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Delivery of β-carotene to the *in vitro* intestinal barrier using nanoemulsions with lecithin or sodium caseinate as emulsifiers

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Abstract

To increase the intestinal delivery of dietary β-carotene, there is a need to develop nanostructured food systems to encapsulate this fat soluble bioactive. The aim of this study was to evaluate the bioacessibility and bioavailability across the intestinal barrier of β-carotene-enriched nanoemulsions stabilised with two emulsifiers (lecithin or sodium caseinate) by coupling an *in vitro* gastrointestinal digestion with two *in vitro* cell culture models (Caco-2 or co-culture of Caco-2/HT29-MTX). Nanoemulsions stabilised with lecithin had significantly higher β-carotene in the gastrointestinal digested micellar fraction, lower β-carotene in the Caco-2 (and Caco-2/HT29-MTX) apical compartment and significantly higher β-carotene in Caco-2 cellular content compared to β-carotene-enriched nanoemulsions stabilised with sodium caseinate. Finally, to assess anti-inflammatory activity of digested nanoemulsions, lipopolysaccharide stimulated macrophages were exposed to Caco- 2 basolateral samples with levels of TNF-α and IL-β, subsequently quantified. A TNF-α response from stimulated THP-1 macrophages was elicited by basolateral samples, regardless the emulsifier used to formulate nanoemulsions. This study demonstrated that β-carotene permeability is influenced by the food derived emulsifier used for stabilising nanoemulsions, indicating that composition may be a critical factor for β-carotene delivery.

Keywords: β-carotene, nanoemulsions, *in vitro* digestion, intestinal barrier

1. Introduction

β-carotene is a vitamin A precursor with poor water-solubility (0.0006 g/L at 25ºC). Positive health benefits associated with β-carotene consumption include lower incidence of cancer, cardiovascular diseases and degenerative disorders (Goralcyk, 2009). These health attributes have been related to β-carotene's antioxidant and immunomodulatory bioactivities, proven both *in vitro* (Bai et al., 2005) and *in vivo* (Zhou et al., 2018).

Although the recommended dietary allowance (RDA) for β-carotene has not been set, the U.S. Food and Drug Administration and the European Food Safety Authority derive a RDA for vitamin A of 900-700 μg of retinol activity equivalents (RAE) daily and a population reference intake of 750-650 μg REA daily, respectively. In addition, β-carotene appears to be degraded by the acidic environment of the stomach (Boon, McClements, Weiss, & Decker, 2010) which undoubtedly reduces β-carotene concentration in the intestine.

As a solution, recent studies have revealed the use of nanostructured delivery systems such as nanoemulsions to encapsulate and protect β-carotene after oral consumption and enhance its delivery to intestinal barrier (Chen, Li, Li, McClements, & Xiao, 2017; Gasa-Falcon et al., 2020; Yi, Zhong, Zhang, Yokoyama, & Zhao, 2015). Β-carotene enriched nanoemulsions stabilised with pectin, lecithin, sodium caseinate, Tween 20 or sucrose palmitate, have been subjected to *in vitro* gastrointestinal digestion (GID) and the subsequent release of β-carotene has been determined (Gasa-Falcon et al., 2019; Salvia-Trujillo et al., 2013; Teixé-Roig et al., 2020). However, less information is currently available about the use of either proteins or phospholipids-based emulsifiers on β-carotene transit across the intestinal barrier and subsequent bioactivity from nanoemulsions post gastrointestinal digestion (GID)**.** For instance, Lu, Kelly, & Miao (2017) described that the permeability of β-carotene in undifferentiated naïve gastrointestinal epithelial cells is depended on the emulsifier type added to the nanoemulsions rather than initial particle size of the nanoemulsions.

To model absorption *in vitro*, the use of differentiated monolayers expressing tight junctions, best represent the morpho-functional features of the intestinal barrier (Guri, Gülseren, & Corredig, 2013). However, *in vitro* monolayers present their own challenges since digestive fluids and nanoemulsions post GID (micellar fractions) are cytotoxic at relatively low concentrations (Arranz, Corredig, & Guri, 2016). As a result, quantification of compounds on the basolateral side can be challenging.

Thus, the aim of this study was to evaluate the permeability of β-carotene-enriched nanoemulsions stabilised with two different emulsifiers across 21 days differentiated Caco-2 and Caco-2/HT29-MTX co-cultures, post *in vitro* static GID. These 21 days old Caco-2/HT29-MTX co-cultures best represent the mature intestinal mucus barrier. Emulsifiers (lecithin and sodium caseinate) were selected based on their different properties (low (758 g/mol) and high (≈10-50 KDa) molecular weight), sources (synthetic and natural) and previous physiochemical characterisation of nanoemulsions stabilised with these emulsifiers in our group (Gasa-Falcon et al., 2019). Furthermore, the subsequent basolateral anti-inflammatory activity was assessed by quantification of TNF-α and IL-β in lipopolysaccharide (LPS) stimulated macrophages (THP-1 cells).

2. Materials and methods

2.1. Materials

Corn oil (Mazola, ACH Food Companies Inc., Memphis, TN) was purchased from a local market. β-carotene (synthetic, ≥93% (UV), powder) was sourced from Sigma–Aldrich (Ireland). Lecithin was obtained from Alfa Aesar (Karlsruhe, Germany). Sodium caseinate (NaCas) (≥92% purity) was from Acros Organics (Geel, Belgium). The Caco-2 cell line was purchased from the European Collection of Cell Cultures (ECACC 86010202) and the human monocyte THP-1 (ATCCTIB-202) and the human colon adenocarcinoma HT-29 cell lines (ATCCHTB-38) were purchased from American Type Culture Collection. This latter cell line was differentiated to HT-29-MTX following the protocol described by Guri et al. (2013). Tissue culture plastics were sourced from Sarstedt Ltd. (Wexford, Ireland). CellTiter 96 AQueus One Solution reagent was purchased from Promega (MyBio, Kilkenny, Ireland). Milli-Q water was used to prepare all nanoemulsions. All other chemicals were sourced from Sigma–Aldrich (Ireland) unless specified otherwise.

2.2. Preparation of nanoemulsions

Primary emulsions were prepared by mixing 4% (w/w) of the lipid phase (corn oil enriched with 0.5% w/w of β-carotene) with 96% (w/w) of the aqueous phase containing the emulsifier (lecithin or NaCas) at 2% (w/w). Both phases were mixed with an Ultra-Turrax (IKA, Staufen, Germany) at 9500 rpm for 3 minutes. Then, primary emulsions were passed through an APV 1000 (SPX Flow Technology, Charlotte, NC, USA) at 500 bars for 3 cycles to obtain nanoemulsions.

2.3. Determination of nanoemulsions properties

Particle size of nanoemulsions was determined using a Mastersizer 3000 (Malvern Instruments Ltd, Worcestershire, UK). The results were reported as the surface mean diameter (d_{43} (µm)) and the width of the distribution. The refractive index of the corn oil and water employed to perform the analysis were 1.34 and 1.33, respectively.

The emulsions ζ-potential was determined using a Zetasizer NanoZS (Malvern Instruments Ltd, Worcerstershire, UK). Samples were previously diluted (1/100) and equilibrated prior to analysis.

Physical stability of β-carotene enriched nanoemulsions was determined with an analytical centrifuge LUMiSizer 6112 (L.U.M. GmbH, Berlin, Germany) that accelerates destabilisation of samples. Results were analysed using the software package SEPView 6.0 (L.U.M. GMBH) that records transmitted light across the sample length and calculates the instability index that ranges from 0 to 1, with the greatest instability at 1. Instrumental parameters used for physical stability analysis were: speed 2,186 rcf; time interval 20 seconds; exposure time 10,000 seconds; temperature 25ºC.

2.4. *In vitro* static simulated digestion

Nanoemulsions were subjected to a simulated *in vitro* static GID (gastric and upper intestinal phases) that mimics the adult human upper gut. The INFOGEST standardised method (Brodkorb et al., 2019) was followed with minor modifications. Briefly, gastric phase consisted of 5 mL of nanoemulsion with simulated gastric fluid containing porcine pepsin (EC 3.4.23.1) (3925.3 U/mg); pH was adjusted to 3.0 using HCl (1 M) and volume to 10 mL (Milli-Q water). The mixture was incubated for 2 h at 37°C with continuous shaking in a rotator. After 2h, pH was increased to 6.5 using NaOH and 20 µL $CaCl_2$ (0.3 M), 4 ml bile (630 g/mol, EC232-369-0), and 2.5 mL pancreatin based on trypsin activity (8.13 U/mg; EC232.468.9) were added. Based on Verkempinck et al. (2017), extra lipase (pancreatin and lipase) was added to reach 420 U/mL. The pH of the mixture was adjusted to 7.0, the volume to 20 mL with milli-Q water, and the mixture was incubated for 2 h at 37°C. The digestion was then stopped by adding protease inhibitor 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (1 mM). To obtain the micellar fraction, the digested fractions were centrifuged (Heraeus Megafuge 1.0, Massachusetts, USA) at 2890 x g for 40 minutes at 4 °C (Garrett, Failla, Sarama, &Craft, 1999). Samples were stored at −80°C for further experiments.

2.5. *In vitro* cell based assays

Caco-2 and HT29-MTX cell lines were grown in 75 cm^2 tissue culture flasks in a humidified 37 °C incubator with a 5% CO_2 air atmosphere. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin. At 80% confluency, cells were trypsinated (0.25% trypsin/EDTA), diluted 1:6 in DMEM medium, and reseeded in flasks. Caco-2 and HT29-MTX cell lines in this study were used at passage number 29-41 and 53-67, respectively.

2.6. Cytotoxicity of micellar fractions

Caco-2 cells were seeded at a density of 8 × 10^4 cells/well in 96-well plate. After 24 h of incubation, cells were washed with PBS. GID micellar fractions were filtrated (0.45 µm), diluted in complete DMEM (between 2-16% v/v) and 80 µL were added to each well. Subsequently, 20 µL of CellTiter 96® AQueous One Solution Cell Proliferation Assay reagent was added to each well and cells were incubated for 2 h. After 2 h, the quantity of formazan produced was measured spectrophotometrically at 490 nm in a microplate reader (Synergy HT BioTek, Winooski, VT, USA). Results were expressed as the percentage of cellular viability relative to a control group (cells with DMEM medium) versus the micellar fraction concentration (%, v/v). Cytotoxicity of pure β-carotene dissolved in DMSO was also evaluated (0.05-10 µg/mL).

2.7. Permeability across intestinal barriers

Caco-2 cells were seeded at a density of 6 × 10^4 cells per insert in 12-well Transwell® plates (0.4 µm pore size, 1.2 cm diameter, Costar, Cambridge, MA). In co-culture experiments, Caco-2 and HT29-MTX were grown separately and then seeded at a ratio of 75:25, to a final density 6 × 10^4 cells per insert.

Culture media of each plate was changed every two days for 21 days. The integrity of the cell monolayer was monitored by measuring the transepithelial electrical resistance (TEER) ($\Omega\cdot cm^2$) using a Millicell-ERS Voltohmmeter (Merck Millipore, Carrigtwohill, County Cork, Ireland). On day 21, apical and basolateral compartments were washed three times with PBS and 470.6 µL and 1500 µL DMEM were added to apical and basolateral compartments, respectively. Then, micellar fractions (29.4 µL) were added to apical compartment and incubated for 2 h. During this 2 h, the TEER value did not change significantly (data not shown).

After permeability experiment, apical and basolateral samples were collected. Moreover, cell monolayer was washed three times with PBS, scraped and collected. Cells were centrifuged (Heraeus Megafuge 1.0) for 3 min at 215 x g and the supernatant discarded. Cells were stored at -80 ºC for further analysis.

2.8. Determination of β-carotene

Extraction of β-carotene from samples (apical, cells and basolateral) was performed as described by Yuan, Gao, Zhao, & Mao (2008) with minor modifications. Briefly, the samples were filtrated (0.45 µm) and mixed with ethanol and hexane, followed by a centrifugation (2890 x g, 5 min, 5ºC) (Heraeus Megafuge 1.0). The upper fraction was collected, dried under N_2 and stored at -80 ºC. Each sample extract was dissolved in 200 µL of the injection solvent acetonitrile (ACN): methanol (MeOH) 7:3 (v/v): acetone 6.7:3.3 (v/v) and filtered through 0.2 µm nylon filters (Millipore, Bedford, MA). High-performance liquid chromatography system (Waters Xevo TQ-S, Milford, USA) equipped with a photodiode array detector (HPLC-PDA) at 450 nm and a column ACQUITY UPLC® (C18 BEH 130 Å, 1.7 µm, 2.1 × 150 mm) (Waters) (30°C and flow rate 0.85 mL/min) were used. . Mobile phase consisted of solvent A: ACN: MeOH 7:3 (v/v) and solvent B: water 100%, and the flow was isocratic (100% ACN/MeOH 7/3). β-carotene was quantified by comparison with external standards. Results were reported as ng/mL and cellular uptake as percentage of detected β-carotene in cells versus apical samples at time 0 h.

2.9. Anti-inflammatory activity of basolateral samples

Human monocytes THP-1 were cultured in RPMI 1640 culture medium supplemented with 10% (v/v) FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37 °C in a 5% CO_2 humidified atmosphere. Cells were added at a density of 5 x 10^5 cells/mL in 24 well plates. Differentiation to macrophages was induced by adding 1 µg/µL 12-O-tetradecanoyl phorbol-13-acetate (TPA) to cells followed by 48 h incubation. After differentiation to macrophages, cells were washed with PBS. LPS (0.05 µg/mL) and Caco-2 basolateral samples were added to each well. After 24 h incubation, the culture medium was collected for subsequent TNF-α and IL-1β quantification. Release of TNF-α and IL-1β was measured in the supernatants of THP-1 cells using ELISA kits (R&D Systems, Minneapolis, USA), according to manufacturer’s instructions. Multiscanner autoreader (Synergy HT BioTek) was used to read the absorbance of the plates at 450 nm.

2.10. Statistical analysis

Experiments were performed in triplicate on at least two different days and data was expressed as the mean with standard deviation. To determine the statistically significant differences between samples, one-way ANOVA followed by Bonferroni test ($p≤0.05$) was conducted with SigmaPlot 11.0.

3. Results and discussion

3.1. Physicochemical properties of nanoemulsions

Both nanoemulsions containing lecithin and NaCas had a monomodal particle size distribution (Fig. 1A) and exhibited particle sizes in the nanometer range (0.35 and 0.29 µm, respectively) (Table 1), in line with our previous published results (Gasa-Falcon et al., 2019). Nanoemulsions had negative ζ-potential values, with lecithin displaying the highest negative value (Table 1).

NaCas-stabilised nanoemulsions exhibited the lowest end point instability index (0.603 ± 0.006) compared with lecithin-stabilised nanoemulsions (0.773 ± 0.001) (Fig. 1B).

Emulsifiers with a low molecular weight and/or with a high hydrophilic–lipophilic balance (HLB) are associated with a high efficiency at producing small particle sizes in oil-in-water emulsions (Jo & Kwon, 2014). Lecithin has an HLB value of 8 while NaCas has a value of 14. Thus, the intermediate-low HLB value of lecithin could explain why nanoemulsions stabilised with this emulsifier exhibited the highest particle size and instability index compared to nanoemulsions with NaCas (Iyer et al., 2015). In addition, the mass of NaCas (≈10-50 KDa) (Ozturk & McClements, 2016), its gelation behaviour (Rodriguez-Patino & Pilosof, 2011) and the thick interfacial layer covering oil droplets (McClements et al., 1993) undoubtedly contributed to its nanoemulsion stability over the accelerated centrifugation process. The large negative ζ-potential values observed for lecithin and NaCas nanoemulsions (-58.81 mV and -53.41 mV, respectively) could be attributed to the phospholipid head groups from lecithin, and the fact that the nanoemulsion pH of ≈6.5 differs to the NaCas isoelectric point (pI=4.6) respectively (Chang & McClements, 2016). Interestingly, ζ-potential of lecithin stabilised β-carotene nanoemulsions became less negative after *in vitro* GID, while NaCas emulsions post GID have a stronger negative value (Gasa-Falcon et al., 2019). In that previous study, particle size after *in vitro* GID in lecithin nanoemulsions was higher compared to NaCas.

3.2. Cytotoxicity of micellar fractions

The maximum non-toxic concentration of micellar fractions was different depending on the emulsifier used (Fig. 2). Micellar fractions with lecithin showed no cell toxicity (>90% cell viability) when Caco-2 cells were exposed to concentrations below 6% (v/v) (Fig. 2A), while for those containing NaCas no cell toxicity was observed at concentrations under 10% (v/v) (Fig. 2B).

In agreement to our results, several studies have demonstrated that emulsifiers are cytotoxic in a concentration-dependent manner and that toxicity of nanoemulsions depends on the nature of emulsifier employed (Buyukozturk, Benneyan, & Carrier, 2010; Ujhelyi et al., 2012). Furthermore, Sadhukha, Layek & Prabha (2018) observed that the aqueous fraction of digested lipid-based delivery systems was responsible for cytotoxicity in MDCK kidney cells, reducing cell viability by 40%. It has been previously reported that monoglycerides induce dose-dependent apoptosis in mammalian cells (murine thymocytes), which consisted on a rapid reduction in mitochondrial transmembrane potential, production of reactive oxygen species, among other processes (Philippoussos, Arguin, Fortin, Steff, & Hugo, 2002). In our study, micellar factions of nanoemulsions were likely to contain lipid digestion products (i.e. free fatty acids and monoglycerides), and together with emulsifiers contributed to damage the cell integrity.

Similar cell viability results were obtained when control micellar fractions (without β-carotene) were tested in Caco-2 cells, but interestingly control micellar fractions derived from lecithin-stabilised nanoemulsions were 60% less toxic compared to micellar fractions with β-carotene. This suggests that β-carotene plays a role in cell cytotoxicity of Caco-2 monolayers. Indeed, Wooster et al. (2017) observed that the presence of β-carotene in LCT nanoemulsions (IC_{50}= 51 µg/mL) increased four times their toxicity in differentiated Caco-2 cells compared to empty nanoemulsions (IC_{50}= 257 µg/mL). In contrast, a preliminary study by our laboratory indicated that pure β-carotene present in nanoemulsions was not cytotoxic to undifferentiated Caco-2 cells (between 0.05 µg/mL and 10 µg/mL) (data not shown), suggesting that Caco-2 cell monolayers with tight junctions are more sensitive to GID β-carotene-enriched nanoemulsions than undifferentiated Caco-2 cells. Certainly, oxidation products of β-carotene could have been generated during *in vitro* GID, specifically due to the acidic pH of the gastric phase (Failla, Chitchumronchokchai, Ferruzzi, Goltz, & Campbell, 2014). Oxidation of β-carotene can produce carotenoid aldehyde breakdown products, which have documented toxic effects on numerous cell lines (K562, RPE 28 SV4 and ARPE-19) at concentrations between 10–20 µM (Hurst, Saini, Jin, Awasthi, & Van Kuijk, 2005).

To investigate the bioavailability of β-carotene from nanoemulsions, permeability experiments were performed with lecithin and NaCas micellar fractions at a concentration of 6% (v/v).

3.3. Permeability of β-carotene nanoemulsions

After *in vitro* GID, β-carotene concentration present in the micellar fractions was significantly higher in lecithin nanoemulsions compared to those prepared with NaCas (Table 2). There was a significant reduction of β-carotene in apical compartment after 2 h incubation compared to time zero regardless of emulsifier used. Interestingly after 2 h incubation, the apical of NaCas-stabilised nanoemulsions had a significantly higher amount of β-carotene compared to lecithin-stabilised nanoemulsions. β-carotene content in cell lysates of Caco-2 cells was 3 times higher than in Caco-2/HT29-MTX co-cultures. In addition, the β-carotene concentration in Caco-2 cells lysates was significantly higher in lecithin-stabilised nanoemulsions (2.28%) compared with nanoemulsions containing NaCas (1.72%). Concentration of β-carotene was significantly lower in Caco-2/HT29-MTX co-cultures cell lysates (≥0.74%), with no significant differences between both emulsifiers.

The reason why NaCas nanoemulsions had less β-carotene in the micellar fraction, the apical sample at time zero and the cell lysate (Caco-2) may be explained by the fact that proteins such as NaCas, can interact hydrophobically with carotenoids and create complexes that act as a physical barrier for gastrointestinal digestive enzymes access (Wackerbarth, Stoll, Gebken, Pelters, & Bindrich, 2009). Hence, β-carotene may have remained entrapped within these complexes and not solubilised within mixed micelles, a process which is essential for permeability across the intestinal barrier (Baskaran, Sugawara, & Nagao, 2003). Moreover, Yang, Decker, Xiao, & McClements (2015) observed that the addition of 36 mg phospholipids (eg. 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine) within the digestive fluids increased the degree of lipid digestion after *in vitro* GID of vitamin E emulsions. This may explain why lecithin-nanoemulsions had higher concentrations of β-carotene within the micellar fraction. Another study observed that the maximum cellular uptake of carotenoids (β-carotene and lutein) in differentiated Caco-2 cells was obtained when micelles contained 50 µmol/L of lysophosphatidylcholine (phospholipid derived from phosphatidylcholine present in lecithin) (Sugawara et al., 2001).

In agreement with the present study, Li. Arranz, Guri & Corredig (2017) reported a lower permeability of β-carotene from liposomes using 21-day old Caco-2/HT29-MTX co-cultures compared to Caco-2 monolayers. Interaction with mucus produced by HT29-MTX cell line reduces permeability of mucoadhesive lipophilic molecules, such as β-carotene (Sigurdsson, Kirch, & Lehr, 2013). Co-culturing Caco-2 cells with HT-29MTX adds a further layer of mucus complexity to more closely resemble the *in vivo* environment (Arranz. Corredig & Guri, 2016), but reduces permeability rates which may hamper compound detection. Thus, in our study, the use of Caco-2/HT29-MTX cell line resulted in lower recovery of β-carotene in the cell lysates regardless of the emulsifier.

The failure to detect β-carotene in basolateral compartment underlined the limitations of the experiment due to upper concentration limits imposed by cytotoxicity data and inadequate sensitivity of detection instrumentation. Also, β-carotene may not have arrived at the basolateral within the 2 h incubation period.

3.4. Immune functionality of basolateral samples

Basolateral samples from permeability assays with β-carotene NaCas nanoemulsions and β-carotene lecithin nanoemulsions significantly increased TNF-α secretion (112% and 124% respectively, $p<0.05$) compared to basolateral sample control (positive control =2291±138 pg/mL of TNF-α) from LPS activated THP-1 macrophages (Fig. 3A). However, IL-1β levels were unchanged regardless of basolateral samples (4769±145 pg/mL) (Fig. 3B).

Previous studies have demonstrated that β-carotene reduces levels of TNF-α and IL-1β levels secreted from LPS-stimulated RAW264.7 cells (murine macrophage cell line) and from LPS-treated peritoneal macrophages (Li, Hong, & Zheng, 2019) as well as from serum of BALB/c mice, intraperitoneally injected with β-carotene (10 mg/kg) plus LPS (4 mg/kg) (Bai et al., 2005). This discrepancy with our results may be explained by the bypass of the gut and the use of different test material (β-carotene alone versus basolateral samples of Caco-2 monolayers treated with β-carotene-enriched nanoemulsions). Applying β-carotene directly to LPS stimulated THP-1 cells will dose dependently reduce secreted levels of TNF-α (data not shown). It is important to note that our results do not confirm the presence of β-carotene or metabolites in the basolateral compartment. It is possible that other GID components in the micellar fraction may be capable of modulating TNF-α, although previous studies have shown that lecithin, sodium caseinate or emulsions with different fatty acid composition do not up-regulate cytokine production (Mukhopadhya et al., 2014, Reimund et al., 2004, Treede et al., 2009). However, β-carotene can be metabolised to high molecular weight products (β-apo-8'-carotenal, β-apo-10'-carotenal, β-apo-12'-carotenal, β-apo-14'-carotenal, β-apo-15'-carotenal) and short-chain products (hcyclocitral, β-ionone, ionene, 5,6-epoxi-β-ionone, dihydroactinidiolide and 4-oxoionone) (Siems et al., 2005), which themselves may directly or indirectly act as pro-inflammatory agents (Yeh, Wang, Chen, & Wu, 2009).

4. Conclusions

Bioaccessibility of GID β-carotene in a Caco-2 model was enhanced when β-carotene-enriched nanoemulsions were stabilised with lecithin compared to those stabilised with NaCas. Caco-2 basolateral samples from both nanoemulsions elicited a TNF-α response from stimulated THP-1 macrophages. This study elucidates the importance of nanoemulsion composition for *in vitro* cellular permeability assays and the hurdles faced by concentration limits. Nanostructured food systems using lecithin as emulsifier might be a potential tool to increase uptake of dietary β-carotene.

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Table 1. Particle size (µm) and ζ-potential (mV) of nanoemulsions stabilised with different emulsifiers (LE: lecithin; NaCas: sodium caseinate). Differences among nanoemulsions were compared using one-way ANOVA followed by Bonferroni test. Different letters indicate statistically significant differences within the parameter tested (*p*<0.05).

Table 2. β-carotene concentration (ng/mL) quantified by high-performance liquid chromatography (HPLC-PDA) in micellar fractions (after *in vitro* digestion), apical samples and basolateral samples from permeability experiments after 2h incubation with micellar fractions obtained after *in vitro* digestion of nanoemulsions stabilised with lecithin (LE) and sodium caseinate (NaCas). ND = not detected, i.e., below detection limit of 10 ng/ml. Within a row, different lowercase letters indicate statistically significant differences (*p*<0.05) between emulsifiers. For an emulsifier, a statistical difference between apical t=0 and apical t=2h is denoted by *. Statistical analysis was performed using one-way ANOVA followed by Bonferroni test (**p*<0.05).

Fig 1. (A) Particle size distribution and (B) instability profile of β-carotene-enriched nanoemulsions (0.02% β-carotene w/w, 4% corn oil w/w) stabilised with 2% of lecithin (LE) or NaCas: sodium caseinate (NaCas).

Fig 2. Cell viability (%) of Caco-2 cells after 2h incubation with micellar fractions (with β-carotene) (% v/v) and control micellar fractions (without β-carotene) (% v/v) obtained after *in vitro* digestion of nanoemulsions stabilised with different emulsifiers. Micellar fractions containing lecithin (A) and NaCas (B) were diluted with complete DMEM. Control cells (Ctrl) were grown in media with no treatment (100% viability). Different uppercase and lowercase letters indicate significant differences to control cells for micellar fractions and control micellar fractions, respectively. Statistical analysis was performed using one-way ANOVA followed by Bonferroni test (**p*<0.05). Percentage of cell viability above 80% was considered as non-cytotoxic.

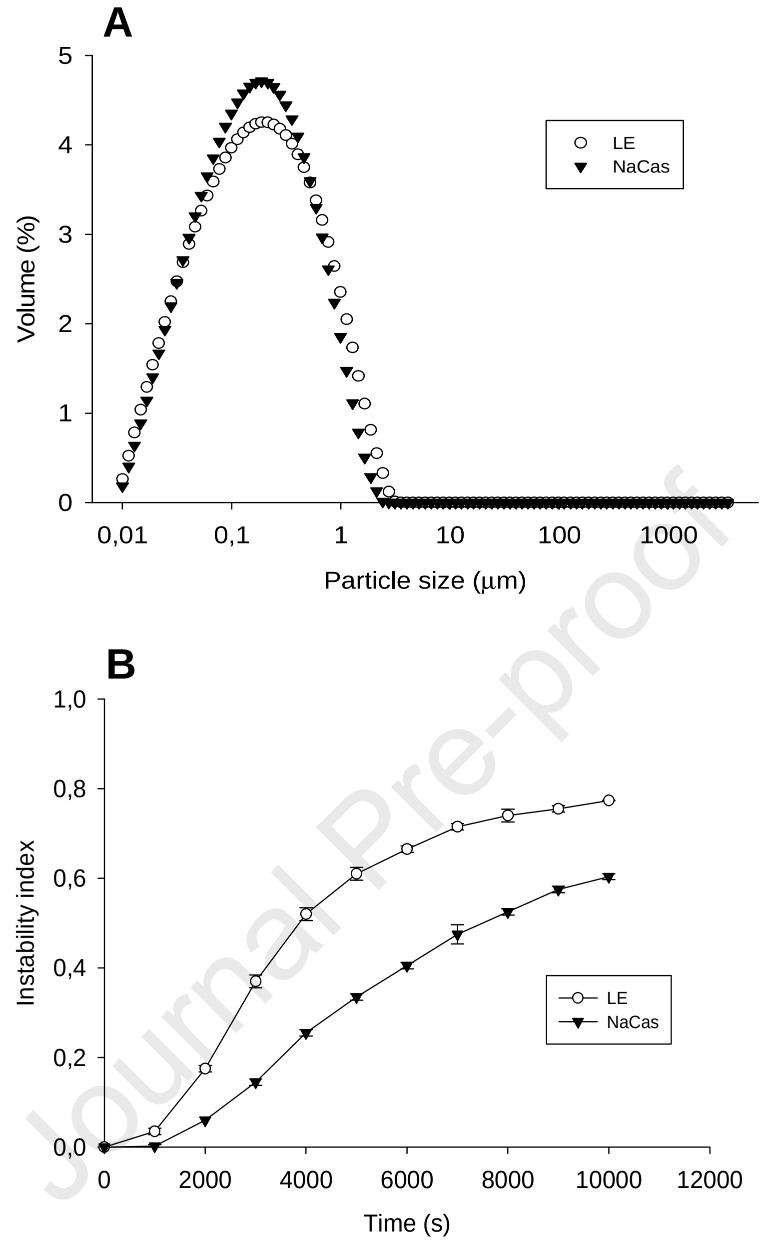
Fig 3. Effects of basolateral samples resulted from permeability experiments with β-carotene-enriched nanoemulsions emulsified with either 2% sodium caseinate (NaCas+) or lecithin (LE+) on the secretion (% ± SEM) of TNF-α (A) and IL-1β (B) in lipopolysaccharide (LPS)-simulated THP-1 cells. Positive controls (Ctrl +) were LPS-stimulated THP-1 cells and negative controls (Ctrl -) were non-stimulated THP-1 cells. Both controls were incubated with basolateral samples collected from control Caco-2 monolayers. Different letters indicate significant differences. Statistical analysis was performed using one-way ANOVA followed by Bonferroni test (*$p<0.05$).

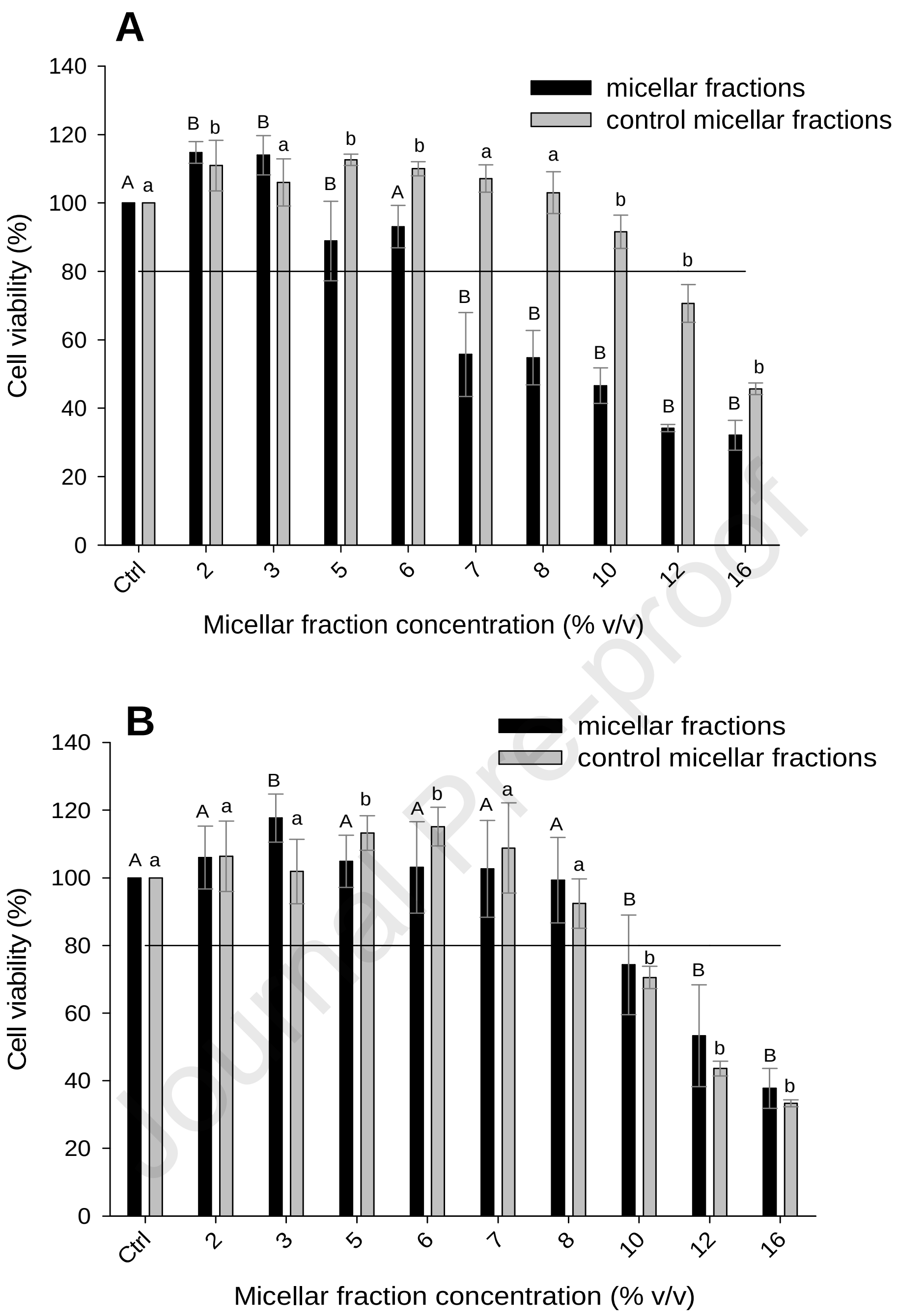
Table 1.

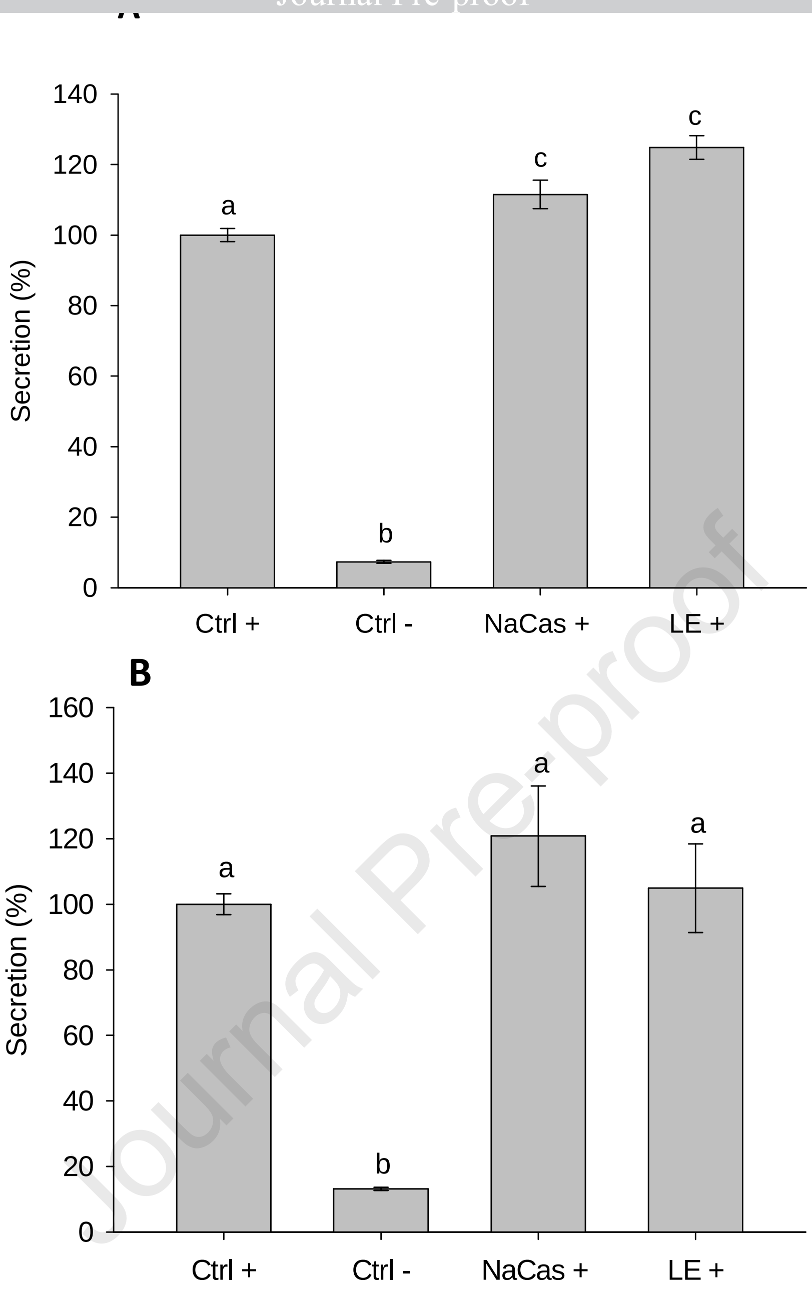
Emulsifier	Particle size (μm)	ζ-potential (mV)
LE	0.35 ± 0.001[a]	-58.81 ± 2.56[a]
NaCas	0.29 ± 0.001[b]	-53.41 ± 1.83[b]

Table 2.

	Caco-2 model (ng/mL)		Caco-2/HT29-MTX model (ng/mL)	
Emulsifier	**LE**	**NaCas**	**LE**	**NaCas**
Micellar fraction	36430 ± 665^{a}	32858 ± 70^{b}	37370 ± 521^{a}	32759 ± 102^{b}
Apical t=0h	$2171 \pm 39^{a*}$	$1930 \pm 4^{b*}$	$2198 \pm 40^{a*}$	$1927 \pm 6^{b*}$
Apical t=2h	251 ± 14^{a}	339 ± 65^{b}	263 ± 36^{a}	365 ± 44^{b}
Cells	49.6 ± 5.4^{a}	33.2 ± 8.4^{b}	15.1 ± 5.7^{c}	18.1 ± 11.2^{c}
Basolateral	ND	ND	ND	ND


A
Volume (%)
0
1
2
3
4
5
0,01
0,1
1
10
100
1000
Particle size (μm)
LE
NaCas
B
Instability index
0,0
0,2
0,4
0,6
0,8
1,0
0
2000
4000
6000
8000
10000
12000
Time (s)
LE
NaCas


A
micellar fractions
control micellar fractions
Cell viability (%)
0
20
40
60
80
100
120
140
A a
B b
B a
B b
A b
B a
B a
B b
B b
B b
Ctrl
2
3
5
6
7
8
10
12
16
Micellar fraction concentration (% v/v)
B
micellar fractions
control micellar fractions
Cell viability (%)
0
20
40
60
80
100
120
140
A a
A a
B a
A b
A b
A a
A a
B b
B b
B b
Ctrl
2
3
5
6
7
8
10
12
16
Micellar fraction concentration (% v/v)


A
Secretion (%)
0
20
40
60
80
100
120
140
a
b
c
c
Ctrl +
Ctrl -
NaCas +
LE +
B
Secretion (%)
0
20
40
60
80
100
120
140
160
a
b
a
a
Ctrl +
Ctrl -
NaCas +
LE +

Highlights

- Permeability of β-carotene was assessed using *in vitro* cell culture models.
- Bioaccessibility of β-carotene was enhanced with lecithin-stabilised nanoemulsions
- Nanoemulsions elicited a TNF-α response from stimulated THP-1 macrophages
- This study elucidates the hurdles faced by concentration limits

The authors declare that there are no conflicts of interest.