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# Labrafac™ MC60 is an efficacious intestinal permeation enhancer for macromolecules: comparisons with Labrasol® ALF in *ex vivo* and *in vivo* rat studies

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**Abstract**

Labrafac™ MC60 (glycerol monocaprylocaprate) is a lipid-based excipient used in oral formulations as a solubiliser. Due to the high proportions of established permeability enhancers, caprylate ($C_8$) and caprate ($C_{10}$), in Labrafac™ MC60, we hypothesised that it might behave as an intestinal permeation enhancer. We therefore evaluated this using two paracellular markers (ex vivo) and insulin (in vivo) as model molecules. Ex vivo studies were conducted in isolated muscle-stripped rat colonic mucosae mounted in Ussing chambers. Apical addition of Labrafac™ MC60 (8, 12, and 16mg/ml) enhanced the apparent permeability coefficients ($P_{app}$) of [$^{14}$C] mannitol and FITC-dextran 4kDa (FD4) across colonic mucosae. Similar effects were observed in isolated jejunal mucosae, but at higher concentrations (40mg/ml). The enhancing capacity of Labrafac™ MC60 was transient due to reversibility of reductions in transepithelial electrical resistance (TEER) upon wash-out and effects on fluxes were molecular weight-dependent (MW) as suggested by fluxes of a set of high MW FITC-dextrans. The permeability enhancing effects of Labrafac™ MC60 ex vivo were maintained in the presence of simulated intestinal fluids, FaSSIF and FaSSCoF, in both jejunal and colonic mucosae, respectively. Following intra-intestinal regional instillations to rats, the relative bioavailability of 50 IU/kg insulin ad-mixed with Labrafac™ MC60 was 5% in jejunum (40mg/ml) and 6% in colon (8mg/ml). When Labrafac™ MC60 was combined with PEG-60 hydrogenated castor oil (1% v/v), this further increased the bioavailability of insulin to 8% in jejunum. Absorption enhancement was also maintained in the presence of FaSSIF in jejunal instillations. Histology after 120 min exposure to Labrafac™ MC60 in vivo for both jejunum and colon was similar to untreated control. Labrafac™ MC60 therefore acts as a non-damaging intestinal permeation enhancer for macromolecules and can be considered as another excipient in screening programmes to develop orally administered macromolecules.

**Key words:** Oral peptide administration; sodium caprate, Labrasol®, Labrafac™ MC60, insulin, Ussing chamber; intestinal epithelial permeability

## 1. Introduction

In recent years, two peptide formulations have been approved for oral administration to achieve a level of bioavailability required for efficacy, the glucagon-like peptide-1 receptor agonist (GLP-1 RA), semaglutide [1] and the somatostatin analogue, octreotide [2]. Both formulations contain intestinal permeation enhancers (PEs). PEs can aid the transport of peptides across the epithelium of the gastrointestinal tract by either opening tight junctions between cells or facilitating transcellular transport or a combination of both [3]. Rybelus® (Novo Nordisk, Malev, Denmark) is approved for the treatment of Type 2 diabetes. It comprises a daily tablet containing up to 14 mg semaglutide combined with the medium chain fatty acid (MCFA) derivative, sodium N-(8-[2-hydroxybenzoyl] amino) caprylate

(SNAC, 300mg) as a PE [4]. Mycapssa® (Chiasma, Jerusalem, Israel) is an enteric-coated capsule containing 20 mg octreotide as the active ingredient with sodium caprylate ($C_8$) as the dominant PE along with other surfactants including polysorbate 80 and glycerol mono/tri caprylate in an oily suspension [5, 6]. The main PEs used in these two approved formulations are therefore MCFAs or derivatives. Despite these FDA approvals, the oral bioavailability for both products is ~1% and this will not suffice for most peptides.

In parallel with the development of Rybelsus® and Mycappsa®, another MCFA PE, sodium caprate ($C_{10}$), was investigated as a PE in numerous oral peptide programmes that reached clinical testing [7]. An oral bioavailability of ~2% was achieved for a long-acting basal insulin (Insulin 138) in a Phase II trial from Novo Nordisk, but the programme was subsequently terminated [8]. $C_{10}$ has recently returned to favour as an important clinically relevant PE for oral administration of macromolecules. Merck (Rahway, NJ) recently completed a successful Phase IIB trial using 180 mg $C_{10}$ in a tablet containing up to 30 mg of the PCSK9 macrocycle peptide inhibitor, MK-0616, where up to 2% bioavailability has been estimated [9]. In another example, Astra Zeneca (Mölndal, Sweden) examined $C_{10}$ as a PE for oral administration of MEDI7219 (a stable GLP-1RA peptide) and demonstrated positive preclinical data (Emeh et al., 2023). Lilly (Indianapolis, IN, USA) have investigated immediate-release gastric tablets containing either SNAC or $C_{10}$ as a PE with a glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GIP/GLP1) dual agonist in cynomolgus monkeys and have achieved oral bioavailability values of 4.2% and 5.7% respectively (Tran et al., 2023). Despite their limitations, SNAC, $C_8$, and $C_{10}$ are the dominant PEs due to experience with them in clinical trials. Nonetheless, there is a hunt for other more efficacious intestinal PEs or combinations thereof to facilitate their transport across the gastrointestinal tract. For example, Astra Zeneca carried out a screen of potential PEs in Caco-2 monolayers and identified a combination in ratios of the bile salt, sodium chenodeoxycholate, and the anti-oxidant food additive, propyl gallate, to enhance bioavailability of MEDI7219 (a stable GLP-1RA peptide) to ~5% in dogs [10].

We previously investigated a lipid-based FDA-approved solubilising oral excipient, from Gattefosse (St. Priest, France), Labrasol® ALF (caprylocaproyl macrogol-8 glycerides (European Pharmacopoeia (Ph. Eur.) / caprylocaproyl polyoxyl-8 glycerides NF (United States Pharmacopeia (USP)) as a PE. Part of the attraction is because Labrasol® ALF contains a mixture of MCFAs, but predominately $C_8$ and $C_{10}$ in either free or glyceride formats [11]. Impressive data achieved in a rat gut instillation model with insulin suggested that Labrasol® ALF could be used as a PE in oral formulations for other macromolecules. Its potential was also confirmed for a range of peptides in µFlux™ and oral gavage rat assays carried out by Merck researchers [12], followed by the testing of an macrocycle PKCS9 inhibitor with Labrasol® ALF in cynomolgus monkeys where therapeutic levels in plasma were achieved following oral administration [13]. Labrasol® ALF (1800 mg/capsule) was then included in a formulation with the macrocycle, MK-0616, in a Phase I study where similar plasma exposures were seen when the molecule was formulated as tablets with $C_{10}$, with oral bioavailability values estimated at 2% with both PEs [14].

Following on from the encouraging discoveries made with Labrasol® ALF, we hypothesised that another solubilising excipient produced by Gattefosse, Labrafac™ MC60, might also behave as a PE as suggested by its MCFA composition. Labrafac™ MC60 (glycerol monocaprylocaprate Type I (European Pharmacopoeia (Ph. Eur.) / glyceryl monocaprylocaprate Type I or Glyceryl Mono and Dicaprylocaprate NF (United States Pharmacopeia (USP)) is a lipid-based excipient that is composed of mono- and diglycerides

of caprylic acid ($C_8$) and capric acid ($C_{10}$), with monoester formats predominating (Fig. 1). Labrafac™ MC60 has low aqueous solubility due to a low hydrophilic: lipophilic balance (HLB) value of 5, however it can be more easily used in lipid-based formulations Type I (oily solutions), Type II (self-emulsifying drug delivery systems, SEDDS) and Type III (self microemulsifying drug delivery systems, SMEDDS) formulations. Despite the lower HLB value of Labrafac™ MC60 compared to Labrasol® ALF (HLB 12), we anticipated that the lower solubility of the former could be managed with additives if necessary.

Glycerol mono and dicaprylocaprate has a drug master file and is included in the US Food and Drugs Administration (FDA) Inactive Ingredients Database for oral delivery in capsules, tablets, and solutions. It has a maximum allowed daily exposure of 502 mg [15]. The maximum potency per unit dose is 765mg. Glycerol monocaprylocaprate is also used in a number of approved formulations on the market. Dutasteride, used to treat prostatic hyperplasia, is available as a soft capsule (0.5 mg) containing dutasteride dissolved in glycerol monocaprylocaprate [16]. In Europe, glycerol monocaprylocaprate is part of the film coatings of sitagliptin (a dipeptidyl peptidase-4 inhibitor), and combinations of sitagliptin and metformin for the treatment of type II diabetes [17].

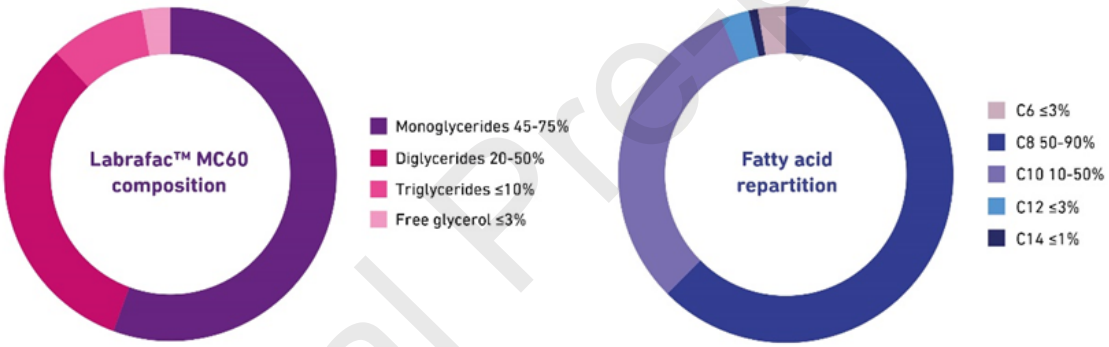


**Fig. 1.** Composition of Labrafac™ MC60 (glyceryl monocaprylocaprate). It is composed of monoglycerides with caprylate ($C_8$) and caprate ($C_{10}$) as the dominant MCFAs.

Labrafac™ MC60 has been used as a co-surfactant in a nanostructured lipid carrier with Gelucire® 44/14 (Gattefossé), olive oil, and distearoyl phosphatidylethanolamine-N-[methoxy poly (ethylene glycol)-2000] designed for the oral delivery of atorvastatin [18]. Another commercial grade, Capmul® MCM, (glycerol monocaprylocaprate, Abitec, Columbus, OH, USA) has been investigated as the oil phase in a SMEDDS for oral administration of the poorly soluble molecule, atorvastatin, with Tween® 20 and Labrasol® ALF as the surfactants [19]. Capmul® MCM has also been used as a permeation enhancer for the oral delivery of dendrimer-N-acetyl-l-cysteine conjugates for paediatric neuro inflammation [20]. After oral administration to rats, the formulation containing Capmul® increased the area under the curve of the conjugates by 47%. Previously Yeh et al., investigated the permeation enhancing abilities of Capmul® MCM ex vivo in the Ussing chambers and found that the Capmul® reduced resistance and increased the permeability of mannitol and PEG4000 across isolated rabbit ileal and colonic mucosae [21, 22]. Oral microemulsions have also been synthesised with Capmul® as one of the components that

increased the bioavailability of a fibrinogen receptor cyclic tetrapeptide antagonist to 27% following intraduodenal rat administration [23]. Capmul® MCM was combined with Labrasol® ALF and Tetraglycol in a SMEDDS for insulin in a hydrophobic ion pair and achieved a pharmacological availability of 3% in diabetic rats following oral gavage [24]. To our knowledge, glycerol monocaprylocaprate itself has not previously been assessed as a PE for insulin in oral administration bioassays.

In this study we investigated Labrafac™ MC60 initially ex vivo in Ussing chambers using isolated rat jejunal and colonic tissue mucosae with paracellular flux markers and then in vivo with insulin as a model peptide using an in vivo rat intestinal instillation model. We were also interested in whether the bile salts in intestinal fluid would interact with the Labrafac™ MC60 and affect its capacity to function as a PE and therefore examined the effects of simulated intestinal fluids in the in vivo studies. Another aspect investigated was whether the digestion of the ester derivative would affect permeability enhancement capacity. We included Labrasol® ALF in our study as a benchmark for comparison. Finally, to improve the performance of Labrafac™ MC60 further, it was also evaluated in combination with other excipients such as Labrasol® ALF, and PEG-60 hydrogenated castor oil (HCO-60). We found that Labrafac™ MC60 was an efficacious PE in both bioassays, with evidence to support a paracellular mechanism of action with acceptable levels of tissue damage at the selected concentrations used.

## 2. Materials and Methods

### *2.1 Materials*

Labrafac™ MC60 (Glycerol monocaprylocaprate or glycerol mono- and dicaprylocaprate,) and Labrasol® ALF (caprylocaproyl polyoxyl-8 glycerides) were gifts from Gattefossé (Saint Priest, France). PEG-60 hydrogenated castor oil (HCO-60) was a gift from Nikko Chemicals. [$^{14}$C] mannitol was obtained from Perkin Elmer, (Ireland), while FITC-dextran analogues (FD4-FD70) were purchased from Merck (Ireland). Human recombinant insulin was purchased from Biosciences (Ireland); a human insulin ELISA kit was purchased from Mercodia (Sweden). Fasted State Simulated Intestinal Fluid-V2 (FaSSIF) and Fasted State Simulated Colonic Fluid (FaSSCoF) were purchased from Biorelevant Ltd (London, UK). Sodium caprate ($C_{10}$), sodium taurodeoxycholate (NaTDC), tributyrin, pancreatin (from porcine pancreas, 8 x USP), and L-phosphatidylcholine (lecithin), and carbachol were purchased from Sigma-Aldrich (Ireland). All solvents used were HPLC grade. All other chemicals were reagent grade. The compositions and HLB values of the investigated excipients and PEs are shown in Table 1.

**Table 1.** Compositions of excipients.

| Excipient | Composition | HLB |
|---|---|---|

| | | |
|---|---|---|
| Labrafac™ MC60 | 50-90% caprylic ($C_8$) and capric acid ($C_{10}$), glycerol | 5 |
| Labrasol® ALF | 50-80% $C_8$ and 20-50% $C_{10}$, PEG-8 | 12 |
| Sodium caprate | $C_{10}$, sodium salt | 21 |
| HCO-60 | Hydrogenated castor oil (PEG 60) | 14 |

## 2.2 Methods

### *2.2.1 Ussing chamber protocol*

Adult Wistar-CRL (male and female) rats were obtained from the Biomedical Facility, UCD and the Charles River Laboratory, UK, and Germany, and were housed in a pathogen-free environment with controlled conditions of humidity and temperature under a 12:12h light/dark cycle with access to laboratory chow and filtered water ad libitum. Ussing chamber studies were conducted as previously described [11, 25]. Rats (unfasted, 250-350g in weight) were euthanised by stunning and cervical dislocation in accordance with the UCD Animal Research Ethics Committee approved protocol, AREC 14-28-Brayden. A mid-line laparotomy was performed, and the colon or jejunum was excised. Jejunal segments were immediately placed in ice cold oxygenated Krebs Henseleit (KH) buffer for 20 min before being opened along the mesentery and washed with KH buffer. The circular and longitudinal muscle layers were stripped from colonic mucosae with a size #5 watchmaker forceps while the jejunal mucosae were left unstripped [26, 27]. Tissue was mounted in pre-equilibrated Ussing chambers (World Precision Instruments, WPI, UK) with a circular diameter window of 0.63 $cm^2$, 5ml KH buffer each side, maintained at 37$^oC$ and oxygenated with carbogen. Tissues were equilibrated for 45 min (colon) and 20 min (jejunum) before addition of paracellular markers and excipients to the apical side. Transepithelial electrical resistance (TEER) was calculated using Ohm's law following periodic voltage clamping (30 sec on and 3 sec off) using a DVC-4000 Voltage Clamp (WPI, UK) and corrected for surface area.

### *2.2.2 Permeability studies across isolated rat intestinal mucosae in Ussing chambers*

Paracellular reference markers, [$^{14}$C]-mannitol (MW: 192 Da, 0.1µCi/ml) and FITC-dextrans (FD, MW: 4-70 kDa, 2.5mg/ml), were added apically and 200µl samples were collected every 20 min over 120 min with replacement with fresh KH at each sampling. Control (Ctrl) refers to untreated tissue. Labrasol® ALF (8mg/ml, colon; 20mg/ml and 40mg/ml, jejunum), sodium caprate ($C_{10}$,2mg/ml, colon; 6mg/ml jejunum) were used as positive controls. Labrafac™ MC60 was evaluated at 2,4,8,12, and 16 mg/ml in colonic mucosae and 20 and 40 mg/ml in jejunal mucosae. Labrafac™ MC60 digested in lipolysis buffer (Section 2.4) was also assessed at 8 and 16mg/ml concentrations. To avoid precipitation, calcium-free KH was used on the apical side when $C_{10}$ was assessed. To assess tissue electrogenic chloride

secretion functionality, the cholinomimetic agent, carbachol (0.1, 1.0 and 10μM), was added basolaterally and changes in short-circuit current (Isc, μA) were measured after 120 min exposure to excipients [28].

To assay permeated markers, basolateral samples containing [$^{14}$C]-mannitol were mixed with scintillation fluid (3 mL) and Disintegrations Per Minute (DPM) were read in a scintillation counter (Tricarb 4910TR, Perkin Elmer). Fluorescence of FD samples was measured in a spectrofluorimeter (MD Spectramax Gemini) with excitation and emission wavelengths of 490/525 nm, respectively. The apparent permeability ($P_{app}$) coefficients of [$^{14}$C]-mannitol and FDs were calculated according to the equation:

**Equation 1**: $P_{app} = dQ/dt(1/A*C0)$,

where dQ/dt is the transport rate across epithelium, A is the exposed area of the tissue (0.63 $cm^2$), and C0 is the initial concentration of marker added to the donor compartment (dpm. $mL^{-1}$ or mg/ml).

TEER recovery studies were also performed. In these studies, the rat colonic mucosae were exposed apically to Labrafac™ MC60 (8, 12, and 16mg/ml) for 30 min before removal and replacement with fresh KH buffer. TEER was measured for a further 90 min. In a variation on the flux protocol, Fasted State Simulated Intestinal Fluid (FaSSIF, pH 6.5, 3 mM taurocholate) and Fasted State Simulated Colonic Fluid (FaSSCoF, pH 7.8, 0.15 mM sodium cholate) were on occasion used on the apical side of mounted jejunal and colonic mucosae respectively, while maintaining KH on the basolateral side [29].

*2.3 In vitro digestion of Labrafac™ MC60*

Digestion of Labrafac™ MC60 was conducted using a pH stat system [11, 30]. Lipolysis buffer and medium were prepared according to the previous descriptions [31]. Lipolysis buffer consisted of 0.47 g/l trismaleate, 8.81 g/l NaCL and 0.21 g/l $CaCl_2$ in MilliQ water. The pH of the buffer was adjusted to 6.5 with either HCL (1 M) or NaOH (1M). The lipolysis medium was prepared by adding 0.056 g/l L-α-phosphatidylcholine and 154 g/l sodium taurodeoxycholate to the lipolysis buffer. The enzymatic solution was prepared by adding 1 g of pancreatin from porcine pancreas in 5 ml lipolysis buffer. After 10 min agitation, the mixture was aliquoted to Eppendorf tubes and centrifuged at 10 min at 2800 g, 5°C (Universal Centrifuge 320R, Hettich). The supernatant was collected to be used in in vitro digestion process and specific activity measurement.

The in vitro lipolysis set up consists in a pH-stat apparatus (Methrom AG), equipped with a Titrando 802 propeller stirrer/804 Ti Stand combination, a double jacketed vessel 60 ml, a glass pH electrode (iUnitrode) and one 800 Dosino dosing unit coupled to a 5 ml autoburette. The enzymatic activity of the pancreatin was evaluated with tributyrin used as model substrate (glyceryl tributyrate. To this end, 28 ml lipolysis medium without L-α-phosphatidylcholine were placed in the vessel and maintained at 37°C, pH 6.5. 500 μL tributyrin were dispersed in the medium. After 150s, 1.5 μl of pancreatin solution were added to the vessel. The specific activity (SA) of the lipase is required to be > 900 U/mg. Five mL of Labrafac™ MC60 were added to 36 ml lipolysis medium heated at 37°C under agitation. After 10 min, 4 ml pancreatin solution were added to the vessel. The pH was regulated at pH 6.5. After 90 min digestion, the content of the vessel was collected in the water used to rinse the equipment. This solution was frozen at -20°C and lyophilized (Heto Drywinner FD3). The freeze-dried samples were rinsed with chloroform and filtered through 0.45 μm glass

filters. Chloroform was further evaporated to obtain the samples of digested Labrafac™ MC60.

*2.4 Combinations of PEs in rat colonic mucosae studies*

Labrafac™ MC60 and Labrasol® ALF were combined either 1:1, 3:7 and 7:3 in PBS and assessed at 1,2 and 4mg/ml in rat colonic mucosae in the Ussing chambers. Labrafac™ MC60 was combined with PEG-60 hydrogenated castor oil (HCO-60, HLB:14) and assessed in rat colon in the Ussing chambers with [$^{14}$C]-mannitol at 8- and 16 mg/ml. HCO-60 comprised a final total of 1% of the combinations by volume. Before combining, HCO-60 was heated to 60$^{o}$C to liquefy it and it was immediately added to Labrafac™ MC60 in PBS.

*2.5 Rat intestinal instillation studies*

Procedures were performed as previously described [11] under a specific project licence AE18982/P215 from the Irish Health Products Regulatory Authority (HPRA) and in compliance with EU Directive 2010/63/EU. Male and female Wistar rats weighing 250-300 g (3 males and 3 females per treatment group) were fasted for 16-18h prior to procedures. All procedures were carried out under anaesthesia which was induced with isoflurane gas (Iso-Vet®, 1000 mg/g isoflurane liquid for inhalation, Piramal Healthcare, UK) at the rate of 5 L/min, mixed with 4 L/min $O_2$ in an induction box and maintained at 2–2.5 L/min mixed with 1-2 L/min $O_2$ via a mask using an anaesthesia vaporising unit (Blease Medical Equipment Ltd., UK). A midline laparotomy was performed, and the jejunum or colon exposed, and a 5-7cm section of the jejunum or colon was tied off at each end with braided suture (Mersilk W212). Topical lidocaine was applied to the incision to provide local analgesia. The excipients ad-mixed with 50IU/kg insulin were injected into the lumen of the segment. Insulin solution (50IU/kg) and PBS or FaSSIF were used as controls.

Human insulin (3.49 mg) was dissolved in HCL (0.1M) before addition of PBS and NaOH (0.1 M) to obtain a clear solution with a concentration of 100 IU insulin/ml. This was then added to excipient in PBS at twice the desired insulin concentration. In the case of studies with FaSSIF in the lumen, the insulin stock was mixed 50:50 with the excipient in 2x FaSSIF. The final concentration was either 8 mg/mL or 40 mg/mL excipient ad-mixed with an insulin dose of 50 IU/kg. Glucose levels were determined using a glucometer (Accu-Chek Performa Meter Inform II, Roche). Retro-orbital blood samples were abstracted at T0 (immediately before surgery), and then at 20, 40, 60, 80, 100, 120 min into heparinised vials, which were kept on ice prior to centrifugation (6500 g, 5 min) and plasma collection. Plasma was stored at -20$^{o}$C until analysis using a human insulin ELISA. At the end of the experiment animals were euthanised by an overdose of pentobarbital by cardiac injection. The segment was removed and fixed in 10% (w/v) formalin.

In order to calculate the relative bioavailability (F) following jejunal and colonic instillations, as a comparator, one group of animals was injected sub-cutaneously (s.c.) with insulin (1 IU/kg) also while under anaesthesia, and equation was used:

**Equation 2:** $$\boldsymbol{Relative\ \%F = \frac{AUC_{(inst.)} \times Dose_{(s.c.)}}{AUC_{(s.c.)} \times Dose_{(inst.)}} \times 100}$$

where AUC (inst.) is the area under the plasma concentration curve over 120 min and AUC (s.c.) is the area under the serum concentration versus time (0-120 min) after s.c. administration. Pharmacokinetic parameters were calculated using PK solver® software [32].

*2.6 Histology*

Rat intestinal tissue from Ussing chamber and instillation studies following 120 min exposures were fixed in 10% (w/v) formalin and embedded in paraffin wax. 5 µm tissue sections were cut on a microtome (Leitz 1512; GMI, USA), mounted on adhesive coated slides, stained with haematoxylin/eosin (H&E), and examined under light microscopy. Slides were examined under a light microscope (BX43, Olympus, Tokyo, Japan) and images were taken with an Infinity 3 high resolution camera (Lumenera, Ottawa, Canada) and assessed with Image® Pro Premiere 9.0 (Media Cybernetics Inc., Rockville, MD, USA).

*2.7 Statistical analysis*

Statistical analysis was conducted using Prism-5® software (GraphPad, San Diego, USA). Statistical analysis was carried using two-way ANOVA with Bonferroni's *post-test* for TEER and in vivo plasma glucose measurements. One-way ANOVA with Dunnett's *post-test* was used for calculations of $P_{app}$. Results are presented as the mean ± standard error of the mean (SEM). A significant difference was considered if P <0 .05.

## 3. Results

*3.1 Permeation enhancing ability of Labrafac™ MC60 in isolated rat colonic mucosae*

The capacity of Labrafac™ MC60 to enhance the permeability of the paracellular marker, [$^{14}$C]-mannitol was measured in rat colonic mucosae in the Ussing chambers in the presence of bilateral KH buffer. $C_{10}$ and Labrasol® ALF were used as positive controls at concentrations that have previously been shown to be efficacious in this assay [11, 25]. Lipid droplets of Labrafac™ MC60 were visible in the gassed apical KH buffer and could be observed moving towards and away from mounted mucosae arising from the air-lift system. Labrafac™ MC60 decreased TEER in a concentration-dependent manner (Fig. 2a, 2b). 2mg/ml Labrafac™ MC60 was without effect on TEER, but both 4mg/ml and 8mg/ml Labrafac™ MC60 decreased TEER from 20 min onwards. By 120 min, TEER decreased by 78% for 4mg/ml and by 92% for 8mg/ml. 12mg/ml and 16mg/ml decreased TEER significantly within 10 min (Table S1). $C_{10}$ and Labrasol® ALF decreased TEER dramatically within 5 min and worked faster than Labrafac™ MC60.

The $P_{app}$ of [$^{14}$C]-mannitol also increased in response to Labrafac™ MC60 exposure also in a concentration-dependent manner, suggesting a reciprocal relationship with TEER (Fig. 2c). The basal $P_{app}$ of [$^{14}$C]-mannitol was 2.3 x 10 $^{6}$ cm $s^{-1}$, similar to previous studies [11, 33] . The $P_{app}$ of [$^{14}$C]-mannitol was increased 2-fold (8mg/ml), 6-fold (12 mg/ml), and 5-fold (16 mg/ml) when exposed to Labrafac™ MC60 compared to KH control (Table 2). The increases were 5-fold for Labrasol® ALF (8mg/ml) and 8-fold for $C_{10}$ (2mg/ml) (Fig. 2c). Carbachol concentrations were added basolaterally after 120 min exposure to excipients, but it was omitted in studies carried out with $C_{10}$ because there is a confounding electrophysiological interaction [34]. With 10µM carbachol, controls showed an increase in Isc of 42 µA (Fig. 2d). For tissue exposed to Labrafac™ MC60, an increase in Isc was seen at all concentrations, with 4mg/ml allowing an increase of 17µA. Though the stimulated Isc in response to carbachol was diminished to an extent in the presence of Labrafac™ MC60, enough electrogenic current was generated to demonstrate epithelial functionality.

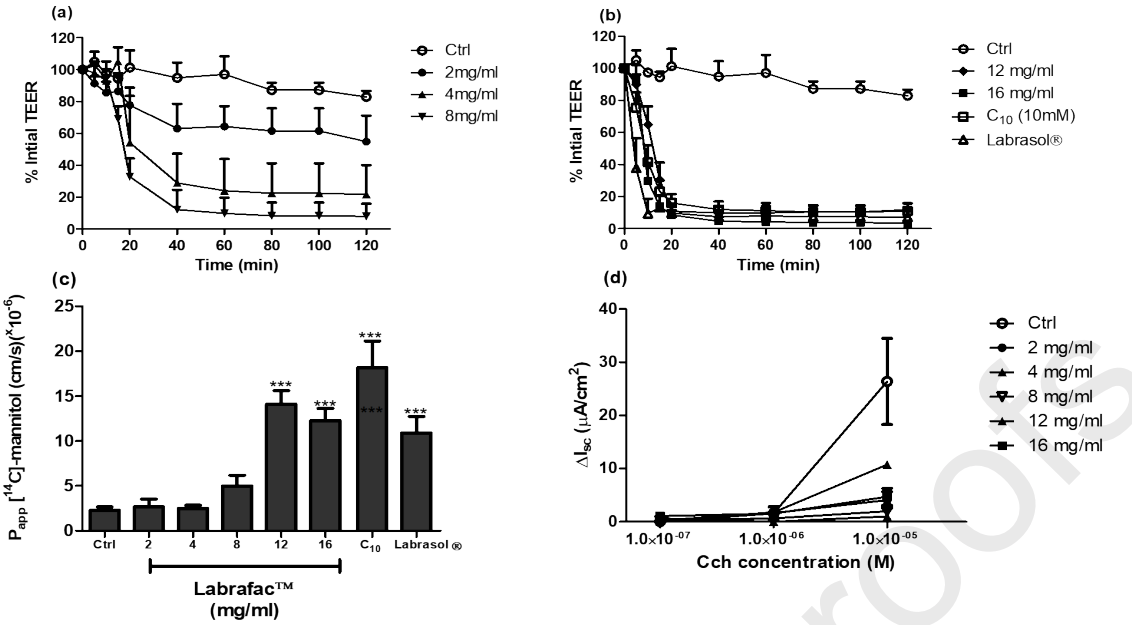


**Fig. 2**. Effect of Labrafac™ MC60 (Labrafac™) on colonic mucosae parameters. (a) % initial TEER upon exposure to 2-8 mg/ml (b) % initial TEER upon exposure to 12mg/ml, 16 mg/ml, and positive controls; (c) $P_{app}$ of [$^{14}$C]mannitol. ***P <0 .001 compared to untreated controls; (d) Isc changes in response to carbachol (Cch). Mean ± SEM, n=3-4 for treatments and n=8 for controls. Labrasol® ALF (8mg/ml) and $C_{10}$, (10 mM) were included as positive controls for other PEs.

**Table 2.** $P_{app}$ of [$^{14}$C]-mannitol across rat colonic mucosae incubated with Labrafac™ MC60.

| Treatment | mg/ml | $P_{app}$ (x10$^{-6}$ cm·s$^{-1}$) | Fold Increase |
|---|---|---|---|
| **Control** | 0 | 2.3 | - |
| **Labrafac™ MC60** | 2 | 2.7 | 1.2 |
| | 4 | 2.5 | 1.1 |
| | 8 | 5.0 | 2.2 |
| | 12 | 14.1 | 6.1 |
| | 16 | 12.3 | 5.3 |

| | | | |
|---|---|---|---|
| **Labrasol® ALF** | 8 | 10.9 | 4.7 |
| **$C_{10}$** | 2 | 18.2 | 8.0 |

Histological examination of colonic tissue after staining with H&E following 120 min exposure to Labrafac™ MC60 at 2 and 4 mg/ml showed an intact epithelium with no damage, similar to untreated controls (Fig. 3i, ii). Labrafac™ MC60 at 8 mg/ml and16 mg/ml (which induced permeability increases) induced slight erosion of the epithelium (Fig.3.iv,v). This type of damage is commonly seen with PEs in the Ussing chambers and, in our experience, when PEs are tested in vivo in the rat intestinal instillation model at even higher concentrations, far less damaging effects are seen on histology [11, 25]. This is because in vivo the blood system is present and there is a greater capacity for epithelial repair in response to membrane perturbation. In addition, the effects on histology ex vivo after exposure to Labrafac™ MC60 were less than those observed for $C_{10}$, which has been in multiple clinical trials as components of tablets containing peptides (Fig. 3viii)[3, 35].

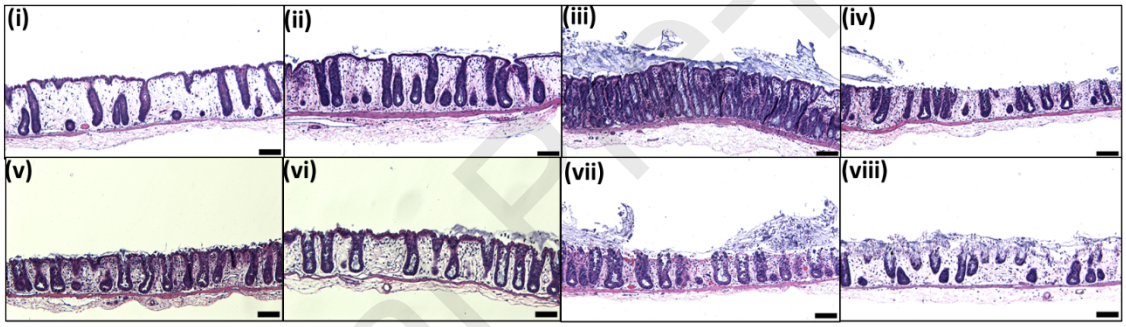


**Fig.3**. Representative H&E histology of rat colonic mucosae exposed to Labrafac™ MC60 concentrations and to positive controls for 120 min. Marker bar =100 µm. (i) Ctrl, (ii) 2mg/ml, (iii) 4mg/ml, (iv) 8mg/ml, (v) 12mg/ml, (vi) 16/mg/ml Labrafac™ MC60, (vii) 8mg/ml Labrasol® ALF and (viii) 2mg/ml $C_{10}$.

*3.2 Effects of digested Labrafac™ MC60 on TEER and the $P_{app}$ of [$^{14}$C] mannitol in KH buffer in rat colonic mucosae.*

Labrafac™ MC60 is made up of esters that are likely to be broken down by intestinal lipases [36, 37]. In an in vitro lipolysis study using pancreatin glycerol monocaprylocaprate (Capmul® MCM), the degree of lipolysis that occurred within 3h was 94-99% [37]. For this reason, it is important to understand if Labrafac™ MC60 is still able to work as a PE after being digested. In our previous study with Labrasol® ALF we took Labrasol® ALF that had been digested in the pH stat system with pancreatin and added it to colonic mucosae mounted in the Ussing chamber and in rat gut instillations and performed lipase inhibition studies [11]. Digested Labrasol® ALF still behaved as a PE in the Ussing chambers and also when lipolysis was pharmacologically inhibited *in vivo*. Here, digested samples of Labrafac™ MC60 were similarly evaluated on colonic mucosae in the Ussing chambers at 8 and 16

mg/ml concentrations and, like digested Labrasol® ALF, caused a decrease in TEER and an increase in the $P_{app}$ of [$^{14}$C]-mannitol (Fig. 4, Table S2). At 8mg/ml, undigested Labrafac™ MC60 enhanced the transport of [$^{14}$C]-mannitol yielding a $P_{app}$ of 5.0 x $10^{-6}$cm·s$^{-1}$ (Fig. 2C), which increased to 14.8 x $10^{-6}$cm·s$^{-1}$ for the digested Labrafac™ MC60. At 16mg/ml Labrafac™ MC60, there was more variability in the $P_{app}$ data and the values were similarly high for both the undigested (Fig. 2C) and digested formats (12.3 x $10^{-6}$cm·s$^{-1}$ vs 17.6 x $10^{-6}$cm·s$^{-1}$ respectively). These data indicate that Labrafac™ MC60 is efficacious in both undigested and digested formats in ex vivo assays. Histological examination of tissue exposed to digested Labrafac™ MC60 showed greater effects on histology compared to the undigested form (Fig. 4cii and iii). Erosion of the epithelial layer could be observed.

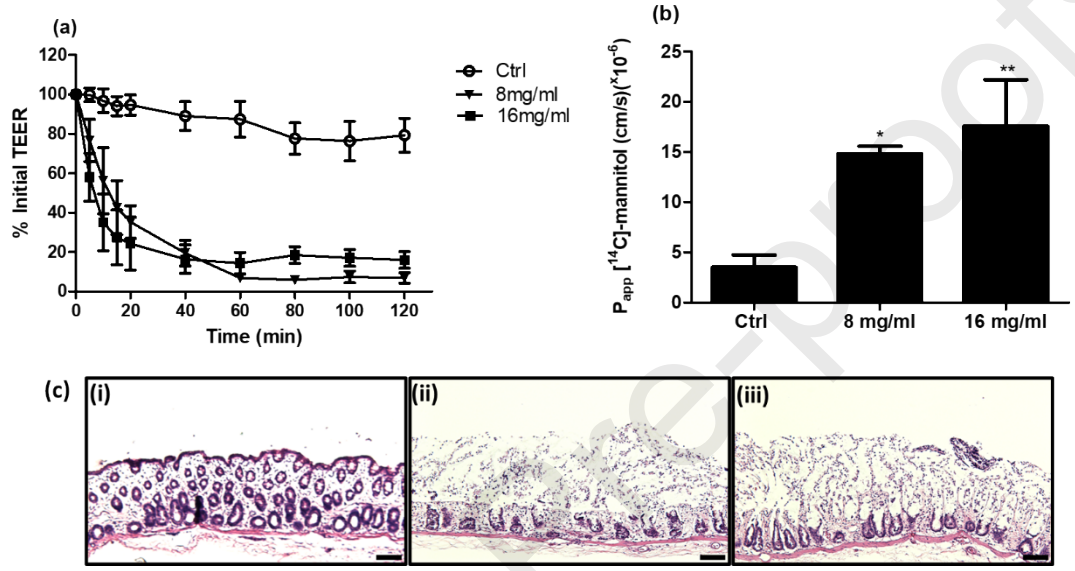


**Fig. 4.** Effect of digested Labrafac™ MC60 (8mg/ml, 16mg/ml) on colonic mucosae (a) % initial TEER and (b) $P_{app}$ of [$^{14}$C]mannitol,*P<0.05 and **P <0 .01 compared to untreated control. Mean ± SEM, n=4-5. (c) representative H&E histology. (i) Ctrl, (ii) 8mg/ml and (iii) 16mg/ml. Marker bar =100 µm.

*3.3 Recovery studies and MW-dependent effects of Labrafac™ MC60 in isolated rat colonic mucosae*

In order to determine if the effects of Labrafac™ MC60 are transient, a wash-out study was performed to see if TEER could recover. Tissue was exposed to Labrafac™ MC60 (8, 12, and 16mg/ml) for 30 min followed by washout and addition of fresh KH buffer. TEER was then recorded for a further 90 min. Concentration-dependent effects were seen in relation to TEER reduction (as seen in Fig. 2A) and recovery (Fig. 5a). The TEER after exposure to 8mg/ml Labrafac™ MC60 was significantly different to control between 15-40 min and 20 min after washout began to recover and was not different to control (Table S3). At 120 min, the TEER of control was 88% of its initial TEER at T0 compared to 74% (8mg/ml), 54% (12mg/ml) and 43% (16mg/ml Labrafac™ MC60). This differs from the results seen in Fig. 2a where no washout was performed. 8mg/ml had a final TEER at 120 min of 8% and 12% 12mg/ml and 3% 16mg/ml. No difference was observed between tissue exposed to Labrafac™ MC60 (8-16mg/ml) for 30 min followed by washout compared to tissue exposed to KH alone (Figure 5b). All tissues showed an intact epithelium. From this experiment, it is

clear that TEER can be recovered at least in part at all three concentrations of Labrafac™ MC60 evaluated.

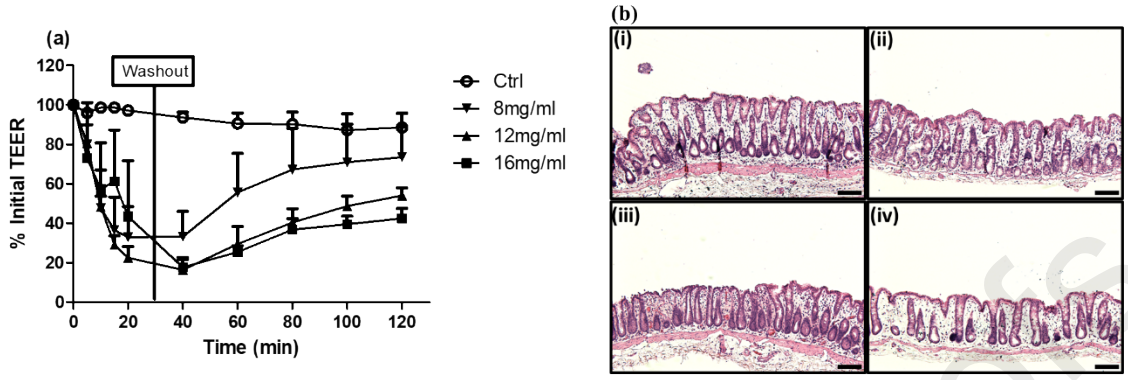


**Fig. 5.** (a) TEER reduction with Labrafac™ MC60 (8, 12, 16mg/ml) and (b) representative histology followed by recovery of TEER in fresh KH. (i) Ctrl, (ii) 8mg/ml, (iii) 12mg/ml and (iv) 16mg/ml. Marker bar= 100 μm.

The capacity of Labrafac™ MC60 to increase the $P_{app}$ of a series of FDs was then evaluated in order to provide evidence of a maximum molecular weight (MW) for molecules whose flux could be enabled and to confirm that the effect was not simply a result of non-specific epithelial barrier breakdown. Labrafac™ MC60 was initially evaluated with FD4 and FD10 at concentrations of 8, 12, and 16 mg/ml Labrafac™ MC60 (TEER results can be seen in Fig. S1). The $P_{app}$ values of FD4 were increased significantly for all concentrations of Labrafac™ MC60, 13-fold, 17-fold, and 11-fold after exposure to Labrafac™ MC60 (8, 12, and 16mg/ml respectively), with 12mg/ml showing the highest increase (Fig 6a, Table 3). For FD10, the $P_{app}$ was significantly increased for 12 and 16mg/ml Labrafac™ MC60, and again 12mg/ml showed the highest increase (Fig. 6b). For this reason, further experiments with the larger MW FDs were conducted using 12mg/ml Labrafac™ MC60. Labrafac™ MC60 increased the $P_{app}$ of each FD, but importantly the effect on flux was reduced as the FD MW increased. While the $P_{app}$ of FD70 was increased with Labrafac™ MC60, this did not occur with Labrasol® ALF at 8mg/ml (McCartney, 2019a). This difference in effect may be due to the higher concentration of Labrafac™ MC60, 12mg/ml, and also due to the fact that the control $P_{app}$ value for FD70 in these experiments was lower than previously observed. The $P_{app}$ of FD70 in the presence of Labrafac™ MC60 was of the same magnitude as that of FD4 alone (Fig. 6c).

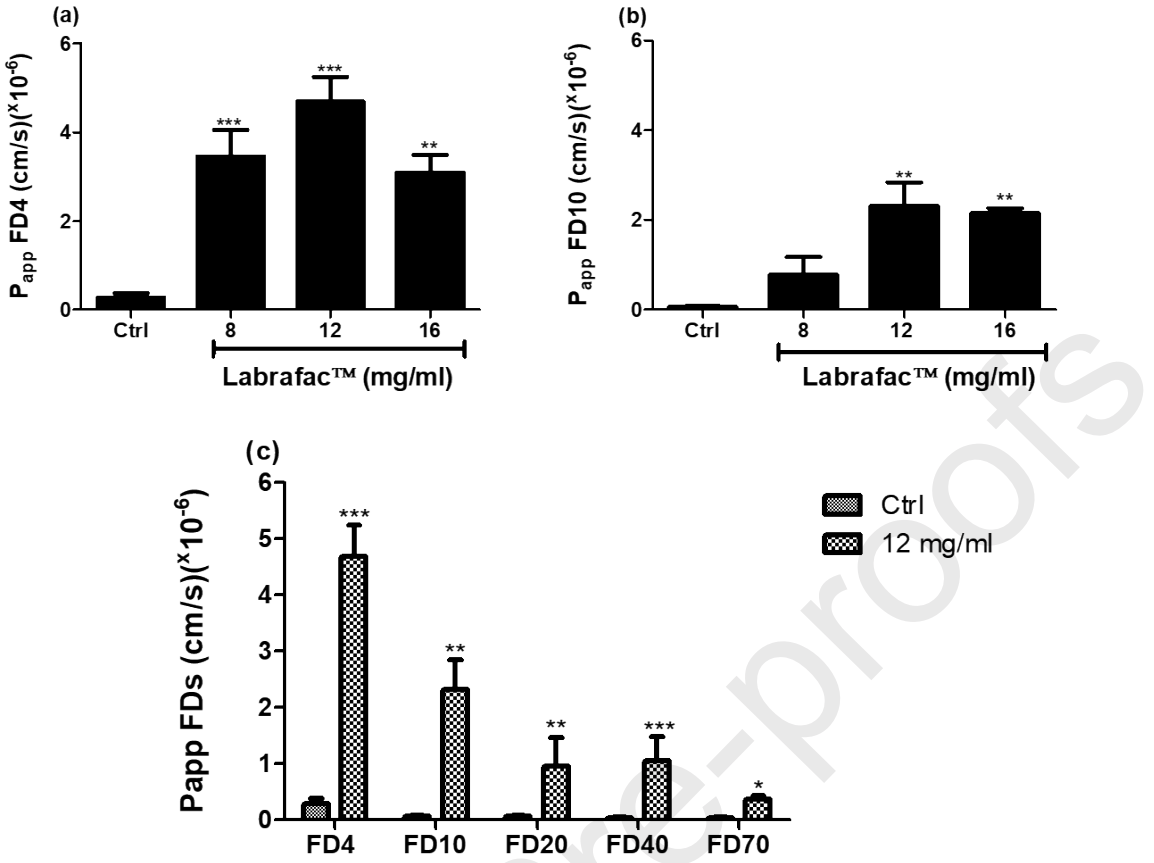


**Fig. 6.** Effect of Labrafac™ MC60 (Labrafac™) (8,12 and 16 mg/ml) on colonic mucosae parameters in KH. (a) $P_{app}$ of FD4; (b) $P_{app}$ of FD10. **P<0.01 and ***P<0.001 compared to untreated control. Mean ± SEM, n=3-4. (c) effect of 12mg/ml on Papp of FDs. Mean ± SEM, n=3-5

**Table 3**. Effect of Labrafac™ MC60 on the $P_{app}$ of FDs across rat colonic mucosae.

| | **$P_{app}$ ($x10^{-6}$ cm·$s^{-1}$)** | | | | |
|---|---|---|---|---|---|
| | **FD4** | **FD10** | **FD20** | **FD40** | **FD70** |
| **Control** | 0.28 ± 0.1 | 0.06 ± 0.02 | 0.07 ± 0.02 | 0.03 ± 0.01 | 0.03 ± 0.01 |
| **8mg/ml** | 3.46 ± 0.6 | 0.7 8± 0.4 | - | - | - |

| | | | | | |
|---|---|---|---|---|---|
| **12mg/ml** | 4.69 ± 0.6 | 2.32 ± 0.5 | 0.96 ±0.5 | 1.1 ± 0.42 | 0.36 ± 0.07 |
| **16mg/ml** | 3.10 ± 0.4 | 2.15 ± 0.1 | - | - | |

*3.4 Effect of Labrafac™ MC60 on TEER and the $P_{app}$ of [$^{14}$C]mannitol in simulated colonic fluid (FaSSCoF)*

It is important to conduct ex vivo studies in models that are as physiologically relevant as possible. For this reason, Labrafac™ MC60 was evaluated in the Ussing chambers with FaSSCoF on the apical side and KH on the basolateral side [29]. The second reason that FaSSCoF was used was that Labrafac™ MC60 did not completely disperse in KH buffer, so we surmised that it might perform better as a PE in FaSSCoF due to the presence of the emulsifying bile salt, sodium cholate (0.15mM). The control $P_{app}$ of [$^{14}$C]-mannitol in FaSSCoF was 5.6 x $10^{-6}$ cm·s$^{-1}$ (Fig. 7b) compared to 2.3 x $10^{-6}$cm·s$^{-1}$ in KH (Fig. 2c). Labrafac™ MC60 (8mg/ml) in FaSSCoF increased the $P_{app}$ by 3-fold, but not significantly (Fig. 7b). In KH, the $P_{app}$ in the presence of Labrafac™ MC60 was 5.0 x$10^{-6}$ cm·s$^{-1}$ compared to 14.3x$10^{-6}$cm·s$^{-1}$ in the presence of FaSSCoF. Colonic mucosae exposed to FaSSCoF for 120 min showed some cellular debris, but the epithelial layer remained intact (Fig. 7c). Tissue exposed to Labrafac™ MC60 in FaSSCoF showed oedema and some minor erosion of the epithelium, similar to exposure in KH (Fig. 3).

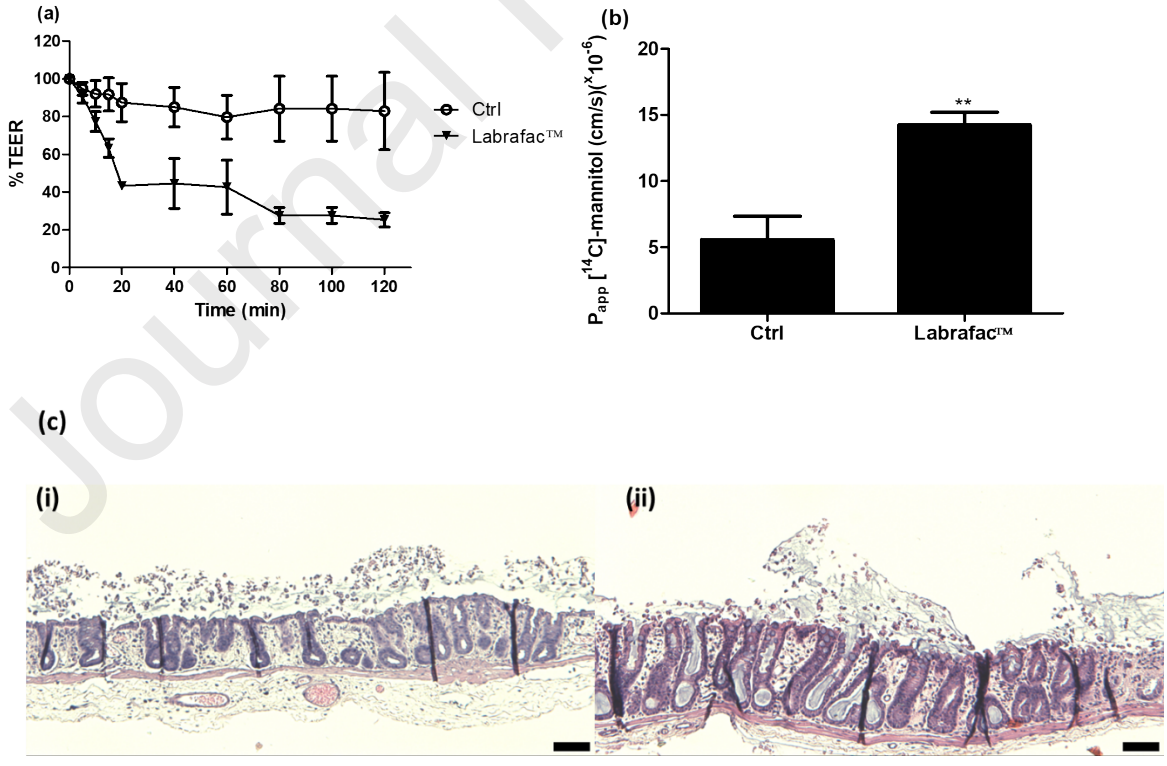


**Fig. 7.** Effect of Labrafac™ MC60 (8mg/ml) on isolated rat colonic mucosae (a) % TEER in FaSSCoF , (b) $P_{app}$ of [$^{14}$C]-mannitol in FaSSCoF and (c) H&E histology in FaSSCoF after

120 min . **P<0.01 compared to untreated control Mean ± SEM, n=3-4 per group. (i)Ctrl, (ii)Labrafac™ MC60.

*3.5 Effects of combinations of excipients in rat colonic mucosae*

To increase the dispersibility of Labrafac™ MC60 and to improve efficacy as a PE, it was combined with Labrasol® ALF which has previously been shown to act as a PE and also has capacity to disperse in KH [11]. The aim was also to see if we could reduce the concentration of PE needed to aid the transport of [$^{14}$C]-mannitol across the colon. Labrasol® ALF and Labrafac™ MC60 were combined in ratios (50/50, 30/70 and 70/30). Table 4 provides the resulting HLB of the combinations. All combinations decreased TEER of rat colonic mucosae to different degrees. Combinations B,C,D,F,G, and H significantly decreased TEER from 10 min compared to untreated control (Table S4). Combination E (30 Labrasol® ALF: 70 Labrafac™ MC60: 1mg/ml) did not. Combination H (100% Labrasol® ALF) decreased TEER from 5 min. The greatest increase in $P_{app}$ was observed with combination D (Labrafac™ MC 60: Labrasol® ALF (70:30), 2mg/ml) which showed a 5-fold increase compared to control (Table 4) and combination H which showed a 6-fold increase. Combination D increased the Papp to 11.9 x10$^{-6}$cm·s$^{-}$ compared to Labrafac™ MC60 alone (2.7 x10$^{-6}$cm·s$^{-1}$ (Table 4). This difference is likely due to the fact that Labrafac™ MC60 is immiscible in aqueous buffers and, by combining with Labrasol® ALF, the HLB was increased from 5 to 9. The combinations did not prevent an increase in Isc in response to the basolateral addition of carbachol, thereby demonstrating retention of function. These results confirm that Labrafac™ MC60 can be combined with other lipid-based excipients when formulating APIs. Compared to Labrafac™ MC60 alone, in examining histology most combinations were similar to control, showing an intact epithelium with minor cell sloughing (Fig.9). Combination B showed the greatest effects on the tissue with the epithelium no longer intact.

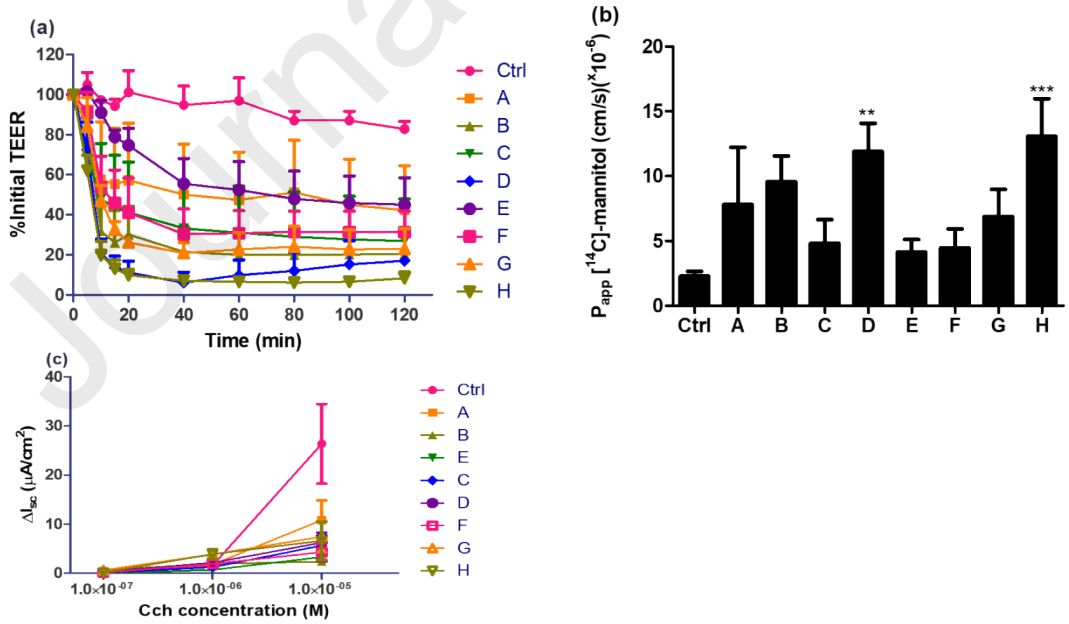


**Fig. 8.** Effect of combinations of excipients on colonic mucosae in the presence of KH. (a) % initial TEER reductions and (b) $P_{app}$ of [$^{14}$C]-mannitol, (c) Isc responses to carbachol.

**P<0.01 and ***P <0.001 compared to untreated controls. Mean ± SEM, n= 8 for control and n=3-7 for combinations. Details for the labelling of the combinations are in Table 4.

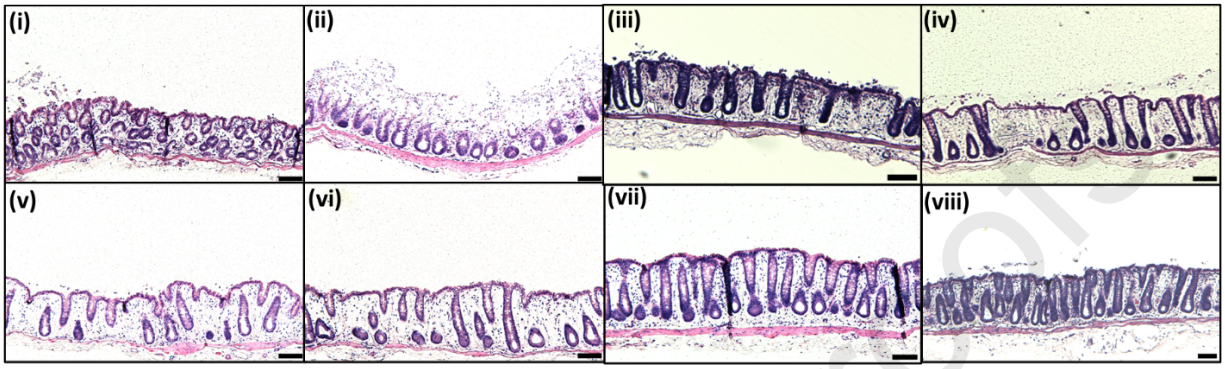


**Fig. 9**. Representative H&E-stained isolated colonic mucosae from Ussing chamber studies exposed to combinations A-H in KH buffer. i A, ii B, iii C, iv D, v E, vi F, vii G, viii H. Scale bar=100 µm.

**Table 4**. Effect of combinations of Labrafac™ MC60 and Labrasol® ALF on the $P_{app}$ of [$^{14}$C] mannitol in rat colonic mucosae in KH buffer. Fold increase is relative to the KH buffer control.

| Combination | Labrasol® ALF (%) | Labrafac™ MC60 (%) | mg/ml | HLB | $P_{app}$ (x $10^{-6}$ cm·$s^{-1}$) | Fold increase |
|---|---|---|---|---|---|---|
| **A** | 50 | 50 | 2 | 8.5 | 7.8 | 3.4 |
| **B** | 50 | 50 | 4 | 8.5 | 9.6 | 4.3 |
| **C** | 70 | 30 | 1 | 9.9 | 4.8 | 2.1 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **D** | 70 | 30 | 2 | 9.9 | 11.9 | 5.2 |
| **E** | 30 | 70 | 1 | 7.1 | 4.1 | 1.8 |
| **F** | 30 | 70 | 2 | 7.1 | 4.5 | 2.0 |
| **G** | 100 | 0 | 1 | 12 | 6.9 | 3.0 |
| **H** | 100 | 0 | 2 | 12 | 13.1 | 5.7 |

Labrafac™ MC60 was then combined with PEG-60 hydrogenated castor oil (HCO-60, HLB 14), a known solubiliser and emulsifier [38] to further promote its solubilisation in aqueous buffers. HCO-60 (1%) alone had no effect on TEER or the $P_{app}$ of [$^{14}$C]-mannitol (Figure 10a). However, Labrafac™ MC60 when combined with HCO-60 decreased TEER and increased $P_{app}$. Labrafac™ MC60 alone at 8mg/ml had a $P_{app}$ of 5.0x10$^{-6}$cm·s$^{-1}$ compared to 21.0 x10$^{-6}$ cm·s$^{-1}$ with HCO (Fig. 10b), which is a 4-fold increase. At 16mg/ml, Labrafac™ MC60 had a $P_{app}$ of 12.30 x10$^{-6}$ cm·s$^{-1}$ alone compared to 18.70x10$^{-6}$cm·s$^{-1}$ with HCO-60 (Fig 10b). Rat colonic mucosae tissue histology when exposed to HCO was similar to tissue exposed to KH alone whereas tissue exposed to Labrafac™ MC60 + HCO-60 at both concentrations showed erosion of the epithelial layer (Fig. 10c).

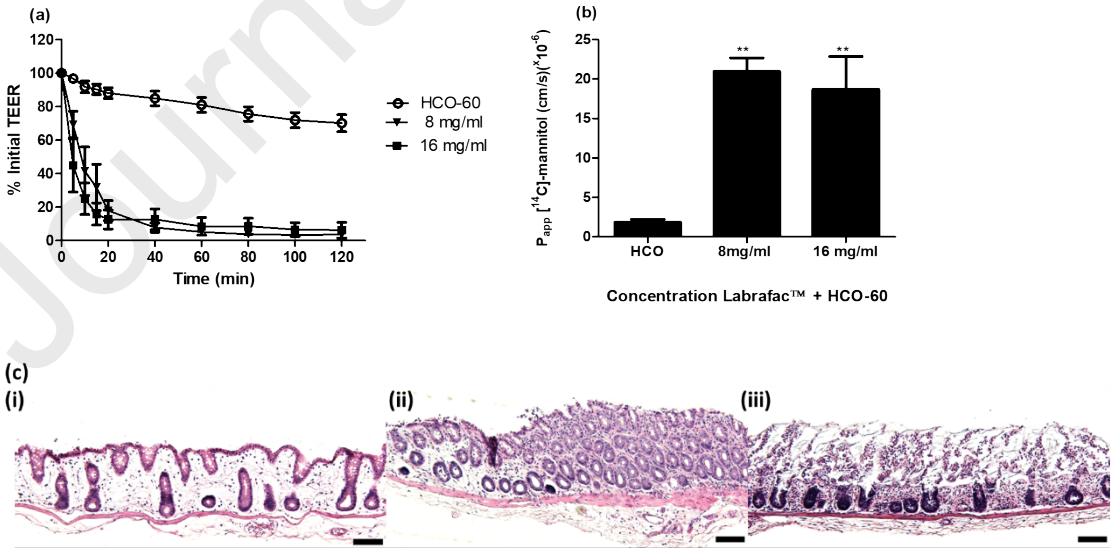


**Fig. 10.** Effect of Labrafac™ MC60 (Labrafac™) (8mg/ml, 16mg/ml) with 1% HCO-60 on (a) % initial TEER and (b) $P_{app}$ of [$^{14}$C]-mannitol in rat colon in Ussing chambers in KH

buffer and (c) H & E staining, (i) Ctrl, (ii) 8mg/ml and (iii) 16mg/ml. Mean ± SEM, n=5. **P<0.01 compared to HCO-60 alone. Scale bar=100 µm.

**Table 5.** Effect of Labrafac™ MC60 with 1% w/v HCO-60 on the $P_{app}$ of [$^{14}$C] mannitol in rat colonic mucosae in KH buffer. **P<0.01 (Student's t-test).

| Concentration | $P_{app}$ (x $10^{-6}$ cm·$s^{-1}$) without HCO-60 | $P_{app}$ (x $10^{-6}$ cm·$s^{-1}$) with HCO-60 | Statistics |
|---|---|---|---|
| **Control** | 2.3 | 1.9 | NS |
| **8mg/ml Labrafac™** | 5.0 | 21.0 | ** |
| **16mg/ml Labrafac™** | 12.3 | 18.7 | NS |

*3.6. Effects of Labrafac™ MC60 on TEER and the $P_{app}$ of [$^{14}$C] mannitol in isolated rat jejunal mucosae in KH buffer*

Due to the more delicate nature of jejunal mucosae, it was not muscle stripped, but tissue was only used if it had a minimum TEER value of 30 Ω.cm$^2$ [26, 39]. Concentrations of excipients assessed with jejunal mucosae were 20 mg/ml and 40mg/ml. These concentrations were chosen as it has previously been seen with other PEs such as $C_{10}$ that higher concentrations are needed in the isolated rat jejunum for efficacy as a PE than for colon [27]. This requirement for higher PE concentrations in jejunal mucosae may be because the jejunum has a higher surface area [40] and has a robustness in epithelial plasma membranes from being normally exposed to bile salts. In isolated rat colonic mucosae, 10mM (or 2mg/ml) $C_{10}$ is the standard effective concentration whereas in jejunum it is threefold higher at 30mM (6 mg/ml) [27]. In addition, Labrasol® ALF increased the bioavailability of insulin in the rat intra-jejunal in vivo model at 40 mg/ml compared to 8mg/ml in the colonic region [11]. Here, in isolated rat jejunal mucosae, Labrafac™ MC60 slowly decreased TEER from 20 min (20mg/ml) and from 80 min (40mg/ml), whereas $C_{10}$ decreased TEER within 5 min (Fig. 11a, Table S5). The $P_{app}$ of [$^{14}$C]-mannitol was increased 1.3-fold (20mg/ml), 2.0-fold (40 mg/ml) Labrafac™ MC60 relative to control compared to 1.6-fold (20mg/ml) and 2.0-fold (40mg/ml) Labrasol® ALF and 2.2-fold for $C_{10}$ (6mg/ml) (Fig. 11b). At all

concentrations of Labrafac™ MC60 assessed tissue responded to carbachol but not to the same extent as control. Histological examination of the tissue after staining with H&E showed an intact epithelium with some cellular debris above the tips of the villi for KH buffer control tissue (Fig. 11d). Due to the delicate nature of jejunal tissue, minor effects are observed even with control tissue in KH for 120 min. For Labrafac™ MC60 and Labrasol® ALF at 20mg/ml, loss of cells at the tips of villi was observed, with a greater effect observed at 40mg/ml. $C_{10}$ showed the greatest amount of damage.

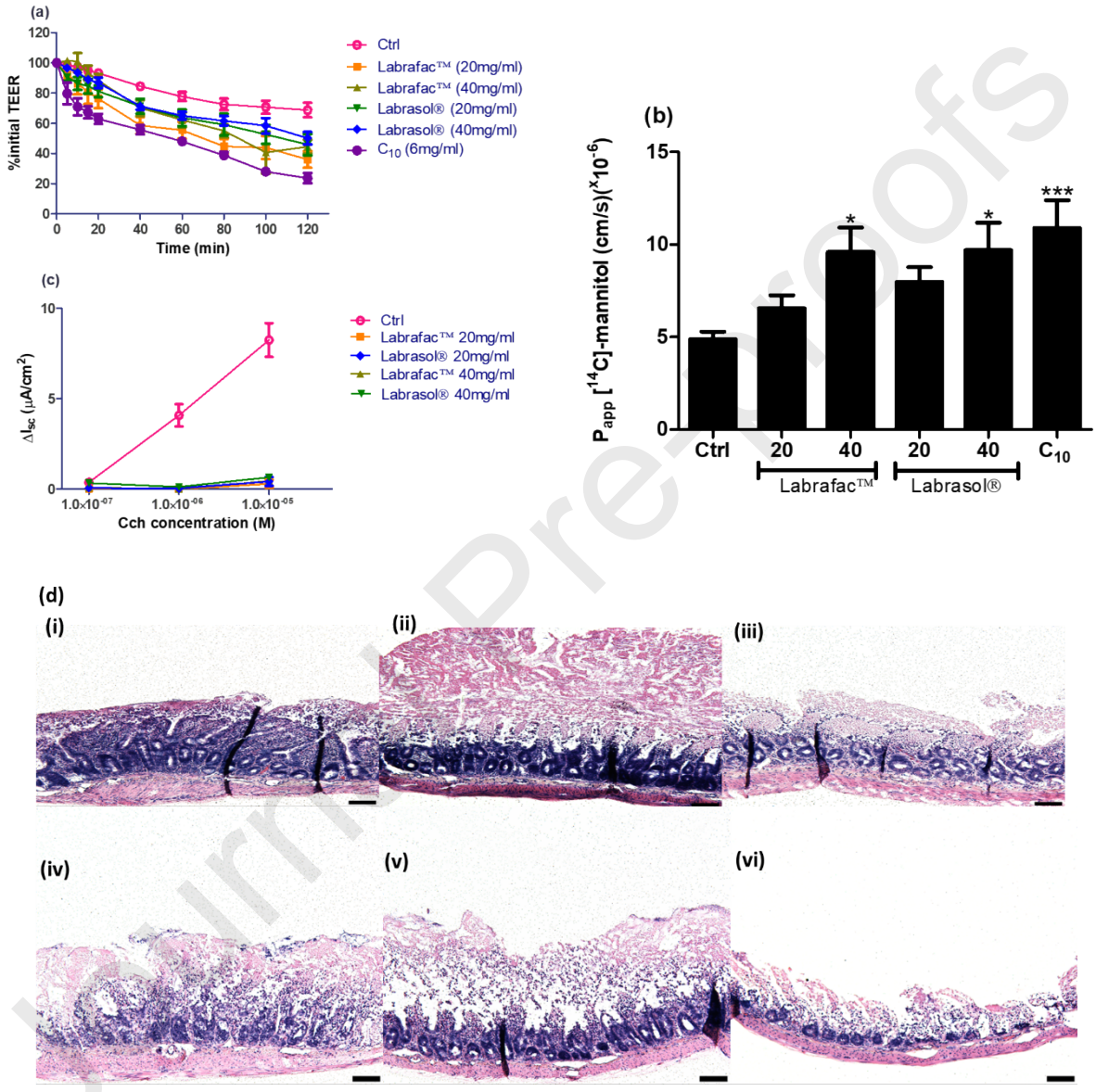


**Fig. 11**. Effect of Labrafac™ MC60 (Labrafac™) on isolated rat jejunal mucosae in KH buffer. (a) % initial TEER reductions and (b) $P_{app}$ of [$^{14}$C] mannitol, (c) Isc changes in response to carbachol (Cch).and (d) H&E histology(i) Ctrl, (ii) 20mg/ml Labrafac™ MC60, (iii) 20mg/ml Labrasol® ALF, (iv) 40mg/ml Labrafac™ MC60, (v) 40mg/ml Labrasol® ALF, (vi 6mg/ml $C_{10}$. *P<0.05 and ***P <0.001 compared to KH control. Labrafac™ MC60 and Labrasol® ALF were assessed at 20mg/ml and 40 mg/ml, with $C_{10}$ at 6mg/ml. Mean ± SEM, n=4-9.

*3.7. Effects of Labrafac™ MC60 on TEER and the $P_{app}$ of [$^{14}$C] mannitol in rat jejunal mucosae in FaSSIF*

The excipients were then assessed in apical side FaSSIF at 40 mg/ml concentrations of Labrasol® ALF and Labrafac™ MC60 in jejunal mucosae as this was the effective concentration for these agents in KH buffer in these tissues. Of the agents assessed, only the PE control agent, $C_{10}$, reduced TEER significantly, and only from 80 min post-addition (Figure 12a) and it was also the only agent to statistically increase the $P_{app}$ (Figure 12b). However, when the $P_{app}$ results in KH were compared with those in FaSSIF, similar patterns were observed for the excipients with no statistical differences (Table 6, Fig. 13). The basal $P_{app}$ for untreated control in KH v FaSSIF were similar: 4.9 x$10^{-6}$cm·s$^{-1}$and 4.8 x $10^{-6}$cm·s$^{-1}$, respectively. For Labrafac™ MC60 and Labrasol® ALF the values in FaSSIF were decreased slightly. However, the $P_{app}$ data in FaSSIF was less variable than that obtained in KH. Rank ordering of the H&E-stained histology in the order from least-to most damage was: Control< Labrafac™ MC60 < Labrasol® ALF < $C_{10}$. $C_{10}$ induced the greatest amount of damage including erosion of the tips of the villi, a result seen in previous studies and this may relate to it being the only one that was efficacious in FaSSIF [25, 27, 41].

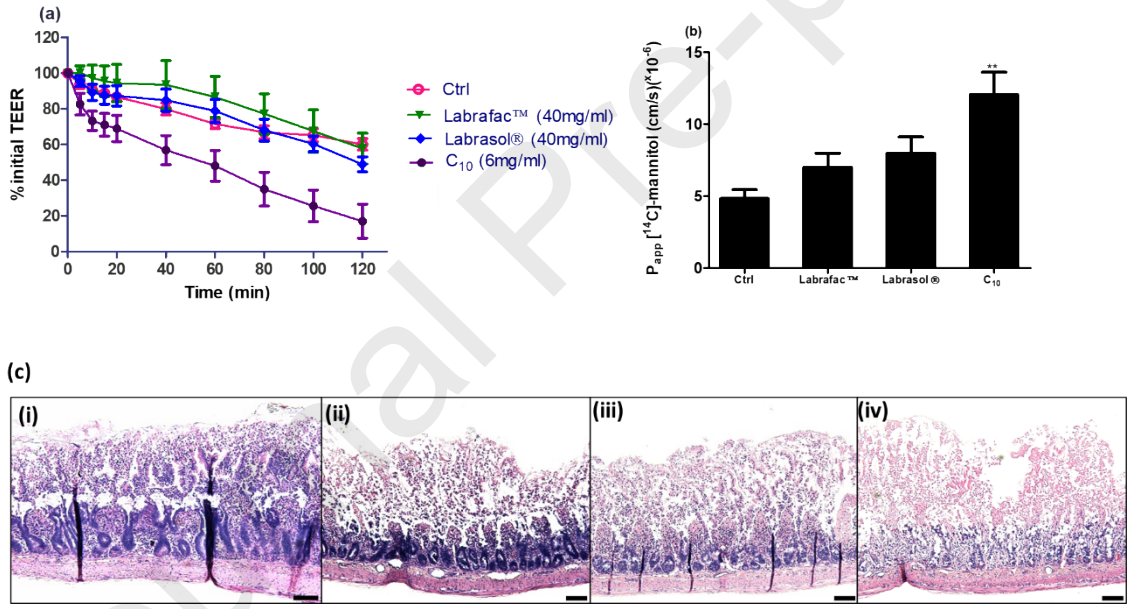


**Fig. 12**. Effect of Labrafac™ MC60 (Labrafac™) on isolated rat jejunal mucosae incubated in apical-side FaSSIF. (a) % initial TEER reductions, (b) $P_{app}$ of [$^{14}$C]-mannitol, and (c) H&E histology (i) Ctrl, (ii) 40mg/ml Labrafac™ MC60, (iii) 40mg/ml Labrasol® ALF. (iv) 6mg/ml $C_{10}$. *P<0.05 and ***P <0.001 compared to KH control. Labrafac™ MC60 and Labrasol® ALF were assessed at 40 mg/ml; $C_{10}$ at 6mg/ml. Mean ± SEM, n=3.

**Table 6**. Comparison of the effect of Labrafac™ MC60 and Labrasol® ALF (both 40 mg/ml) on the Papp of [$^{14}$C] mannitol across rat jejunal mucosae in apical-side FaSSIF. Numbers in brackets (fold increase compared to control). $C_{10}$ (30mM) was used as a positive control.

| Treatment | $P_{app}$ (x $10^{-6}$ cm·$s^{-1}$) | $P_{app}$ (x $10^{-6}$ cm·$s^{-1}$) |
|---|---|---|
| **Control** | 4.9 | 4.8 |
| **Labrafac™ MC60** | 9.6 (2.0) | 7.0 (1.5) |
| **Labrasol® ALF** | 9.7 (2.0) | 8.0 (1.7) |
| **$C_{10}$** | 10.9 (2.2) | 12.1 (2.5) |

*3.8 Effect of Labrafac™ MC60 on intestinal bioavailability of insulin in PBS in the rat intra-jejunal in vivo instillation model*

The capacity of Labrafac™ MC60 to increase the bioavailability of human insulin was compared with Labrasol® ALF. 40mg/ml Labrafac™ MC60 or Labrasol® ALF were mixed with insulin in PBS to produce a final dose of insulin of 50IU/kg body weight. Subcutaneous (s.c.) injection of insulin (1 IU/kg) decreased blood glucose levels and increased insulin plasma levels. Jejunal instillation of 50 IU/kg insulin in PBS containing Labrasol® ALF produced similar glucose reductions to that of s.c. administration. (Fig. 13). Insulin ad-mixed with Labrafac™ MC60 reduced blood glucose levels by 43% compared to a 76% reduction with Labrasol® ALF (Fig. 13a). Labrasol® ALF admixed with insulin decreased blood glucose significantly compared to insulin alone from 30 min onwards ($P<0.001$). However, despite trends, Labrafac™ MC60 ad-mixed with insulin did not significantly decrease blood glucose levels (Table S6).

Differences were also seen in terms of plasma insulin levels (Fig. 13b). Labrafac™ MC60 ad-mixed with insulin significantly increased plasma insulin levels when instilled to give a relative bioavailability of 4.8%, compared to 6.6% with Labrasol® ALF (Table 7). This data confirmed the reproducibility of the effects of Labrasol® ALF as an absorption enhancer for insulin because a bioavailability of 6.7% was achieved in our previous study using the same model [11] . The Tmax for Labrasol® ALF was 23 min compared to 43 min for Labrafac™ MC60 and the Cmax was 267mU/L for Labrafac™ MC60 versus 420mU/L for Labrasol® ALF. The Tmax of 43 min for Labrafac™ MC60 reflects the variability of this excipient as out of 6 rats, 3 rats had a Tmax of 20 min, 1 rat at 40 min and 2 rats at 80 min. Histological examination of H&E-stained tissue showed that exposed tissues with either Labrafac™ MC60 or Labrasol® ALF were similar to control tissue exposed to insulin alone. All tissue showed intact villi with mild cellular debris (Fig. 13c).

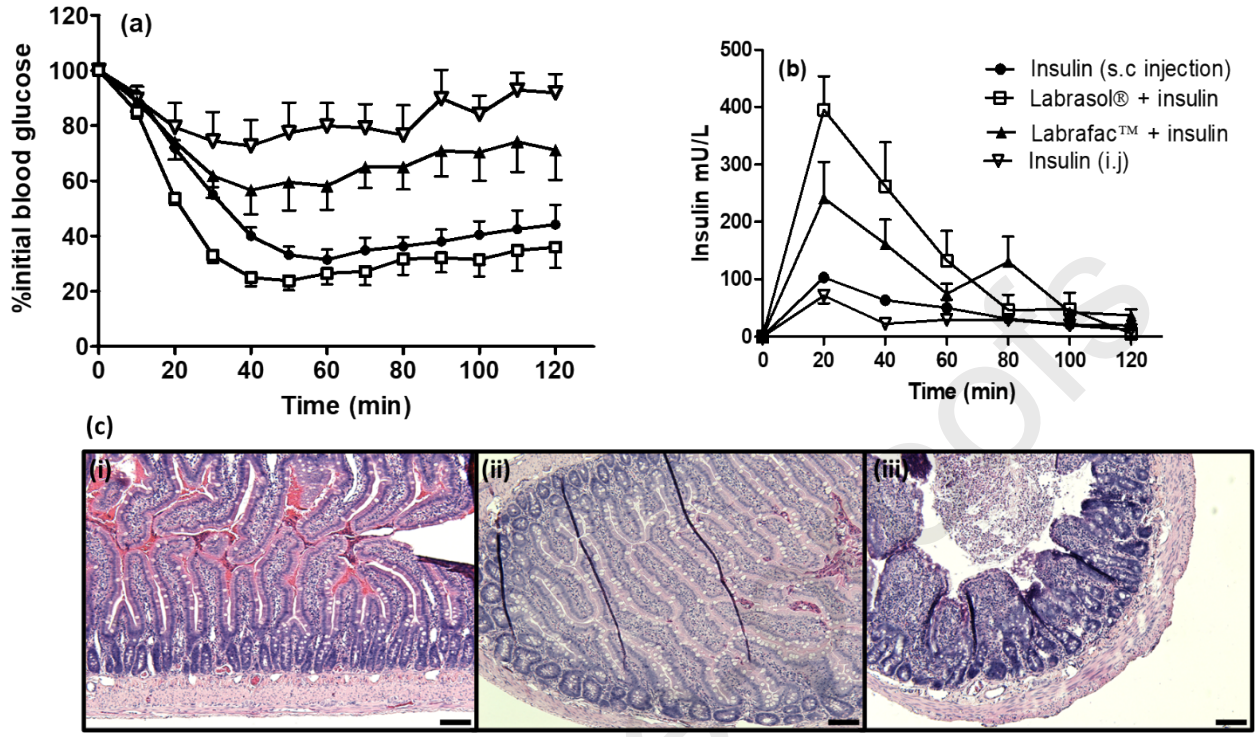


**Fig. 13.** Rat intra-jejunal (i.j.) instillations of Labrafac™-MC60 with insulin. (a) Blood glucose levels and (b) plasma insulin levels (c) H & E-stained rat jejunal tissue: (i) insulin (i.j. 50 IU/kg), (ii) Labrafac™ MC60 (Labrafac™) (40 mg/kg) + insulin and (iii) Labrasol® ALF (40 mg/kg) + insulin after 120 min instillation in PBS. Insulin (1 IU/kg) was administered by the s.c. route (a, b). Mean ± SEM and n=6. Scale bar=100 µm.

**Table 7**. PK parameters from rat intra-jejunal (i.j.) instillations of 50 IU/kg insulin with PEs (both at 40 mg/ml).

| Treatment | Tmax (min) | Cmax (mU/L) | AUC (0–120) (mU/L.min) | %F |
|---|---|---|---|---|
| **Insulin (1 IU/kg) (s.c.)** | 27±7 | 104±3 | 5544±642 | - |
| **Insulin in PBS (i.j.)** | 30±10 | 74±13 | 3568±691 | 1.3 |

| | | | | |
|---|---|---|---|---|
| **Insulin + Labrafac™ MC60 in PBS (i.j.)** | 43±12 | 267±60 | 13377±2144 | 4.8 |
| **Insulin + Labrasol® ALF in PBS (i.j.)** | 23±3 | 420±58 | 18272±3800 | 6.6 |
| **Insulin in FaSSIF (i.j.)** | 40±13 | 58±18 | 3684±1197 | 1.3 |
| **Insulin + Labrafac™ MC60 in FaSSIF (i.j.)** | 30±7 | 316±75 | 14297±3007 | 5.2 |
| **Insulin + Labrasol® ALF in FaSSIF )i.j.)** | 23±3 | 380±69 | 16340±2537 | 5.9 |

*3.9 Effect of Labrafac™ MC60 on bioavailability of insulin in FaSSIF in the rat intra-jejunal instillation model*

In order to make the studies more physiologically relevant they were also conducted using FaSSIF in the jejunal lumen. The bile salts in the buffer did not interfere with the capacity of the two excipients to function as PEs *in vivo*. The bioavailability of insulin solution was not altered in FaSSIF compared to PBS (Table 7). Both Labrafac™ MC60 and Labrasol® ALF reduced blood glucose levels and increased plasma insulin levels in FaSSIF similar to PBS (Fig. 14). Compared to insulin alone, Labrafac™ MC60 significantly decreased blood glucose levels at 30-50 min and 70-80 min in FaSSIF, which was an improvement over PBS (Table S7). In terms of plasma insulin levels, no difference was seen between the AUC obtained for Labrafac™ MC60 in PBS or FaSSIF (Figure 14c). The $C_{max}$ of plasma insulin in the presence of Labrafac™ MC60 increased from 267mU/L to 316mU/L and the Tmax shifted from 43 min to 30 min (Table 7). Labrasol® ALF also showed no difference between the AUC achieved for insulin in PBS vs FaSSIF. Histology after exposure to excipients in luminal PBS or FaSSIF showed no difference between the buffers (Figure 13c and 14d) with healthy tissues observed after 120 min incubation.

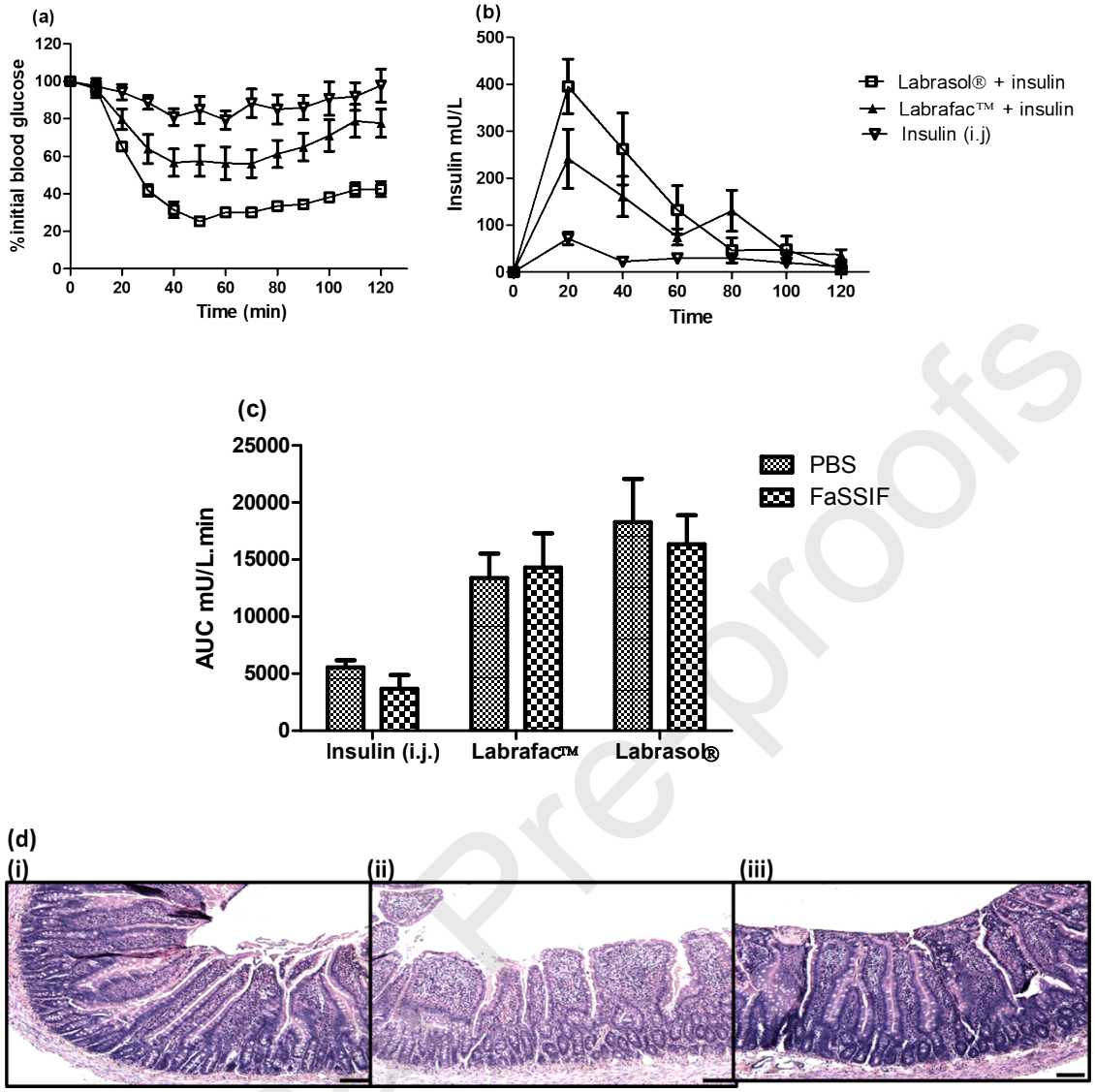


**Fig. 14.** (a) Blood glucose levels and (b) plasma insulin levels after intra-jejunal (i.j.) instillation of Labrafac™ MC60 (Labrafac™) or Labrasol® ALF (40 mg/mL) with insulin (50 IU/kg) in FaSSIF. (c) AUC of plasma insulin concentration versus time of excipients in PBS v FaSSIF.. (d) H&E-stained histology, (i) insulin (i.j.), (ii) Labrafac™ MC60 and (iii) Labrasol® ALF Mean ± SEM and n=6.

*3.10 Effects of Labrafac™ MC60 in combination with HCO-60 in PBS on insulin bioavailability in the rat intra-jejunal model.*

As for the Ussing chamber studies, Labrafac™ MC60 was combined with HCO-60 to create an emulsion in PBS that might result in an increase in dispersibility and therefore enable a further improvement in bioavailability from instillations. An emulsion was formed when the combination of Labrafac™ MC60 with HCO-60 was added to PBS. Combining of HCO-60 with Labrafac™ MC60 improved the capacity of Labrafac™ MC60 to deliver insulin across the small intestine in vivo. Labrafac™ MC60 co-presented with HC0-60 and insulin

decreased blood glucose by ~40% within 20 min, by 60% between 40-60 min and by 46% at 120 min (Fig. 15a). Comparing the bioavailability of insulin ad-mixed with Labrafac™ MC60 with that of insulin combined with Labrafac™ MC60 and HC0-60, the relative F increased from 5% to 8%, the highest bioavailability of all the variations evaluated in intra-jejunal instillations. The Tmax of plasma insulin shifted from 43 min to 27 min and the Cmax increased from 267 mU/L to 500 mU/L with the combined excipients (Table 8). No negative histological effects on the colonic epithelium were seen with the combination of Labrafac™ MC60 and HCO-60 (Figure 15d).

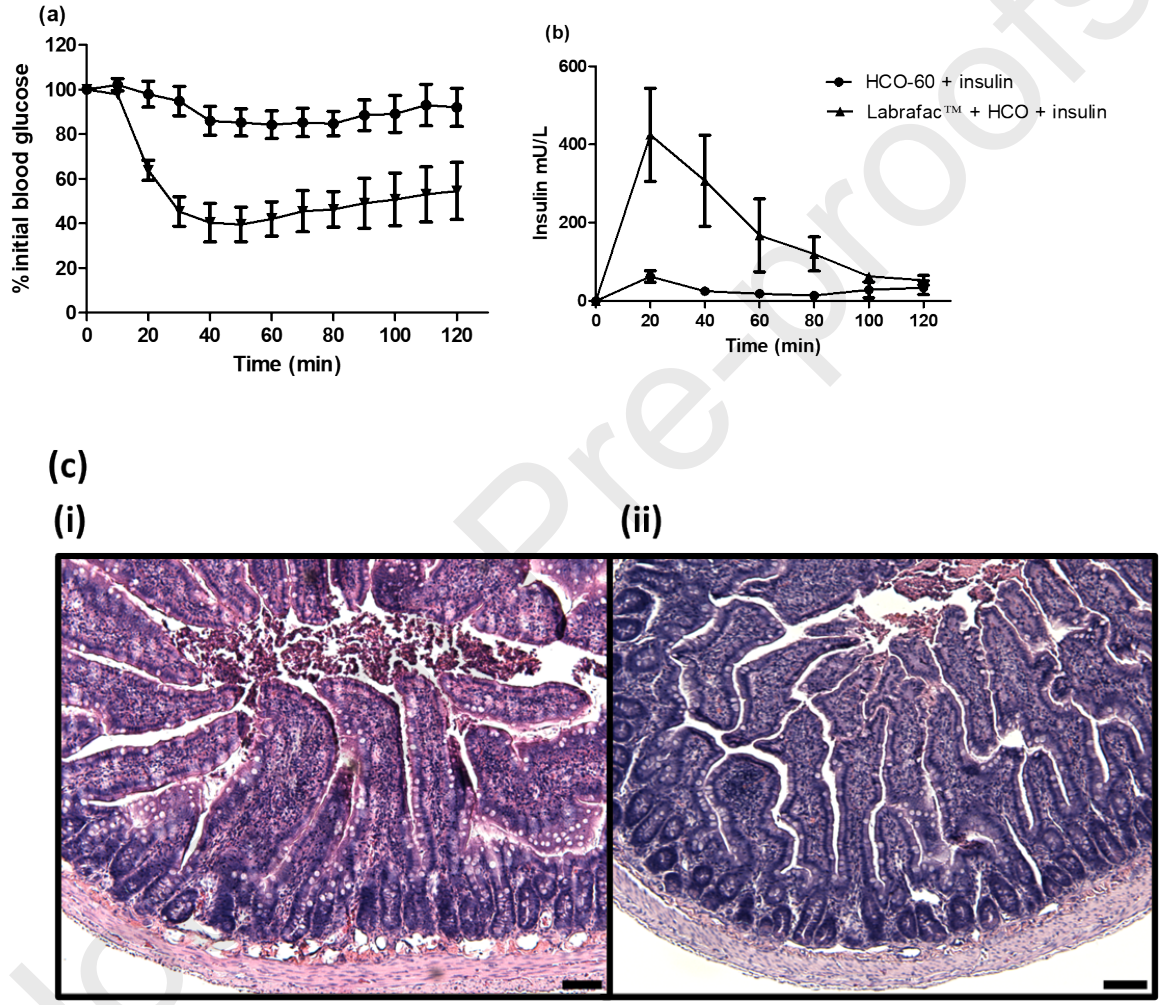


**Fig.15**. (a) Blood glucose levels, (b) plasma insulin levels, and (c) H&E staining 120 min after i.j. instillations. (i) Insulin + HCO-60 (1% w/v) (ii) Insulin + Labrafac™ MC60 (40 mg/ml) + HCO-60 in PBS. Mean ± SEM and n=6.

**Table 8**. Pharmacokinetic parameters from rat jejunal instillations using ad-mixtures of Labrafac™ MC60 (40 mg/ml) with and without HCO-60 (1% w/v) ad-mixed with insulin (50 IU/kg) in PBS.

| Treatment | Tmax (min) | Cmax (mU/L) | AUC (0–120) (mU/L.min) | % F |
|---|---|---|---|---|
| Insulin + HCO-60 | 53±18 | 68±15 | 3340±936 | 1.2 |
| Insulin + Labrafac™ MC60 | 43±12 | 267±60 | 13377±2144 | 4.8 |
| Insulin + Labrafac™ MC60 + HCO-60 | 27±4 | 500±109 | 22109±5853 | 8.0 |

*3.11. Effect of Labrafac™ MC60 in PBS on the bioavailability of insulin in the rat intra-colonic instillation model*

Although most oral formulations of peptides are targeted to the small intestine (with some designed for stomach release), we had previously demonstrated that Labrasol® ALF (8mg/ml) increases the bioavailability of insulin to a remarkable 12% in the rat colonic instillation model [11], consistent with a large dataset showing that many surfactant-based PEs work better in that GI region [42]. Here, we explored whether Labrafac™ MC60 would behave similarly. HCO-60 was not used in this set of experiments. When presented with insulin in colonic instillations both Labrafac™ MC60 and Labrasol® ALF decreased blood glucose levels to a similar extent from 30 min onwards. They also increased plasma insulin levels but Labrasol® ALF did so to a greater extent (Fig. 16a). Both ad-mixtures induced a Tmax of 20 min for insulin, but Labrafac™ MC60/insulin had a lower Cmax of 368 mU/L for plasma insulin compared to 973 mU/L for Labrasol® ALF /insulin (Fig. 16b, Table 9), reflecting the solubility advantage of Labrasol® ALF. The bioavailability results for Labrasol® ALF with insulin in colonic instillations (13%) were similar to those we obtained previously for it [11]. Labrafac™ MC60 ad-mixed with insulin produced a bioavailability of 6%, similar to the value obtained with the agent in jejunum (5%), but at five times lower concentration (8mg/ml in colon vs 40mg/ml in jejunum). Rank ordering of the relative bioavailability values obtained in jejunum (40mg/ml) for insulin were: Labrafac™ MC60+HCO-60 (PBS, 8.0%)> Labrasol® ALF (PBS, 6.6%)> Labrasol® ALF (FaSSIF, 5.9%)>Labrafac™ MC60 (FaSSIF, 5.2%)>Labrafac™ MC60 (PBS, 4.8%). In colonic instillations (40mg/ml) the order was: Labrasol® ALF (PBS, 13.2%)> Labrafac™ MC60 (PBS, 6%). Had we evaluated HCO-60 with Labrafac™ MC60 and insulin in intra-colonic instillations, the value would have been increased further. The histology of the colon upon exposure to the two excipients showed an intact layer of epithelium with only minor cellular debris, similar to controls (Fig. 16c).

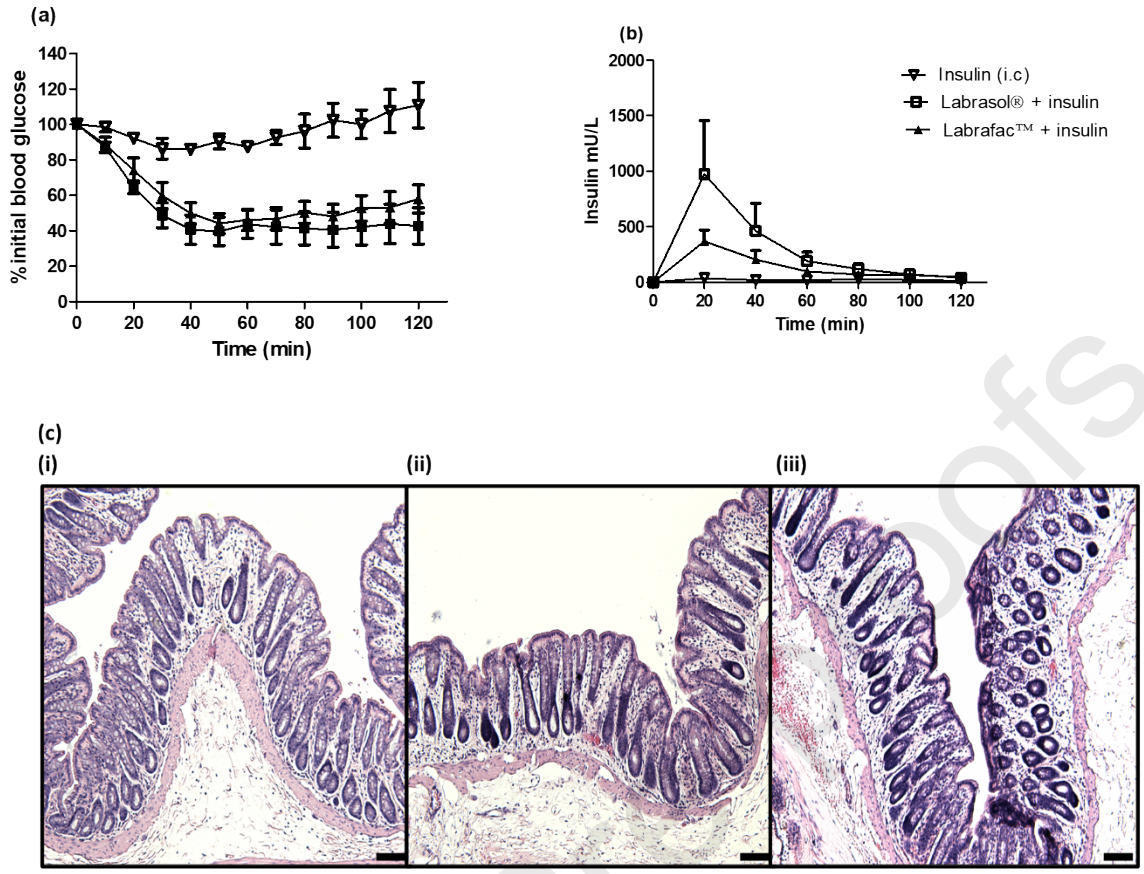


**Fig. 16**. (a) Blood glucose levels and (b) plasma insulin levels after intra-colonic (i.c.) instillation of Labrafac™ MC60 (Labrafac™) or Labrasol® ALF (8 mg/mL) with insulin (50 IU/kg). (c) H&E-stained colonic histology after 120 min: (i) insulin, (ii) Labrafac™ MC60 + insulin, (iii) Labrasol® ALF + insulin Mean ± SEM and n=6.

**Table 9**. Pharmacokinetic parameters after intra-colonic administration of ad-mixtures of insulin (50 IU/kg) with the two excipients (8 mg/kg).

| Treatment | Tmax (min) | Cmax (mU/L) | AUC (0–120) (mU/L.min) | %F |
|---|---|---|---|---|
| Insulin (i.c.) | 40±13 | 46±12 | 2515±622 | 0.9 |
| Labrafac™ MC60 + insulin | 20±0 | 368±103 | 16569±5049 | 6.0 |
| Labrasol® ALF + insulin | 20±0 | 973±482 | 36707±17237 | 13.2 |

## 4. Discussion

The data in this study shows that Labrafac™ MC60 can function as an intestinal PE for insulin. To date, Labrafac™ MC60 has been used only as a solubiliser and as a component of oral lipid-based formulations Type I (oily solutions), Type II (SEDDS) and Type III (SMEDDS) for small molecules. Our study has demonstrated that Labrafac™ MC60 can act as an intestinal PE ex vivo in both jejunal and colonic tissue using marker flux molecules and in vivo in intra-jejunal and intra-colonic instillations using insulin. Labrafac™ MC60 was compared to another lipid-based excipient Labrasol® ALF, now well established as a leading intestinal PE [11, 13, 43]. While both excipients contain predominantly $C_8$ and $C_{10}$ moieties, they differ in their HLB values, reflected in solubility capacity in aqueous media. Labrafac™ MC60 with a HLB of 5 has low aqueous solubility, making it challenging to use in the Ussing chamber physiological buffer, KH. For this reason, we also investigated improvements in dispersibility by combining it with other excipients such as Labrasol® ALF and HCO-60.

A major advantage of Labrafac™ MC60 as a PE is that it is already in approved formulations that are currently on the market including dutasteride [16], and separately, it is listed on the FDA inactive ingredients database. This makes it attractive for the pharmaceutical industry to use in oral peptide programmes from a regulatory perspective, providing reduced toxicological and commercial risk compared to investing in new chemical entity PEs with no history of use in humans. An extensive toxicology programme would be required for new PEs, along with a requirement to synthesise them in large quantities for clinical trials in accordance with Good Manufacturing Practice (GMP) guidelines.

It is important to compare the results obtained for Labrafac™ MC60 and Labrasol® ALF with those previously obtained data for established PEs in the same ex vivo and in vivo bioassay models. Data published by Twarog et al, had similar control values in the colon ($3.0x10^{-6}$ $s^{-1}$) and in the jejunum ($3.5x10^{-6}$ $s^{-1}$ ) to this study (Table 2 and 6) in Ussing chamber with the paracellular marker, [$^{14}$C]-mannitol [27]. In their study they investigated the PE, SNAC, in rat jejunal mucosae and showed a 1.9-fold increase in the $P_{app}$ of [$^{14}$C]-mannitol for 20mM (6mg/ml) SNAC which did not increase with the higher concentration of 40mM (12mg/ml) SNAC. For $C_{10}$ (30mM;6mg/ml) the increase was 3.2-fold versus the 2.2-fold increase observed in our study. Compared to this, Labrafac™ MC60 and Labrasol® ALF (40mg/ml) increased the $P_{app}$ 2-fold. In colon, in the Twarog study, SNAC showed the highest increase in the $P_{app}$ of , [$^{14}$C]-mannitol at 40mM with a 5-fold increase compared to a 9-fold increase for $C_{10}$ (10mM). In our study we observed an increase of 2-fold for 8mg/ml Labrafac™ MC60 and 6-fold for 12mg/ml which increased to 9-fold for 8mg/ml Labrafac™ MC60 combined with 1% HCO-60. Studies in rat intra-jejunal instillations demonstrated that the non-ionic surfactant PE, sucrose laurate ($C_{12}$), could achieve a relative F for insulin of 1.3% at a concentration of 50mM (20mg/ml), while the relative F was 2.5 % at 100mM (40 mg/ml). For $C_{10}$, the F of insulin was 4.4% (50 mM, 10mg/ml) and 3.3% (100mM; 20mg/ml) [25]. The relative F of Labrafac™ MC60 and Labrasol® ALF (40mg/ml) were higher than this at 4.8% and 6.6% respectively in the current study. The highest value in i.j. instillations obtained was for insulin delivered with Labrafac™ MC60 combined with HCO-60 (8%). Comparable results were achieved with Labrafac™ MC60 and Labrasol® ALF in these models compared to known PEs including SNAC which is in a formulation on the market (Rybelsus®). In fact, the combination of Labrafac™ MC60 and HCO-60 performed better than $C_{10}$ in intra-jejunal instillations, noting that the latter is in a formulation of an oral macrocycle peptide that has reached Phase III trials [8]. In the colonic instillations, the %F results for Labrafac™ MC60 and Labrasol® ALF were comparable with sucrose laurate (25mM/10mg/ml) and $C_{10}$ (10mM, 2mg/ml) at 6.7% and 11.7% respectively [25].

Many PEs work by opening the tight junction between intestinal cells which raises concerns about this facilitating pathogens and lipopolysaccharide fragments to cross the epithelium [3, 44]. Both $C_8$ and $C_{10}$ interact with TJ proteins such as occluding, claudin 5, and zonula occludens-1 (ZO-1) [5, 45]. Since Labrafac™ MC60 contains caprylate and caprate it was important to show that the effects of it were MW- dependent in order to rule out non-specific effects that could relate to toxicity. In rat colonic mucosae in the Ussing chamber the enhancing capacity of a range of different molecular weight FITC-dextrans (4-70 kDa) were evaluated. As the FITC-dextrans increased in MW, the capacity of Labrafac™ MC60 to function as a PE decreased. The largest dextran assessed 70kDa is smaller than the bystanders of concern. These results were similar to those seen before with Labrasol® ALF and also by Tuvia et al (2014) in a rat study where the Transient Permeation Enhancement™ (TPE™) technology was used to enhance the oral delivery of octreotide (Mycapssa™). Similar to Labrafac™ MC60, TPE™ contains caprylate and glycerol mono/tri caprylate [5]. It is also important that the effects of PEs are transient and for this reason we performed a washout study showing that TEER values of rat colonic mucosae in Ussing chambers were able to recover after removal of Labrafac™ MC60.

Studies were initially performed in rat colon in the Ussing chambers as it has been shown that some enhancers particularly ones that contain medium chain fatty acids perform better there [46]. This pattern was also observed in the current study, where similar or greater enhancement was observed in the colon at lower concentrations of Labrafac ™ MC60 compared to jejunal mucosae. This is likely due to the fact that small intestinal epithelia has a larger surface area and is exposed to high concentrations of bile salts, which may promote some resilience to surfactants compared to the colonic region [25, 47]. Performing studies in the colon can establish whether an excipient acts as a PE and at what concentration it does this, a useful initial screen. By comparison, when testing PEs in jejunal mucosae the efficacious concentration may be increased several fold. For Labrafac™ MC60 and Labrasol® ALF, the effective concentrations were 8mg/ml for colonic mucosae versus 40mg/ml for jejunal. The profile of TEER reduction was also changed depending on the region of origin. In colonic mucosae reductions were seen quickly and, particularly for Labrasol® ALF and $C_{10}$, initiated within 5-10 min and remained decreased. In jejunal mucosae changes in TEER were not as dramatic, but basal values were a lot lower and variable, and this made it difficult to make clear distinctions in the TEER profiles in the presence of the PEs. Nonetheless, in vivo studies were performed mostly in the jejunum as this is main target site following oral administration of peptides.

Differences were observed in intestinal tissue histology from ex vivo experiments in rat colonic and jejunal mucosae compared to the same regions after intestinal instillations in vivo, all at 120 min. Following exposure of colonic mucosae in Ussing chambers to low mM concentrations of $C_{10}$ in a previous study, the tissue revealed erosion of the epithelium, whereas in vivo in an instillation model tissue damage was not observed even at higher concentrations [25]. In histological samples of canine intestinal tissue after they were orally administered three tablets a day for seven days containing 330mg of $C_{10}$, no negative effects on histology of intestinal epithelia were observed [48]. The reasons for this may be that in vivo, intestinal tissue is not exposed to the PE for long enough to be affected by it and the intestinal epithelium is constantly renewing. Berg et. al have shown that the effects of $C_{10}$ on the epithelium of perfused rat intestinal segments in vivo may only be about 30–120 min depending on the concentration of $C_{10}$ [49, 50]. Consistent with these reports, negative histology effects seen with Labrafac™ MC60 in ex vivo mucosae were not evident in the in vivo samples. The main reason for the difference between the bioassays in respect of tissue

histology is that in the instillation model there is an intact blood supply which supports the epithelium with nutrient supply and in its capacity to resist perturbation. Jejunal administration of a GI patch containing erythropoietin (EPO) and Labrasol® ALF for 6h also showed no evidence of damaged intestinal epithelial histology similar to the results in our study [51]. Labrasol® ALF was dosed at 143mg/kg and the weight of the rats was 380-400g which would be a dose of 54-57 mg/ml, slightly above the dose in our study. In their study they compared Labrasol® ALF (% F =12), Gelucire® 44/14 (% F=10) and HCO-60 (% F= 3) as PEs and found Labrasol® ALF to have the greatest capacity to increase the F of EPO (100 IU/kg), with HCO-60 performing the worst. The HCO-60 (8mg/ml) dose was higher than what was included in our study (0.4mg/ml), so this would support our data showing no enhancement effects from HCO-60.

It has previously been shown that combining Labrasol® ALF with MCFAs allowed lower concentrations overall of the PEs to be used, while still increasing the $P_{app}$ of FD4 across rat colonic mucosae in Ussing chambers [52]. There also seems to be an interest from industry in pursuing combination approaches, as recently Astra Zeneca researchers screened PE combination in Caco-2 monolayers and combination of propyl gallate and sodium chenodeoxycholate in animal trials in a 1:2 ratio by weight and produced F values of 6% in dogs in tablets with a stable GLP-1 RA. [10]. Here. we combined Labrafac™ MC60 and Labrasol® ALF and the best results were obtained in isolated rat colonic mucosae when they were mixed in a ratio of 30:70. The rationale was that while Labrasol® ALF performs independently as a PE, it can aid the emulsification of Labrafac™ MC60 [19]. In jejunal instillations however, the highest % F obtained was when insulin was administered in a combination of Labrafac™ MC60 with HCO-60. Due to its low HLB of 5 , Labrafac™ MC60 is water insoluble and therefore, unlike Labrasol® ALF with HLB of 12,it is not very soluble in either PBS or KH buffers. The addition of HCO-60, a known emulsifier, allowed the Labrafac™ MC60 to form an emulsion in the PBS or KH buffer, thus improving its capacity to function as a PE. Importantly, At the concentration used (1% w/v) HCO-60 did not act independently as a PE. Another lipid based excipient with a HLB of 5, Capryol® 90, was also combined with HCO-60 by other researchers to enhance the permeation of insulin across rat intestine [53]. The paper did not investigate the effects of HCO-60 alone nor did they provide a rationale as to why they combined these two excipients.

Labrafac™ MC60 and Labrasol® ALF both contain $C_8$ and $C_{10}$, which interact with bile salts present in fasted-state small intestinal fluid [54]. Above their critical micelle concentration (CMC), these MCFAs form micelles thereby reducing the amount of free monomer that can interact with the lipid bilayer of intestinal epithelial plasma membranes. The CMC values can be shifted in intestinal fluid and the MCFAs can also form mixed micellar structures with bile salts and phospholipids. It has been suggested that this means that the capacity of MCFAs to function as PEs may be reduced in intestinal fluid as the free concentrations are dispersed into micellar structures (Berg et al. 2022, Maher et al. 2023). In Caco-2 monolayer studies, the capacity of n-dodecyl-β-d-maltopyranoside (DDM) to act as PE was also decreased in simulated intestinal fluid due to interactions with bile salts and micelles [55]. A study carried out using coarse-grained molecular dynamics investigated $C_8$, $C_{10}$-, and SNAC predicted that monomers of caprate and caprylate were incorporated into the lipid membrane, whereas for SNAC, its monomers were adsorbed on the surface of the membrane [56]. On the contrary, our study demonstrated that the effects on permeability of Labrafac™ MC60 and Labrasol® ALF were unaffected by simulated intestinal fluid ex vivo, likely due to the relatively high concentrations used. We also showed that % F of insulin delivered inter-jejunally in vivo for both agents was not altered when administered in FaSSIF. This result is similar to one seen

with sodium caprate where in vivo the presence of bile salts had no effect on ability of high concentrations of $C_{10}$ to act as a PE [50].

It is important when comparing PEs to think about the amount of PE that will be needed in the final oral dosage form. Currently there is 300mg SNAC in the Rybelsus® formulation. In humans, studies have been carried out using $C_{10}$ in amounts of up to 500-700mg per tablet [57], but recent human studies showed that 180mg per tablet was efficacious with the macrocycle, MK-0616. In contrast to SNAC and $C_{10}$, Labrafac™ MC60 and Labrasol® ALF are oily liquids and therefore the final dosage form will be expressed as a volume in a capsule. A concentration of 40mg/ml is the equivalent of 40µl of these agents. In a recent Merck Phase 1 trial, 1800 mg of Labrasol® ALF with a macrocyclic peptide PCSK9 inhibitor was used in a capsule [14]. Labrafac™ MC60 is well suited to the delivery of lipophilic drug or drugs that have been made more lipophilic through the use of hydrophobic ion pairing and formulated in SEDDS [58]. Labrafac™ MC60 can be used as an oily solution in which a drug is dissolved in, which is the case in the formulation of dutasteride currently on the market, or as the co-surfactant in microemulsions and as a component in SEDDS. Efforts are also being made to create solid dosage forms of lipid-based excipients using 3D printing [59, 60] and spray drying [61]. Labrasol® ALF also has been used as the delivery vehicle for a nanocomplex containing insulin hydrophobically ion paired with the bile salt, sodium glycodeoxycholate. Intra-jejunal administration of this formulation to rats resulted in a % F of 14 [62]. The data obtained in our study in the intestinal instillation model presents a best-case scenario for PEs, so the question is whether we can relate the data for Labrafac™ MC60 to performance in large animals and humans. By comparison, Labrasol® ALF has still functioned as an efficacious PE in monkeys [13] and humans [14] for an orally administered macrocycle peptide [14]. The data obtained with Labrafac™ MC60 ex vivo and in vivo in the current study is comparable to $C_{10}$ and Labrasol® ALF, so similar trends might be expected for Labrafac™ MC60 in humans.

## 5. Conclusions

Labrafac™ MC60 increased the flux of paracellular markers across excised rat jejunal and colonic tissue mucosae in a reversible and MW dependent manner. Using the in-situ intestinal instillation technique in vivo, the relative bioavailability of insulin in both rat colon and jejunum regions was increased without damaging the tissue. Combining Labrafac™ MC60 with a dispersing and solubilising excipient, HCO-60, further improved its enhancing capacity. Labrafac™ MC60 therefore acts as an efficacious PE and may be considered as a candidate to enhance the oral delivery of macromolecules formulated in capsule dosage forms, particularly incorporating compatible oily solutions and SEDDs.

## Acknowledgements

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## Declaration

DB acts as a consultant to Pharma companies working on oral formulations of peptides that were mentioned in the paper.

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**Supplementary Files**

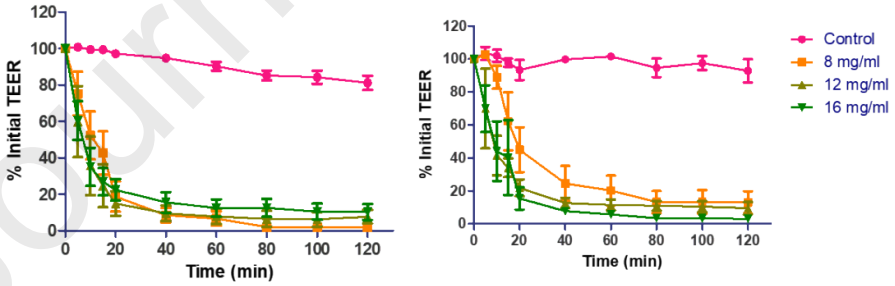



**Figure S1**: TEER reductions across rat colonic mucosae exposed to Labrafac™ MC60 (8, 12, 16 mg/ml), with (a) FD4 (Fig. 6a); (b) (FD10) (Fig. 6b).

**Table S1**. Statistical analysis of TEER values across rat colonic mucosae mounted in Ussing chambers following exposure to Labrafac™ MC60. (Fig 2a). Two-way ANOVA with

Bonferroni's post-test. *P<0.05, **P<0.01 and ***P<0.001 versus KH control. ns = non-significant.

| Time | 2mg/ml Labrafac™ MC60 | 4mg/ml Labrafac™ MC60 | 8mg/ml Labrafac™ MC60 | 12mg/ml Labrafac™ MC60 | 16mg/ml Labrafac ™ MC60 | 8 mg/ml Labrasol® ALF | 2mg/ml $C_{10}$ |
|---|---|---|---|---|---|---|---|
| 0 | ns | ns | ns | ns | ns | ns | ns |
| 5 min | ns | ns | ns | ns | ns | *** | * |
| 10 min | ns | ns | ns | ** | *** | *** | *** |
| 15 min | ns | ns | ns | *** | *** | *** | *** |
| 20 min | ns | ** | *** | *** | *** | *** | *** |
| 40 min | ns | *** | *** | *** | *** | *** | *** |
| 60 min | ns | *** | *** | *** | *** | *** | *** |
| 80 min | ns | *** | *** | *** | *** | *** | *** |
| 100 min | ns | *** | *** | *** | *** | *** | *** |
| 120 min | ns | *** | *** | *** | *** | *** | *** |

**Table S2**. Statistical analysis of TEER across rat colonic mucosae mounted in Ussing chambers after exposure to digested Labrafac™ MC60 (Fig. 4a). Two-way ANOVA with Bonferroni's post-test. **P<0.01 and ***P<0.001 versus KH control. ns = non-significant.

| Time | 8mg/ml Labrafac™ MC60 | 16mg/ml Labrafac™ MC60 |
|---|---|---|
| 0 | ns | ns |
| 5 min | ns | ** |
| 10 min | ** | *** |
| 15 min | *** | *** |
| 20 min | *** | *** |
| 40 min | *** | *** |
| 60 min | *** | *** |
| 80 min | *** | *** |
| 100 min | *** | *** |
| 120 min | *** | *** |

**Table S3**. Statistical analysis of TEER values across rat colonic mucosae mounted in Ussing chambers after exposure to Labrafac™ MC60 in three concentrations before washout at 30 min (Fig.5a). Two-way ANOVA with Bonferroni's post-test. *P<0.05, **P<0.01 and ***P<0.001 versus KH controls. ns = non-significant.

| Time (min) | 8mg/ml Labrafac™ MC60 | 12mg/ml Labrafac™ MC60 | 16mg/ml Labrafac™ MC60 |
|---|---|---|---|
| 0 | ns | ns | ns |
| 5 | ns | ns | ns |
| 10 | ns | ns | ns |
| 15 | * | ** | ns |
| 20 | ** | ** | * |
| 30 | Wash-out | | |
| 40 | * | *** | *** |
| 60 | ns | * | ** |
| 80 | ns | ns | * |
| 100 | ns | ns | ns |
| 120 | ns | ns | ns |

**Table S4**. Statistical analysis of TEER values across rat colonic mucosae after exposure to Labrafac™ MC60 and Labrasol® ALF in different ratios (Fig.8a). Two-way ANOVA with Bonferroni's post-test. *P<0.05, **P<0.01 and ***P<0.001 versus KH controls. ns = non-significant. See Table 4 for specifications of the different treatment groups.

| Time (min) | A | B | C | D | E | F | G | H |
|---|---|---|---|---|---|---|---|---|
| 0 | ns | ns | ns | ns | ns | ns | ns | ns |
| 5 | ns | ns | ns | ns | ns | ns | ns | ** |
| 10 | ns | *** | ** | *** | ns | * | * | *** |
| 15 | ns | *** | ** | *** | ns | * | ** | *** |
| 20 | ns | *** | *** | *** | ns | *** | *** | *** |
| 40 | ns | *** | *** | *** | ns | *** | *** | *** |
| 60 | * | *** | *** | *** | ns | *** | *** | *** |
| 80 | ns | *** | ** | *** | ns | ** | ** | *** |
| 100 | ns | *** | *** | *** | ns | ** | ** | *** |
| 120 | ns | *** | ** | *** | ns | ** | ** | *** |

**Table S5**. Statistical analysis of TEER values across rat jejunal mucosae mounted in Ussing chambers after exposure to Labrafac™ MC60, Labrasol® ALF, and $C_{10}$ (Fig.11a). Two-way ANOVA with Bonferroni's post-test. *P<0.05, **P<0.01 and ***P<0.001 versus KH controls. ns = non-significant.

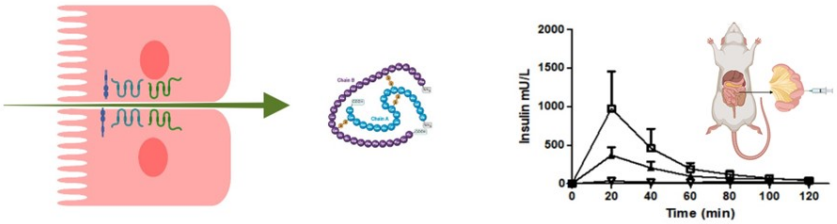
| Time (min) | Labrafac™ (20mg/ml) | Labrasol® (20mg/ml) | Labrafac™ (40mg/ml) | Labrasol® (40mg/ml) | $C_{10}$ (6mg/ml) |
|---|---|---|---|---|---|
| 0 | ns | ns | ns | ns | ns |
| 5 | ns | ns | ns | ns | * |
| 10 | ns | ns | ns | ns | *** |
| 15 | ns | ns | ns | ns | *** |
| 20 | * | ns | ns | ns | *** |
| 40 | *** | ns | ns | ns | *** |
| 60 | ** | ns | ns | ns | *** |
| 80 | *** | ns | * | ns | *** |
| 100 | *** | * | *** | ns | *** |
| 120 | *** | *** | *** | * | *** |

**Table S6.** Statistical analysis of reduction in blood glucose after intra-jejunal instillation of either Labrafac™ MC60 or Labrasol® ALF (both 40mg/ml) with insulin (50 IU/kg) in PBS (Fig. 13a). Two-way ANOVA with Bonferroni's post-test. *P<0.05, **P<0.01 and ***P<0.001 versus free insulin. ns = non-significant.

| Time (min) | Labrafac™ MC60 + insulin in PBS | Labrasol® ALF + insulin in PBS |
|---|---|---|
| 0 | ns | ns |
| 10 | ns | ns |
| 20 | ns | ns |
| 30 | ns | *** |
| 40 | ns | *** |
| 50 | ns | *** |
| 60 | ns | *** |
| 70 | ns | *** |
| 80 | ns | *** |
| 90 | ns | *** |
| 100 | ns | *** |
| 110 | ns | *** |
| 120 | ns | *** |

**Table S7.** Statistical analysis of reduction in blood glucose after intra-jejunal instillation of Labrafac™ MC60 or Labrasol® ALF (both 40mg/ml) with insulin in FaSSIF (Fig. 14a). Two-way ANOVA with Bonferroni's post-test. *P<0.05, **P<0.01 and ***P<0.001 versus insulin controls. ns = non-significant.

| Time (min) | Labrafac™ MC60 + insulin in FaSSIF | Labrasol® ALF + insulin in FaSSIF |
|---|---|---|
| 0 | ns | ns |
| 10 | ns | ns |
| 20 | ns | ** |
| 30 | * | *** |
| 40 | * | *** |
| 50 | * | *** |
| 60 | ns | *** |
| 70 | ** | *** |
| 80 | * | *** |
| 90 | ns | *** |
| 100 | ns | *** |
| 110 | ns | *** |
| 120 | ns | *** |




**Labrafac™ MC60 and Labrasol ALF® act as intestinal permeation enhancers and increase bioavailability of insulin in rats**