Received: 27 January 2024 | Revised: 3 April 2024 | Accepted: 25 April 2024

DOI: 10.1111/1750-3841.17113

Journal of Food Science WILEY

ORIGINAL ARTICLE

Integrated Food Science

Performance of β-carotene-loaded nanostructured lipid carriers under dynamic in vitro digestion system: Influence of the emulsifier type

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Funding information
Fundação de Amparo à Pesquisa do Estado de São Paulo; Sao Paulo Research Foundation (FAPESP), Grant/Award Numbers: #2018/03172-0, #2019/05176-6, #2019/27354-3, #2021/02271-8; Iceland Liechtenstein Norway Grants, Grant/Award Number: PT-INNOVATION-0105; Foundation for Science and Technology (FCT), Grant/Award Numbers: 2022.00788.CEECIND/CP1718/CT0024, UIDB/04469/2020, SFRH/BD/147286/2019, SFRH/BD/140182/2018; LABBELS - Associate Laboratory in Biotechnology, Bioengineering and Microelectromechnaical Systems, Grant/Award Number: LA/P/0029/2020

Abstract: A better understanding of how emulsifier type could differently influence the behavior of nanostructured lipid carriers (NLC) under the gastrointestinal digestion process, as well as at the cellular level, is of utmost importance for the NLC-based formulations' optimization and risk assessment in the food field. In this study, NLC composed by fully hydrogenated soybean and high-oleic sunflower oils were prepared using soy lecithin (NLC L_β) or Tween 80 (NLC T_β) as an emulsifier. β-Carotene was entrapped within NLC developed as a promising strategy to overcome β-carotene's low bioavailability and stability. The effect of emulsifier type on the digestibility of β-carotene-loaded NLC was evaluated using an in vitro dynamic digestion model mimicking peristalsis motion. The influence of β-carotene-loaded NLC on cell viability was assessed using Caco-2 cells in vitro. NLC T_β remained stable in the gastric compartment, presenting particle size (PS) similar to the initial NLC (PS: 245.68 and 218.18 nm, respectively), while NLC L_β showed lower stability (PS > 1000 nm) in stomach and duodenum phases. NLC T_β also provided high β-carotene protection and delivery capacity (i.e., β-carotene bioaccessibility increased 10-fold). Based on the results of digestion studies, NLC T_β has shown better physical stability during the passage through the in vitro dynamic gastrointestinal system than NLC L_β. Moreover, the developed NLC did not compromise cell viability up to 25 μg/mL of β-carotene. Thus, the NLC developed proved to be a biocompatible structure and able to incorporate and protect β-carotene for further food applications.

KEYWORDS
carotenoids, cell viability, dynamic in vitro gastrointestinal system, lipid nanoparticles

Practical Application: The findings of this study hold significant implications for industrial applications in terms of developing nanostructured lipid carriers from natural raw materials widely available and used to produce other lipid-based products in the food industry, as an alternative to synthetic ones. In this respect, the β-carotene-loaded NLC developed in this study would find a great industrial application in the food industry, which is in constant search to develop functional foods capable of increasing the bioavailability of bioactive compounds.

1 | INTRODUCTION

In recent years, significant efforts have been made on the development of new functional foods mainly due to the health benefits associated with their consumption (Fathi et al., 2013; Salvia-Trujillo, Verkempinck, Zhang, et al., 2019; Zardini et al., 2018). In this regard, the incorporation of carotenoids as bioactive components into functional foods has been raising great interest in the food industry. Among carotenoids, β-carotene is considered one of the most nutritionally active compounds, presenting a high provitamin A activity and antioxidant activity, which can block free radical-mediated reactions and reduce the risk of many disorders, such as cancer and cardiovascular diseases (Rehman et al., 2020; Yuan et al., 2008). However, the same beneficial β-carotene functional properties also create some challenges, namely, its fast degradation once incorporated into food products.

To fulfill its biological functions, β-carotene needs to be bioaccessible, that is, it must be released from the food matrix and incorporated into mixed micelles for absorption in the gastrointestinal tract (GIT) and thus be able to be absorbed by the intestinal cell epithelium, becoming bioavailable (Jafari et al., 2017; Kopec & Failla, 2018; McClements et al., 2015). β-Carotene incorporation within a lipid phase before digestion can considerably increase β-carotene bioaccessibility as it facilitates its transfer to the micellar phase during digestion (Salvia-Trujillo et al., 2017). So, lipid-based delivery systems can be used as an approach to encapsulate β-carotene, improving their stability and bioaccessibility (Gomes et al., 2019; Salvia-Trujillo et al., 2017).

Composed of a binary mixture of a solid lipid and a spatially distinct liquid lipid, nanostructured lipid carriers (NLC) present a great potential to increase the β-carotene bioaccessibility. In this type of nanocarrier, the bioactive compounds are dissolved in the lipid phase with the lowest melting point and are then incorporated into the less organized structures of the lipid matrix with the highest melting point (solid). The oil incorporation in the solid matrix leads to the formation of an amorphous nanostructure with many imperfections in its matrix, which allows a greater space for bioactive compounds' incorporation. The type of lipid matrix used to obtain NLC directly influences (i) the bioaccessibility of the encapsulated lipid-soluble bioactive compounds (Tan & McClements, 2021), (ii) the solubilization capacity of mixed micelles (Salvia-Trujillo et al., 2013a), and (iii) the oxidative stability of chemically labile bioactive compounds during food processing and storage, as well as during their passage through the GIT (Tan & McClements, 2021). High-oleic oils, such as high-oleic sunflower oil (HOSO), have demonstrably higher oxidative stability values when compared to oils with high polyunsaturated fatty acids content (O'Brien, 2008; Oliveira et al., 2016). Thus, the use of this lipid matrix to obtain NLC can avoid chemical transformations, allowing higher β-carotene protection and delivery in GIT. Fully hydrogenated vegetable oils, such as fully hydrogenated soybean oil (FHSO), can be used as a high-melting-point lipid matrix to obtain NLC, mainly due to high stearic acid (C18:0) levels, which have a neutral atherogenic effect (Valenzuela et al., 2011). Both raw materials, in addition to their potential for the production of stable NLC, have low cost and wide availability, since they are already used to produce other lipid-based products in the oil and fat industry. Despite all their potential, these oils are not typically used to produce NLC. Nevertheless, in a recent work, Lüdtke, Stahl, Grimaldi, Cardoso, et al. (2022) produced NLC using FHSO and HOSO as lipid matrices in different ratios and different emulsifiers and observed that most of the FHSO:HOSO systems evaluated form stable particles, with crystallinity properties suitable for food incorporation.

The interfacial properties also influence the gastrointestinal fate of lipid particles, once they influence (i) the size of the particles obtained (surface area) and their aggregation status in the GIT, (ii) the adsorption of bile salts and lipases on their surface, (iii) the removal of products of lipid digestion, and (iv) the mixed micelles formation and their properties (Singh et al., 2009). Since the emulsifier

used to stabilize NLC affects their properties and behavior during digestion (Hur et al., 2009; Park et al., 2018; Zhang et al., 2015), the choice of the most adequate emulsifier is crucial to produce NLC able to carry, protect, and deliver β-carotene.

Derived from polyethoxylated sorbitan and oleic acid, Tween 80 is a nonionic emulsifier largely used to stabilize lipid nanostructures (Mohammadi et al., 2019). Due to their smaller molecular size, Tween 80 can quickly coat the droplet surfaces, rapidly decreasing the surface tension; so, this emulsifier is very effective in producing and stabilizing smaller particles (Håkansson et al., 2013). However, the replacement of synthetic emulsifiers by natural ones is an ongoing challenge in NLC production. Owing to their wide availability, soybean lecithin (SL) represents an effective alternative to produce NLC (Katouzian et al., 2017; Mohammadi et al., 2019). Commercial SLs are mainly composed of phospholipids, whose chemical structure is very similar to triacyclglycerols (TAG), which can facilitate the recovering of the droplets during the homogenization process (Lüdtke, Stahl, Grimaldi, Forte, et al., 2022). Since the composition of the emulsifier and their specific interaction with the lipid/aqueous phases have been proved to affect NLC characteristics (Lüdtke, Stahl, Grimaldi, Forte, et al., 2022), the emulsifiers used in this study were chosen based on previous study for our research group, which evaluated the effect of synthetic and natural emulsifiers over the NLC stability (Lüdtke, Stahl, Grimaldi, Forte, et al., 2022) and over the β-carotene entrapment into NLC structures (Lüdtke et al., 2023).

At the nanoscale, the biological fate of delivery systems and bioactive compounds incorporated can be modified, influencing their absorption, distribution, metabolism, excretion, and, thus, their potential toxicity. Understanding the biological fate of nanostructures after passing through the GIT as well as their potential toxicity is essential to predict and increase their functionality and bioactive compound bioavailability (Pinheiro et al., 2017). Several studies have been performed to evaluate the bioaccessibility and/or bioavailability of β-carotene entrapped in emulsions (Ashkar & Davidovich-Pinhas, 2024; Gomes et al., 2023; Hu et al., 2024), nanoemulsions (NE) (Chen et al., 2020; Gasa-Falcon et al., 2019; Liang et al., 2013; Salvia-Trujillo & McClements, 2016), solid lipid nanoparticles (LN) (de Abreu-Martins et al., 2020; Salvia-Trujillo, Verkempinck, Rijal, et al., 2019), and nanoliposomes (Li et al., 2017; Tan et al., 2014). However, the reports on the evaluation of the bioaccessibility of β-carotene entrapped in NLC are still very limited. Moreover, most of the studies use synthetic or unfeasible priced lipid raw materials (Gomes et al., 2017, 2019) to produce NLC and/or assess their digestibility using simplified static systems that do not accurately mimic the complex conditions found in the human GIT (Rohmah et al., 2022). Therefore, the aim of this study was to go further and evaluate the in vitro digestibility of β-carotene-loaded NLC (composed of HOSO and FHSO as a lipid matrix, and Tween 80 or SL as an emulsifier) using a dynamic in vitro GIT model that more closely reproduces the in vivo behavior. Also, cell viability studies of the developed NLC have been conducted in order to evaluate their safety. The results obtained from this study will provide relevant information about the behavior of lipid delivery systems under digestion, β-carotene bioaccessibility, NLC biocompatibility and safety, and the potential use of conventional lipids derived from the oil industry to obtain NLC. In fact, as far we know, there is no scientific work that evaluates the bioaccessibility and cell viability of β-carotene entrapped in NLC produced using HOSO and FHSO.

2 | MATERIALS AND METHODS

2.1 | Materials

HOSO and FHSO were supplied by Cargill Foods. β-Carotene (purity: 95%) and ethoxylated sorbitan monooleate (Tween 80) were purchased from Sigma–Aldrich, and enzymatically modified soybean lecithin (SL) SOLEC AE IP was supplied by Solae. Pepsin from porcine gastric mucosa, lipase and pancreatin (8 × USP - unit of potency of drugs, used in United States Pharmacopeia) from porcine pancreas, bile extract porcine, and other reagents of analytical grade were purchased from Sigma–Aldrich. Caco-2 cell line (ATCC), obtained from human colon carcinoma, was kindly provided by the Department of Biology, University of Minho. Dulbecco's modified Eagle's medium (DMEM), nonessential amino acids (NEAA), and phosphate-buffered saline (PBS) were obtained from Lonza. Penicillin/streptomycin, trypsin-EDTA, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and dimethyl sulfoxide (DMSO) were purchased from Sigma–Aldrich; fetal bovine serum (FBS) was obtained from Merck.

2.2 | NLC production

The pre-emulsion was composed by FHSO and HOSO (60/40, w/w), aqueous phase (88%) (ultrapure water) and emulsifier (2%) (Tween 80–NLC T_β or SL–NLC L_β) (Lüdtke, Stahl, Grimaldi, Cardoso, et al., 2022). β-Carotene (0.5% in the lipid phase) was mixed in the lipid melted phase and heated at 85°C. On the other hand, the emulsifier and water were mixed together and then heated at

85°C. Afterward, the oil phase and aqueous phase were mixed and homogenized at 10,000 rpm for 3 min by a high-speed Ultra-turrax homogenizer (T18; IKA Werke). Subsequently, the pre-emulsion passed through a high-pressure homogenizer (HPH) (NanoDeBee; Bee International) at 20,000 psi and 20 cycles of homogenization (conditions previously optimized). The nanoemulsion was cooled at 5°C for 24 h and stored at 25°C in covered glasses.

For the bioaccessibility tests, a control emulsion was prepared using the same composition of NLC, but not submitted to HPH. The phases were heated at 85°C, mixed with high-speed Ultra-turrax homogenizer (T18; IKA Werke) at 5000 rpm/5 min, and immediately submitted to in vitro digestion.

2.3 | in vitro digestion

The digestion process was based on the work of Pinheiro et al. (2016) and Mulet-Cabero et al. (2020), with some modifications. A dynamic in vitro gastrointestinal system (DIVGIS) was used in the digestion experiments (Fernandes et al., 2021). The DIVGIS consists of four compartments—stomach, duodenum, jejunum, and ileum—that simulate the main conditions that occur during digestion in the human GIT. Each compartment is composed of two connected acrylic reactors with a flexible wall (stomacher bags) and connected by silicone tubes. The simulation of peristaltic movements was carried out by the alternate compression and relaxation of the flexible walls, through the passage of water (37°C) in each reactor. Gastric and intestinal secretions were freshly prepared and secreted continuously inside the reactors by syringe pumps at preset flow rates. The jejunum and ileum compartments were connected to hollow fibers (Repligen Minikros, S02-S05U-05-P; Breda) to simulate the absorption that occurs in the small intestine. These fibers divided the fluids into three different fractions: jejunum filtrate, ileum filtrate, and non-ileum filtrate (Pinheiro et al., 2017).

A volume of 100 mL of each sample (NLC L_β, NLC T_β, and a control sample obtained as described in Section 2.2) was used as the initial sample. The oral phase was carried out in a static digestion bath at 37°C, by the addition of the simulated salivary fluid (SSF) (KCl 15.1 mM, KH_2PO_4 3.7 mM, $NaHCO_3$ 13.6 mM, $MgCl_2(H_2O)_6$ 0.15 mM, $(NH_4)_2CO_3$ 0.06 mM, $CaCl_2(H_2O)_2$ 1.5 mM) and agitation at 120 rpm for 2 min (Brodkorb et al., 2019). After the oral phase, the sample was introduced into the system through the gastric compartment, and the simulated gastric fluid (SGF) (KCl 6.9 mM, KH_2PO_4 0.9 mM, $NaHCO_3$ 25 mM, NaCl 47.2 mM, $MgCl_2(H_2O)_6$ 0.12 mM, $(NH_4)_2CO_3$ 0.5 mM, $CaCl_2(H_2O)_2$ 0.15 mM) and pepsin (3940 U/mL) and lipase (65 U/mL) solution were continuously secreted at the flow rate 0.4 mL/min. A constant volume was transferred to the duodenum compartment, and then the simulated intestinal fluid (SIF) (KCl 6.8 mM, KH_2PO_4 0.8 mM, $NaHCO_3$ 85 mM, NaCl 38.4 mM, $MgCl_2(H_2O)_6$ 0.33 mM, $CaCl_2(H_2O)_2$ 0.6 mM), bile salts, and pancreatin solution were continuously secreted at the flow rate 0.8 mL/min. At a predefined time, a constant volume of chyme was transferred to the subsequent compartment. The pH was continuously adjusted with HCl (1 mol/L) or NaOH (1 mol/L) to reach pH 2.0 or 7.0 in the gastric and intestinal phases, respectively. The digestion experiments lasted approximately 4 h. During in vitro digestion, samples were collected directly from the lumen of the different compartments, from the jejunal and ileal filtrates, and from the ileal delivery in order to determine the changes in the NLC structure during the digestion and the bioaccessibility of β-carotene entrapped in NLC. All samples were kept in an ice bath during the digestion process.

Samples collected from the jejunum and ileum filtrates were used to determine particle size (PS), polydispersity index (PDI), and ζ-potential (ZP). Enzyme inhibitor Pefabloc (1 mmol/L) was added (10 μL of 1 mL of sample) in intestinal filtrates (jejunum, ileum, and unfiltered) to stop the reaction. These samples were also used to determine β-carotene bioaccessibility, and in this case, the samples were stored in falcon tubes wrapped in aluminum foil and subsequently frozen, remaining until the moment of analysis. The measurements of PS, PDI, and ZP were conducted on samples collected after the oral phase, stomach, and duodenum in the time of half digestion, as well as on the filtered jejunum, ileum, and unfiltered samples (collected at the end of digestion), immediately after the end of the in vitro digestion. The samples were collected in the time of half digestion for comparison purposes, as the dynamic nature of GIT system fluids is always in and out. The simulation of digestion was done at least in triplicate.

2.4 | PS, PDI, and ZP

PS, PDI, and ZP of the NLC were measured at different digestion stages by dynamic light scattering (DLS) (Zetasizer Nano ZS; Malvern Instruments). All samples were diluted at 1:100 with a buffer solution of the same pH as the samples before analysis.

2.5 | β-Carotene bioaccessibility

The β-carotene bioaccessibility percentages in the jejunum and ileum were calculated in relation to the initial amount present in NLC L_β and NLC T_β and in the control sample. As some studies on β-carotene bioaccessibility use foods rich in carotenoids as a control, a sample that presents the same materials and proportions as the NLC (but without

undergoing the HPH process) has been chosen as the control. β-Carotene bioaccessibility at different stages of digestion was determined by liquid–liquid extraction and spectrophotometric analysis, based on the methodology described by Berni et al. (2020) with some modifications. Aliquots (5 mL) of each sample (i.e., jejunum and ileum filtrate) were vortexed with 10 mL of acetone with butylayed hydroxytoluene (0.01%) for 30 s; afterward, 10 mL of hexane was added and vortexed for 10 s. Distilled water was added until the final volume of 45 mL, and then the solution was centrifuged (Allegra 64R; Beckman Coulter Inc.) at 10,000 rpm at 4°C for 5 min. The upper phase containing β-carotene was collected and dried with N_2 flow, resuspended in hexane, and analyzed by UV-VIS spectrophotometer (V-560; Jasco) at 450 nm (absorbance peak). The concentration of β-carotene was determined from a previously prepared calibration curve of absorbance versus β-carotene concentration in hexane. The β-carotene bioaccessibility (%) was calculated based on the following equation:

$$\beta\text{-carotene bioaccessibility} = \frac{\beta\text{-carotene on the filtrates}}{\beta\text{-carotene on the initial sample}} \times 100, \quad (1)$$

where "β-carotene on the filtrates" is the total β-carotene (μg) in the jejunum and ileal filtrates (considering the total volume of sample collected in each filtrate).

2.6 | Free fatty acid release

The amount of free fatty acid (FFA) released from the lipid digestion of NLC was determined using a pH-stat automatic method (Titrando 902; Metrohm). This method was also used to monitor the pH that was maintained at 7.0 via the titration of 0.05 N NaOH solution. The volume of NaOH added to the NLC was recorded, which was used to calculate the concentration of FFA released from the lipid phase during lipolysis.

NLC T_β, NLC L_β, and a control sample were first submitted to the oral and stomach phase in the DIVGIS. After 1 h 45 min of stomach phase, 20 mL of each sample was collected and placed into a glass reactor connected with a water bath at 37°C. Intestinal fluids and bile were added to the sample under stirring, and then the pH was adjusted to 7.0 using NaOH or HCl solutions. Pancreatin was added, and the titration was started. Blank experiments (no added pancreatin) were performed to determine the volume of NaOH required to achieve the pH of 7.0. The quantity of FFA released was calculated based on the following equation adapted from AOCS (2009) and Li and McClements (2010):

$$\text{FFA}\ (\%) = \frac{(V\text{NaOH sample} - V\text{NaOH blank}) \times m\text{NaOH} \times M\text{Elipid}}{W\text{lipid} \times 2} \times 100, \quad (2)$$

where V_{NaOH} sample and $V_{\text{NaOH blank}}$ are the NaOH titrated volume in the sample and blank assays, respectively; m_{NaOH} is the NaOH titrant molar concentration, in this case 0.05 M; M_{Elipid} is the equivalent molecular weight of lipid phase, calculated proportionally according to the fatty acid composition of FHSO and HOSO (28.32) (AOCS, 2009); and W_{lipid} is the mass of lipid (g) presented in NLC structures.

2.7 | Cell viability assay

Regarding the cellular viability assay, samples of free β-carotene, β-carotene-loaded NLC (obtained with Tween 80 and SL), and NLC (obtained with Tween 80 and SL) without β-carotene (free β-carotene NLC) were diluted to the following β-carotene concentrations: 1, 2.5, 5, 15, and 25 μg/mL. The components used to produce NLC (i.e., HOSO, Tween 80, and SL emulsifiers) were also tested individually. FHSO was not tested due to its high melting point, which makes it difficult to dilute to perform the assay. β-Carotene stock solution was prepared in pure ethanol (99.9%), and Tween 80 and SL stock solutions were prepared in ultrapure water. MTT assay was conducted according to Gonçalves et al. (2021) with some modifications. Briefly, Caco-2 cells were cultured at 2×10^5 cells/well (in a 96-well microplate) in DMEM supplemented with 10% (v/v) FBS, 1% (v/v) NEAA, and 1% (v/v) penicillin/streptomycin. Caco-2 cell line was chosen in our study because it is a widely used line to study the cytotoxicity and absorption of bioactive compounds and nanoparticles (Jafari & McClements, 2017; Reboul et al., 2006). Cell culture was grown in a humidified 5% CO_2 incubator at 37°C during 48 h. After incubation, the cell culture medium was replaced by 200 μL of fresh culture medium containing tested samples. At least three replicates of each sample were analyzed. After 24 h of incubation, the samples were removed, and 100 μL of MTT

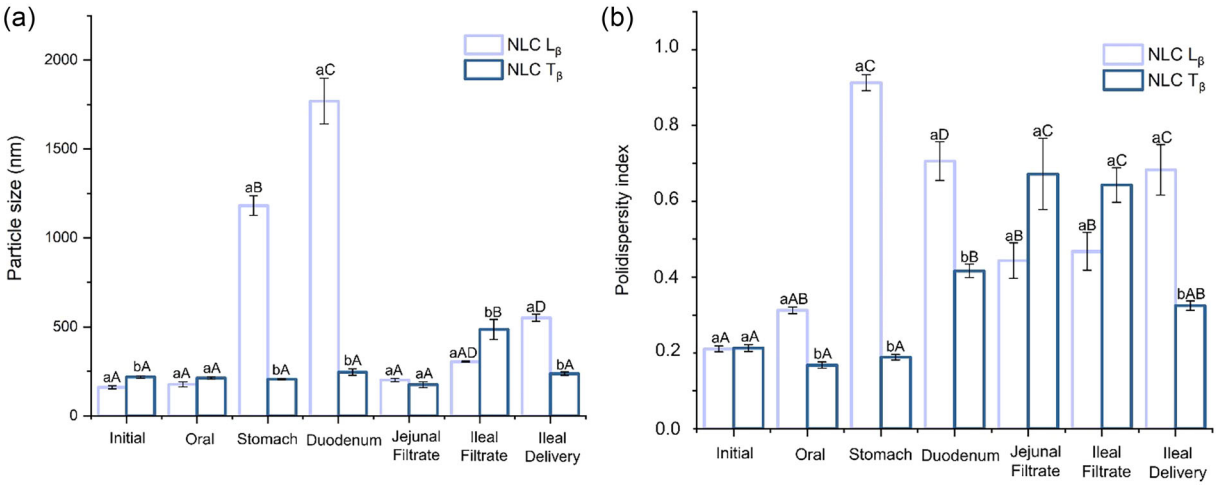

FIGURE 1 (a) Particle size and (b) polydispersity index of β-carotene-loaded nanostructured lipid carriers (NLC) samples collected at different stages of in vitro digestion. Errors bars represent the standard deviation of $n = 3$ replicates. Different capital letters indicate significant difference between simulated gastrointestinal phases ($p < 0.05$) for the same NLC. Different lower-case letters indicate significant difference between NLC in the same gastrointestinal phase ($p < 0.05$).

solution (0.5 mg/mL in PBS) was added to the wells and incubated at 37°C for 3 h. Then, formazan crystals generated were solubilized with 200 μL of DMSO, followed by gentle stirring for 30 min on an orbital shake. Cell viability was assessed at 570 nm (reference wavelength set at 630 nm), and the results were expressed as cell viability (%) relative to the control (i.e., untreated cells in DMEM medium).

2.8 | Statistical analysis

All experiments were carried out at least in triplicate. Experimental data were analyzed for significant differences using ANOVA. Means were compared using the Tukey test by 0.05% of significance using the OriginPro2018 Statistic Software (Origin Lab Corporation).

3 | RESULTS AND DISCUSSION

3.1 | PS, PDI, and ZP

It is well-known that lipid particles coated with unstable emulsifiers in an acidic environment undergo strong aggregation and creaming in the stomach, often resulting in faster gastric emptying. On the other hand, particles coated with acid-stable emulsifiers tend to remain individualized in the stomach (Tan & McClements, 2021; Wang et al., 2019). The NLC considered in this study showed different behavior during the passage through the GIT. NLC T_β remained stable in the gastric compartment as can be seen in Figure 1a, showing a PS similar to the one observed in the oral phase, which indicates the absence of particle aggregation. Tween 80 is a nonionic emulsifier that forms a compressed film around particle surfaces, stabilizing NLC formulations by creating steric repulsion (Souto et al., 2004). Therefore, NLC T_β stability under gastric conditions can be explained by the steric repulsion presented by nonionic emulsifiers, like Tween 80, in an acidic medium (Golding & Wooster, 2010).

Our results are in agreement with previous studies, which reported that the size of particles coated by certain types of nonionic emulsifiers, such as Tween 80, was not significantly altered under gastric conditions (Mun et al., 2007; Park et al., 2018; Salvia-Trujillo et al., 2013a; van Aken et al., 2011). In particular, Verkempinck et al. (2018) claimed that Tween 80 is not sensitive to pH changes in the gastric phase (pH 1–3), thus providing higher stability to emulsified delivery systems when compared to other emulsifiers. Also, a study carried out by Salvia-Trujillo et al. (2017) reported that the use of Tween 80 as an emulsifier provided emulsions' stability under in vitro acidic conditions, even though these emulsions had different PS (15.1, 1.93, and 0.72 μm) before in vitro digestion, indicating that this emulsifier provides particles stability regardless of their size.

Regarding NLC L_β, the PS significantly increased ($p < 0.05$) after being submitted to gastric conditions (Figure 1a). This nanocarrier was produced using an enzymatically hydrolyzed lecithin, obtained by the hydroxylation of commercial SLs. Several studies have shown that emulsified systems stabilized with SL have less stability under GIT conditions compared to synthetic ones (Park et al., 2018; Verrijssen et al., 2015). A drastic destabilization of droplets under in vitro gastric conditions has been previ-

ously reported in emulsions stabilized with phospholipids (Verrijssen et al., 2015), whereas in emulsions stabilized with nonionic emulsifiers, the PS remained stable during the gastric phase (Salvia-Trujillo et al., 2013b, 2017). Similar results were reported by Park et al. (2018). These authors carried out an in vitro digestibility study using NE stabilized with different emulsifiers, namely, natural SL and Tween 20 (similar to those used in our study). The authors found that NE stabilized with Tween 20 was the only one stable (aggregation was not observed) in the gastric phase, indicating that those stabilized with SL were more apt to coalesce when subjected to gastric conditions. Therefore, the size increase observed in the present study during the passage through the GIT can be attributed to aggregation, coalescence, or flocculation due to the action of digestive enzymes and changes in pH and ionic strength (Pinheiro et al., 2013).

In this study, a significant PS increase of NLC L_β from the gastric phase to the duodenum was observed (Figure 1a), while NLC T_β maintained their PS in the duodenum and jejunum, showing an increase in PS only after passing through the ileum (i.e., the final small intestine compartment). Most of the lipid digestion takes place in the small intestine through the action of pancreatic lipase, which binds to the oil/water interface. In general, the size of the oil droplets together with the composition of the interfacial layer is fundamental for the activity of pancreatic lipase (McClements et al., 2009). The PS increase in NLC T_β can be attributed to the reduced physical stability of these particles at the end of the small intestine, which could be related to several physicochemical mechanisms that happened when the sample was exposed to small intestinal conditions. NLC T_β entered the duodenal compartment presenting a relatively high surface area for pancreatic lipase adsorption to the particle surface. Thus, some molecules of the nonionic emulsifier (Tween 80) that originally covered the particle may have been displaced by lipase or other superficially active substances present in the intestinal environment (e.g., bile salts, phospholipids). The adsorption of these substances may have promoted a change in NLC composition, structure, and surface properties, reducing the physical stability of these particles and thus allowing the digestion of lipids present in the NLC structure. Additionally, the digestion of TAG (present in the NLC structure) by lipase promotes FFA and monoacylglycerols (MAG) production, which changes the structure's internal composition and surface properties, since FFA and MAG are also surface-active species (Qian et al., 2012). These results agree with a study carried out by de Abreu-Martins et al. (2020), who reported an increase in PS of solid LN and liquid LN at the end of the small intestine. Similar results were obtained by Qian et al. (2012), who found a large increase in PS of NE stabilized by a non-anionic emulsifier (Tween 20) after incubation in simulated small intestine fluids.

PDI is a parameter that indicates the size distribution and homogeneity of the analyzed particles. Homogeneous and dispersed particles indicate a PDI value in the range between 0.1 and 0.25, whereas values higher than 0.5 represent high PDI and wide particle distribution (Lakshmi & Kumar, 2010). The PDI values found after the different digestion stages are shown in Figure 1b. In general, it can be inferred that the PDI values confirmed NLC L_β destabilization in the gastric phase, because PDI values were higher than 0.9. This value is indicative of a very heterogeneous distribution and agrees with the PS found for this nanocarrier. On the other hand, the NLC T_β presented PDI values lower than 0.5 in the oral and gastric phases and in the duodenal compartment, confirming NLC T_β stability. Samples collected from the jejunum and ileum filtrate presented PDI values higher than 0.6, probably due to the presence of mixed micelles resulting from the lipid digestion process, which made the PS distribution heterogeneous.

The ZP is a very important characteristic since the electrical charge is responsible for the electrostatic interactions and subsequent stability of the lipid-based system. In addition, electrical charge can also affect droplet/particle interactions within the intestinal lumen and emulsion digestibility (Singh et al., 2009). This parameter provides relevant information about the electrical charge of the lipid particle surface depending on the species adsorbed at the oil–water interface (Salvia-Trujillo et al., 2017). In this study, both NLC presented differences in ZP values during the passage through the GIT as can be seen in Figure 2, probably due to the initial charge of these nanostructures. Park et al. (2018) also reported that the electrical charge (ZP) and stability to aggregation of lipid droplets from NE submitted to in vitro digestibility tests changed according to the type of emulsifier used for NLC stabilization.

The NLC T_β was produced using Tween 80, a nonionic emulsifier, that stabilizes NLC formulations by creating steric hindrance resulting from the complex structure of this emulsifier (McClements & Rao, 2011; Souto et al., 2004). Thus, the particles stabilized with Tween 80 normally show neutral ZP values (McClements & Rao, 2011). This could explain the initial ZP value found in this study for NLC T_β, which was approximately −14 mV. During the passage through the GIT, NLC T_β showed changes in the ZP value. Although not statistically significant, there was an increase (in modulus) in ZP values of NLC T_β after 2 h of passage through the gastric cavity, in contrast to some studies that reported a decrease in the electrical charge of the particles when subjected to gastric condi-

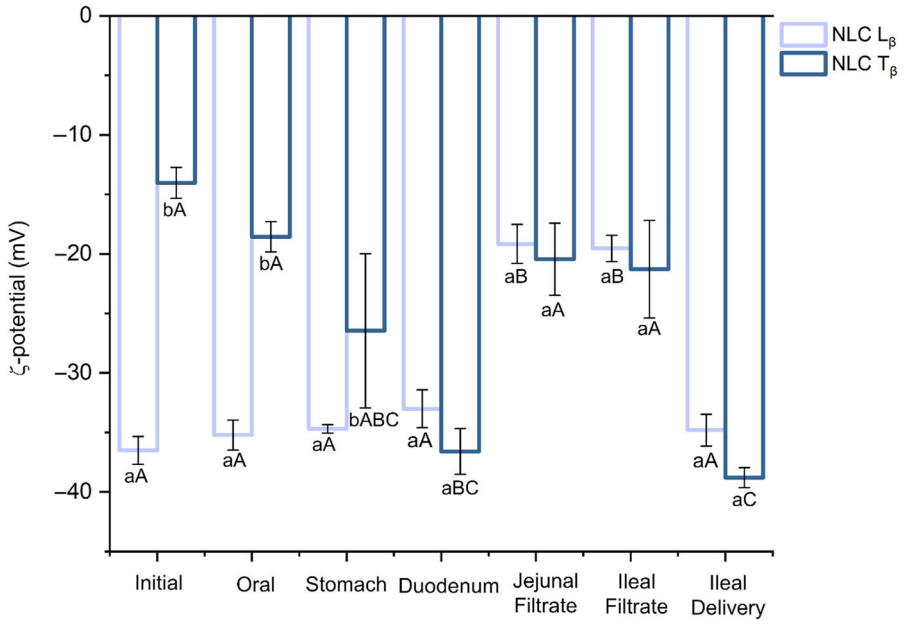

FIGURE 2 ζ-Potential of β-carotene-loaded nanostructured lipid carriers (NLC) samples collected at different stages of in vitro digestion. Errors bars represent the standard deviation of $n = 3$ replicates. Different capital letters indicate significant difference between simulated gastrointestinal phases ($p < 0.05$) for the same NLC. Different lower-case letters indicate significant difference between NLC in the same gastrointestinal phase ($p < 0.05$).

tions (de Abreu-Martins et al., 2020). After 2 h of passage through the duodenum, the ZP value of NLC T_β significantly increased ($p < 0.05$). Singh et al. (2009) reported that the negative charge of emulsified systems after the intestinal phase can be attributed to the presence of anionic FFA and other products of lipid digestion from lipolysis or complex colloidal structures formed from bile salts and phospholipids. Not only does the concentration of lipid species released at the end of the intestinal phase determine the electrical charge of emulsified systems subjected to digestion, but also the chain size of the FFA released in the process. Salvia-Trujillo et al. (2013a) reported that the negative charge of digested samples decreased with increasing chain length of released FFA.

Regarding NLC L_β, the ZP value remained stable and above (in modulus) −30 mV in the oral and gastric phases, and in the duodenal compartment, as shown in Figure 2. The ZP values of samples collected at the end of the intestinal phase (i.e., jejunum and ileum filtrates) showed a significant ($p < 0.05$) reduction (in modulus) to values close to −20 mV. The results found for this NLC agree with the study carried out by Salvia- Trujillo et al. (2017), who reported a significant decrease in ZP value of carotenoid-enriched oil-in-water emulsions after the intestinal phase. The authors highlighted that these changes in ZP suggest a relationship between the electrical charge characteristics during in vitro digestibility and the degree of lipolysis.

3.1.1 | Bioaccessibility

The type of emulsifier can affect the rate and extent of lipid digestion (Park et al., 2018). Thus, the bioaccessibility of β-carotene incorporated into NLC was evaluated to assess the effect of emulsifiers (SL and Tween 80) on the β-carotene delivery for intestinal absorption. We also explored if the lipid matrices considered in this study were able to promote the protection and delivery of bioactive compounds in the GIT, since studies on the β-carotene bioaccessibility incorporated in NLC obtained using FHSO and/or HOSO were not reported to date. The bioaccessibility of β-carotene incorporated into NLC L_β and NLC T_β, and in the control sample, is shown in Figure 3.

The total β-carotene bioaccessibility values found were 0.5%, 0.23%, and 2.27% for the control, NLC L_β, and NLC T_β samples, respectively. Different hypotheses were proposed to explain the low β-carotene bioaccessibility values obtained in this study. Carotenoids bioavailability in foods is typically very low, mainly due to low chemical stability of these compounds, which partially elucidates the values found in the in vitro dynamic digestibility experiments carried out in the present study. Significant β-carotene loss has probably occurred as a result of its degradation (e.g., oxidation, isomerization) during the passage through the GIT due to enzymatic action, pH, oxygen, presence of pro-oxidants, and photodegradation. Additionally, some

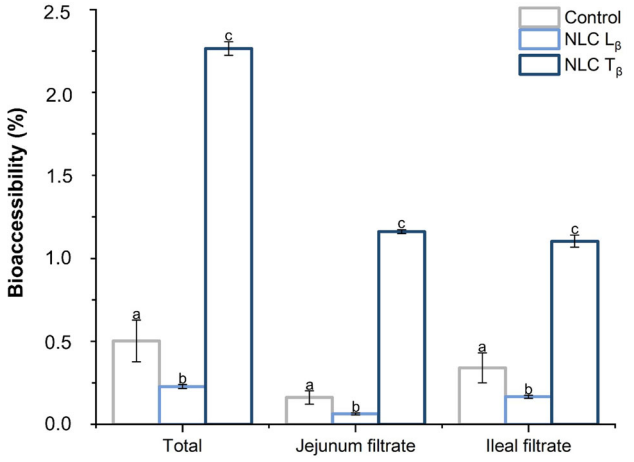

FIGURE 3 Bioaccessibility of β-carotene-loaded nanostructured lipid carriers (NLC) samples collected at different stages of in vitro digestion. Errors bars represent the standard deviation of $n = 3$ replicates. Different letters indicate significant differences between the samples ($p < 0.05$).

intrinsic characteristics of the in vitro dynamic GI system used in this work promoted the adsorption of carotenoids in the plastic bags (used inside the reactors to allow the peristaltic movements) and in the hollow fiber membranes (used to simulate the intestinal absorption) (Berni et al., 2020). Therefore, even though this dynamic in vitro digestibility model allows a better simulation of the conditions found in the human GIT, we verified that it is not possible to state that the β-carotene bioaccessibility values found in this study will reflect the in vivo values, mainly due to β-carotene losses that occurred during the passage through the dynamic GIT model system.

Most of the studies carried out to assess β-carotene bioaccessibility used static in vitro digestibility systems. As in vitro static digestion models do not consider some physical processes that take place during the digestion, such as mixing, shear, changes in conditions over time, or peristalsis (Lin et al., 2018), and normally the particles remain in the same vessel throughout all the stages of digestion, the compounds' losses are not so significant. Therefore, the β-carotene bioaccessibility assessed in static GIT systems is usually much higher than the values found in our study. For example, Salvia-Trujillo et al. (2013a) investigated the bioaccessibility of β-carotene incorporated in emulsions with different drop sizes (ranging from 0.2 to 23 μm) using a static digestibility system. The β-carotene bioaccessibility was around 59% for the small-size emulsion and 34% for the large-size emulsion. Han et al. (2019) studied the effect of the carrier oil, medium-chain triglycerides (MCT), and long-chain triglycerides (LCT) on the β-carotene bioaccessibility of emulsion-based delivery systems, using a static digestibility system. The β-carotene bioaccessibility found was 23.1% and 65.5% for the MCT and LCT emulsions, respectively. In another study, Yi et al. (2015) evaluated the bioaccessibility of β-carotene incorporated in NE (produced with different vegetable oils), using a two-stage in vitro digestion method. The authors found a β-carotene bioaccessibility close to 70% in some NE delivery systems, positively correlated to the length of carrier oil. de Abreu-Martins et al. (2020) studied the bioaccessibility of β-carotene incorporated in solid LN and liquid LN obtained from different lipid matrices (i.e., medium-chain TAG, hydrogenated palm oil, glyceryl stearate) through a static digestibility method. The authors found that β-carotene bioaccessibility values ranged between 22.6% and 28.1%.

The differences in the bioaccessibility of β-carotene-loaded NLC found using static and dynamic in vitro digestion systems were studied by Gomes et al. (2017). These authors prepared NLC with cupuacu butter and a mixture of two emulsifiers (Span 60 and Cremophor RH40). The β-carotene bioaccessibility in the static system was 92%, while in the dynamic system, β-carotene bioaccessibility was nearly 20%. These differences were attributed mainly to the more realistic digestion conditions simulated by the dynamic in vitro system and to β-carotene losses (11.8%) along the process. The authors concluded that the dynamic system appeared to be more reliable to determine the bioaccessibility of bioactive compounds incorporated in the LN. In another study, Gomes et al. (2019) evaluated the bioaccessibility of β-carotene loaded in NLC, produced with murumuru butter and a mixture of two nonionic surfactants (Span 60 and Cremophor RH40) using a dynamic gastrointestinal system similar to the one used in our study. β-Carotene bioaccessibility found in the dynamic system was about 42%. β-Carotene losses have also been reported in a study carried out by Berni et al. (2020), using a similar dynamic gastrointestinal system to assess the bioaccessibility of pitanga and buriti carotenoids (β-carotene and lycopene)-loaded microemulsions. The β-carotene bioaccessibility values were approximately 2% and 2.5% for pitanga and buriti carotenoids-loaded microemulsions, respectively. The authors attributed the low β-carotene bioaccessibility values found to the carotenoid low stability and losses inside the dynamic gastrointestinal system.

The digestion and absorption of carotenoids in the human GIT can be more realistically predicted by dynamic in vitro digestion models that are suitable to indicate the micellization process of these bioactive compounds (Geng et al., 2023). So, the bioaccessibility values found in studies using a dynamic gastrointestinal system can be more related to the β-carotene bioavailability found in studies in vivo, suggesting that the in vitro static digestion models overestimate the β-carotene bioaccessibility.

One of the great advantages of nanoscale lipid-based delivery systems is the possibility to maintain its high surface area until reaching the small intestine, thus promoting an increase in lipolysis rate. Lipase has a globular shape of approximately 5 nm; so, the smaller the size of the lipid particle/oil droplet, the higher the surface area available for lipase binding, and consequently, the higher the rate and extent of lipid digestibility (TAG conversion to FFA and MAG) (Salvia-Trujillo et al., 2017). The increase in lipolysis can generate more hydrolysis products (e.g., MAG and FFA), thereby increasing mixed micelles production and the release and solubilization of bioactive compounds incorporated into the lipid phase, thus making it more bioaccessible (Li & McClements, 2010; Salvia-Trujillo et al., 2017; Verrijssen et al., 2015). In our study, despite β-carotene losses observed throughout digestion, it was possible to verify β-carotene bioaccessibility differences between NLC L_β and NLC T_β. The differences observed in our study can be related to the chemical structure and hydrophilic/lipophilic balance (HLB) of the emulsifiers used to produce NLC. SL used in this study is an enzymatically hydrolyzed lecithin that presents an HLB of around 7–8 (Fernandes et al., 2012; Tanno, 2012), indicating a lower interaction with the aqueous medium compared to emulsifiers with higher HLB, such as Tween 80, which has an HLB of 15 (Mohammadi et al., 2019). The lower interaction with the aqueous medium exhibited by the NLC stabilized with SL may have resulted in less stability during the passage through the GIT, especially in the gastric phase. So, the NLC L_β instability under gastric conditions was reflected in a lower bioaccessibility of β-carotene incorporated into these structures when compared to NLC T_β. As reported previously, the use of SL to stabilize NLC resulted in a drastic droplets' destabilization under in vitro gastric conditions, promoting the PS increase from 176.2 (oral phase) to 1181.17 nm (gastric phase). PS, followed by the composition of the lipid matrix, is the factor that most affects the bioaccessibility of the compounds incorporated into emulsion-based delivery systems. This is mainly related to the surface area of lipid particles. In general, emulsifiers that lead to coalescence or flocculation of particles in the small intestine decrease the bioaccessibility of bioactive compounds incorporated into the particles (Tan et al., 2020).

Also, the β-carotene bioaccessibility found for the NLC L_β was lower ($p < 0.05$) than the value found for the control sample. As the use of Tween 80 has been shown to provide higher stability to the particles, the control sample was prepared using the same materials and the same proportions used to obtain the NLC T_β but without the HPH step. Through these results, it was possible to verify that the use of SL to stabilize NLC did not promote the increase of the β-carotene bioaccessibility, even though NLC L_β had a much smaller size than the control sample.

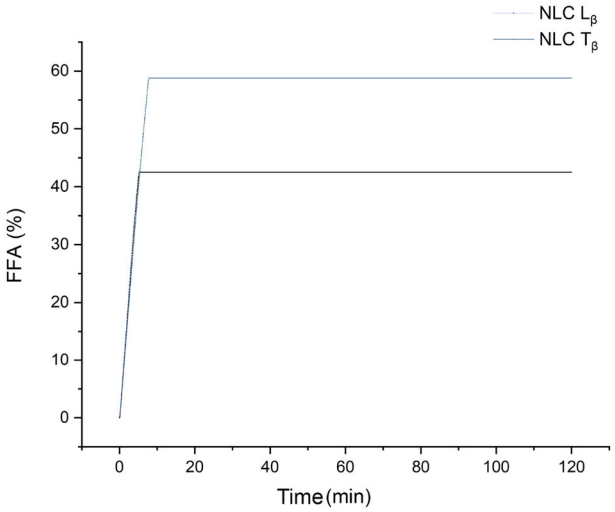

FIGURE 4 Free fatty acids release from β-carotene-loaded nanostructured lipid carriers (NLC) during in vitro digestion. Graphic points represent the media of $n = 3$ replicates. Standard deviation was between 0.5 and 1.1 for NLC L_β and between 0.8 and 1.0 for NLC T_β.

Moreover, the use of Tween 80 to stabilize NLC proved to be effective in increasing β-carotene bioaccessibility compared to NLC L_β and the control sample, due to the stability showed by these NLC under gastric conditions. As explained previously, emulsifiers with a higher HLB have greater affinity with the aqueous phase, promoting the formation of more stable emulsions in aqueous medium, such as gastrointestinal fluids. Thus, NLC T_β surface area was maintained until reaching the small intestine, allowing a higher area for lipase binding and consequently increasing the release of β-carotene entrapped in the structure. A study carried out by Wang et al. (2012) demonstrated that the lower the PS, the higher the inclusion efficiency of β-carotene in micelles. This fact reinforces our study results where NLC T_β promoted a significantly higher β-carotene bioaccessibility ($p < 0.05$) than NLC L_β.

3.1.2 | Free fatty acids release

The concentration of FFA released during the lipolysis of NLC obtained with different emulsifiers was evaluated using the pH-stat method. Lipolysis assays were also performed on NLC without the addition of pancreatic lipase to demonstrate that the observed lipolysis occurred due to enzyme action only and not to particle autolysis. The lipolysis reaction occurred immediately after the onset of the intestinal phase (Figure 4). Up to 10 min of reaction, an

exponential increase was observed in FFA release. After this point, a stationary phase was reached, with a residual increase in FFA until the end of the intestinal phase, which is in accordance with a study carried out by de Abreu-Martins et al. (2020). The results obtained in our study suggest that the emulsifier molecules that originally covered the surface of the particle were rapidly displaced by bile salts and phospholipids present in the intestinal fluids, allowing the binding of lipase to lipid droplets' surface and consequently FFA release (Qian et al., 2012).

The FFA concentration resulting from lipolysis is shown in Figure 4. Results showed that Tween 80 used as an emulsifier to obtain NLC resulted in higher FFA release during the lipolysis process when compared to the use of SL. The high FFA production observed may be associated with the higher NLC T_β stability obtained with Tween 80 under gastric conditions. This is demonstrated through the PS value obtained after the passage of this NLC through the GIT, which results in a higher surface area for lipase and bile salts action (Pinheiro et al., 2013). Regarding NLC L_β, as previously discussed, there was a destabilization during the gastric phase, leading to a significant increase in PS, which resulted in a reduction in the surface area for lipase binding and consequently for lipolysis.

Similar behavior was observed in studies performed to evaluate the effect of different emulsifiers on the lipolysis rate of emulsion-based delivery systems. For example, Mun et al. (2007) investigated the influence of the interfacial composition of corn oil-in-water emulsions coated by different emulsifiers (sodium caseinate, whey protein isolate, lecithin, and Tween 20) on their in vitro digestion behavior. The authors found that the lipid droplets; resistance to lipid digestion increased in the following order: nonionic surfactant (Tween 20) > phospholipids (lecithin) > protein (caseinate or whey protein isolate). Pinheiro et al. (2013) studied the lipid digestion of curcumin-loaded NE prepared using corn oil and three different emulsifiers—Tween 20 (nonionic), sodium dodecyl sulfate (SDS; anionic), and dodecyltrimethylammonium bromide (DTAB; cationic)—through a pH-stat automatic titration unit. The Tween 20-stabilized emulsion presented the highest total FFA produced, mainly due to the reduced emulsion droplet size throughout the simulated digestion. Park et al. (2018) evaluated the effect of two emulsifiers, Tween 20 and soy lecithin, used to coat emulsion droplets containing β-carotene, on lipid digestion. Soy lecithin-stabilized emulsions showed the lowest rate and extent of lipid digestion compared to Tween 20-stabilized emulsions, which is in accordance with our work results. The authors attributed these results to the low emulsifying capacity presented by soy lecithin, showing the coalescence of droplets after exposure to the gastric phase.

In general, the higher the FFA concentration released during lipid digestion, the higher the concentration of lipid species in the micellar fraction, and subsequently, the higher the carotenoid micellarization capacity and bioaccessibility (Salvia-Trujillo et al., 2017). However, the type of lipid species formed during digestion has also a strong influence on the solubilization capacity of mixed micelles and, consequently, on bioactive compound bioaccessibility. The presence of long-chain monounsaturated fatty acids, such as oleic acid, and long-chain monoacylglycerols in the micellar fraction contributes to the increased β-carotene bioaccessibility (de Abreu-Martins et al., 2020).

The results obtained in our study reinforce that the lipid digestion degree can be affected by the emulsifier used to stabilize NLC and highlight the direct relationship between bioaccessibility and the amount of FFA released during digestion. The NLC T_β, stabilized by a nonionic emulsifier (Tween 80), presented the highest FFA release value during simulated lipolysis compared to NLC L_β, which allowed a higher β-carotene delivery and superior amount of lipid products in mixed micelles. Thus, β-carotene bioaccessibility observed for NLC T_β was also higher than the one observed for the NLC L_β, due to a higher bioactive compound solubilization capacity (Lin et al., 2018; Wang et al., 2012), as discussed above. These findings hold significant implications for industrial applications in terms of developing NLC capable of increasing the bioavailability of bioactive compounds for use in functional foods.

3.2 | Cell viability

The knowledge related to cytotoxicity and absorption of bioactive compounds and nanoparticles is important to establish delivery system effectiveness and safety. It was observed that free β-carotene did not have a negative effect on cell viability (which was close to 100%) at the concentrations tested (Figure 5a). Similar results were obtained by Yi et al. (2014), who demonstrated that β-carotene in its pure form at 0.1% did not show cytotoxicity against Caco-2 cells. Han et al. (2019) also reported that pure β-carotene, within the concentration range of 0.1–0.005 μg/mL, was not cytotoxic to Caco-2 cells. Also, the results of the present study (Figure 5b,c) show that all the individual materials used to produce NLC did not have a cytotoxic effect at the highest β-carotene concentration tested (25 μg/mL).

Regarding NLC samples, both non-β-carotene-loaded NLC presented no negative effect on cell viability at all the concentrations tested (Figure 5d,e), suggesting that these NLC did not significantly decrease cell viability, even at the highest concentration tested (25 μg/mL). The interfa-

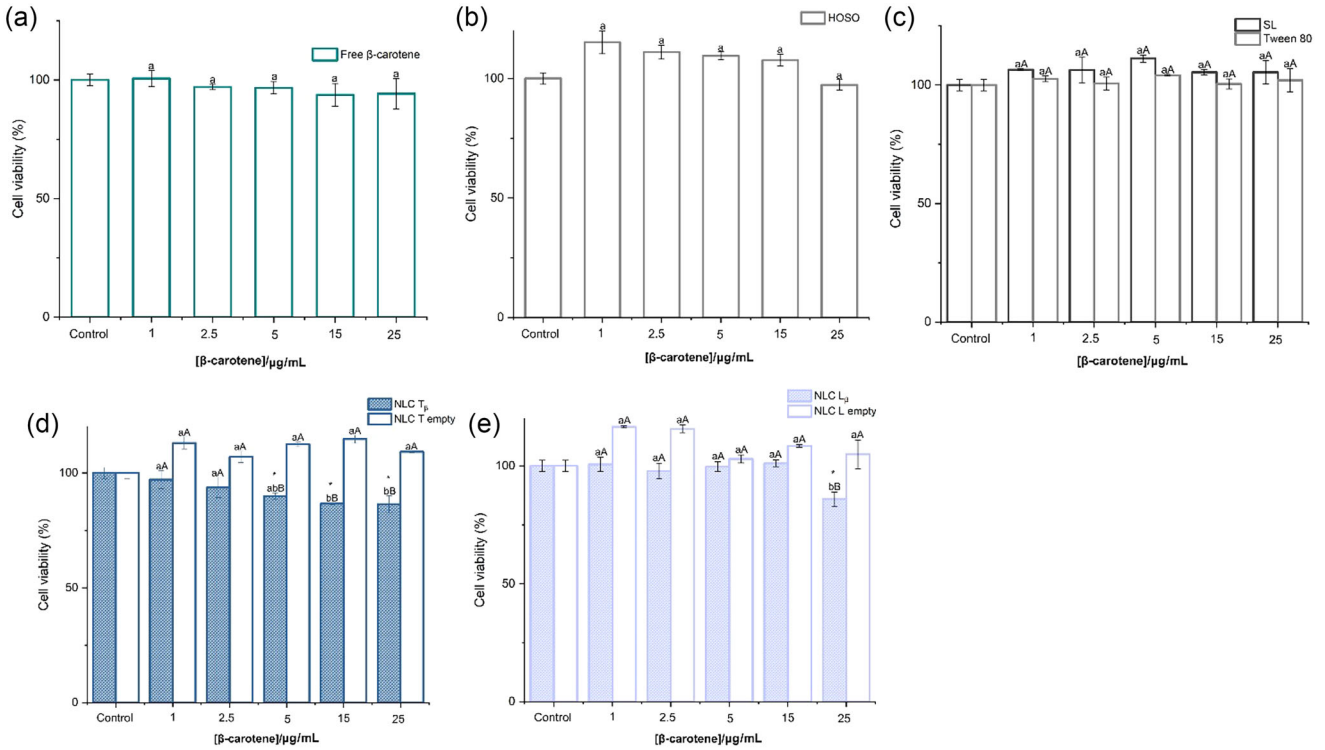

FIGURE 5 In vitro cell viability results for (a) free β-carotene, (b) high-oleic sunflower oil (HOSO), (c) emulsifiers (soybean lecithin and Tween 80), (d) NLC T_β and empty NLC T, and (e) NLC L_β and empty NLC L measured by MTT assay. Errors bars represent the standard deviation ($n = 3$ replicates). Different capital letters indicate significant differences between different samples tested at the same concentration ($p < 0.05$). Different lower-case letters indicate significant differences between concentrations for the same sample ($p < 0.05$). Asterisks indicate significant difference relative to the control group ($p < 0.05$).

cial NLC properties have an influence on their interactions with living cells and tissues. Thus, the emulsifier type used to stabilize NLC, its amount, and interaction with the lipid core will have an effect on NLC performance when in contact with cells (Doktorovova et al., 2014). For example, Gomes et al. (2019) evaluated Caco-2 cell viability when in contact with β-carotene-loaded NLC (produced using murumuru butter and a mixture of Span 60 and Cremophor RH40). The results indicated that NLC were toxic to the cells, probably due to the type of emulsifier used and to the extremely reduced PS (ca. 35 nm). So, the authors concluded that a mixture of nonionic surfactants and NLC with reduced average size can lead to a high level of cytotoxicity. In this sense, we verified that the emulsifiers (type and concentration) used in this study to obtain NLC, SL, and Tween 80 allowed the production of biocompatible and nontoxic particles. However, the incorporation of β-carotene in the NLC decreased cell viability for both NLC (Figure 5d,e). Regarding NLC T_β, a significant ($p < 0.05$) reduction in cell viability was observed with increasing β-carotene concentration when compared to control, reaching values close to 86% for the sample with the higher concentration tested (25 μg/mL). A significant ($p < 0.05$) reduction of cell viability was also observed in 25 μg/mL NLC L_β sample, reaching values similar to those observed for NLC T_β at the same dilution (ca. 86%). The effect of increased cytotoxicity in β-carotene-loaded NLC can be explained by the better solubilization of the bioactive compound within the NLC structure, thus being available in higher concentration in the system (Doktorovova et al., 2014). Most authors who performed cell viability assays in LN consider that values above 70% indicate that the particles do not present toxicity and that the material can be considered safe (Doktorovova et al., 2014). In this context, although a reduction in cell viability (ca. 86%) for β-carotene-loaded NLC was observed, the values found indicate that these nanostructures are nontoxic and biocompatible.

4 | CONCLUSION

The in vitro digestibility and cytotoxicity studies of NLC provided relevant information about the behavior of these nanostructures in GIT, as well as their safety and biocompatibility. The results of the present work showed that the lipid digestion degree can be affected by the emulsifier used to stabilize NLC, since the emulsifier directly

influences the particles' stability during digestion. It was possible to verify that NLC obtained using Tween 80 (NLC T_β) as an emulsifier showed better physical stability during the passage through the in vitro dynamic gastrointestinal system, compared to the other samples. Although the β-carotene losses in the dynamic gastrointestinal system were significantly high, the bioaccessible fraction found for NLC T_β was relatively higher than the other samples tested, which is in line with the higher release of FFA observed for this delivery system during the lipolysis process.

The results obtained demonstrated that the lipid materials and emulsifiers tested in this study allow for obtaining nontoxic, safe, and biocompatible NLC at the concentrations tested (1–25 μg/mL of β-carotene). Both NLC developed and all the materials used for their production showed cell viability values above 85% at 25 μg/mL, thus indicating a reduced risk of cell cytotoxicity of the NLC obtained. However, the cell viability values obtained must be considered for the application of NLC in foods, and concentrations of β-carotene that allow cell viability closer to 100% must be chosen. Moreover, only by understanding the digestive fate of NLC and β-carotene will be possible to improve their performance and to have conclusive information about NLC safety.

The results obtained in this study are important for designing effective lipid-based nanodelivery systems, using conventional lipids derived from the oil industry. In this context, the lipid materials considered, FHSO and HOSO, and the emulsifier Tween 80 have great potential for obtaining nontoxic, biocompatible, and safe NLC, capable of promoting increased bioaccessibility of bioactive compounds, such as β-carotene.

AUTHOR CONTRIBUTIONS

Fernanda L. Lüdtke: Conceptualization; methodology; data curation; investigation; writing—original draft. **Jean-Michel Fernandes**: Methodology; writing—review and editing. **Raquel F. S. Gonçalves**: Methodology; writing—review and editing. **Joana T. Martins**: Methodology; data curation; writing—review and editing. **Paulo Berni**: Methodology; writing—review and editing. **Ana P. B. Ribeiro**: Funding acquisition; supervision; writing—review and editing. **Antonio A. Vicente**: Funding acquisition; writing—review and editing. **Ana C. Pinheiro**: Conceptualization; funding acquisition; writing—review and editing; validation.

ACKNOWLEDGMENTS

F.L.L. and P.B. acknowledge the Sao Paulo Research Foundation (FAPESP) for the financial support (grants #2018/03172-0, #2019/05176-6, #2019/27354-3, and #2021/02271-8). F.L.L. also thanks Iceland Liechtenstein Norway Grants (PT-INNOVATION-0105) for the fellowship. J.-M.F. and R.G. acknowledge the Foundation for Science and Technology (FCT) for their fellowships (SFRH/BD/147286/2019 and SFRH/BD/140182/2018, respectively). J.T.M. acknowledges FCT for her Assistant Research contract obtained under the scope of Scientific Stimulus Employment with reference 2022.00788.CEECIND/CP1718/CT0024 (https://doi.org/10.54499/2022.00788.CEECIND/CP1718/CT0024). This study was supported by FCT under the scope of the strategic funding of UIDB/04469/2020 unit and by LABBELS – Associate Laboratory in Biotechnology, Bioengineering and Microelectromechnaical Systems, LA/P/0029/2020.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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How to cite this article: Lüdtke, F. L., Fernandes, J.-M., Gonçalves, R. F. S., Martins, J. T., Berni, P., Ribeiro, A. P. B., Vicente, A. A., & Pinheiro, A. C. (2024). Performance of β-carotene-loaded nanostructured lipid carriers under dynamic in vitro digestion system: Influence of the emulsifier type. *Journal of Food Science*, *89*, 3290–3305. https://doi.org/10.1111/1750-3841.17113