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In vitro bioaccessibility and uptake of β-carotene from encapsulated carotenoids from mango by-products in a coupled gastrointestinal digestion/Caco-2 cell model

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ABSTRACT

β-carotene is a carotenoid with provitamin A activity and other health benefits, which needs to become bioavailable upon oral intake to exert its biological activity. A better understanding of its behaviour and stability in the gastrointestinal tract and means to increase its bioavailability are highly needed. Using an *in vitro* gastrointestinal digestion method coupled to an intestinal cell model, we explored the stability, gastrointestinal bioaccessibility and cellular uptake of β-carotene from microparticles containing carotenoid extracts derived from mango by-products. Three types of microparticles were tested: one with the carotenoid extract as such, one with added inulin and one with added fructooligosaccharides. Overall, β-carotene was relatively stable during the *in vitro* digestion, as total recoveries were above 68 %. Prebiotics in the encapsulating material, especially inulin, enhanced the bioaccessibility of β-carotene almost 2-fold compared to microparticles without prebiotics. Likewise, β-carotene bioaccessibility increased proportionally with bile salt concentrations during digestion. Yet, a bile salts level above 10 mM did not contribute markedly to β-carotene bioaccessibility of prebiotic containing microparticles. Cellular uptake experiments with non-filtered gastrointestinal digests yielded higher absolute levels of β-carotene taken up in the epithelial cells as compared to uptake assays with filtered digests. However, the proportional uptake of β-carotene was higher for filtered digests (24 – 31 %) than for non-filtered digests (2 – 8 %). Matrix-dependent carotenoid uptake was only visible in the unfiltered medium, thereby pointing to possible other cellular transport mechanisms of non-micellarized carotenoids, besides the concentration effect. Regardless of a filtration step, inulin-amended microparticles consistently resulted in a higher β-carotene uptake than regular microparticles or FOS-amended microparticles. In conclusion, encapsulation of carotenoid extracts from mango by-products displayed chemical stability and release of a bioaccessible β-carotene fraction upon gastrointestinal digestion. This indicates the potential of the microparticles to be incorporated into functional foods with provitamin A activity.

1. Introduction

Vitamin A deficiency is one of the most common micronutrient deficiencies worldwide, affecting almost a third of children under 5 years old, primarily living in low- and middle-income countries. The main cause of vitamin A deficiency is probably related to a low dietary diversity (Wirth et al., 2017). If the consumption of animal sources with preformed vitamin A is limited, carotenoid intake, mostly from plant origin, is necessary to meet vitamin A requirements (Moran et al., 2018). Among the carotenoids, β-carotene has the highest provitamin A activity because of its similar structure. Its adequate dietary intake through vegetables and fruits is positively associated with eye health, growth, tissue differentiation and reduced prevalence of chronic diseases (Bohn et al., 2019). While the carotenoid content in food can be used as a reference to quantify the daily intake of vitamin A and hence consumption recommendations, an assessment of the oral bioavailability,

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the fraction of carotenoids that reach the systemic circulation upon oral intake, is much more difficult. Oral bioavailability may be driven by host differences through genetic polymorphisms in intestinal transport proteins, but also through dietary behaviour, food matrix composition or any gastrointestinal digestion process that affects release of carotenoids from its matrix (Bohn, 2018; Böhm et al., 2020).

The latter process constitutes the term bioaccessibility, defined as the amount of a compound that is released from its food matrix into the gastrointestinal tract and becomes available for intestinal absorption (Santos et al., 2019). Specifically for carotenoids, the released compound must be incorporated into mixed micelles to be absorbed by enterocytes (Kopec & Failla, 2018). Several variables related to food (e.g. matrix and presence of other nutrients) and to the host (e.g. bile acid secretion and physicochemical interactions with gastrointestinal secretions) have shown to modulate carotenoids bioaccessibility (Bohn et al., 2017; Maurya et al., 2020). For instance, β-carotene bioaccessibility decreased more in a yogurt than in a pudding matrix, a result that was attributed to the protein content of yogurt (Donhowe et al., 2014). Adding dietary fibre (e.g. inulin) to tomato sauce negatively affected the carotenoid bioaccessibility; inulin physically sequestered carotenoids through strong interactions, altering emulsification efficiency (Tomas et al., 2018). Also, decreased levels of bile salts have previously been shown to diminish carotenoid bioaccessibility (Biehler et al., 2011; Hedrén et al., 2002), since they play a key role in the incorporation of carotenoids in mixed micelles (Bohn et al., 2017; Kopec & Failla, 2018).

Bioaccessibility is commonly assessed by *in vitro* digestion methods, which estimate the fraction of compounds available for intestinal absorption (Santos et al., 2019). *In vitro* digestion models simulate the *in vivo* gastrointestinal digestion processes, without the invasive and ethically constraint processes involved in animal and human studies (Lucas-González et al., 2018). Although *in vitro* digestion models are often a simplified representation of *in vivo* conditions, they provide insight into the interactions and effect of the digestive conditions, and food matrix interferences on β-carotene bioaccessibility (Lin et al., 2018). Over the last decade, to improve comparability of results from different research groups, the COST INFOGEST network proposed a standardized *in vitro* digestion protocol (Brodkorb et al., 2019; Minekus et al., 2014). As this protocol was initially focused on protein digestion, it showed some limitations for its use with carotenoids. Therefore, several adaptations were proposed including different bile concentrations, enzymatic activities, separation of micelles by centrifugation and/or filtration and carotenoid extraction from micelles (Cano et al., 2019; Estévez-Santiago et al., 2016; Rodrigues et al., 2016; Wen et al., 2018). Among these modifications, the isolation of the micellar phase (e.g. by filtration or centrifugation) remains debatable, as no consensus is reached about the methodology to obtain the bioaccessible fraction. Some describe it as the total content of carotenoid available in the supernatant after centrifugation (Barman et al., 2020; Gasa-Falcon et al., 2020; Lyu et al., 2021) or ultra-centrifugation (Cano et al., 2019; Petry & Mercadante, 2019) of the digests; whereas others define it as the amount of carotenoid in mixed micelles after microfiltration (Corte-Real et al., 2014; Hayes et al., 2020; Song et al., 2021) or ultrafiltration of the digest supernatant (Corte-Real et al., 2014). Although filtration ensures the presence of mixed micelles of particle sizes similar to true micelles obtained in *in vivo* digestion, we quantified the bioaccessibility in centrifuged-only samples, as the impact of the filtration step on β-carotene uptake was also investigated.

Following assessment of carotenoid bioaccessibility, prediction of oral bioavailability requires an assessment of the intestinal absorption. Differentiated Caco-2 monolayers are applied to simulate the apical uptake *in vitro* due to its morphology, expression of marker enzymes and permeability characteristics which are comparable with human enterocytes (Ding et al., 2021). The Caco-2 cell line is a useful model to study the apical uptake, metabolism and transport of diverse nutrients across the intestinal epithelium (Failla & Chitchumronchokchai, 2005). Normally, prior to conducting absorption experiments with cell models, filtering digested samples with 0.20 μm pore size filters is a common practice to remove non-solubilized carotenoids and reduce microbial contamination (Corte-Real et al., 2014). However, filtration is likely to affect β-carotene uptake in Caco-2 cell models, as seen for anthocyanins, whose transport rates through the intestinal monolayer are increased when the mixture containing them has been previously filtered (Cardona et al., 2015).

In recent years, interest in agro-industrial by-products as an unexplored pool of natural bioactives has grown (Kumari et al., 2018; Rodriguez-Lopez et al., 2020; Szabo et al., 2018). Peels and seeds of various fruits such as orange, passion fruit, and mango have been chemically characterised, identifying important amounts of bioactive compounds (Hatami et al., 2020; Lenucci et al., 2022; Marcillo-Parra et al., 2021; Mercado-Mercado et al., 2018; Savic Gajic et al., 2021; Sánchez-Camargo et al., 2019). Especially, mango peel has gained attention due to its valuable components such as carotenoids, polyphenols, enzymes, and vitamins E and C (Jahurul et al., 2015; Marcillo-Parra et al., 2021; Mercado-Mercado et al., 2018; Sánchez-Camargo et al., 2019). Considering that by-products from mango processing can represent up to 60 % of the total weight of the fruit (Jahurul et al., 2015), the world production of mango was estimated to be over 51 million tons in 2019 (Lenucci et al., 2022), and that total carotenoid content determined in mango peels ranged between 1.75 and 5.69 mg per 100 g dry weight (Lenucci et al., 2022; Marcillo-Parra et al., 2021), mango peel represents an important source of carotenoids. However, in an *in vitro* digestion model, bioaccessibility of mango peel was limited by dietary fibre which retained these compounds within its structure (Mercado-Mercado et al., 2018). Therefore, extraction methods that use solvents or supercritical fluids, assisted or not with ultrasound or microwaves (Marcillo-Parra et al., 2021; Villacís-Chiriboga et al., 2021; Vélez-Erazo et al., 2021) can produce carotenoid-enriched extracts that may result in better bioaccessibility. Yet, these extracted carotenoids require adequate storage to avoid considerable degradation, since they are highly sensitive to environmental conditions such as heat, light and oxygen (Chuyen et al., 2019). To face these unfavourable environmental conditions, carotenoid microencapsulation offers physical barrier protection, since it comprises enveloping the core material (e.g. carotenoids) with a wall material, thus increasing storage stability and improving the bioaccessibility of bioactives through controlled release during digestion (Eun et al., 2020; Zhou et al., 2018). Several materials such as maltodextrin, starch, inulin, alginate, gum arabic, whey protein concentrate, among others, have been successfully used to encapsulate carotenoids through spray drying (Chuyen et al., 2019; Etzbach et al., 2020). Likewise, inulin (prebiotic) and cellobiose (potential prebiotic) were also considered as carotenoid encapsulation materials with additional benefits on the intestinal microbiota (Etzbach et al., 2020; Rivas et al., 2021). However, the effect of encapsulation materials on the bioaccessibility of bioactives is still poorly studied (Chen et al., 2020; Fu et al., 2019; Lin et al., 2018).

Bioaccessibility of carotenoids such as fucoxanthins and astaxanthins significantly improved by microencapsulation, both in *in vitro* and *in vivo* experiments, since the wall materials effectively protected the carotenoids in the gastric environment, while increasing the release rate in the intestinal phase (Sun et al., 2018; Zhou et al., 2018). Therefore, in the current study, we wanted to assess the effect of prebiotics (inulin or fructooligosaccharides) incorporated in the wall material of the microparticles, containing carotenoids extracted from mango by-products, on the bioaccessibility of β-carotene. In addition to the good stability that microencapsulation would provide to β-carotene, effective bioaccessibility is desired. Thus, the bioaccessibility of β-carotene from microparticles containing encapsulated carotenoids extracted from mango by-products, and the carotenoid uptake by intestinal cells, using an *in vitro* digestion model coupled with a Caco-2 cell model were investigated. Furthermore, the effect of the concentrations of bile salts set in the *in vitro* digestion model, since they facilitate the solubilization of carotenoids, were evaluated. Finally, the effect of filtering the diluted

digestion supernatants through a pore size of 0.22 μm, prior to use in a Caco-2 cell model, on β-carotene uptake was also assessed.

2. Materials and methods

2.1. Chemicals, enzymes, and encapsulated carotenoids

β-carotene, *trans*-β-apo-8′-carotenal, tert-butylhydroquinone (TBHQ), triethylamine, ammonium carbonate, magnesium carbonate, Clara-Diastase, porcine pepsin, porcine pancreatin and porcine pancreatic lipase were purchased from Sigma-Aldrich (St. Louis, MO, United States). Rapidase solution was from DSM (Delft, the Netherlands) and Difco™ Oxgall, from BD Bioscience (Erembodegem, Belgium). Magnesium chloride hexahydrate was from Carl Roth GmbH + Co.KG (Karlsruhe, Germany) and glacial acetic acid from Fisher Scientific (Merelbeke, Belgium). MEM non-essential amino acid solution (NEAA), penicillin/streptomycin (P/S), trichloroacetic acid (TCA), tris(hydroxymethyl)aminomethane, dimethyl sulphoxide (DMSO), and sulforhodamine B (SRB) were from Sigma-Aldrich (Overijse, Belgium). Thiazolyl blue tetrazolium bromide (MTT) was obtained from Amresco (Zottegem, Belgium). Dulbecco's modified Eagle's medium with GlutaMAX™ and sodium pyruvate (DMEM), Dulbecco's phosphate-buffered saline (DPBS) with and without calcium and magnesium, and trypsin-ethylenediaminetetraacetic acid (EDTA) solution were from Life Technologies (Merelbeke, Belgium). Fetal bovine serum (FBS) was from VWR (Leuven, Belgium). All other reagents of both analytical and HPLC grade were purchased from Chem-Lab Analytical (Zedelgem, Belgium).

Three different types of microparticles with encapsulated carotenoids derived from mango by-products were provided by Escuela Politécnica Nacional - EPN - (Quito-Ecuador). The microparticles were produced as described by Marcillo-Parra (2022). Briefly, the microcapsules were produced by spray-drying an emulsion containing a mixture of maltodextrin, gum arabic, lecithin, and the carotenoid extract. Additionally, it included either fructooligosaccharides, inulin or no prebiotics in the wall material. The physicochemical characteristics of the microparticles used in this study are available in the same reference of the production methodology (Marcillo-Parra, 2022).

2.2. In vitro digestion of encapsulated carotenoids

The encapsulated carotenoids were digested accordingly to the standardized INFOGEST method (Brodkorb et al., 2019; Minekus et al., 2014), with adaptations for carotenoid analysis (Rodrigues et al., 2017). Rodrigues et al. (2017) used enzyme concentrations rather than activities in the intestinal phase when screening the carotenoid bioaccessibility in raw mixed vegetable salads with or without animal products, showing similar results to those of the INFOGEST method. Then, in this study, concentrations of pancreatin (1.2 mg/mL) and pancreatic lipase (0.6 mg/mL) per final volume (Rodrigues et al., 2017) were used to better simulate the fed state. Our own adaptations consisted in reducing volumes in the digestion model (final volume after intestinal phase = 10 mL) due to the limited amount of encapsulated carotenoid microparticles, and in protecting the digests from light to avoid carotenoid degradation.

The simulated salivary, gastric, and intestinal fluids containing electrolytes were prepared as described by Brodkorb et al. (2019). Briefly, 1 g sample was weighed into a 15 mL screw cap polyethylene tube, hydrated with 1.25 mL distilled water and vortexed for 1 min. The oral phase was limited to the mixing of the hydrated sample with 1.25 mL of the simulated salivary fluid; α-amylase was omitted since the samples did not contain starch. The mixture was shaken manually for 1 min. For the gastric phase, 2.5 mL of simulated gastric fluid, recently acidified with HCl (1 M) and containing porcine pepsin (6.3 mg/mL), was added to the tube. When needed, the pH of the mixture was adjusted to 3 by adding HCl (5 M). The tubes were then transferred to a shaking incubator (Edmund Bühler GmbH, Bodelshausen, Germany) at 37 °C and 200 rpm, for 2 h. To simulate small intestinal digestion, the samples were mixed with 5 mL of simulated intestinal fluid containing pancreatin (2.4 mg/mL) and pancreatic lipase (1.2 mg/mL) to reach the final concentrations mentioned above. Additionally, three different bile salt concentrations, 2, 10, and 15 mM (2 mM is the critical micellar concentration (CMC) of the bile salts sodium glycocholate and sodium taurochenodeoxycholate (Moghimipour et al., 2015), 10 mM is the typical concentration in the fed state (Brodkorb et al., 2019; Minekus et al., 2014), and 15 mM was chosen as a concentration higher than physiological), were applied to analyse their effect on the β-carotene bioaccessibility. The pH was increased to 7 by NaOH (1 M) addition, and the samples were then incubated at 37 °C and 200 rpm, for 4 h. Finally, the suspension obtained at the end of the small intestinal digestion was centrifuged at 4000 g and 4 °C for 30 min. The supernatant was transferred to a glass tube with screw cap to measure the bioaccessible content of β-carotene, whereas the pellet was preserved in the original tube to later quantify the β-carotene that was not released or solubilized. Both supernatant and pellet were flushed with nitrogen and immediately stored at −20 °C until further analysis.

2.3. Microstructure of microparticles upon digestion using Cryo-SEM

The microstructure of encapsulated carotenoid microparticles (i.e. with and without inulin) and their digestion fluids were visualized during the *in vitro* digestion process after the oral phase, 1 h after the gastric phase, and 4 h after the small intestinal phase began, using a JSM-7100F TTLS LV TFEG-SEM (JEOL ltd, Tokyo, Japan) under vacuum conditions ($1e^{-6}$ mbar) and at an accelerated voltage of 3 keV. Prior to cryo-SEM imaging, drops of homogenised digest suspension, sampled at the times stated above, were placed on aluminium SEM stubs covered with a carbon adhesive tape, and dried completely under a stream of nitrogen. Later, the samples were vitrified in a nitrogen slush (-210 °C) and transferred under vacuum conditions into a PP3010T cryo-SEM preparation stage (Quorum Technologies ltd., East Sussex, U.K.) conditioned at −140 °C, where they were fractured. The samples were sublimated at −70 °C for 30 min and then sputter-coated with platinum using argon gas, transferred to the SEM stage at −140 °C to finally be visualized in the scanning electron microscope.

2.4. Uptake of β-carotene by Caco-2 cells

2.4.1. Cell culture

The human colorectal carcinoma cell line Caco-2 (HTB-37™) was obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were maintained in 25 cm^2 canted neck tissue culture flasks (Sarstedt Co., Essen, Belgium) in an incubator (Memmert GmbH & Co., Nurnberg, Germany) at 37 °C and 10 % CO_2. The culture medium was a high glucose DMEM, supplemented with 10 % heat-inactivated FBS, 1 % NEAA and 1 % P/S. The medium was replaced three times per week, and the cells were subcultured upon a confluency of 80 – 90 %, using 0.25 % trypsin-EDTA solution.

2.4.2. Uptake experiments

Digest supernatants (i.e. micellar fraction with the carotenoids) obtained at 15 mM bile salts were used for uptake experiments using a Caco-2 cell model. Prior to the uptake experiments, cytotoxicity assays including MTT and SRB were performed to define the dilution conditions of the digests and the exposure time (see Supplementary Material). Uptake experiments were performed in T25 flasks, as we wanted to evaluate only the apical uptake of β-carotene (Rodrigues & Failla, 2021). Caco-2 cells were seeded at a concentration of 1×10^6 cells per T25 flask. The supplemented growth medium was replaced three times per week, until differentiation (14 days post-confluence) was achieved. Then, the medium was removed, and the cells were washed with warm serum-free exposure medium twice. 3 mL of a 1:8 diluted digest in exposure medium, filtered (0.22 μm pore diameter, Millex-GP) or

unfiltered, was added to the Caco-2 cells and incubated at 37 °C and 10 % CO_2 for 4 h. Next, the exposure medium was collected in a 20 mL headspace vial to study the remaining carotenoid concentration in the medium after uptake. These vials were pretreated with antioxidants by drying inside 1 mL of TBHQ (2.5 % in ethanol) to avoid carotenoid degradation. The monoculture was washed with 1 mL of warm PBS (with Ca^{2+} and Mg^{2+}), 1 mL of cold PBS (without Ca^{2+} and Mg^{2+}) was added, and cells were mechanically detached using a cell scraper. Next, the cells suspension was transferred to a second 20 mL headspace vial with dried TBHQ. The flask was rinsed twice with cold PBS and the suspensions were added to the same vial. All vials were covered with aluminium foil, flushed with nitrogen, and stored at −80 °C until further analysis.

2.5. β-carotene analysis

2.5.1 β-carotene extraction from encapsulated materials and pellets

β-carotene was extracted following the method of Grootaert et al. (2021), under dim light to avoid the photo-oxidation of the carotenoids. The main variation of this method was the amount of material used for the extractions, 50 mg of encapsulated carotenoid microparticles or the full pellet stored in the conical flasks after digestion. Briefly, samples were subjected to an enzymatic pre-treatment at 30 °C using Clara-Diastase, Rapidase, NaCl and acetone/pentane. The organic phase containing the carotenoids was recovered after centrifugation and re-extractions with acetone/pentane, and then dried. The dried organic fraction was saponified by potassium hydroxide, magnesium carbonate and ethanol, overnight at 30 °C. Then, the carotenoids from the mixture were exhaustively extracted with hexane and then washed with NaCl and distilled water. Next, anhydrous Na_2SO_4 was used to remove excess of water from the carotenoid-rich extract before drying it completely. The dried residue obtained was redissolved in 500 μL of a methanol/acetonitrile mixture (9:1 *v/v*) and 500 μL of an ethyl acetate with triethylamine (0.25 % *v/v*) solution, filtered (0.45 μm pore diameter, Millipore) and transferred to a HPLC vial.

2.5.2. β-carotene extraction from gastrointestinal digests

To measure the β-carotene released from the encapsulated materials during *in vitro* digestion, the method from Biehler et al. (2011) was adapted. 1 mL digest was transferred to a 20 mL glass headspace vial together with 1 mL TBHQ (2.5 % in ethanol), 4 mL of acetone/hexane (1:1 *v/v*) and 200 μL *trans*-β-apo-8′-carotenal (17 μg/mL in dichloromethane) as internal standard. The mixture was vortexed for 1 min and centrifuged at 4000 g for 2 min at 4 °C. The upper carotenoid-containing organic phase was transferred to a 20 mL test tube with screw cap, whereas the aqueous phase was re-extracted twice with 4 mL hexane. The combined hexane phases were dried completely under nitrogen. The residue was redissolved in 500 μL of methanol/acetonitrile (9:1 *v/v*) and 500 μL of ethyl acetate with triethylamine (0.25 % *v/v*), filtered (0.45 μm pore diameter, Millipore) and transferred to a HPLC vial.

2.5.3. β-carotene extraction from exposure medium and exposed cells

For β-carotene extraction from the cell medium, the procedure was as follows: 1 mL TBHQ (2.5 % in ethanol), 1 mL distilled water, 4 mL acetone/hexane (1:1 *v/v*) and 100 μL *trans*- β-apo-8′-carotenal (4 μg/mL in dichloromethane), were added to the exposure medium, vortexed (1 min) and centrifuged (4000 g, 4 °C, 5 min). The upper carotenoid-containing phase was transferred to a 20 mL test tube with screw cap, whereas the aqueous phase was re-extracted twice with 3 mL of hexane and 2 mL of water, vortexed and centrifuged each time. The hexane phases were pooled and dried completely under nitrogen. The residue was redissolved in 100 μL methanol/acetonitrile (9:1 *v/v*) and 100 μL ethyl acetate with triethylamine (0.25 % *v/v*), filtered (0.45 μm pore diameter, Millipore) and transferred to a HPLC vial with insert. Carotenoid extraction from the cells was performed according to this method, although for this case 50 μL *trans*-β-apo-8′-carotenal (0.4 μg/L in dichloromethane) was used as internal standard.

2.5.4. β-carotene analysis by RP C30-HPLC-DAD

Carotenoids were separated by high-performance liquid-chromatography with diode-array detection (HPLC-DAD) in an Ultimate 3000 System (Dionex, Thermo Scientific, Landsmeer, The Netherlands), using a reversed phase YMC-pack C30 column (Waters Inc., Schermbeck, Germany, 250 mm × 4.6 mm i.d., S-5 μm), as described by Grootaert et al. (2021). Samples were placed in an autosampler with light-cover and maintained at 4 °C. The gradient program consisted of mobile phase A) methanol/acetonitrile (9:1 *v/v*) and mobile phase B) ethyl acetate with 0.25 % triethylamine which were combined as follows: the gradient started at 100 % A, linear gradient to 40 % A and 60 % B over 25 min, isocratic for 5 min, linear gradient to 100 % A over 5 min and isocratic for 5 min. The flow rate was 1 mL/min, and the column temperature was maintained at 30 °C. The sample injection volume was 25 μL. Carotenoids were detected at 450 nm and identified by retention time as compared to pure standards or by their HPLC-DAD spectra.

Quantitative determinations were performed using analytical standard curves of β-carotene, as described by Grootaert et al. (2021). In our study, the concentration ranges depended on the type of sample analysed, microparticles, supernatants and pellets, exposure medium, and treated cells. The need to use different calibration curves was based on the expected β-carotene concentrations of the samples, as low as 20 ng/mL in the cells or as high as 2000 ng/mL in the pellets. For the treated cells, the analytical standard curve was constructed by 5 points that covered a linear range of 20–100 ng/mL of β-carotene with 100 ng/mL of internal standard added to each point; its limit of detection (LOD) = 6.03 ng/mL, limit of quantification (LOQ) = 18.27 ng/mL, and R^2 = 0.98 were obtained from 3 calibration curves. For the exposure medium, a 5-point calibration curve was done in a linear range of 40–300 ng/mL of β-carotene with 2000 ng/mL of internal standard; its LOD = 10.02 ng/mL, LOQ = 30.35 ng/mL and R^2 = 1.00 were calculated from 3 calibration curves. For the microparticles, supernatants, pellets and unfiltered exposure medium, the analytical curve had 5 points in a linear range of 1200 – 5000 ng/mL of β-carotene with 3000 ng/mL of internal standard; its LOD = 46.56 ng/mL, LOQ = 141.08 ng/mL and R^2 = 1.00 were calculated from 6 calibration curves. LOD and LOQ were calculated as explained by Grootaert et al. (2021). All standard curves were constructed in methanol/acetonitrile (9:1 *v/v*). The percentages of β-carotene recovery for the supernatant, exposure medium, and cells were calculated at different time points, and the variability of the results was assessed in a small-scale validation experiment in which the intraday (n = 3) and interday (n = 3) variability was assessed (see Supplementary Material).

2.6. Statistical analysis

Tests were performed in triplicate. The results of β-carotene quantification are reported as median with interquartile range. Statistical differences between samples treated under different conditions were established using Mood's median tests, except for the differences between filtered and unfiltered samples during uptake experiments that were determined by Kruskal-Wallis. Non-parametric tests were used for the statistical analysis as the sample size was small (n = 3). Cytotoxicity tests were performed with 6 replicates (wells) for each condition and the results are expressed as median with 95 % confidence intervals. The statistical significance of the effect of several digest dilutions on the cells was analysed with a Mood's median test. The SPSS statistical software (version 27, SPSS Inc., Chicago, IL, USA) was used for the statistical analysis ($p < 0.05$), Sigmaplot software (version 13, Systat Software Inc., San Jose, USA) and RStudio software (version 1.4.1717) were used for the graphics. BioRender was used for the original figure of β-carotene assemblies and cellular uptake.

3. Results

3.1. β-carotene content of the microparticles

β-carotene content of the encapsulated materials was 48.56 ± 8.94 μg/g in microparticles without prebiotics, 48.33 ± 1.25 μg/g in microparticles with fructooligosaccharides and 41.72 ± 2.33 μg/g in microparticles with inulin. A significant difference was observed only between microparticles with inulin and with fructooligosaccharides.

3.2. β-carotene stability upon in vitro digestion

Because of β-carotene instability during *in vitro* gastrointestinal digestion (Petry & Mercadante, 2019; Tan et al., 2020), we first verified whether it could be fully recovered from the digests. The recovery of β-carotene, as an indicator of its stability, was quantified by comparing the total amount of β-carotene determined after the *in vitro* digestion (see Supplementary Material) to the amount in the undigested material. Recovery of β-carotene upon small intestinal digestion was generally efficient and ranged from 68 to 102 % (Fig. 1). The presence of prebiotics in the encapsulation matrix resulted in high β-carotene recoveries, reaching ~ 100 % for microparticles with inulin and fructooligosaccharides, whereas recoveries of up to 84 % were determined for microparticles without prebiotics. High β-carotene recoveries were also related to high concentrations (10 – 15 mM) of bile salts. Microparticles without prebiotics achieved the highest recovery at 15 mM, whereas both microparticles with prebiotics achieved their maximum recoveries a 10 mM. Yet, there were no significant differences between the β-carotene recoveries of the three types of microparticles, neither at the same nor at different concentrations of bile salts.

3.3. β-carotene bioaccessibility from microparticles

The β-carotene liberated from the microcapsules upon gastrointestinal digestion may be incorporated into mixed bile salt micelles, thus becoming available for absorption by intestinal cells, but it could also precipitate, making it unavailable for absorption. In this study, we calculated the β-carotene bioaccessibility as the ratio between the β-carotene released in the supernatant of the digest (i.e. the mixed micelle fraction) after *in vitro* digestion in the small intestine, and its concentration in the initial sample.

The β-carotene bioaccessibility depended on the concentration of bile salts and the composition of the microparticles; bioaccessibility increased with rising concentrations of bile salts, with the highest values being those related to the presence of prebiotics (Fig. 1). The highest increases were found for inulin, reaching values of up to 3.3-fold (at 10 mM) and 3.8-fold (at 15 mM) higher compared to 2 mM bile salts. For these microparticles, β-carotene bioaccessibilities at 10 and 15 mM were not significantly different from each other, but they did differ significantly from those at 2 mM. For microparticles containing fructooligosaccharides, less sharp increases of bioaccessibility were observed, i.e. 2.2-fold (at 10 mM) and 2.3-fold (at 15 mM) compared to that determined with 2 mM bile salts. Although β-carotene bioaccessibilities increased as a function of the bile salts concentration, they were not significantly different between each other according to the non-parametric statistical test used. Bioaccessibilities from the

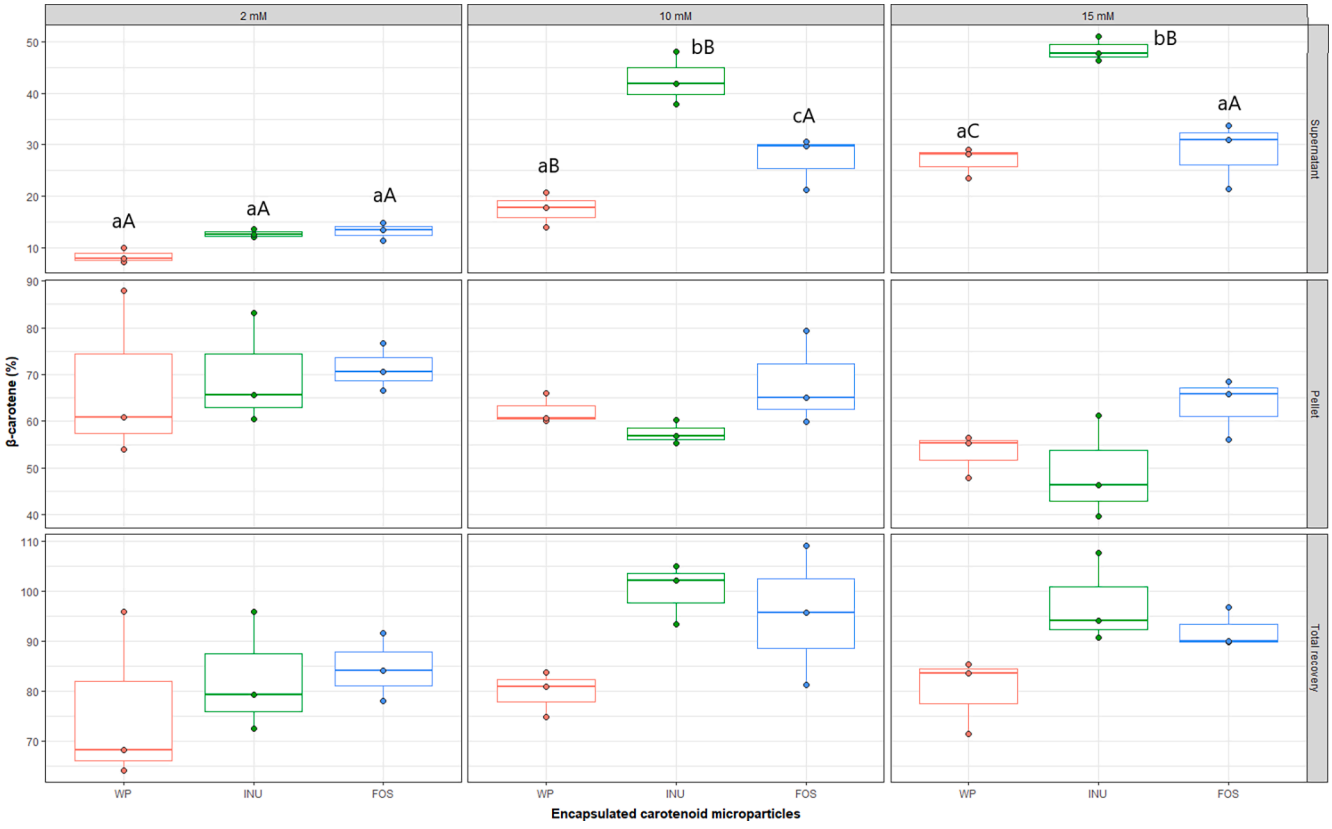

Fig. 1. β-carotene percentages compared to the initial β-carotene amount, determined in the unfiltered digest supernatant and pellet after completing the *in vitro* digestion of microparticles with fructooligosaccharides (FOS), with inulin (INU) and without these prebiotics (WP), using concentrations of bile salts equal to 2 mM, 10 mM, and 15 mM. Medians and interquartile ranges (n = 3) of the supernatants, pellets and total recovery are reported in the box plots. For the supernatants, different capital letters indicate statistically significant differences between the same material when the concentrations of bile salts differ, while different lower-case letters represent statistically significant differences between the materials when the bile salts concentration is constant. There were no significant differences between the percentages of β-carotene recovered in the pellet, nor between the percentages of total β-carotene recovered, regardless the type of microparticles or the concentrations of bile salts.

microcapsules without prebiotics were 2.2-fold (at 10 mM) and 3.6-fold (at 15 mM) higher than that determined at 2 mM bile salts, being significantly different in all cases. Overall, increasing the bile salts concentration from 2 mM to 15 mM resulted in 2- to almost 4-fold increases in β-carotene bioaccessibilities. Then, as β-carotene bioaccessibilities were also influenced by the composition of the microparticles matrices, they were evaluated with the same bile salts concentration. The highest β-carotene bioaccessibility determined in this study, at the highest bile salts concentration tested (15 mM), was from microparticles with inulin (47.7 %). It was significantly higher than bioaccessibilities from microcapsules with fructooligosaccharides (30.9 %) or without prebiotics (28.2 %). At a bile salt concentration of 10 mM, which is the typical concentration in the fed state (Brodkorb et al., 2019; Minekus et al., 2014), significant differences between the β-carotene bioaccessibilities from the three powders were observed, with the highest values for microparticles with inulin (41.8 %) and the lowest values for microparticles without prebiotics (17.8 %). At a low concentration of bile salts (2 mM), although β-carotene bioaccessibilities of microparticles with inulin (12.6 %), and with fructooligosaccharides (13.4 %) were higher than without prebiotics (7.9 %), there were no significant differences between them. Overall, the addition of inulin in the composition of the microparticles offered a nearly 2-fold increase in the β-carotene bioaccessibility when compared to powder without any prebiotics, when evaluated at the same bile salts concentration.

3.4. *β-carotene uptake from filtered and unfiltered digests by Caco-2 cells*

Upon gastrointestinal digestion, the characteristics of the structures associated with β-carotene will determine its epithelial uptake and eventually bioavailability. To better discern the absorbable from non-absorbable β-carotene fraction, we performed a filtration step on the different gastrointestinal digest supernatants of 15 mM bile salts. The three supernatants (replicates) of each type of digested microparticles were pooled to obtain a representative sample for uptake experiments. β-carotene accumulation was assessed in Caco-2 monolayers, that were exposed to filtered or unfiltered digest supernatants. Prior to the accumulation experiments, cytotoxicity of the digests was assessed using MTT and SRB assays on several dilutions and exposure times (see Supplementary Material). It was concluded that an 8-fold dilution applied for 4 h was the most appropriate condition for further uptake experiments.

To assess the effect of filtration on β-carotene accumulation, the proportional uptake, as a percentage, was calculated comparing the amount of β-carotene recovered in cells exposed to digest supernatants to the initial amount applied. Although filtration reduced the concentration of β-carotene in the samples prior to the accumulation studies, the proportional uptake of β-carotene from filtered samples was significantly higher than those from unfiltered samples (Table 1). The differences in proportional uptake between both filtration conditions depended on the composition of the microcapsules, being higher when they included fructooligosaccharides and lower when they contained inulin. In addition, when evaluating the β-carotene uptakes from the three types of microcapsules under the same filtration conditions (i.e. filtered or unfiltered), they varied significantly from each other depending on the composition of the microcapsules in the unfiltered medium, but not in the filtered medium. In the unfiltered medium, inulin positively affected β-carotene uptake by Caco-2 cells, obtaining the highest percentage, in contrast to fructooligosaccharides, which got the lowest uptake percentage.

3.5. *Carotenoid profiles of the microparticles before and after in vitro digestion, and after Caco-2 cellular uptake*

Carotenoid profiles of the microparticles (undigested samples) were similar regardless of whether they contained inulin or fructooligosaccharides (Fig. 2). The peaks representing the main carotenoids found in the undigested samples were tentatively identified by their DAD spectra as follows: 1. lutein (max. 444/470 nm, %III/II = 56 %), 3. antheraxanthin (max. 444/472 nm, %III/II = 54), 4. 13-*cis*-β-carotene or 15-*cis*-β-carotene (max. 444/475 nm, %III/II = 3), 5. α-carotene (max. 444/472 nm, %III/II = 51), 6. β-carotene (max. 452/477 nm, %III/II = 18) and, 7. 9-*cis*-β-carotene (max. 447/471 nm, %III/II = 17) (de Faria et al., 2009; Rodriguez-Amaya & Kimura, 2004; Rodriguez-Amaya, 2001). Peak 2 corresponded to *trans*-β-apo-8′-carotenal (max. 461 nm), the internal standard used in the carotenoid extractions. Compounds whose standards were available in the laboratory, *trans*-β-apo-8′-carotenal, β-carotene, lutein and α-carotene, were confirmed by both their DAD spectrum and retention time. These same six peaks were also detected in the chyme supernatants after small intestinal digestion, along with an additional peak 8 (Fig. 2), which is presumably β-cryptoxanthin or zeaxanthin (max. 450, 480 nm,% III / II = 26) (Rodriguez-Amaya & Kimura, 2004; Rodriguez-Amaya, 2001). The carotenoid profiles of the gastrointestinal digest supernatants were equivalent (Fig. 2), irrespective of the type of microparticles.

Chromatographic profiles of the carotenoids obtained from Caco-2 cells after exposure to the diluted digests depended on whether the supernatant was filtered. A greater diversity of carotenoids taken up (i.e. number of peaks) was observed in cells exposed to filtered than unfiltered supernatants (Fig. 2), although the variety of available carotenoids in the apically applied filtered media was less (see Supplementary Material). Three carotenoids, lutein, β-carotene and β-cryptoxanthin or zeaxanthin, were found in cells exposed to all types of filtered digests. In the case of exposure to unfiltered digests, the accumulated carotenoids differed according to the digested microparticles, only β-carotene was taken up from microparticles without prebiotics, β-carotene and lutein from microparticles with fructooligosaccharides, and lutein, β-carotene

Table 1
Total β-carotene content in i) filtered and unfiltered diluted digests applied to Caco-2 cells (Total applied), ii) cells after 4 h of treatment (Total in cells), and iii) cells and medium after 4 h of treatment (Total recovery).

Amount of β-carotene								
	Filtered digests				Unfiltered digests			
	Total applied (ng)	Total in cells (ng)	Total in cells (%)	Total recovery (%)	Total applied (ng)	Total in cells (ng)	Total in cells (%)	Total recovery (%)
WP	23.66	6.14 [4.70–6.99]^{aA}	25.93 [19.85–29.55]^{aA}	86.58 [85.13–88.04]^{aA}	494.80	25.38 [24.74–26.59]^{aB}	5.13 [5.00–5.38]^{aB}	77.74 [76.81–81.83]^{aA}
INU	23.99	7.31 [5.72–7.80]^{aA}	30.45 [23.84–32.51]^{aA}	78.41 [69.80–79.14]^{aA}	705.40	55.34 [55.13–55.41]^{bB}	7.85 [7.82–7.86]^{bB}	102.06 [98.36–120.67]^{bB}
FOS	21.26	5.09 [4.51–5.30]^{aA}	23.96 [21.20–24.94]^{aA}	69.55 [53.63–72.31]^{aA}	468.15	9.98 [9.61–10.46]^{cB}	2.13 [2.05–2.24]^{cB}	15.45 [13.54–15.48]^{cB}

Values are expressed as median [interquartile range] of three independent samples. Distinct capital letters indicate statistically significant differences between filtered and unfiltered digests of the same material, while different lower-case letters represent statistically significant differences between the materials at the same filtration condition.
WP: microparticles without inulin or fructooligosaccharides; FOS: microparticles with fructooligosaccharides; INU: microparticles with inulin.

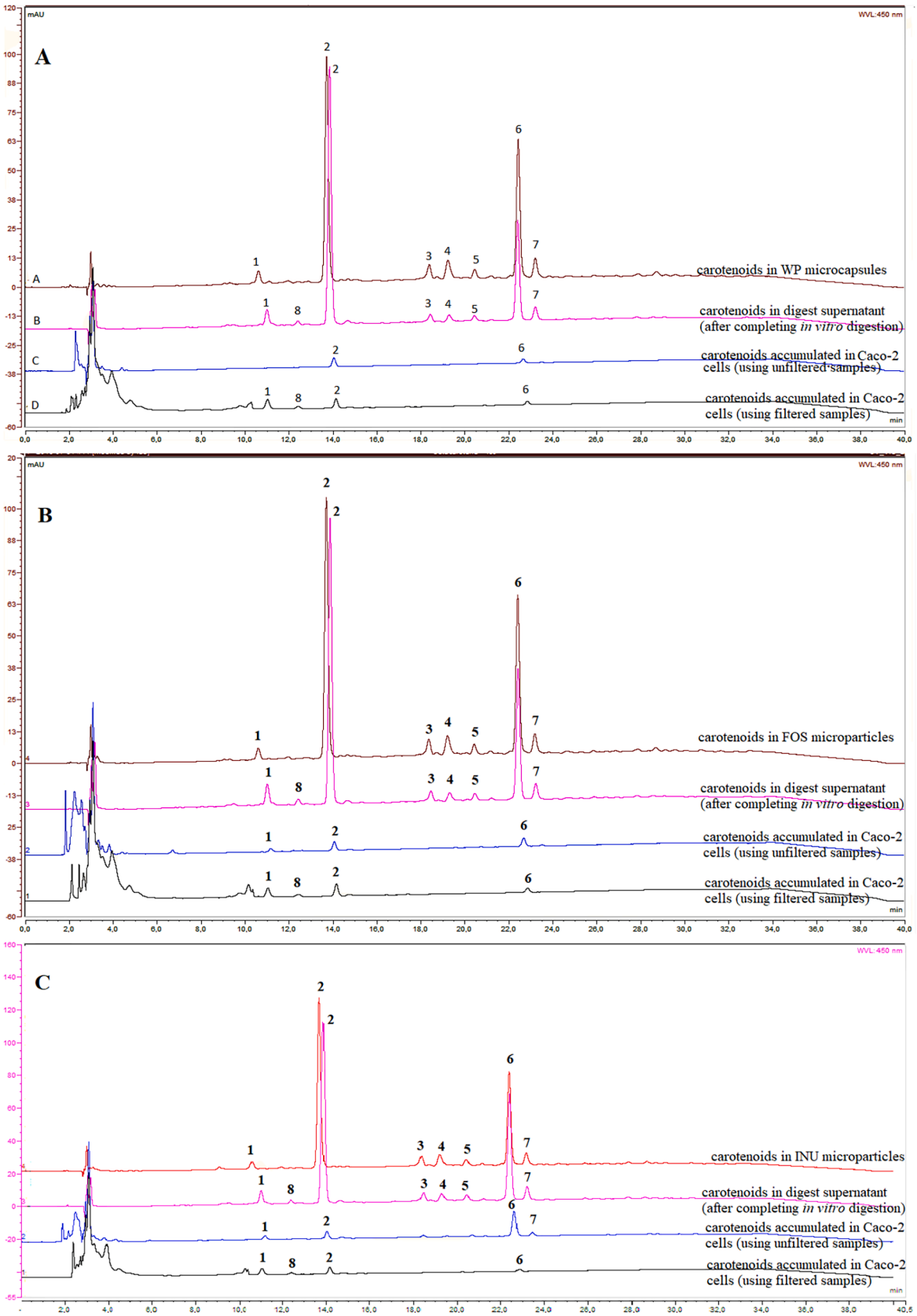

Fig. 2. Carotenoid profiles of encapsulated carotenoid microparticles without prebiotics -WP- (A), with fructooligosaccharides -FOS- (B), and with inulin -INU- (C), before and after *in vitro* digestion and cellular uptake. Peaks identification based on DAD spectra: 1. lutein, 2. *trans*-β-apo-8′-carotenal, 3. presumably antheraxanthin, 4: presumably 13-*cis*-β-carotene or 15-*cis*-β-carotene, 5. α-carotene, 6. β-carotene, 7. presumably 9-*cis*-β-carotene, and 8. presumably β-cryptoxanthin or zeaxanthin. Chromatograms were processed at 450 nm.

and 9-*cis*-β-carotene from microparticles with inulin. Overall, filtration allowed the uptake of more types of carotenoids despite the smaller variety of carotenoids offered by the filtered digest.

3.6. Changes in morphology of microparticles during in vitro digestion

According to the scanning electron microscope (SEM), both spray dried samples (i.e. encapsulated carotenoid microparticles with and without inulin) showed spherical shapes with a mostly smooth surface with some oil droplets (Fig. 3 A, E). After oral phase, undissolved surface microparticles, few broken structures and amorphous aggregates were observed (Fig. 3 B, F). Notably, the surface of the particles showed some irregular roughness. After 1 h of gastric digestion, the microparticles with intact structure were scarce in both cases. Broken surface structures (Fig. 3 C) and few microparticles, some with shrunken shape (Fig. 3 G) were observed. Finally, after 4 h of intestinal digestion, small assemblies (i.e. sphere-like, clumps) were observed within the digest fluids of both type of microparticles (without and with inulin) (Fig. 3 D, H).

4. Discussion

In the present study, we assessed how the inclusion of prebiotics, inulin and fructooligosaccharides, in the microparticles matrix, and the concentration of bile salts affect the *in vitro* bioaccessibility of β-carotene. Additionally, the effect of digest filtration on β-carotene uptake by Caco-2 cells was evaluated.

A good chemical stability for β-carotene (i.e. total recoveries > 68 %) was observed in all three digested materials, regardless of the bile salts concentration. Similar values were reported by Rodrigues et al. (2017), where about 75 – 98 % of β-carotene contained in mango, spinach, carrot, and tomato remained after *in vitro* digestions, in a setup like ours. In our study, it is presumed that the protection against oxidation during the *in vitro* digestion was associated with the microparticles composition. Although SEM images showed that almost all microparticles were disintegrated into visible particles after one hour of gastric digestion (Fig. 3), it is likely that β-carotene was not released into the digest as such yet but was still integrated in the capsule fragments. In the following gastrointestinal phases, as β-carotene was released, prebiotics (inulin or fructooligosaccharides) exerted a protective effect. β-carotene recoveries from digested microparticles containing prebiotics were the highest (~100 %) but did not differ statistically from recoveries from microparticles without the prebiotics. We hypothesized that the greater protection of β-carotene against degradation may be due to the flexible molecular structure of these prebiotics and the hydrophobic nature of their backbones. Such properties have been observed for inulin (Cooper, 1993; Vereyken et al., 2003) and leads to random coil structure formation in solution (Vereyken et al., 2003) and hydrophobic interactions with nonpolar molecules such as α-casein, β-casein and β-lactoglobulin (Esmaeilnejad Moghadam et al., 2019; Schaller-Povolny & Smith, 2002). As fructooligosaccharides are largely similar to inulin, but with lower number of repeating furanose units, it is likely that in our study, similar random coil structures incorporated β-carotene through non-covalent hydrophobic interactions with their backbones, thus protecting it from unfavourable conditions such as acidic pH during the gastric phase (Cooper, 1993; Vereyken et al., 2003). In addition, no carotenoid degradation products were observed in the chromatograms (Fig. 2), which is similar to a previous study that evaluated the loss of lutein, lycopene, and β-carotene through *in vitro* digestions (Kopec et al., 2017). Nevertheless, it is also possible that β-carotene derivatives could not be extracted or separated by the analytical method used in this study. Peak 8 in the supernatant chromatogram (Fig. 2) is probably not a β-carotene derivative produced upon *in vitro* digestion, but a xanthophyll. Unlike the extraction method of the raw material, no saponification step with xanthophyll losses (Rodriguez-Amaya, 2015) was applied during extraction of digests.

Most studies of encapsulated carotenoid microparticles evaluate the encapsulation efficiency, characterization of microparticles and storage stability. Instead, we performed bioaccessibility studies to assess β-carotene potentially available for further uptake by intestinal cells. Our results showed maximum β-carotene bioaccessibilities of 28 – 48 % for maltodextrin/ gum arabic microparticles (with or without inulin and fructooligosaccharides), whereas Liu et al. (2018) reported bioaccessibilities of up to 68 % for caseinate/alginate microparticles. Differences are probably related to the encapsulation material, although slightly different *in vitro* digestion conditions were also applied. Sun et al. (2018) also demonstrated the impact of matrix on fucoxanthin bioaccessibility, which was 46 – 58 % when using maltodextrin, gum arabic and whey protein isolate as encapsulating materials instead of hydroxypropyl-β-cyclodextrin, gelatin and isolated pea protein. As maltodextrin and gum arabic were part of all microparticles in our study, we may conclude that differences in bioaccessibility depended on the presence of prebiotics (i.e. inulin and fructooligosaccharides). Carotenoid encapsulation with prebiotics, such as inulin and cellobiose, has recently received some attention (Etzbach et al., 2020; Rivas et al., 2021), although their impact on carotenoid bioaccessibility was not described yet.

Inulin increased the β-carotene bioaccessibility to almost double compared to microparticles without prebiotics, thereby suggesting a particular role of inulin in the formation of mixed β-carotene and bile salts micelles during *in vitro* small intestinal digestion. These micelles are the result of the amphiphilic properties of bile acids that allow their self-association in water when the critical micellar concentration is exceeded (Pavlović et al., 2018). Also, micelle solubilisation capacity depends on the volume of the hydrophobic site in the micelle core; the higher the volume, the better the solubilisation of hydrophobic compounds (Dima et al., 2020). Since inulin possesses self-aggregating characteristics (Dan et al., 2009) and has hydrophobic sites (Vereyken et al., 2003), it may have been incorporated into mixed micelles, thus increasing the volume of the hydrophobic core of the micelle and, consequently, improving the amount of β-carotene that was solubilized within. This mechanism is supported by the larger spherical structures, possibly micelles, in the SEM images of the digests of inulin-containing microparticles (Fig. 3 H) compared to inulin-free microparticles (Fig. 3 D).

Higher bile salts concentrations during *in vitro* digestion significantly improved β-carotene bioaccessibility, and is consistent with other studies with carrots, spinach, meat, tomato paste, and from β-carotene enriched in canola oil (Garrett et al., 1999; Hedrén et al., 2002; Wright et al., 2008). Bile salts act mainly as emulsifiers of dietary fats when their critical micellar concentration of 2 mM is exceeded (Tyssandier et al., 2001), facilitating their further intestinal uptake (di Gregorio et al., 2019). In our study, 2 mM bile salts was sufficient to initiate the micelle formation but insufficient for a substantial transfer of β-carotene to the aqueous phase, as the β-carotene bioaccessibilities ranged between 8 and 13 %. At a physiological concentration of bile salts in the fed state (10 mM), the β-carotene bioaccessibility improved remarkably, especially that of the microparticles with inulin and fructooligosaccharides whose values reached 42 and 30 %, respectively. After this, although the effect of a higher concentration (15 mM) further improved bioaccessibilities, these values did not differ significantly from those achieved at 10 mM. This is in line with other studies in which an initial increase in β-carotene bioaccessibility was observed at bile salt concentrations above the critical micellar concentration, but this effect was less pronounced at the highest concentrations tested, >4 mM sodium glycodeoxycholate and > 10 mM sodium taurocholate (Rich et al., 2003), and 9 mM porcine bile extract (Garrett et al., 1999). Rich et al. (2003) associated this effect with the preferred solubilization of other compounds, such as lutein or lipids, rather than β-carotene. In our case, the micellization of β-carotene at 15 mM is possibly restricted by inulin or fructooligosaccharides, and therefore, we may carefully conclude that less bile salts (10 mM) are sufficient to complete the micellization of β-carotene equivalent to that obtained at a higher concentration (15 mM).

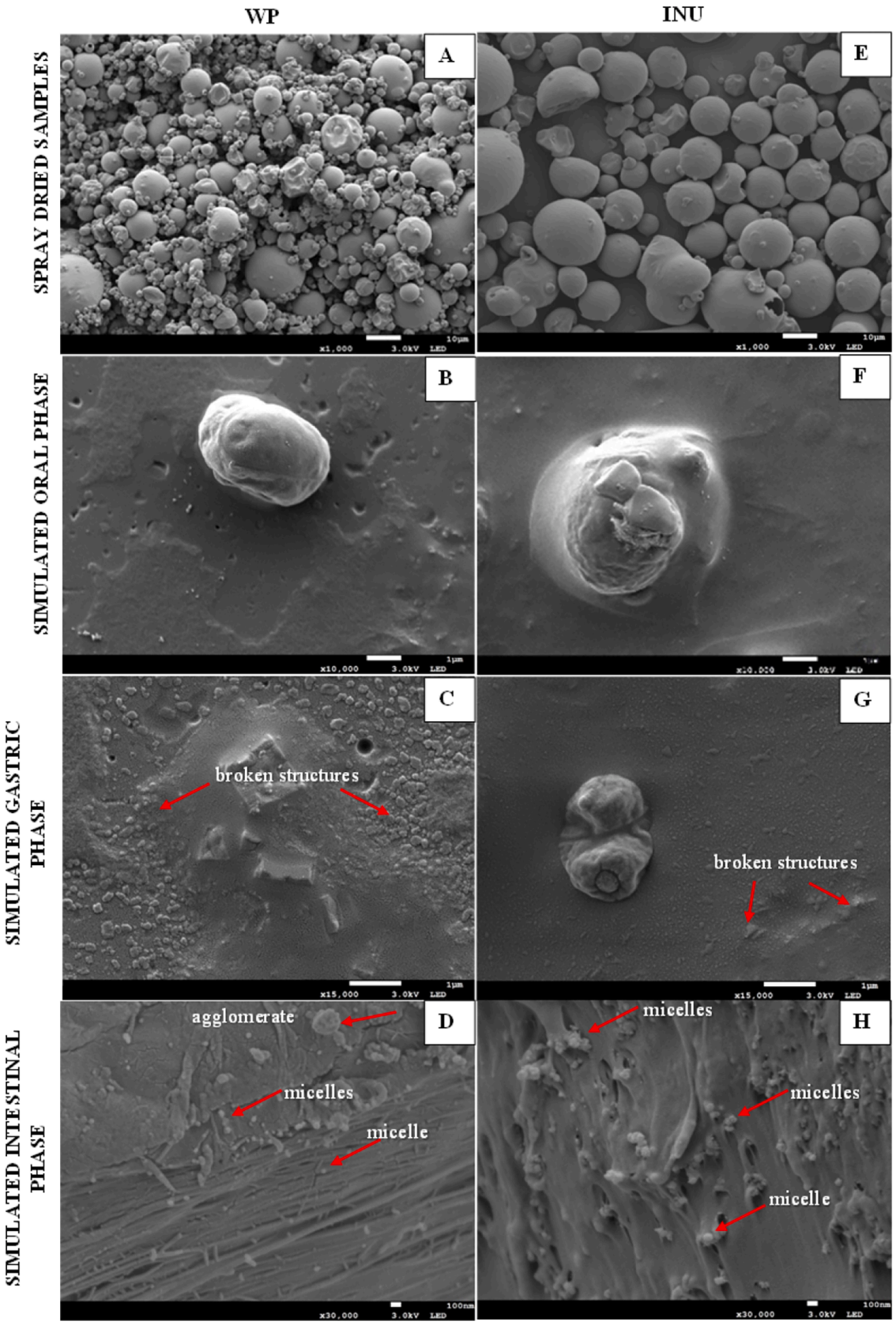

Fig. 3. Changes in morphology of microparticles during *in vitro* digestion. Spray dried samples (A, E) represent the samples before digestion; encapsulated carotenoid microparticles without inulin or fructooligosaccharides (WP) and encapsulated carotenoid microparticles with inulin (INU). The images of the simulated phases represent digested samples taken after oral phase (B, F), 1 h of gastric phase (C, G), 4 h of intestinal phase (D, H). Magnifications of samples were × 1 000 (A, E), × 10 000 (B, F), × 15 000 (C, G) and × 30 000 (D, H). Pictures D and H were visualized after slicing, and the spherical-like structures are presumably micelles.

Our results showed that filtration strongly decreased (21- to 29- fold) β-carotene amounts in the exposure medium, which is in line with studies using filters with pore sizes of 0.02 and 0.2 μm (Corte-Real et al., 2014). This may be due to the presence of β-carotene in structures of different shapes and sizes in the digest. Indeed, SEM images showed that at the end of the *in vitro* digestion, some spherical-like structures together with some agglomerates were present (Fig. 3 D, H). Bile salt micelles exhibit ellipsoidal, spherical and disc shapes (Kristo et al., 2015; Pabois et al., 2021), so we presume that some of the structures observed in SEM images are bile micelles. According to these observations and based on structures previously described after simulating intestinal conditions with and without lipolysis products (Fatouros et al., 2009), and also proposed after the *in vitro* digestion of β-carotene encapsulated in alginate-based hydrogel beads (Zhang et al., 2016), we hypothesize that β-carotene in our digest supernatant can be distributed over i) mixed micelles smaller than 0.22 μm, ii) micellar assemblies larger than 0.22 μm, iii) other types of colloidal structures, and iv) insoluble or crystallized structures (Fig. 4). Some particles (e.g. vesicles) would not be dense enough to be removed by the centrifugation step (Ozturk et al., 2015), but the filtration step may remove these larger particles containing β-carotene and other carotenoids, that are not expected to penetrate through the mucus layer. This mechanism is supported by the presence of fewer carotenoid peaks and the reduction of their areas in the chromatograms of the filtered samples (see Supplementary Material).

Carotenoid uptake was matrix-dependent only in unfiltered media, suggesting other possible cellular transport mechanisms of non-micellarized carotenoids, besides the concentration effect. In our study, the maximum β-carotene concentration tested was 0.44 μM, within the physiological range in which During et al. (2002) proposed an intestinal transport mediated by apical membrane proteins rather than passive diffusion, as traditionally believed. The scavenger receptor class B type I (SR-BI), the cluster determinant 36 (CD36) and the Niemann-Pick C1-Like 1 (NPC1L1) would participate in the β-carotene absorption, although their role is not yet clear (Bohn et al., 2019; Reboul, 2019). As, in contrast to unfiltered digests, no significant impact of inulin and fructooligosaccharides was observed in β-carotene cellular uptake after filtered digests incubation, we may hypothesize that the cellular uptake was determined by three different mechanisms. First, our results demonstrate that carotenoid concentration impacts cellular uptake, with a possible saturation effect at higher exposures, as reported by During et al. (2002) and O'Sullivan et al. (2007). Second, it is likely that transporter expression or other cellular events were impacted by the presence of inulin and/or fructooligosaccharides; although this was not tested, and no evidence from literature is present. Third, we suggest that specific relatively large structures (colloidal structures >0.22 μm), as visible in the SEM pictures, and β-carotene crystals may have restricted the matrix-dependent carotenoids uptake in the unfiltered samples, whereas the smaller particles that passed through the 0.22 μm filter, improved cellular bioavailability, especially in the inulin-containing digests. This latter process is supported by the number of carotenoid peaks in the chromatograms; in the cells exposed to filtered digests, lutein, presumably β-cryptoxanthin or zeaxanthin, and β-carotene were found, whereas a less variety in carotenoids was detected in cells exposed to unfiltered digests. Although this effect of filtration on uptake has not yet been described for carotenoids, it was reported for anthocyanins, as filtration favoured anthocyanin transport in a Transwell system with Caco-2 cells due to the reduced size (<0.25 μm) of the particles (Cardona et al., 2015).

Then, considering that cellular uptake of β-carotene was higher from the unfiltered digests, it is questioned whether samples filtration underestimates the bioaccessibility of β-carotene. Although applying methodologies that include filtration would lead to a better estimation of oral bioavailability due to particle size discrimination of carotenoid-

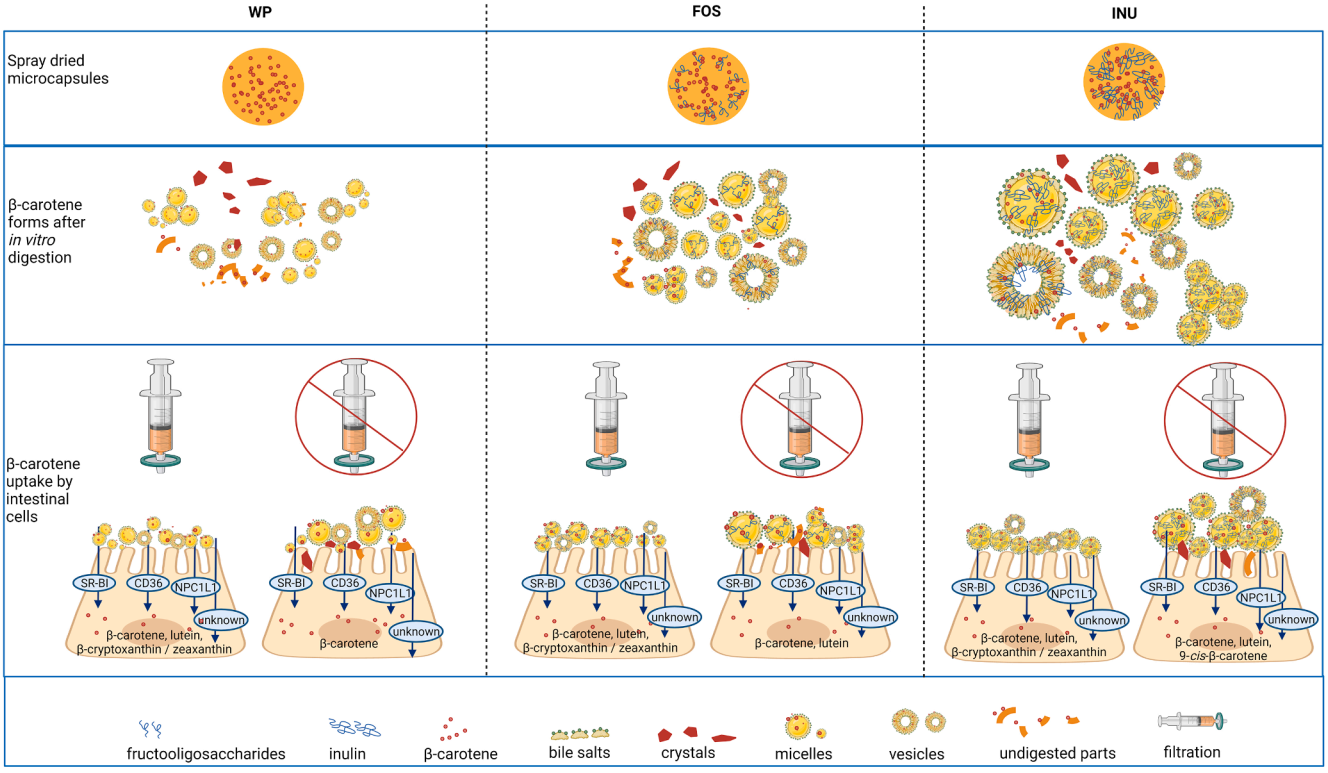

Fig. 4. Proposed assemblies associated with carotenoids, especially β-carotene, obtained after *in vitro* digestion of encapsulated carotenoid microparticles without prebiotics (WP), with fructooligosaccharides (FOS) and with inulin (INU). The panel with the intestinal cells shows: i) the possible facilitated diffusion via membrane proteins SR-BI, CD36, NPC1L1, or unknown proteins, ii) the interference of the unfiltered structures in the uptake, and iii) the carotenoids taken up by the Caco-2 cells and found in the chromatograms. Created with BioRender.com.

containing structures, those methods may be too stringent depending on the pore size selected for filtration. Including the filtration step may allow *in vitro* methodologies harmonization. However, it does not mimic *in vivo* carotenoid uptake since no filtration prior absorption happens in human organisms.

Future research could focus on the development of harmonized procedures for obtaining bioaccessible fractions prior carotenoid bioaccessibility analysis. This approach should also include an in-depth chemical and structural characterization of the micellar and particulate materials and aggregates originating from the microcapsules, and their subsequent contribution to the association to the cell membrane, impact on specific carotenoid transporter expression, and the mode-of-action on the selective uptake of specific carotenoid types.

4.1. Conclusions

We conclude that the *in vitro* bioaccessibility of β-carotene depends on the composition of the microparticles containing the carotenoid extract, and that especially inulin has the strongest impact on this process. Moreover, the impact of bile salts concentration on β-carotene bioaccessibility is stronger when inulin is incorporated in the microparticles, thereby indicating an effect of this prebiotic on solubilization. Although encapsulating material and the concentrations of bile salts did interfere with the bioaccessibility of β-carotene, these did not affect its chemical stability. Filtration had the highest impact on carotenoid presence in the exposure medium, and hence affected cellular β-carotene uptake through a concentration-dependent mechanism. Nevertheless, in unfiltered digests, β-carotene uptake was dependent on the presence of prebiotics in the microparticles, thereby pointing to the contribution of other factors to this process. Finally, the filtration of the exposure medium enhanced the relative amount of β-carotene accumulated in the cells, despite the lower concentration of β-carotene in the filtrated digest. Whether this finding is due to other compounds released during the *in vitro* digestion is not clear. Overall, this study showed that encapsulated carotenoid extracts from mango by-products improved both the chemical stability and bioaccessibility of β-carotene. Thus, those encapsulated materials could be considered as a potential source of provitamin A to be used for the development of novel functional foods.

CRediT authorship contribution statement

Katty Cabezas-Terán: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Visualization. **Charlotte Grootaert:** Methodology, Writing – review & editing, Supervision. **Johana Ortiz:** Writing – review & editing. **Silvana Donoso:** Writing – review & editing, Funding acquisition. **Jenny Ruales:** Resources. **Filip Van Bockstaele:** Methodology, Writing – review & editing. **John Van Camp:** Funding acquisition. **Tom Van de Wiele:** Conceptualization, Methodology, Writing – review & editing, Supervision.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2022.112301.

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