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An *ex vivo* intestinal absorption model is more effective than an *in vitro* cell model to characterise absorption of dietary carotenoids following simulated gastrointestinal digestion

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ABSTRACT

To get the most accurate food digestion-related data, and how this affects nutrient absorption, it is critical to carefully simulate human digestion systems using model settings. In this study, the uptake and transepithelial transportation of dietary carotenoids was compared using two different models that have previously been used to assess nutrient availability. The permeability of differentiated Caco-2 cells and murine intestinal tissue were tested using all-*trans*-β-carotene and lutein prepared in artificial mixed micelles and micellar fraction from orange-fleshed sweet potato (OFSP) gastrointestinal digestion. Transepithelial transport and absorption efficiency were then determined using liquid chromatography tandem-mass spectrometry (LCMS-MS). Results showed that the mean uptake for all-*trans*-β-carotene in the mouse mucosal tissue was 60.2 ± 3.2% compared to 36.7 ± 2.6% in the Caco-2 cells with the mixed micelles as the test sample. Similarly, the mean uptake was higher in OFSP with 49.4 ± 4.1% following mouse tissue uptake compared to 28.9 ± 4.3% using Caco-2 cells for the same concentration. In relation to the uptake efficiency, the mean percentage uptake for all-*trans*-β-carotene from artificial mixed micelles was 1.8-fold greater in mouse tissue compared to Caco-2 cells (35.4 ± 1.8% against 19.9 ± 2.6%). Carotenoid uptake reached saturation at 5 μM when assessed with the mouse intestinal cells. These results demonstrate the practicality of employing physiologically relevant models simulating human intestinal absorption processes that compares well with published human *in vivo* data. When used in combination with the Infogest digestion model, the Ussing chamber model, using murine intestinal tissue, may thus be an efficient predictor of carotenoid bioavailability in simulating human postprandial absorption *ex vivo*.

1. Introduction

The absorption of carotenoids by intestinal tissue is a key step in their bioavailability. Unfortunately, evaluation of carotenoid bioavailability has long been hampered by the limited knowledge of their absorption mechanisms as well as by the limitations presented by the experimental approaches involving laboratory animals or humans (Yonekura & Nagao, 2007). While human studies represent the gold standard to investigate bioavailability, challenges associated with food structure, complexity of behaviour during digestion and inter-individual variations in response often lead to misinterpretation of study results (Desmarchelier & Borel, 2017; Faulks & Southon, 2005; Reboul, 2019). Furthermore, human studies are expensive, time consuming, often invasive and have ethical implications (Chacon-Ordonez, Carle, & Schweiggert, 2019). Although no animal model accurately reflects human physiology, previous studies have demonstrated that animals can be essential in elucidating mechanisms by which food components can modulate metabolic pathways and translate information to human models (Carvalho et al., 2018; Chalvon-Demersay, Blachier, Tomé, & Blais, 2017; Schmaelzle et al., 2014). Lately, with the use of *in vitro* cell culture systems and molecular techniques, the mechanisms of carotenoid absorption at cellular level have started to be unveiled (Desmarchelier et al., 2017; Durojaye, Riedl, Curley, & Harrison, 2019; O'Sullivan, Aisling, & Brien, 2009; Reboul, 2019). For instance, delivery of carotenoids across the apical membrane is believed to be facilitated by scavenger receptor class B type I (SR-BI), cluster determinant 36 (CD36), and possibly Niemann-Pick C1-like 1 (NPC1L1) (Kopec & Failla, 2018). What is however not clear, is whether these proteins are indeed

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involved in the direct transfer of carotenoids from the extracellular space to the interior or whether they are components of membrane complexes responsible for internalization of carotenoids (Reboul, 2013). O'Sullivan, Aisling, & Brien, (2009) reported that carotenoids from the same food or meal have been found to interact with each other and at any stage during the absorption, metabolism and transport process. To date, mechanisms of carotenoid interaction during both cellular and tissue uptake systems when administered at equal concentration are yet to be elucidated.

The current widely accepted method using Caco-2 cells (derived from colon adenocarcinoma) to evaluate permeation after intestinal digestion have several limitations, from epithelial cell composition to expression of enzyme and protein transporters (Bohn et al., 2018; Desmarchelier & Borel, 2017; Failla, Rodrigues, & Chitchumroonchokchai, 2019; Kopec & Failla, 2018). To compensate for the shortcomings associated with Caco-2 cell monolayers, the Ussing chamber technique, an *ex vivo* approach using mouse intestinal tissue has successfully been used to evaluate nutrient absorption (Liu, Zhou, & Chen, 2019; Mulet-Cabero et al., 2020). One of the challenges reported for dietary carotenoids is their low and variable bioavailability which may be linked to poor release from the food matrix and limited micellisation, cellular uptake and transportation (Bohn, 2019). Coupled to this, is the problem of effective detection and quantification of carotenoids in both food and biological samples.

The aim of the research reported here was to determine the fate of dietary carotenoids following simulated upper GI digestion of biofortified orange fleshed sweet potato (OFSP) and exposure of the resulting micellar fraction to two different absorption models (differentiated Caco-2 cells and murine intestinal tissue). Experiments included comparison of the absorption efficiency of dietary carotenoids prepared in artificial micelles and at equal concentration. The uptake and *trans*-epithelial transportation of dietary carotenoids from the OFSP micellar fraction and artificial micelles were determined from aliquots obtained from the apical and basolateral chambers of both models and analysed using LCMS-MS.

2. Materials and methods

2.1. Materials

Only chemicals and reagents of analytical and HPLC grade were used. Extraction chemicals sodium chloride, butylated hydroxytoluene (BHT), potassium hydroxide, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Formazan crystals) and crystalline ammonium acetate were obtained from Sigma-Aldrich (Poole, UK) as well as solvents used during extraction such as ethanol, 1,2-dichloroethane, 2-propanol and hexane. Analytical solvents methanol and methyl *tert*-butyl ether (MTBE) were obtained from Fisher Scientific (Loughborough, UK). High purity carotenoid standards (>95%): all-*trans*-β-carotene and β-apo-8′-carotenal were purchased from Sigma-Aldrich; lutein came from Extrasynthese (Lyon, France). All chemicals and products used in the preparation of artificial micelles were purchased from Sigma-Aldrich while sodium taurocholate was obtained from Fisher Scientific. Unless otherwise stated, all cell culture medium components were purchased from Gibco (Life Technologies, Warrington, UK).

2.2. Preparation of artificial micelles

For the preparation of artificial micelles, the original protocol of (Homan & Hamelehle, 1998) was adapted with minor modifications. Briefly, 1-oleoyl-*rac*-glycerol (0.3 mM), oleic acid (0.5 mM), phosphatidylcholine (0.04 mM), lysophosphatidylcholine (0.16 mM) and cholesterol (0.1 mM) were dissolved in chloroform/methanol (2:1, v/v) and carotenoids, all-*trans*-β-carotene and lutein (0–20 μM for each) dissolved in hexane placed in 15 mL glass tubes and the solvent mixture evaporated to dryness under a flow of nitrogen gas. The dried residue was then reconstituted in Dulbecco's modified eagle medium (DMEM) containing 5 mM sodium taurocholate and incubated at 37 °C for 30 min. The solution was then placed in a sonicating bath (Deco – FS100, UK) for 30 min followed by incubation at 37 °C for 1 h to ensure complete solubilisation. After this incubation period, the solution was filtered through 0.2 μm pore size cellulose filters (Whatman®, Spartan). Aliquots of the resulting solutions were flushed with nitrogen gas and stored at −20 °C to be used within 5 days.

2.3. In vitro digestion

The simulated *in vitro* digestion of test foods in the present experiment was carried out according to a combination of protocols as proposed by the INFOGEST network and the recently developed semi-dynamic digestion models (Minekus et al., 2014; Mulet-Cabero et al., 2020) with adaptations for carotenoid analysis (Rodrigues, Mariutti, & Mercadante, 2016). Briefly, using the static *in vitro* digestion model, 5 g of the biofortified test food (OFSP) was minced in the electric mincer and mixed with 3.5 mL of SSF, 0.5 mL of α-amylase prepared in SSF to achieve a final concentration of 75 U/mL, and $CaCl_2$ at a final concentration of 0.3 mM in a 50 mL brown and low light permitting centrifuge tube. Water (0.975 μL) was added to achieve a 50:50 (v/v) final ratio of food to SSF. The mixture was then incubated at 37 °C for 2 min to simulate oral transit time. A blank test tube was included in the experiment that had water in place of the test food but contained all the other fluids and enzyme. The test food (10 mL) from the oral phase was then exposed to the gastric phase where the pH was adjusted to 3 using 1 M HCl and 10 mL of previously warmed (at 37 °C) SGF containing porcine pepsin and $CaCl_2$ in final concentrations of 2000 U/mL and 0.75 mM respectively. The sample was then incubated at 37 °C in a shaking incubator set at 100 rpm for 2 h. Prior to this, a test experiment was conducted to determine the amount of acid required for use during digestion. Upon completion of the incubation period of 2 h at 37 °C, the gastric sample-chyme was delivered to the intestinal phase and mixed with pancreatic suspension from porcine pancreas (assessed as trypsin activity), $CaCl_2$ and bile salts prepared in SIF to achieve final concentrations of 100 U/mL, 0.3 mM and 10 mM in the final mixture. The concentration of bile acid from the extract was ascertained using an enzyme assay (bile acid kit, ref 1 2212 9990 313, DiaSys Diagnostic System GmbH, Germany) and using the manufacter's protocol. The pH of the final mixture was adjusted to 7 in order to neutralise the mixture and addition of water to dilute the SIF stock and also achieve 50:50 ratio of the gastric sample-chyme to SIF. Incubation for the intestinal digestion was set for 2 h at 37 °C on the shaking incubator at 100 rpm.

After intestinal digestion, the digesta was placed on ice and centrifuged (Beckman Coulter, UK) at 35,000 × *g* at 4 °C for 68 min. The centrifugation process allowed a 3-phase separation composed of the oil droplets and other suspensions on top, followed by the aqueous micellar fraction on the middle and the residual solids and sediments forming a pellet at the bottom of the centrifuge tube. Separation of the micellar fraction containing the bioaccessible carotenoids from the non-bioaccessible aqueous fraction was achieved using 13 mm cellulose regenerated syringe filters of 0.22 μM pore size (Whatman®, Spartan, UK). An aliquot of the filtered aqueous phase containing mixed micelles was transferred to a foil-covered 15 mL glass test tube, flushed with nitrogen gas and stored at −80 °C until further analysis.

2.4. Cell culture

2.4.1. Caco-2 cell line and experiments

Human colon adenocarcinoma Caco-2 cells were purchased from the European Collection of Animal Cell Cultures (Salisbury, UK). These were routinely maintained in 75 cm^2 plastic flasks (Corning®, Corning Inc., UK) in a CO_2 incubator (Sanyo, UK) at 37 °C and 5% CO_2. The cells were grown in DMEM (Gibco, UK) containing 4.5 g/L glucose. The medium

was supplemented with 10% fetal bovine serum (FBS) (Gibco), 1% non-essential amino acid solution (NEAA, Gibco) and 1% penicillin–streptomycin (Sigma Aldrich). Cells were passaged upon reaching 80% confluence. Medium was aspirated and the cells washed with phosphate buffered saline (PBS) before adding trypsin. After incubation for 5–10 min when the cells were detached, full medium was added to stop enzyme activity. A cell counter (Cyto, Corning, UK) was used to count viable cells.

2.4.2. Cellular uptake experiments

Cells at passages 15–20 were seeded on a flat-bottomed 6-well plate (Corning®) at a density of 5.0×10^4 cells/cm^2. To differentiate, they were continuously grown for 21 days and the culture medium changed every second day. On the day of the experiment, the apical side received 2 mL of serum-free medium (DMEM with 1% NEAA) and cells treated with carotenoids from either the micellar fraction from digesta or artificial micelles while the basolateral side received 2 mL of serum-free medium alone (Juan, Montesano, Mañes, & Juan-García, 2022). Micelles generated from the simulated digestion were diluted in DMEM in the ratio 1:2 to avoid the toxic action of bile salts on the cells. Cells treated with micelles containing carotenoids were then incubated for 4 h at 37 °C and 5% CO_2. After the incubation period, the plates were placed on ice and apical medium removed with the glass Pasteur pipette (Sigma-Aldrich, UK) connected to an automatic aspirator (Grant Bio, UK). Cells were then washed with 1 mL DPBS to remove the buffer, safely discarded before scraping them in the presence of 0.5 mL ice-cold DPBS. Carotenoid uptake was calculated as the quantity of carotenoid present in the harvested cells divided by the sum of the quantity of carotenoid remaining in the apical chamber and that present in the harvested cells expressed as a percentage. Similarly, cellular absorption was calculated as a ratio of carotenoids accumulated in the cells and those secreted to the basolateral section against what was initially present, expressed as a percentage (Rošul et al., 2022).

2.4.3. Cell integrity measurements

The integrity of the Caco-2 cell monolayer grown on the semi-permeable membrane of the transwell plate was evaluated using the Trans-epithelial Electrical Resistance (TEER) (Millicell®ERS-2, UK) voltohmmeter. TEER, a non-destructive method, is a widely accepted quantitative technique used to measure the tight junction dynamics in cell culture models for epithelial monolayers (Srinivasan et al., 2015). TEER values above 100 Ω.cm^2 following subtraction of the background electrical resistance on differentiated Caco-2 cell monolayers was considered satisfactory.

2.4.4. Cell viability assay

Cell viability was determined using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay, based on the protocol by Wang et al. (2022) with some minor modifications. The Caco-2 cells were seeded at 5.0×10^4 cells/cm^2 on a 48-well plate (Corning®, UK) and incubated at 37 °C, 5% CO_2 for 48 h to allow cells to adhere to the plate and reach 90% confluence. The concentration of carotenoids were determined spectrophotometrically using solvent specific extinction coefficients (Rodriguez-Amaya & Kimura, 2004) and dried down under nitrogen gas. The dried carotenoid residual, at concentrations between 0 and 20 μM and bile salts diluted at final concentration range of between 0 and 10 mM was reconstituted with cell culture medium containing 1% Dimethyl sulfoxide (DMSO). The control cells contained DMEM medium only. At the end of the incubation period, the medium was discarded, and the cells carefully washed with Dulbecco's phosphate buffer saline (DPBS). The medium was replaced with 100 μL MTT working solution (0.5 mg/mL) and the plate incubated in the dark for 45 min and 1 mL DMSO was added into each well and the plate shaken for 10 min at 35 rpm using a horizontal mini orbital shaker (Stuart, UK) to dissolve the formazan crystals. In triplicate, 200 μL of the solubilised formazan solution was transferred into a 96-well plate and cell viability measured at 570 nm and background subtraction using 620 nm reading via Tecan Spark 10 M plate reader.

2.4.5. Permeability experiments

To assess the cell permeability of carotenoids, Caco-2 cells were grown on semi-permeable membrane inserts (Transwell® 0.4 μm, polyester) at a density of 5.0×10^4 cells/cm^2 on 6-well plates. Permeability was then determined in differentiated cells after 21 days of incubation. At the beginning of each experiment, the apical side of the cell monolayer received 2 mL of either artificial micelles or micelles obtained from orange fleshed sweet potato digesta after GI digestion while the basolateral chamber received a similar volume of serum-free medium. Cell monolayers on the transwell plates were then incubated at 37 °C, 5% CO_2, for 16 h. This is the period during which chylomicron production is expected to be complete, while allowing maximum absorption and measurement of carotenoids in the basolateral chamber (Reboul et al., 2005). After the incubation period, medium was collected from both the apical and basolateral chambers for analysis, together with the cells on the transwell inserts that were harvested by scraping. The resulting extract was then stored at −80 °C until LCMS analysis.

2.5. Tissue system

2.5.1. Ussing chamber experiment

To investigate the absorption kinetics of carotenoids by *ex vivo* small intestinal tissue, the Ussing chamber methodology was used, consisting of two half chambers with fresh intestinal tissue sample mounted between. The method as described previously (Mackie et al., 2019), involved the collection of the whole length of the digestive tract from 6 to 8 weeks C57BL/6 mice. The collected intestine was flushed through with ice-cold 10 mmol/L glucose solution. To avoid the lymphoid follicles, the most distal part (1 cm) was discarded and a section of duodenum taken and cut along the mesenteric attachment. The cut section was washed with 10 mmol/L glucose and the serosal and muscularis layers stripped off using fine forceps. The tissue segment was then mounted on the slider (P2404, Physiologic instruments, San Diego, USA), and inserted in an Ussing chamber system (EM-CSYS-4 with low volume P2400 chamber), separating the chamber into the apical and the basolateral compartments leaving the exposed tissue area of 0.25 cm^2. The apical compartment was filled with 1 mL Ringer's solution containing 10 mmol/L mannitol to maintain osmotic balance while the basolateral compartment was filled with a similar volume of Ringer's solution containing 10 mmol/L glucose. The buffers in both compartments were continuously bubbled with Carbogen (95% O2 and 5% CO2) and maintained at 37 °C.

2.5.2. Tissue viability measurements

After stripping and mounting, mouse tissue was then equilibrated for 30 min to achieve steady state conditions in transepithelial potential difference (PD). This open-circuit transepithelial potential difference was monitored on a continuous basis using a DVC-1000 multichannel voltage clamp unit (World Precision Instruments, New Haven, USA) with Ag-AgCl electrodes and 150 mmol/L NaCl salt bridges. At 20-minute intervals, a short circuit current (Isc) was applied to zero the PD with recordings collected using Spike2 v8.08 software (Cambridge Electronic Design, Cambridge, UK) and used to calculate transmucosal resistance using Ohm's law. The calculated resistance serves as a measure of tissue integrity throughout the experimental period. During the initial equilibration period, transepithelial resistance was assessed by measuring voltage changes in response to micro-amp pulses lasting a few seconds, applied every 2 min.

2.5.3. Tissue permeability studies

When electrical parameters stabilized, buffers from both compartments were removed and Ringer's solution in the apical compartment was replaced with 500 μL Ringer's + 500 μL of the micellar phase from

digesta as described in section 2.3 whose final volume contained 10 mmol/L mannitol. Similarly, the buffer in the basolateral compartment was replaced with 500 μL Ringer's + 500 μL simulated intestinal fluid salt solution with the final volume containing 10 mmol/L glucose. During the course of digestion, a 200 μL aliquot was collected from both apical and basolateral compartments at 0, 30, 60, 90 and 120 min and the same volume replaced with fresh and previously warmed (37 °C) Ringer's solution containing 10 mmol/L mannitol and glucose respectively. Upon completion of the experiment, transepithelial conductance was checked and 10 μM forskolin was added to the compartments to measure tissue viability.

2.6. Extraction of carotenoids

Extraction of carotenoids from scraped cells, apical and basolateral solutions followed the procedure described by Reboul et al. (2005) with modifications. To the glass tube containing scrapped cells or sample solution, 3 mL of ethanol containing 0.1% (v/v) butylated hydroxytoulene (BHT) was added to denature the cells and 100 μL of β-apo-8′-carotenal (abs = 0.204) added as an internal standard before closing the glass tube and vortexing for 30 s. Six mL of hexane was added to the tube and it was vortexed for 30 s. This was followed by centrifugation (Eppendorf, UK) at 400 *g* for 5 min at 4 °C. The hexane (upper layer) containing partitioned carotenoids was removed with a clean glass Pasteur pipette. Exhaustive extraction was completed by repeating the extraction with 6 mL of hexane twice before combining the extracts and drying under a steady flow of nitrogen gas. The dried extract was then stored at −80 °C until LCMS analysis.

2.7. LCMS-MS analysis of carotenoids

Previously extracted and dried carotenoids were reconstituted in methanol:MTBE (60:40, v/v) mobile phase. For every batch of samples run, 1 μL sample volume of different concentrations of each carotenoid was injected into the ACQUITY UPLC I-Class PLUS system (Waters, Wilmslow, UK) LCMS-MS system interfaced with the Xevo TQ-XS tandem mass spectrometer for mass detection. Data acquisition and processing used MassLynx software version 4.1. Optimised MS conditions were as follows: Electrospray Ionisation (ESI) performed in the positive ionisation mode, Capillary voltage 1.73 Kv, cone voltage: 41.27 V, source temperature: 150 °C, desolvation temperature: 300 °C, cone gas flow: 1 L/hr, desolvation gas flow: 798L/hr, collision gas flow on.

For ESI+, high purity nitrogen was used as the nebulization and desolvation gas at flow rates of 50 and 300 L/hr respectively. Optimal ESI + -MS in the selected ion mode (SIM) and full scan (*m/z* 200–650) modes, as well as ESI + -MS-MS in the multiple reaction monitoring mode (MRM) mode, were assessed and compared for the quantification of carotenoids. Chromatographic separation of carotenoids was achieved using the ACQUITY UPLC BEH 130 Å C18 column (1.7 μm, 2.1 × 150 mm, Waters, Wilmslow, UK) at a flow rate of 0.360 mL/min and column temperature set at 30 °C following the protocol by Li, Tyndale, Heath, and Letcher (2005) with modifications. Reverse phase elution of carotenoids was performed using a gradient method with mobile phases of 95% methanol containing a mixture of 0.01 M Ammonium acetate and 0.1% v/v formic acid (A) and *tert*-butyl-methyl ether (MTBE) (B) with a column temperature set at 30 °C. The following was the gradient elution programme: An isocratic mixture of methanol (87%) and MTBE (13%) was maintained from time 0 to 12 min, followed by a linear gradient until 25 min at which time the mobile phase composition was methanol (63%) and MTBE (37%). From 25 to 35 min, MTBE increased to 63% and was maintained for 1 min before returning to initial concentrations. After each sample run, the LCMS system was flushed with 50% methanol for lines that used 95% methanol containing ammonium acetate while lines using a stronger mobile phase (MTBE) were flushed with 100% methanol to remove strongly retaining residuals. Carotenoids were identified according to their relative retention time and accurate mass (*m/z* 568.43 for lutein, *m/z* 536.44 for β-carotene and *m/z* 552 for β-cryptoxanthin for precursor ions and corresponding product ions at *m/z* 463 and 551 for lutein, *m/z* 460 for β-cryptoxanthin and *m/z* 536 for β-carotene). Calibration curves of pure carotenoid standards were constructed for carotenoid quantification, results normalised per surface area of the tissue and expressed in μg/g.

2.8. Statistical analysis

All experimental results are presented as means of triplicates ± standard deviations. Normality of residuals was assessed by Shapiro-Wilk's test. For data with normally distributed residuals, outcomes of interest were evaluated using 1- or 2-way analysis of variance (ANOVA) followed by multiple comparisons with Tukey's test. Significance was set at $P < 0.05$. All statistical analyses were performed using GraphPad Prism version 8.0.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com.

3. Results

3.1. Tissue and cell viability evaluation

Permeability of the tissue to Lucifer Yellow (LY) was found to be < 3 %. Cell viability was determined after exposure to different concentrations of bile acids. Cell viability was 87 ± 6.5 % after 24 h exposure to 1.25 mM and 10.7 ± 4.3% after 10.0 mM bile acid. These results (Fig. 1) indicate that bile acid concentration at 1.25 mM did not cause significant damage to the cells within the incubation period and thus did not adversely affect cell viability (Fig. S1 and S2).

3.2. Identification and quantification of carotenoids

The isolation, identification and quantification of carotenoids was conducted using LCMS-MS with ESI in the positive ionisation mode following a procedure described elsewhere (Li, Tyndale, Heath, & Letcher, 2005) with modifications. Given that carotenoids lack keto-groups such as oxygen to which protons might attach (Lacker, Strohschein, & Albert, 1999; van Breemen, 1995), the current method involved the use of the LCMS-MS with ESI^+ with the modification of the mobile phase to include the addition of 0.1% formic acid in methanol in order to induce solution-phase oxidation as described elsewhere (Cortés-Herrera, Chacón, Artavia, & Granados-Chinchilla, 2019). Under these conditions, β-carotene yielded molecular ion $[M]^{\cdot+}$ at *m/z* 536.8; lutein at *m/z* 568.4 and β-cryptoxanthin at *m/z* 552.4 (Fig. S1, Table S1).

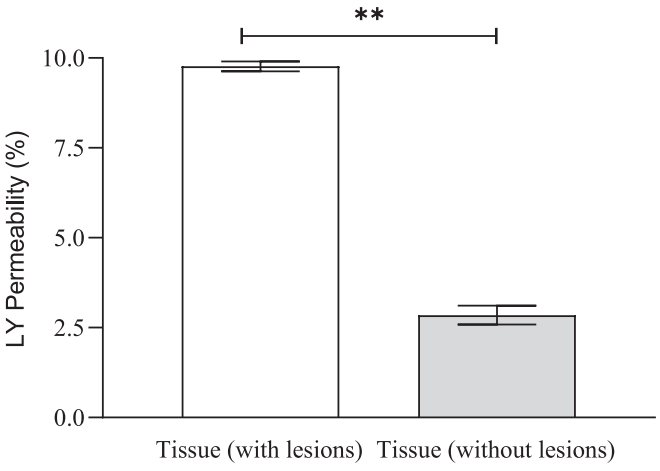

Fig. 1. Permeability of Lucifer Yellow (LY) through mouse intestinal tissue (□ damaged tissue with lesions) and (■ intact tissue without lesions). Values are means ± SD (n = 3).

3.3. Carotenoid uptake by Caco-2 cells

The mean cellular content of β-carotene estimated as a proportion of total carotenoids added was 36.7 ± 2.5% (3.67 ± 0.09 μM) from artificial micelles and 7.2 ± 0.8% (0.72 ± 0.01 μM) from the micellar fraction from OFSP. The pattern was similar for lutein which had 19.9 ± 2.5% (1.99 ± 0.05 μM) from artificial micelles compared to 3.7 ± 0.6% (0.37 ± 0.02 μM) from the OFSP micellar fraction. In artificial micelles, the cellular uptake of β-carotene exceeded that of lutein (Fig. 2A). In the second part of the experiment, carotenoids prepared in artificial micelles were used to investigate their transportation upon incubation with Caco-2 monolayers for 16 h, simulating the 16–18 hrs it takes for complete assembly of chylomicrons in the endoplasmic reticulum, eventually obtained as triglyceride-rich fraction (Failla, Chitchumronchokchai, Ferruzzi, Goltz, & Campbell, 2014). Surprisingly, no carotenoids were detected in the basolateral chamber (Fig. 2B). However, using carotenoids prepared in artificial micelles, there was a significant accumulation of β-carotene representing 44.5 ± 1.6% of the original concentration compared to the amount of lutein accumulated 24.2 ± 1.8% ($P < 0.05$) in the cells. Furthermore, when micelles generated from OFSP chyme were used to investigate carotenoid transport, very low concentrations of carotenoids were detected in the cells and again none was detected in the basolateral chamber (Fig. 2C). After 16 h incubation, the distribution of carotenoids in the cells were 9.8 ± 0.5% for β-carotene and 5.1 ± 0.6% for lutein. Despite the longer incubation time, the apical chamber still had high concentrations of carotenoids with β-carotene having 82.7 ± 2.9% while lutein had 79.4 ± 5.2% of the initial concentration. It is not clear whether the remaining ~ 17% of the carotenoids had degraded or had been adsorbed to the walls of the transwell plates.

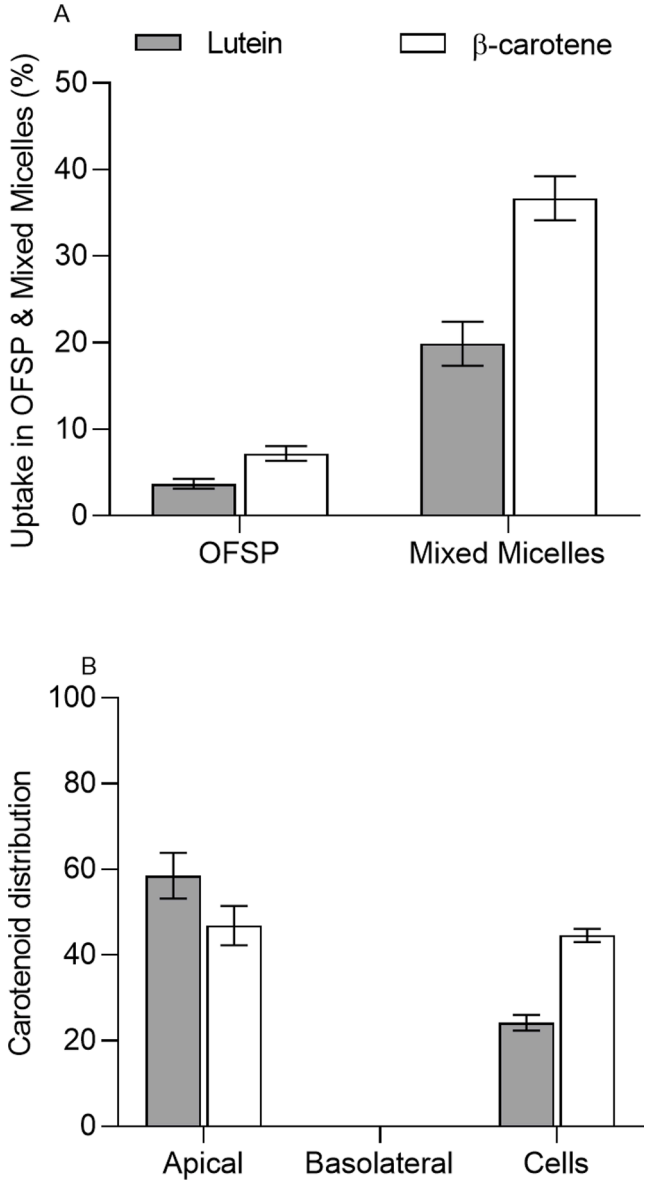

Fig. 2. Uptake and distribution of carotenoids in OFSP and artificial micelles after different incubation times. Results show total uptake of (A) carotenoids in OFSP and artificial micelles after 4 h of incubation, (B) distribution of carotenoids in artificial micelles across differentiated Caco-2 cell monolayers following incubation of 16 h. Data for carotenoids in OFSP and mixed micelles are means ± SD (n = 3) of triplicate assays from two independent treatments.

3.4. Mouse tissue permeability studies

In order to determine the fate of dietary carotenoids once exposed to the intestinal mucosa, Ussing chambers containing murine intestinal mucosa had the apical chamber filled with a two-fold dilution of OFSP sample following *in vitro* digestion (Fig. 3). Transportation and absorption of carotenoids was assessed by measuring the concentration of carotenoids in both the apical and basolateral chambers as a function of

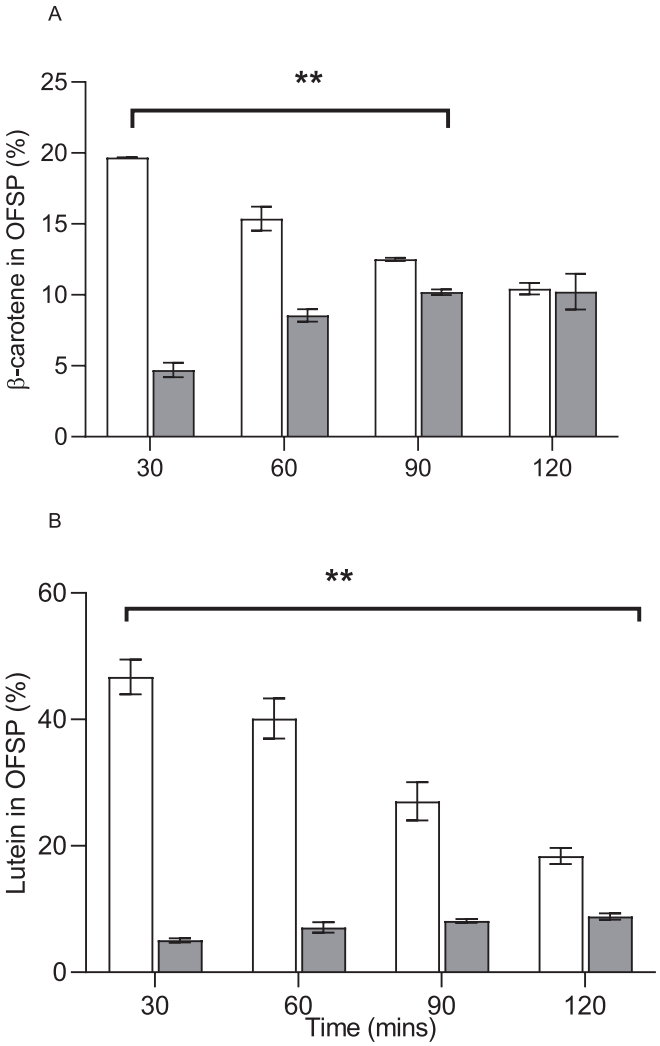

Fig. 3. Carotenoid absorption determined by concentration change from the apical (□) to the basolateral (■) chamber in (A) β-carotene and (B) lutein in OFSP after exposure to murine intestinal mucosa. Data are expressed as percentage change from initial concentration. $N = 3$ (**$P < 0.05$).

time. The carotenoids in the apical chamber represent content that was digested and therefore became accessible whereas the carotenoids in the basolateral side represents content that had been absorbed and transported across the intestinal wall and were available for physiological functions and storage. Results in Fig. 3 show that a progressive increase in the concentration of both lutein and β-carotene in the basolateral chamber over 90 min when it stabilised until the end of the 2 hrs incubation period. However, the rate and extent of absorption were significantly different between the two carotenoids ($P < 0.05$). For instance, after 30 mins incubation, 10.8% of lutein and 23.9% of β-carotene had translocated to the basolateral chamber. The lutein concentration in the basolateral chamber increased to 15.2% after 60 mins incubation and 17.4% after 90 mins. In contrast, β-carotene increased 2-fold after 60 mins. Similar observations were made with β-carotene where an increase of 51.8% was recorded after 90 mins with no further increases at the end of the incubation period.

3.5. Model-based uptake efficiency

The mean uptake for all-*trans*-β-carotene in the mouse mucosal tissue was 60.2 ± 3.2% compared to 36.7 ± 2.6% in the Caco-2 cells with the mixed micelles as the test sample (Fig. 4). Similarly, the mean uptake was higher in OFSP with 49.4 ± 4.1% following mouse tissue uptake compared to 28.9 ± 4.3% using Caco-2 cells for the same concentration. In relation to the model uptake efficiency, the mean percentage uptake for β-carotene in the mixed micelles was 1.8-fold greater with the mouse tissue than that obtained with the use of Caco-2 cells (35.4 ± 1.8% against 19.9 ± 2.6%). The trend was similar with OFSP as mean values obtained indicated 31.3 ± 1.5% with the mouse mucosal tissue compared to 15.6 ± 2.0% from the Caco-2 cells. Our results further indicate that the uptake of carotenoids in both cases was higher when assessed with the tissue system compared to the Caco-2 cells. For this experiment uptake was used to measures absorption of test compounds while uptake efficiency was meant to assess absorption performance in the two different models.

4. Discussion

The primary objective of this study was to determine the fate of dietary carotenoids following simulated upper GI digestion of OFSP and exposure of the resulting micellar fraction to differentiated Caco-2 cells and murine intestinal tissue. The second objective was to compare absorption efficiencies of dietary carotenoids prepared in artificial micelles. In the present study, carotenoids prepared in artificial micelles and those generated from the OFSP micellar fraction were successfully delivered to both differentiated Caco-2 cell monolayers and mouse intestinal tissue. *In vitro* digestion models have traditionally been used to study the bioaccessibility and thus predict bioavailability of nutrients. However, for these models to produce reliable data, their applicability in simulating real physiological processes during intestinal digestion needs proper assessment if the prediction of bioavailability under *in vitro* conditions are to remain relevant. Results of such tests are important in giving credence to the validity and interpretation of obtained data (Nejdfors, Ekelund, Jeppsson, & Westrom, 2000; Sjögren, Eriksson, Vedin, Breitholtz, & Hilgendorf, 2016).

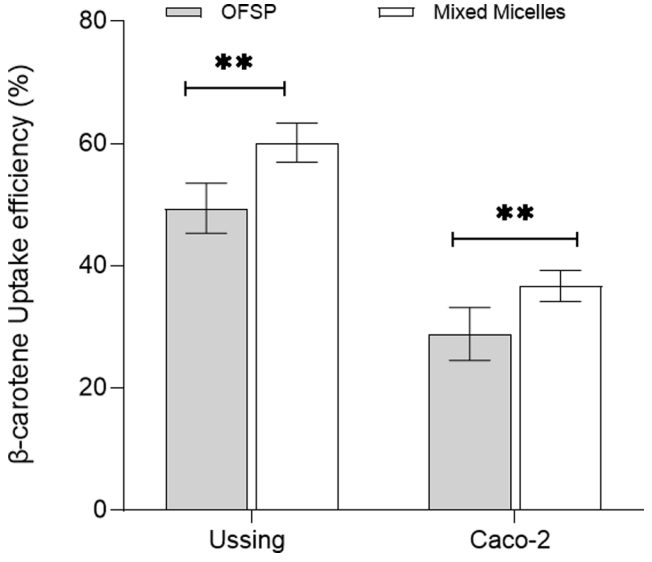

Fig. 4. Transport efficiency of β-carotene from artificial micelles and OFSP digesta on mouse intestinal tissue (□) and differentiated Caco-2 cells (■). Results are presented as mean ± SD ($n = 3$).

4.1. Uptake and transport of carotenoids in Caco-2 cells

The use of Caco-2 cell monolayers in simulating intestinal absorption is well established and has previously been used to study the uptake and secretion of dietary carotenoids (Desmarchelier & Borel, 2017; Durojaye et al., 2019; Garrett, Failla, Sarama, & Craft, 1999; O'Sullivan, Ryan, & O'Brien, 2007; Reboul et al., 2005). In the present study, concentrations of carotenoids added to the cells were similar to those that have been used elsewhere (1–10 μM) to assess their cellular uptake (During, Hussain, Morel, & Harrison, 2002; Reboul, 2013). Results obtained in the present study showed that cellular uptake for all-*trans*-β-carotene was greater than that of lutein in both OFSP and artificial micelles in the uptake and transport models. For instance, incubation of Caco-2 cells with a combination of all-*trans*-β-carotene and lutein prepared in artificial micelles resulted in cellular uptake of 3.67 μM and 1.99 μM, representing a percentage uptake of 36.7% for β-carotene and 19.9% for lutein, respectively. Although current cellular uptake results were relatively high compared to previously published data (O'Sullivan et al., 2007; Rošul et al., 2022), the preferential uptake of β-carotene at the expense of lutein was similar to previous findings. O'Sullivan, Aisling, & Brien, (2009)) reported that supplementation of Caco-2 cells with 1 μM β-carotene or lutein under similar conditions to those presented in the current study resulted in cellular uptake of 12% β-carotene and 8% lutein. Similar findings have been reported by Sugawara et al. (2001) who found that the cellular uptake of β-carotene was higher than that of lutein from micelles containing 1 mM carotenoids. The preferential uptake of β-carotene contrasts with previous studies that demonstrated preferential uptake of lutein at the expense of β-carotene (Yao et al., 2019).

The probable explanation for the higher intracellular β-carotene levels could be due to its stability when formed as an emulsion compared with the stability of lutein. In support of this argument, Borel et al. (1996) found that the emulsified form of β-carotene was indeed more stable compared to more polar carotenoids resulting in more efficient uptake. According to Reboul (2019), uptake efficiency correlates with carotenoid polarity as well as structural flexibility. The greater affinity that polar flexible carotenoids exhibit for lipid transporters may help explain this observation. Interestingly, the present study observed that carotenoids in artificial micelles were more absorbable than those from the micelles generated by digesting OFSP. These findings are supported by previous works (Chitchumroonchokchai, Schwartz, & Failla, 2004; Dhuique-Mayer et al., 2007; Sugawara et al., 2001) suggesting that the composition of artificial micelles such as differences in particle size and surface charge as well as competition from other lipids *in vitro* digestion may play a critical role in their interaction with the brush border surfaces of the enterocytes and therefore, absorption. This finding is supported by a human study whose aim was to assess the differences in absorption performance between β-carotene and lutein. In this human study, serum responses of single doses of β-carotene and lutein, both alone and as equimolar mixture, Kostic, White, & Olson (1995) found β-carotene significantly reduced the serum responses for lutein to

53–61% of control values when taken as a combined mixture (Kostic, White, & Olson, 1995; Liu, Glahn, & Liu, 2004). This therefore suggests an interactive effect that carotenoids have on each other and possibly explains the competition that exists during uptake, absorption and subsequent metabolism. Furthermore, the preferential uptake of carotenoids could be due to the matrix effect, taking into account localization of carotenoids in the food matrix as organelles in which carotenoids are located may determine the extent of carotenoid liberation and solubility. Failla et al. (2019) suggested that carotenoids in the food chyme may not solubilise to the same extent compared to pure compounds during digestion resulting in decreased bioavailability of the former. This could be explained by the difference in the food matrix and the amount of lipid present in the chyme. Regarding simulations of carotenoid secretion into the lymphatic system, the amount of carotenoids transported to the basolateral chamber correlates linearly with the amount absorbed by the Caco-2 cell monolayers regardless of the initial compound concentration at the apical side (During et al., 2002). Until recently, the long-held view regarding carotenoid absorption has been that of occurring through passive diffusion (Hollander & Ruble, 1978). However, research in the last decade has seen developments supporting the theory that several distinct proteins are involved in facilitating transfer of carotenoids present in the micelle into enterocytes after digestion. These proteins include scavenger receptor class B type 1 (SR-B1), NPCI-like intracellular cholesterol transporter (NPCILI), the liver fatty acid binding protein (L-FABP) and cluster of differentiation 36 (CD36) (Bohn et al., 2019; Desmarchelier et al., 2017; During & Harrison, 2004; Failla et al., 2019; Reboul, 2013; Reboul, 2019) which are known to be highly expressed at the apical side of the differentiated Caco-2 cell monolayers (O'Sullivan, Aisling, & Brien, 2009; Reboul et al., 2005). The concentration of 10 μM used in the current study for preparation of artificial micelles was well within the acceptable physiological concentrations as previous studies have reported values of around 7 μM in human duodenal lumen for lutein (Reboul et al., 2005; Tyssandier et al., 2003) whereas an instantaneous absorption of a 5 mg dose of β-carotene, a typical amount of β-carotene found in a carotenoid-rich diet, would therefore result in an approximate intestinal concentration of 25 μM (Borel et al., 1998; Novotny et al., 2010). In examining transport mechanisms across differentiated Caco-2 cells, carotenoid enriched micelles from both artificial and digested OFSP preparations were incubated for 16 h and their secretion into the basolateral chambers assessed. The present study was unable to detect basolateral secretion of carotenoids from either micelle preparations. This is in agreement with a previous study (Dhuique-Mayer et al., 2007) where, despite using similar methods with the present study except for differences in the membrane pores in the transwell plates, no compounds could be detected in the basolateral chamber. In contrast, some other reports have indicated carotenoid presence in the basolateral compartment of the transwell system (Aherne, Daly, Jiwan, O'Sullivan, & O'Brien, 2010; Chitchumroonchokchai, Schwartz, & Failla, 2004; During, Hussain, Morel, & Harrison, 2002; Kostic, White, & Olson, 1995; O'Sullivan, Ryan, & O'Brien, 2007; Reboul et al., 2005). Discrepancies in these findings may be related to differences in membrane pore sizes on the transwell plates and the use of taurocholate and oleic acid for the stimulation and secretion of chylomicrons into the basolateral chamber in some studies (Aherne et al., 2010; Chitchumroonchokchai et al., 2004; O'Sullivan et al., 2007). During et al. (2002) and Reboul et al. (2005) used membrane pore sizes of 3 μm and 1 μm respectively. The rationale for the use of membranes with large surface pore sizes in these studies is not entirely clear but the basis for using the 0.4 μm in the present study stems from previous studies demonstrating that transwell membranes with larger pore sizes tend to allow the migration of cells from the apical side to the basolateral side (Seeballuck, Ashford, & O'Driscoll, 2003; Seeballuck, Lawless, Ashford, & O'Driscoll, 2004). Our method, although similar in design to Reboul et al. (2005), did not add chylomicron stimulating compounds as the aim was to reproduce physiochemical conditions in the intestinal lumen involving mixed lipid micelles containing bile salts, fatty acids, monoglycerides and phospholipids. Carotenoids being lipophilic in nature, follow the fate of lipid digestion, particularly during the process of emulsification with bile salts from the gall bladder and pancreatin from the pancreas, presumably with the correct physiological concentrations, to produce a micellar fraction which goes on to be absorbed across the epithelial cell membrane. It is therefore surprising to observe that differentiated Caco-2 cells in the indicated studies were supplemented further with taurocholate and oleic acid to enhance chylomicron secretion in contrast to what would adequately mimic the full scope and physiology of human digestion and absorption.

4.2. Transport of carotenoids across murine intestines

The extent to which humans would derive maximum health and nutritional benefits from dietary carotenoids and their intakes is dependent on their bioavailability. While experiments involving human subjects constitute the gold standard, and their results provide the best scientific evidence of the bioavailability or bioactivity of a particular compound, there are limitations related to the experimental design, ethical considerations, high costs in conducting the study and challenges in interpretation of results (Cilla, Bosch, Barberá, & Alegría, 2018). Furthermore, it has been demonstrated that determination of bioactive compounds in foodstuff is insufficient for the prediction of potential *in vivo* effects, as metabolites reaching the circulation may be different from the original compounds found in the food, as a result of intensive metabolism that takes place during absorption (Carbonell-Capella, Buniowska, Barba, Esteve, & Frígola, 2014). The permeability of carotenoids was determined using mouse intestinal mucosa with the Ussing chamber that facilitates more accurate prediction of percentage absorption *in vivo* (Mackie, Mulet-Cabero, & Torcello-Gómez, 2020). For the present study, the initial hypothesis of carotenoid bioaccessibility was tested using the static digestion model before extending the assay to the semi-dynamic digestion model owing to the complexity of the test sample. Insights gained from the semi-dynamic digestion model were then fed back into the design of physiologically relevant screening methods to assess carotenoid permeability. During tissue uptake using the Ussing chamber, a linear increase in the absorption of both carotenoids was observed for 90 min before recording a plateau in the absorption curve. These results suggest that the carotenoids reached a saturation point with 51.8% of all-*trans*-β-carotene and 17.4% of lutein from the initial concentration being absorbed and transported across the intestinal wall. This is in agreement with previous human studies where plasma response after ingestion of nutritional doses of β-carotene and lutein have been reported to respond linearly before the onset of carotenoid transport saturation (During et al., 2002; Garrett et al., 1999). More recently, and although working on different compounds from test samples used in the present study, Mulet-Cabero et al. (2020) observed lower concentrations of some amino acids in the basolateral chamber when compared to the apical section after incubation and attributed this to the reduced area of the mouse tissue in the Ussing chamber that could have led to the saturation in the tissue and therefore hampering amino acid transport.

In the present study, the tissue uptake for lutein was particularly low. Similar results were obtained in an experiment by Reboul et al. (2005) where the lutein uptake rate was found to be saturable under physiological conditions (37 °C, 30 min) described by a hyperbolic equation. Using the Michaelis-Menten equation, the authors found K value (concentration required to reach half of the maximum rate of absorption) to be 2.78 μM, reaching saturation at approximately 5.56 μM for lutein. This value is in agreement with previous findings where measured lutein concentration in the human duodenal lumen was around 5–7 μM after a spinach-rich meal (Tyssandier et al., 2003). A human study that investigated intestinal absorption of β-carotene, lycopene and lutein found that the absorption of β-carotene or bioaccessibility maybe a saturable process considering that only about 3.5% of a 50 mg dose was able to be

detected in the TAG-rich lipoprotein fraction (O'Neill & Thurnham, 1998). They concluded that <2 mg of β-carotene from the supplement was absorbed irrespective of the dose, confirming the β-carotene saturation process. Results from the tissue experiment in this study, which found the bioavailability of β-carotene from test meal to be 10.3% are supported by previous studies that used more accurate isotopic tracer methods to assess bioavailability of β-carotene in test meals and pure sources to be in the range of 3 and 16 % (Edwards, You, Swanson, & Parker, 2001; Lin, Dueker, Burri, Neidlinger, & Clifford, 2000).

4.3. Model-based absorption efficiencies of carotenoids

The Ussing chamber is a technique that has in the recent past found increasing use to investigate the transport of chemical agents through the intestinal barrier as well as drug metabolism in enterocytes, both of which are key determinants for the bioavailability of orally administered drugs and food compounds (Gotoh, Kamada, & Momose, 2005; Kisser et al., 2017; Sjögren et al., 2016; Thomson et al., 2019; Westerhout, Wortelboer, & Verhoeckx, 2015). Moreover, the use of *ex vivo* models with animal or human segments of the gut on the Ussing chamber have been found to provide a better representation of the morphological and physiological features of the intestinal walls. Unlike Caco-2 cells, the animal segments has all relevant cell types and architecture, and the presence of mucus layer allowing simulation of further possible hydrolysis of brush border enzymes (Mackie, Mulet-Cabero, & Torcello-Gómez, 2020). In the present study, the carotenoid absorption efficiency was 1.8 times higher when assessed with mouse intestinal mucosa than with the differentiated Caco-2 cell monolayers. The fact that carotenoids were not detected in the basolateral section of the transwell system of the Caco-2 cell provides strong evidence on the shortcomings of the model, highlighting the importance of using more physiological digestion models in investigating nutrient digestion. This finding is however not surprising as the tissue system on the Ussing chambers is considered to provide physiologically relevant conditions for measuring the transport of nutrients across various epithelial tissues (Mulet-Cabero et al., 2020). The failure to detect carotenoids in the basolateral chamber of the Caco-2 transwell system could be because of exclusion of the supplementation step involving the addition of taurocholate and oleic acid to induce chylomicron secretion. Interestingly, of the total β-carotene secreted by Caco-2 cells, 80% is associated with chylomicrons, pointing to the importance of chylomicron assembly for β-carotene secretion into the lymph *in vivo* (Harrison & Kopec, 2018). It has been reported elsewhere that under normal cell culture conditions, Caco-2 cells are unable to form and secrete chylomicrons (During et al., 2002). The high oleic acid and taurocholate has been reported to induce intracellular triglyceride synthesis and thus, facilitates chylomicron formation (During & Harrison, 2004). Secondly, due to their hydrophobic nature, it is unlikely that carotenoids could cross the aqueous intracellular compartment without being bound to specific transport proteins. Indeed, L-FABP, one of the intracellular proteins reported in the transportation of large molecules in its hydrophobic pocket, seems to be the likely candidate (Desmarchelier et al., 2017) for the transportation of carotenoids in physiological conditions. Unfortunately, this protein is not expressed in the Caco-2 cell monolayers and a likely reason for these carotenoids not to be secreted in the basolateral compartment.

4.4. Conclusion

To the best of our knowledge, this is the first study to use biofortified test food digesta generated from a semi-dynamic digestion model that takes into account relevant physiological parameters and compares the carotenoid absorption kinetics using Caco-2 cell monolayers with a more physiologically relevant system employing mouse intestinal mucosa. The fact that carotenoids were detectable in the basolateral section of the Ussing chamber demonstrate two things: (1) that carotenoids are bioavailable under tissue system experimental conditions and (2) that, unlike the use of the Caco-2 cell model, the Ussing chamber model can be used as a more realistic approach to study the absorption and transport of carotenoids through mouse intestinal tissue simulating human intestinal absorption processes. In this study, carotenoids were detectable in the basolateral chamber of the Ussing system and not in the transwell system of the differentiated Caco-2 cells. Secondly, based on the absorption efficiency, there's a preferential uptake of all-*trans*-β-carotene at the expense of lutein even when these compounds are prepared in artificial micelles. These findings add to the increasing body of knowledge demonstrating the practicality of employing the Ussing chamber model in obtaining carotenoid permeability data that compare very well with published human *in vivo* data. Despite model limitations related to the technical difficulties in the skills to conduct the experiment; limited availability of fresh and viable fresh animal intestines and also that, the use of mice intestinal tissue results in the death of animals, the Ussing chamber can be considered a suitable model to represent carotenoid bioavailability in humans.

CRediT authorship contribution statement

Ng'Andwe Kalungwana: Conceptualization, Formal analysis, Investigation, Writing – original draft. **Lisa Marshall:** Supervision, Writing – review & editing. **Alan Mackie:** Methodology, Investigation, Supervision, Writing – review & editing. **Christine Boesch:** Conceptualization, Investigation, Supervision, Writing – review & editing.

Declaration of Competing Interest

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

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2023.112558.

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