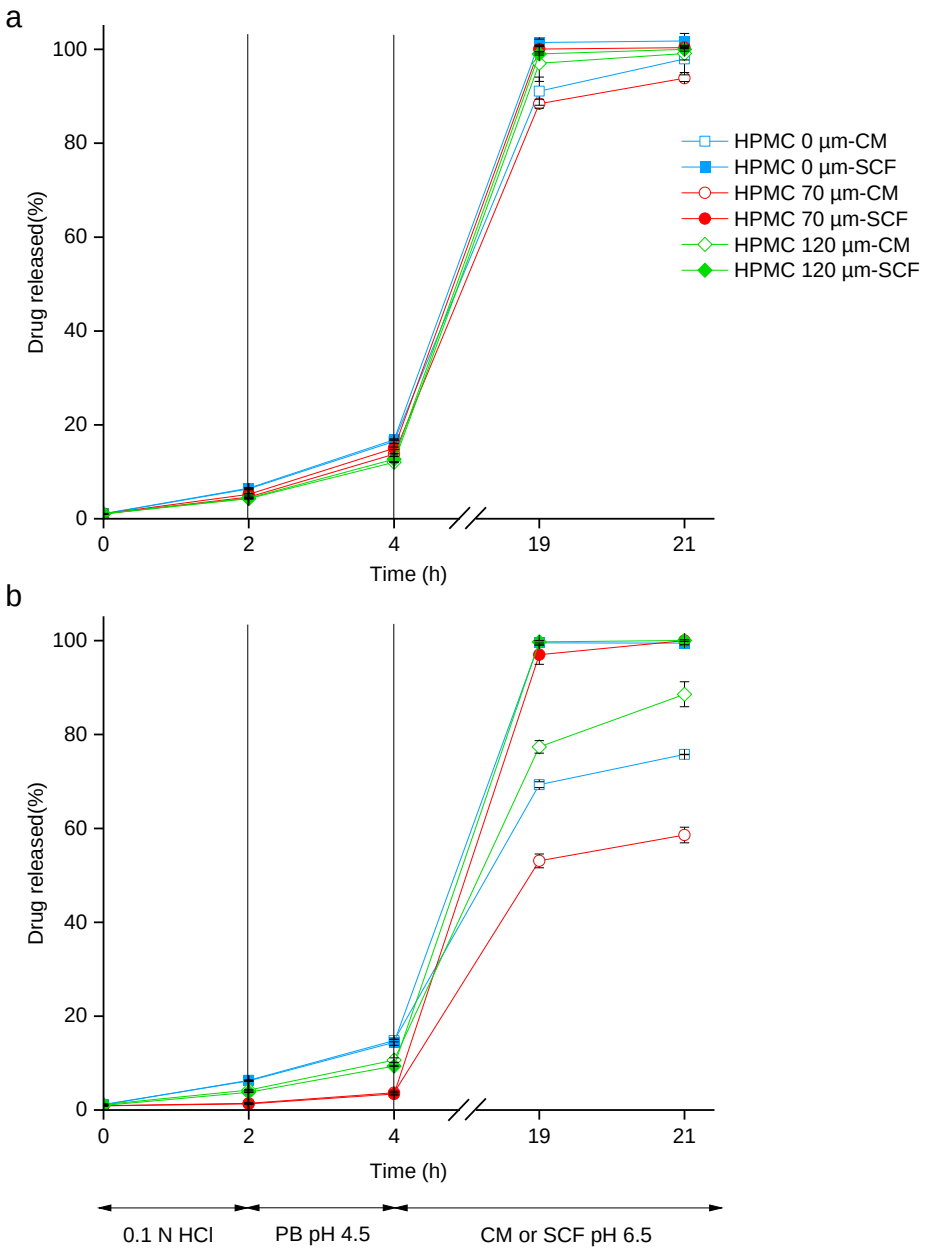

Figure 3: Paracetamol release from tablets coated with an inner HPMC and an outer (a) Eudragit® S : pectin or (b) Eudragit® S : pectin : chitosan layer, upon exposure to 0.1 N HCl, phosphate buffer (PB) pH 4.5 and simulated colonic fluid (SCF), inoculated with feces, or culture medium (CM), not inoculated. The thickness of the inner HPMC layer was varied as indicated.

3.2 *In vitro* and *in vivo* evaluation of coated 5-ASA-containing minitablets

The proposed delivery platform was also evaluated in a rat colitis model [28]. For this study, minitablets containing 5-ASA were coated with HPMC up to a thickness of 70 µm and with Eudragit® S : pectin : chitosan up to an amount of polymethacrylate of 14 mg/cm^2, based on the above-discussed *in vitro* results. 5-ASA is the first-line and most widely used drug treatment for mild-to-moderate ulcerative colitis [33-35]. By selective colonic delivery, the efficacy and tolerability of the therapy can be improved.

Only minor adjustments of the operating conditions for film coating were required, due to the smaller size of the tablet cores: batch size, product temperature, inlet air temperature and drying air volume were set at 35 g, 31 °C, 40 °C and 39 - 41 m^3/h, respectively. Table V shows applied amounts of polymers and achieved coating thicknesses.

Table V: Amounts of coating materials applied to 5-ASA tablets and corresponding coating thicknesses

Batch code	HPMC inner layer		Eudragit® S/polysaccharides outer layer		
	Amount per unit area (mg/cm^2)	Thickness (µm)	Total amount applied (mg/cm^2)	Eudragit® S amount applied (mg/cm^2)	Thickness (µm)
HPMC$_{70}$-EuS/pec-chit	9.3	70.9 ± 6.2	31.2	13.8	212.3 ± 7.6

Figure 4 illustrates the *in vitro* release profiles of 5-ASA from the minitablets upon exposure to 0.1 N HCl for 2 h, followed by phosphate buffer pH 7.4. Clearly, drug release was pulsatile, as desired. Moreover, it was much faster in SCF containing fecal samples compared to pure culture medium (Figure 5).

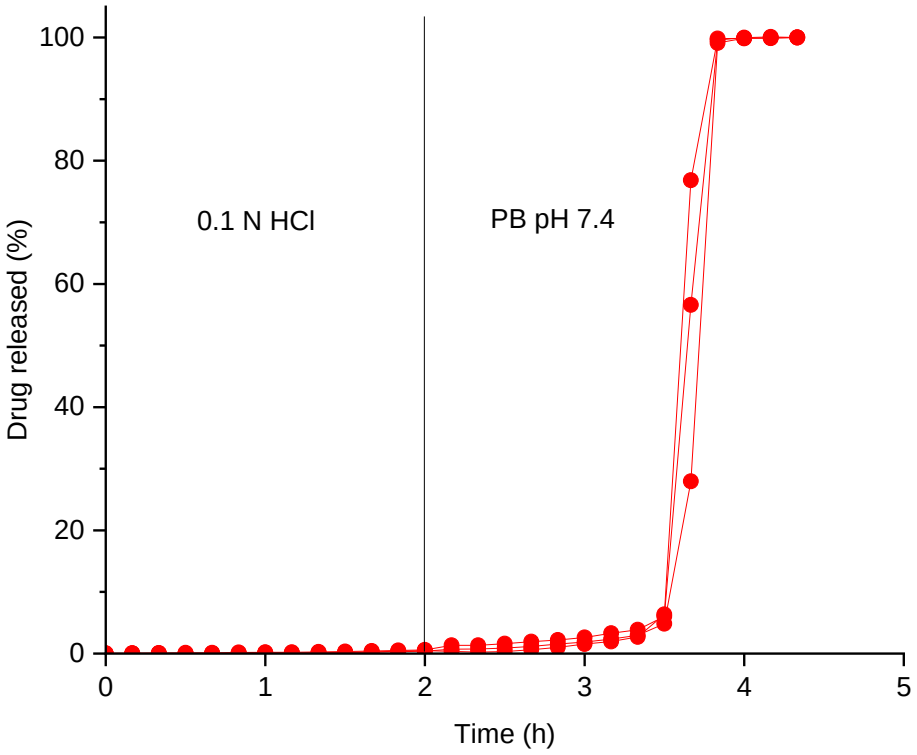

Figure 4: 5-ASA release from minitablets (7 units per vessel) coated with an inner HPMC and an outer Eudragit® S : pectin : chitosan layer upon exposure to 0.1 N HCl and phosphate buffer (PB) pH 7.4. Each curve corresponds to the results obtained from 7 units.

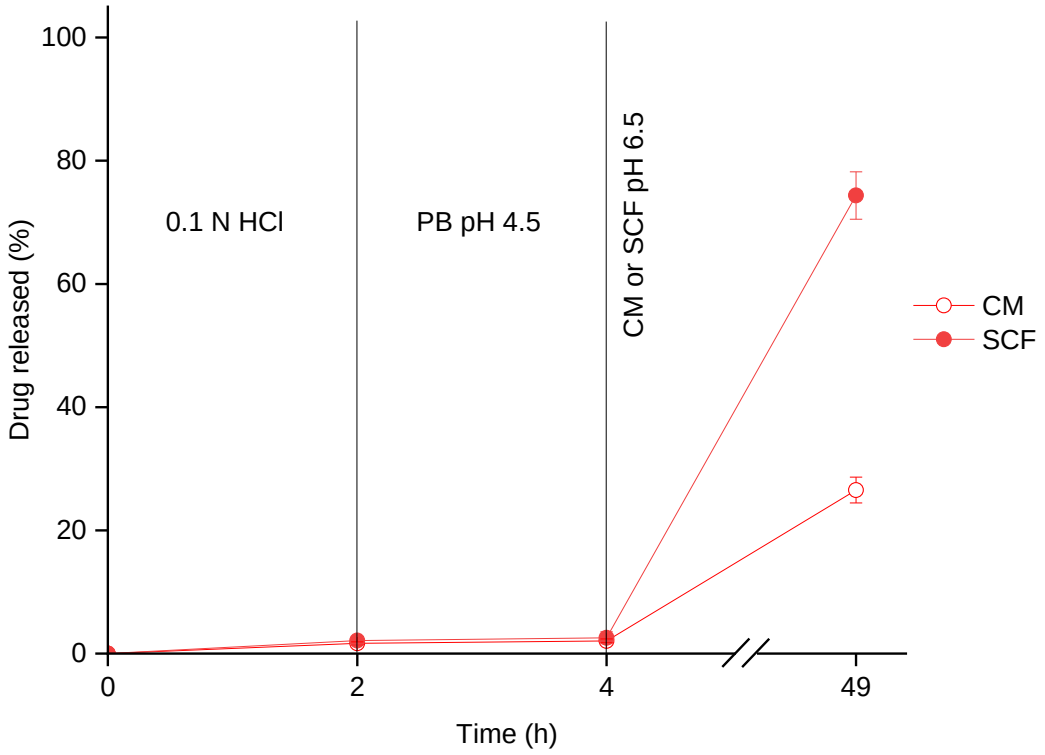

Figure 5: 5-ASA release from minitablets coated with an inner HPMC and an outer Eudragit® S : pectin : chitosan layer upon exposure to 0.1 N HCl, phosphate buffer (PB) pH 4.5 and simulated colonic fluid (SCF), inoculated with feces, or culture medium (CM), not inoculated.

Subsequently, the delivery system proposed was evaluated *vs.* Pentasa®, a long-standing oral 5-ASA product marketed for the therapy of IBD, upon administration to rats that had developed pre-established colitis signs. The animals received 37.5 mg/day 5-ASA, either as the double-coated minitablets (Test formulation) or in the form of Pentasa® (Reference formulation) over a time frame of 15 days.

E. coli was quantified in fecal samples collected before and after completion of the treatment, as a marker of intestinal inflammation [36]. The number of these bacteria at the end of the study period was generally higher than assessed on day 0, irrespective of the dosage form administered, due to progression of the disease (Figure 6). However, the

numbers were on average lower in samples from rats treated with the Test as compared to the Reference formulation ($5.5 \cdot 10^6 \pm 4.2 \cdot 10^6$ *vs.* $14.7 \cdot 10^6 \pm 26.1 \cdot 10^6$). Interestingly, a six-fold greater mean difference between the initial and final bacterial count was found with the marketed product compared to the double-coated minitablets ($12.2 \cdot 10^6 \pm 26.7 \cdot 10^6$ *vs.* $2.0 \cdot 10^6 \pm 1.9 \cdot 10^6$). Moreover, less data variability was observed in the case of the Test formulation, possibly highlighting consistency of its *in vivo* performance. These results would indicate that the proposed formulation may be more effective in preventing overgrowth of *E. coli*. When a broader range of typical bacterial species was considered, the relative number of *E. coli* was proved to be diminished by the Test formulation, whereas a marked proliferation was observed with the Reference 5-ASA product (Figure 7). In addition, *Bacteroides* were less reduced with the double-coated minitablets than with Pentasa®, and lactic acid bacteria were augmented in both groups of rats during the treatment.

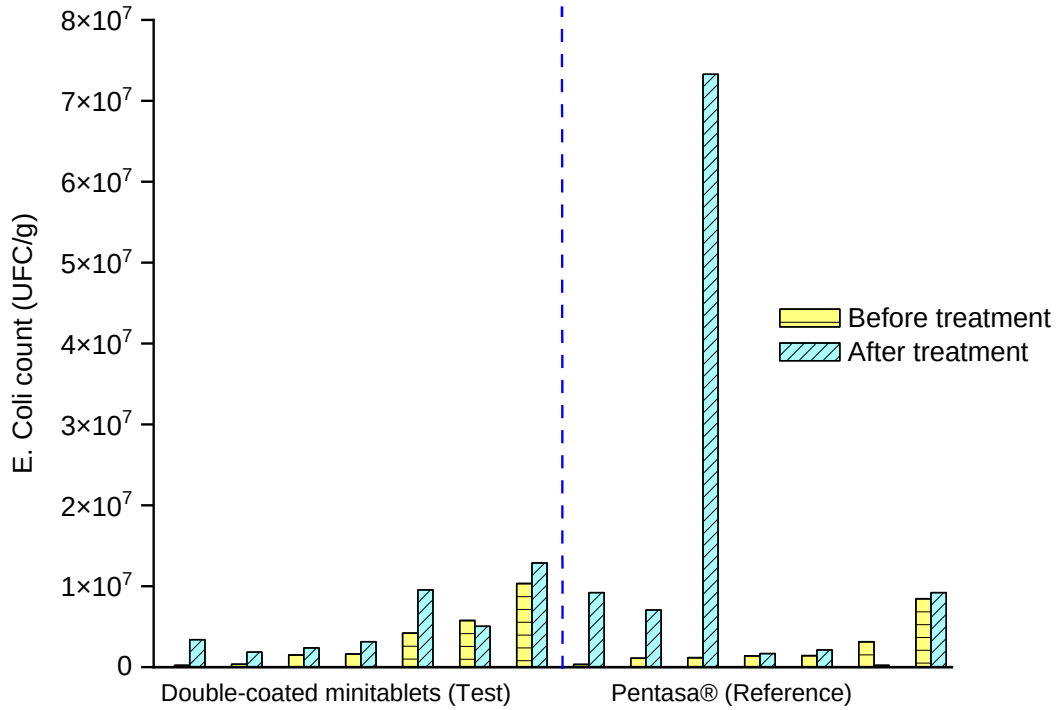

Figure 6: E. coli counts on Drigalski agar in fecal samples from rats before and after treatment with Pentasa® and double-coated minitablets.

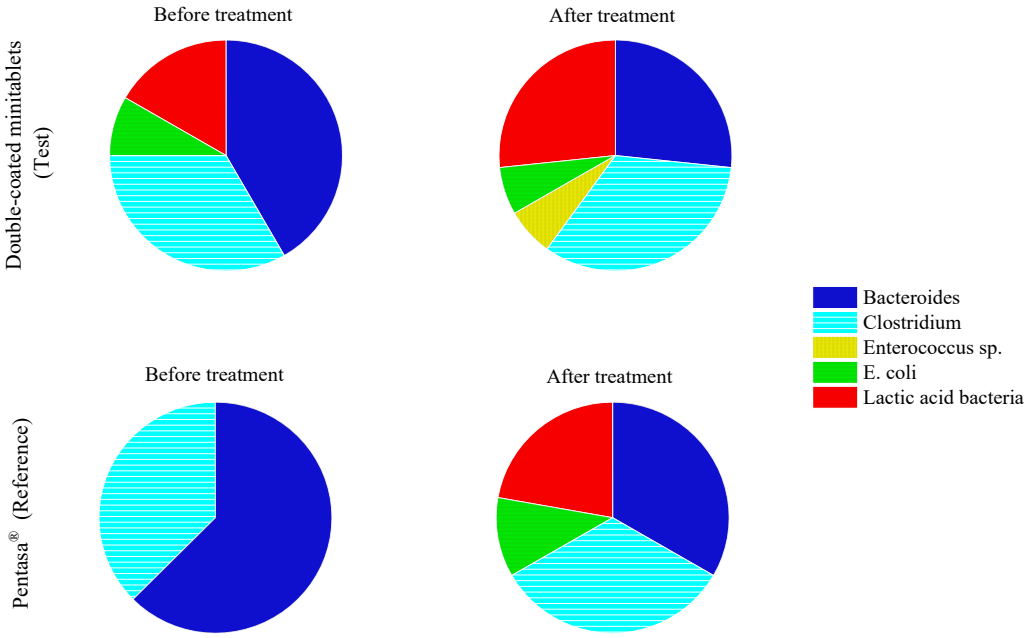

Figure 7: Relative bacterial counts on Columbia blood agar in fecal samples from rats before and after treatment with Pentasa® and double-coated minitablets.

Histological analysis of colonic and ileal tissue samples collected at the end of treatment revealed that most of the rats treated with the Test formulation showed a low level of inflammation (Table VI). On the other hand, a greater frequency of medium to severe lesions was observed following administration of Pentasa®. Interestingly, signs of severe inflammation were found in the untreated rat, both colonic and ileal specimen reaching a score of 5.

Therefore, based on these preliminary results, the *in vivo* performance of the Test formulation was proved at least not to be inferior to that of the Reference commercial one.

Table VI: Frequency in number of inflammation levels based on a scoring system of histological lesions in colon and ileum samples from rats after treatment with Pentasa® and double-coated minitablets.

	Colon		Ileum	
Inflammation	Pentasa® (Reference)	Double-coated minitablets (Test)	Pentasa® (Reference)	Double-coated minitablets (Test)
low (<3)	1	5	2	4
medium to severe (≥3)	6	2	5	3

4. CONCLUSIONS

An oral delivery platform, comprising a drug-containing core, a swellable inner HPMC coating and an outer layer based on enteric Eudragit® S and pectin (optionally with chitosan) as a microbially-degradable component, was proposed to achieve more reliable colon targeting, leveraging multiple physiological variables of the intestine. The pH-dependent outer coating barrier prevents drug release in the upper gastrointestinal tract and assures the onset of drug release when the dissolution threshold value for the enteric polymer is reached for a sufficient period of time. If this does not happen, pectin (and chitosan) serves as a back-up mechanism, undergoing enzymatic degradation by colonic bacteria. The inner HPMC layer provides additional protection in case the dissolution pH value for the enteric polymer is reached too early. If so, drug release is delayed because of the time taken for HPMC swelling/dissolution. Therefore, this formulation design addresses both issues faced with pH-responsive colonic delivery systems: *(i)* premature release in the small bowel, and *(ii)* release failure in the colon.

When pectin and chitosan were combined to form a polyelectrolyte complex, the outer coating exhibited improved barrier properties against the aqueous fluids. The release performance of pectin : chitosan-containing systems, tested in simulated colonic fluid, was clearly affected by fecal bacteria strains.

In an HLA-B27 transgenic rat colitis model, double-coated systems based on 5-ASA minitablet cores, manufactured according to this delivery technology, were shown to limit overgrowth of *E. coli,* alteration of the healthy gut microbiota and also progression of inflammation. These effects were assessed *vs.* a reference 5-ASA commercial product (Pentasa®), which has long been used for the treatment of mild to moderate active ulcerative colitis and for maintenance of its remission.

DECLARATION OF COMPETING INTEREST

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

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