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Impact of the physiological stage on the bioaccessibility of α-tocopherol from fortified yogurts using dynamic *in vitro* digestion simulating young and old adults' gastrointestinal conditions☆

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ABSTRACT

Age-related physiological decline negatively impacts nutrient digestion and absorption, making older adults more susceptible to diet-related diseases. Fortified and functional foods are promising strategies to enhance the delivery of nutraceuticals and bioactive compounds. However, understanding how these foods behave under age-specific gastrointestinal conditions is essential. Despite the relevance of *in vitro* gastrointestinal models, there is a lack of dynamic systems that accurately replicate the digestive physiology of older adults.

This study employed the DIDGI® dynamic *in vitro* gastrointestinal model, following the INFOGEST guidelines, to simulate the digestive processes of both young and old adults. Fortified yogurts containing α-tocopherol encapsulated in oil-in-water nanoemulsions were tested to assess the impact of age-related digestive conditions on digestibility and release kinetics of α-tocopherol.

The intestinal recovery of α-tocopherol was significantly higher in the young adult model (97.3 ± 5.9 %) compared to the older adult model (79.8 ± 5.2 %). Although bioaccessibility during the intestinal phase was comparable in both models (ranging from 60.54 ± 7.38 % to 78.90 ± 8.88 %), the overall estimated bioavailability was significantly greater in the young adult model (67.76 ± 7.15 %) than in the older adult model (57.59 ± 4.50 %). Distinct release kinetics were also observed between models, indicating that physiological stage significantly affects nutrient release and absorption. This study demonstrates that aging alters digestive function, impacting the bioavailability of encapsulated bioactives.

These findings underscore the importance of developing age-specific fortified foods and digestion models to optimize nutritional support for older adults.

1. Introduction

The older adult population (i.e., people over 65 years old) has been reported to be the fastest-growing age group, being expected to represent ca. 1.6 billion of the global population by 2050, with the population aged 80 years or older growing even faster (United Nations: Department of Economic and Social Affairs, 2020). Life quality and expectancy worldwide have been greatly improved due to scientific and technological advances, among others. However, longer life expectancy does not necessarily mean healthy aging. In fact, several age-related changes occur in the human body that have a profound impact on the quality of life of older adults. Among other things, aging leads to sensory impairments, sarcopenia, immunosenescence, somatic diseases or chronic conditions, psychological and cognitive difficulties (Fernandes et al., 2021).

Advances in medicine and nutrition allow the set up of methods to promote or reduce undesirable age-related changes, especially non-communicable chronic diseases. In pair, appropriate dietary choices in older adults have a proved positive impact on healthy aging. For instance, protein-rich diets provide better muscle mass maintenance and

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better muscle function; the Mediterranean diet is associated with better cognition and the Okinawan diet is associated with increased longevity (Bauer et al., 2015; Karstens et al., 2019; Willcox et al., 2014). In addition to these diets, functional foods (either fortified or enriched) have been extensively used to produce and commercialize tailored food products aimed at providing higher content in specific macronutrients (e.g., protein), nutraceuticals and bioactive compounds (García et al., 2019; Keršienė et al., 2020; Mills et al., 2018). In particular, lipophilic vitamins are fundamental to human health and are particularly important in older adults, as these bioactive compounds have great antioxidant and anti-inflammatory capacity in addition to contributing to the preservation of lipid membranes and essential fatty acids (Csapó et al., 2017; Fernandes et al., 2021). Despite this, older adults struggle to achieve their recommended daily allowance (Millen et al., 2016). For instance, α-tocopherol (a type of Vitamin E) deficiency is common in older adults, and has been associated with the occurrence of several diseases and chronic conditions (e.g., sarcopenia, vision loss, atherosclerosis, immunosenescence, cardiovascular diseases, cancers, among others) (Casas et al., 2018; Lorenzo-López et al., 2017; Rémond et al., 2015). Vitamin E sources are mainly vegetable oils, seeds, fruits and vegetables and presents a high quenching capacity and removal of reactive oxygen species, playing an important role in the regulation of genetic expression, modulation of cellular functions, inhibition of lipoxygenases and anti-inflammatory action (Boccardi et al., 2016). However, like other bioactive compounds their stability and functionality are affected by the environmental conditions, reducing their functionality and bioaccessibility. The development of functional foods containing α-tocopherol can provide food products with improved vitamin bioaccessibility (Lv et al., 2019). The development of such functional foods often relies on the application of nanostructured delivery systems, such as nanoemulsions (Gonçalves et al., 2022). These structures have been extensively studied for their ability to preserve and improve the bioaccessibility of liposoluble compounds during the gastrointestinal tract (Zhang et al., 2020). Therefore, these food products are designed to improve the delivery extent of nutrients, nutraceuticals and bioactive compounds, and consequently decrease the risk of diet-related diseases.

As important as their development, it is necessary to understand how these food products and biologically-active molecules interact with other food components, how they act in the gastrointestinal tract and if the formulation modulates their bioaccessibility and estimated bioavailability. This is particularly important in older adults, as age-related changes directly affect gastrointestinal function and motility, secretion production, sensory perception, and absorption efficiency in older adults (Amarya et al., 2015). In addition, older adults have a lower tolerance for *in vivo* experiments due to their fragility. To go further in simulating different physiological stages, the recently published INFOGEST static *in vitro* digestion model has been adapted to the older adult population (Menard et al., 2023). This model based on *in vivo* data proposes adaptations concerning the duration, pH, enzyme levels and bile salts concentration compared to the adult model (Brodkorb et al., 2019) to simulate the less effective digestive capacity of the older adult population. However, static digestions models do not simulate the gastrointestinal motility which plays an important role in food digestion and absorption and is significantly affected by age-related changes. As such, more complex dynamic *in vitro* digestion models have been proposed that are able to mimic other physiological aspects of the digestion process, such as peristalsis, changes in the physiological environment and absorption of compounds (Makran et al., 2022). Different approaches simulating such parameters have been described, still, there are no harmonized dynamic *in vitro* gastrointestinal models that mimic the digestive conditions of old adults (Denis et al., 2016; Levi & Lesmes, 2014; Shani-Levi et al., 2017). For instance, the *in vivo* digestive conditions of old adults were recently considered to set up the DIDGI® *in vitro* dynamic model and to evaluate the kinetics of protein and lipid digestion of high-protein milk formulas (Nebbia et al., 2023). The DIDGI® *in vitro* dynamic model is among the most easily modulated *in vitro* dynamic systems. This system is composed by two main compartments that simulate the stomach and the small intestine and is operated through the StoRM software, which allows to input digestive parameters (i.e., pH curves, fluids secretion rates, stomach and intestine emptying rates, etc.) of data obtained from in vivo studies (Ménard et al., 2015).

Despite the increasing interest in adapting dynamic *in vitro* digestion models to mimic the gastrointestinal conditions of the older adult population, there has been a lack of understanding of how these changes affect the extent and kinetics of food digestion as compared to the adult. It is important to understand if the digestive conditions applied significantly modulate the digestion of functional foods. Therefore, the objectives of the present study were: i) to adapt the digestive conditions suggested by the latest INFOGEST to the dynamic *in vitro* model DIDGI® (Menard et al., 2023); ii) to compare and evaluate the impact of the physiological stage on the digestibility and α-tocopherol release kinetics of yogurts fortified with nanoemulsions encapsulating α-tocopherol.

2. Materials and methods

2.1. Materials

LIPOID® P75, composed by phospholipids from soybean with 70 % phosphatidylcholine (non-GMO), was kindly provided by Lipoid GmbH (Ludwigshafen, Germany). Fat-free stirred yogurts (Carrefour Classic) and sunflower oil (Rustica, E.Leclerc) were purchased at a local supermarket (Rennes, France). Materials were all standard analytical grade. Chemicals, vitamin E (i.e., α-tocopherol, T3251), analytical standard (i.e., squalene, 442,785), gastric enzymes (i.e., porcine pepsin, P6887), intestinal enzymes (i.e., porcine pancreatin, P7545 8 USP) and bile salts (i.e., bovine bile, B3883) were purchased from Sigma Aldrich (Saint Quentin Fallavier, France).

2.2. Nanoemulsions formulation and yogurt fortification

The fortified food product developed for *in vitro* digestion in the DIDGI® was obtained by combining fat-free natural stirred yogurt with oil-in-water (O/W) nanoemulsions encapsulating α-tocopherol. The selected food matrix was stirred to reduce structural changes caused by addition and the homogenization of ingredients. The oil-in-water nanoemulsions were produced by combining the aqueous and organic phases in a ratio of 19:1 (*v/v*) and processing them using an Ultra Turrax® (T25, Ika-Werke, Staufen, Germany) (UT) for 15 min at 15,000 rpm. The aqueous phase contained 3 % of lecithin that was solubilized at 80 °C for 1 h under stirring. The organic phase was composed of sunflower oil and α-tocopherol with a final concentration of 0.75 mg/mL (i.e., 15 mg per 20 mL formulation). The fortified food product was obtained by combining 125 g of yogurt with 20 mL of nanoemulsions. The nanoemulsions were produced the day before the *in vitro* digestions and stored in the dark at 4 °C. Yogurt fortification was performed on the day of the *in vitro* digestions.

2.3. Adult dynamic in vitro digestion model

Fortified yogurts were submitted to *in vitro* digestion using the DIDGI® bi-compartmental dynamic system to simulate yogurt digestion and understand the influence of the methodologies on the kinetics of α-tocopherol release during digestion (Ménard et al., 2014). The digestive conditions used are summarized in Table 1. Simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared as described by (Lindahl et al., 1997). Basal gastric volume on the fasted state was 24 mL of SGF at pH 2.2 (Egger et al., 2019). Gastric acidification followed the curve defined by the equation: $pH = 1.68 + 3.52e(-time/45)$ and the gastric emptying conditions followed the Elashoff's equation with ($t_{1/2}$) set to 70 min and the shape of the curve at $\beta = 2$ (Ménard et al., 2018). The remaining parameters, specifically gastric concentration and flow rate, bile salts concentration and flow rate, intestinal pH and intestinal

Table 1
Gastrointestinal parameters set in DIDGI® for dynamic *in vitro* of young adult's and old adults' digestion protocols.

		Young adult	Old Adult
Gastric conditions (37 °C)			
Simulated Gastric Fluid (SGF) (stock solution adjusted at pH 6.6)	**NaCl**	**68 mmol/L**	
	$CaCl_2$	**0.6 mmol/L**	
	K^+	**13.4 mmol/L**	
Fasted state	**SGF**	**24 mL**	
	pH	**2.2**	
Ingested amount		**145 g**	
Gastric pH	**Acidification Curve**	**pH = 1.68 + 3.52e (−time$_{(min)}$/45)**	**pH = 5e (−0.005*time$_{(min)}$)**
SGF + pepsin	**Pepsin**	**2000 U/mL of gastric content**	**1200 U/mL of gastric content**
	Flow rate	**1 mL/min from 0 to 10 min**	
	Flow rate	**0.5 mL/min from 10 to end**	
Gastric emptying (Elashoff fitting)	$t_{(1/2)}$	**70 min**	**98 min**
	β	**2**	**2**
Intestinal conditions (37 °C)			
Simulated Intestinal Fluid (SIF) (stock adjusted at pH 6.6)	**NaCl**	**142 mmol/L**	
	$CaCl_2$	**0.5 mmol/L**	**1 mmol/L**
	K^+	**5.4 mmol/L**	
Intestinal pH		**6.6**	
SIF + Pancreatin	**Pancreatin**	**7 %**	**5.5 %**
	Flow rate	**0.25 mL/min**	
SIF + Bile	**Bile**	**4 %**	**2.7 %**
	Flow rate	**0.5 mL/min from 0 to 30 min**	
	Bile	**2 %**	**1.3 %**
	Flow rate	**0.5 mL/min from 30 min to end**	
Intestinal emptying (Elashoff fitting)	$t_{(1/2)}$	**160 min**	
	β	**1.6**	

emptying were set up as described by (Ménard et al., 2018).

Dynamic digestions were performed in triplicate for 270 min. Initial samples were collected in the gastric phase (G0) and after 35, 70 and 105 min. The selected gastric sampling points correspond to fractions of gastric emptying half-time (i.e., $t_{2/8}$, $t_{4/8}$ and $t_{6/8}$), corresponding to 35, 70 and 105 min, respectively. Particle size analyses were performed on gastric samples right after collection. In the intestinal phase, samples were collected after 80, 120, 160, 200, 240 and 270 min of digestion (i.e., I80, I120, I160, I200, I240 and I270, respectively) for α-tocopherol analyses. The intestinal emptying (IE, representing the sample that was emptied from the intestine reactor) was collected in intervals from 0 to 120 min, 120 to 180 min, and 180 to 270 min (i.e., IE120, IE180, IE270, respectively). Intestinal samples were stored at −20 °C before lipid extraction.

2.4. Old adult dynamic in vitro digestion model

In order to simulate the digestive conditions of the older adult population (i.e., people over 65 years old), some modifications were applied in the system set up (parameters are summarized in Table 1). Due to a lack of literature, SGF composition and fasted gastric conditions remained the same as proposed for the adult digestion model (Egger et al., 2019; Nebbia et al., 2023). Based on the recent recommendations proposed by the INFOGEST international consensus for the adaptation of the digestion model to the older adult population, the gastric emptying half-time was increased by 40 % and lead to a $t_{1/2} = 98$ min ($\beta = 2$), the porcine pepsin was reduced by 40 % (i.e., 1200 U/mL of gastric content) and in the intestine, the calcium chloride concentration of the SIF was increased to 1 mmol/L and pancreatin and bile concentrations reduced (Menard et al., 2023). The remaining conditions, namely acidification curve in the gastric compartment, intestinal enzymes, bile salts concentration and flow rate, as well as intestinal emptying, followed the conditions proposed by (Nebbia et al., 2023).

As in the adult model, digestions were performed in triplicate for 270 min. Because gastric emptying was longer in the old adult model, the first samples were collected in the gastric phase (G0) and after 49, 98 and 147 min, corresponding to the same fractions of gastric emptying half-time (i.e., $t_{2/8}$, $t_{4/8}$ and $t_{6/8}$, respectively). Intestinal samples were similar in both models, being collected for α-tocopherol analyses after 80, 120, 160, 200, 240 and 270 min (i.e., I80, I120, I200, I240 and I270, respectively), as well as the intestinal emptying, collected between 0 and 120 min, 120 to 180 min, and 180 to 270 min (i.e., IE120, IE180, IE270, respectively). Intestinal samples were stored at −20 °C before lipid extraction.

2.5. Particle size measurements

The size distribution of the nanoemulsions was assessed after production and on the following days using a Mastersizer 2000 laser light scattering (Malvern Instruments, Malvern, UK). The particle size distribution of the fortified yogurts particles before and during the gastric phase was also measured. The refractive index used was 1.462 for vegetable oil at 633 nm and 466 nm and 1.33 for water (dispersing solution in the measurement cell). Analyses were performed with and without sodium dodecyl sulphate (SDS) to evaluate a possible aggregation effect.

2.6. α-Tocopherol quantification

Quantification of α-tocopherol during the intestinal phase was performed to compare the kinetic release during the digestion of young and old adults. Total and soluble samples (obtained after a centrifugation step at 18000 *g* during 30 min) were analysed at each sampling time. α-Tocopherol was extracted from the samples using a liquid-liquid protocol described by (Lv et al., 2019). Briefly, 1 volume (i.e., 500 mL) of hexane:ethanol (1:1, *v/v*) was added to samples, followed by vortex and centrifugation at 1500 *g* for 10 min. Squalene was used as an internal standard and was added to each sample at a fixed concentration (i.e., 1 mg/mL) before extraction. The hexane fraction was collected and the extraction procedure was repeated 3 times. The combined hexane fractions were dried under nitrogen. The dried samples were resuspended in methanol before quantification. α-Tocopherol quantification was performed using a HPLC (35900E, Agilent, USA) coupled with a ELSD detector (PL-ELS 1000, Polymer Laboratories, UK). The column used was a Vydak® 218TP54 C18 with 5 μm (Grace Discovery Sciences, Australia). The HPLC mobile phase used was methanol-water (95:5, *v/v*) eluted in an isocratic mode at 0.4 mL/min, the oven temperature was set to 50 °C and the injection volume was 10 μL. Concerning the ELSD parameters, the evaporator temperature was set to 55 °C, the nebulizer temperature to 40 °C and the nitrogen gas flow to 1.5 bar. Due to non-linear response of ELSD detector, calibration curves were obtained using power regression curves as described in (Slavin & Yu, 2012).

α-Tocopherol (*αtoc*) quantification allowed to determine the α-tocopherol recovery, bioaccessibility and estimated bioavailability in the DIDGI® digestion protocol for young and old adults. The recovery considers the amount of compound quantified in HPLC-ELSD, which remained untransformed during the digestion, as described in Eq. 1.

$$\alpha toc_{recovery} = [\alpha toc]_{IE} \times mass_{IE} \quad (1)$$

Where $[\alpha toc]_{IE}$ is the concentration of α-tocopherol in each intestinal emptying sample, $mass_{IE}$ is the intestinal delivered mass (g) in each intestinal emptying sample. The recovery percentage was determined as the percentage of the compound recovered in each IE sample in regard to the initial compound amount added into the yogurt, as described in Eq. 2.

$$Recovery\ (\%) = \left(\alpha toc_{recovery} / \alpha toc_{yogurtbeforedigestion}\right) \times 100 \quad (2)$$

Where $\alpha toc_{micelle}$ is the α-tocopherol concentration in the mixed micelles. The bioaccessibility was calculated as the percentage of α-tocopherol recovery in each IE sample in regard to the soluble fraction of the same IE sample (i.e., micellar phase, obtained from centrifugation after sampling), that theoretically indicates the percentage of available compound that was solubilized into mixed micelles, as described in Eq. 3.

$$Bioaccessibility\ (\%) = \left(\alpha toc_{micelle} / \alpha toc_{recovery}\right) \times 100 \quad (3)$$

Finally, the estimated bioavailability, which estimates the α-tocopherol absorption, was determined as the product between recovered α-tocopherol and α-tocopherol solubilized into mixed micelles (i.e., bioaccessible), as described in Eq. 4.

$$Estimated\ Bioavailability\ (\%) = (Recovery \times Bioaccessibility)/100 \quad (4)$$

2.7. Statistical analysis

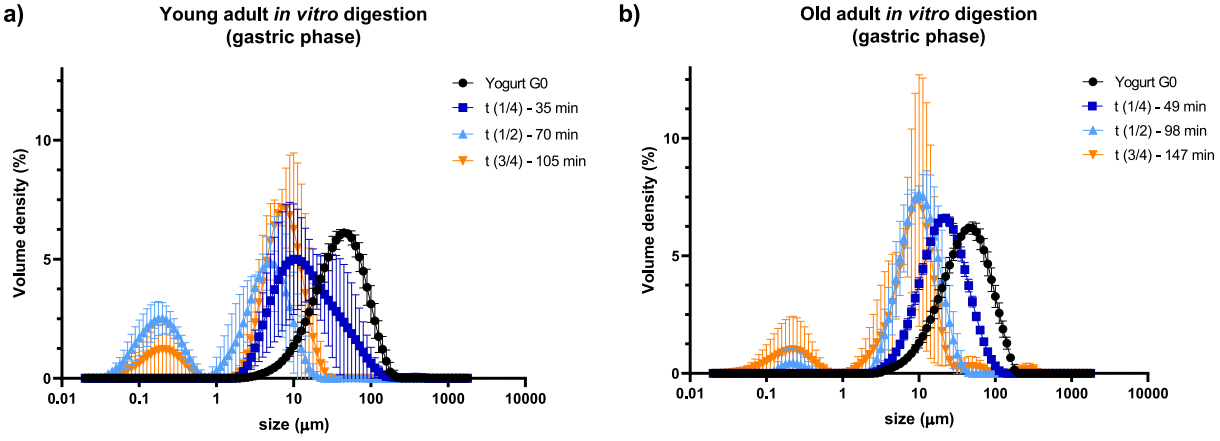
Statistical analysis and graph plotting from the data obtained in this study were performed using Prism version 9.0.0 (GraphPad Software Inc., California USA). A two-way ANOVA followed by post-hock Tukey's honestly significant difference (HSD) test were selected to identify statistically significant differences at 95 % significance level ($p\ value < 0.05$). Results are presented as mean values of experiments performed in triplicate ± standard deviation (SD).

3. Results and discussion

3.1. Nanoemulsions size distribution and stability during in vitro digestion

The O/W nanoemulsions particle size distributions were similar throughout all experiments and stable between the day of production and the following day. The O/W nanoemulsions presented a unimodal distribution and surface weighted mean (i.e., D[3,2]) of 156 ± 10 nm. This outcome proved that the homogenization process had good reproducibility. Despite some misconceptions, the size of a nanoemulsion can be defined by particle size less than 200 nm (McClements, 2021). Nanoemulsions were also analysed after storage overnight at 4 °C in the dark, which showed a insignificant increase of the D[3,2] to 163 ± 14 nm caused by the formation of a small secondary population at ca. 800 nm. The production of O/W nanoemulsions is preferably based on high-energy processes with high enthalpy and energy transfer. Therefore, longer processing periods will result in increased homogeneity and stability. As the UT uses the principle of a rotor-stator mixer, its efficiency falls behind high pressure systems, hence its particles' stability is typically lower compared to more potent processing techniques, such as high-pressure homogenizer or microfluidizer (Liu et al., 2019; Mendes et al., 2020; Schroën et al., 2020). Additionally, these changes could also be related to the surfactant properties. Because lecithin was used, a zwitterionic surfactant, it's self-assembled hydrogen bonds may have undergone re-structuration during overnight storage. In this case, originally less stable bounds at the oil-water interface may have been destabilized in favour of more stable interaction (Fuentes et al., 2021).

During the gastric phase of *in vitro* digestion experiments, particle size distribution was also analysed as presented in Fig. 1. Since nanoemulsions were incorporated into yogurts, the initial particle sizes were substantially higher than those of the nanoemulsions alone (Fig. 1), being also reported the dimensions of yogurt constituents, as well of possible interactions between nanoemulsions and yogurt constituents (mainly proteins and minerals). Nonetheless, the initial particle size distribution in young and old adult digestion experiments did not present statistically significant differences ($p\ value > 0.8$, D[3,2] values presented in Fig. 1), indicating that the incorporation of nanoemulsions into yogurts was reproducible and that the obtained food product was homogeneous. These results were in accordance with the structural properties of stirred yogurt reported in the literature, which suggests that the diameter of the concentrated microgel particles (typical of stirred yogurts) range between 10 and 100 μm (Moussier et al., 2019). The particle size distribution of the formulations used in this study was comparable to results obtained for similar products (i.e., stirred fat-free



		D [3,2] (μm)		pH	
	Young adult	Old adult	*p* value	Young adult	Old adult
Yogurt G0	28.8 ± 2.9	29.8 ± 1.3	0.804 (ns)		
t (1/4)	11.6 ± 3.1	17.2 ± 0.8	0.002 (**)	3.30	3.95
t (1/2)	0.5 ± 0.3	7.1 ± 4.3	0.045 (*)	2.42	3.06
t (3/4)	3.5 ± 4.2	5.2 ± 5.1	0.898 (ns)	2.02	2.40

Fig. 1. Particle size distribution stability during gastric phase of the *in vitro* digestion using the young adult (a) and the old adult (b) model. Statistically significant differences are indicated by * ($p < 0.05$) and ** ($p < 0.01$).

yogurts), regardless the addition of O/W nanoemulsions (Huc et al., 2016).

Once again, because the proposed protocols used different gastric phase durations, the analyses were carried at fractions of the gastric emptying $t_{(1/2)}$. The particle size distribution showed statistically significant differences at gastric emptying $t_{(1/4)}$ (i.e., 35 and 49 min on the young and old adult model, respectively), shifting its D[3,2] to 11.61 ± 3.05 and 17.20 ± 4.29 μm for the young and old adult model, respectively (Fig. 1). This outcome may have been related to delayed gastric acidification on the old adult model and the reduced proteolytic activity, reflected in a less pronounced hydrolysis of larger proteins and peptides into smaller molecules.

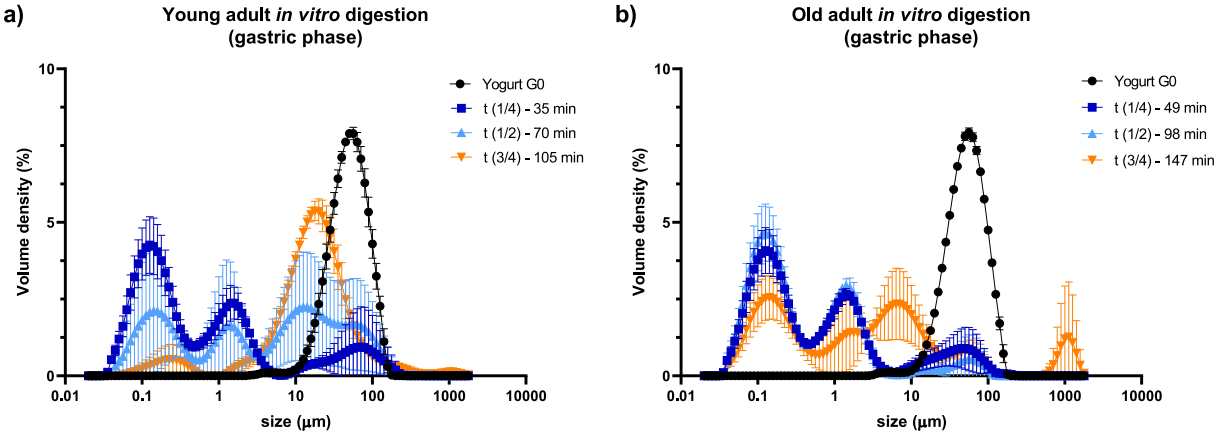
After $t_{(1/4)}$, samples digested using the young adult model presented a bimodal distribution, with a secondary population with a mean diameter peak at ca. 200 nm, that became more pronounced in the final sample. This peak could be representative of casein micelles, that are solubilized during the gastric phase by the action of pepsin and also due to the gradual acidification of the medium (Day et al., 2015). In fact, similar results were obtained when infant formulas were digested in the DIDGI®, suggesting the occurrence of pepsinolysis of κ-casein (Chauvet et al., 2023). For both models, particle size distribution at $t_{(3/4)}$ did not present statistically significant differences and showed a bimodal distribution constituted by the casein micelles at ca. 200 nm and the remaining yogurt constituents. However, as gastric conditions were less aggressive in the old adult model, the volume density of the casein micelles population was also lower. Thus, it can be concluded that different protocols produced similar levels of particle alterations, even though protein degradation was faster and more pronounced in the young adult model.

The analysis of the particle size distribution in SDS, are presented in Fig. 2. The particle size distribution profiles after SDS dilution allow to evaluate whether aggregation or nanoemulsion coalescence occurred in the gastric phase due to the reducing effect of SDS. Once again, the stability of the samples was similar between models until $t_{(1/4)}$, presenting a trimodal distribution with mean diameter peaks at ca. 0.125 (i.e., 125 nm), 1.5 and 45 μm. The smallest population should represent the structures described previously, but the secondary population observed in Fig. 2 was, in fact, representative of the aggregation between two populations. In this study the fat content of the formulation was very low, since the yogurts used were fat-free and nanoemulsions only contained 5 % of oil. In this sense, the population with a mean diameter peak of 1 μm could represent the rearrangement of nanoemulsion constituents, such as interactions between lecithin molecules or even the formation of complexes between the milk proteins available. Aggregation effects were more pronounced in the young adult model after $t_{(1/2)}$, since the volume density of the smaller population started to decrease and a larger population with mean diameter peak at ca. 18 μm began to form, representing ca. 5.4 % of volume density in the final sampling point. These effects were not as severe in the old adult model, since statistically significant differences were observed in D[3,2] (Fig. 2) and the multimodal distribution was maintained with several populations representing ca. 2.5 % of volume density each.

To conclude, the lower enzymatic activity and slower acidification profile applied in the old adult model caused a similar destabilization of the fortified yogurt at earlier stages of digestion. Conversely, after $t_{(1/2)}$, the young adult model was substantially more effective in the hydrolysis of the gastric content (i.e., chyme), and led to more pronounced aggregation effects.

3.2. Kinetics of α-Tocopherol release

The kinetics of α-tocopherol release were evaluated in the intestinal phase of *in vitro* digestions to assess a possible influence of the physiological stage. As mentioned in **section 2.3**, samples were collected from the intestinal compartment to analyse both total and micellar (obtained after a centrifugation step) α-tocopherol concentration at the different sampling times. α-Tocopherol quantified by HPLC-ELSD is presented in Fig. 3a. As expected, the concentration of α-tocopherol in the digesta was higher than that obtained in the micellar phase, since insoluble matter will precipitate when centrifuging. The micellar phase is the soluble fraction of the samples and represents the bioaccessible fraction, corresponding to the estimated absorbed fraction in vivo. As such, the concentration of bioactive compounds in the micellar phase will be strongly influenced by the components available on the digesta. As α-tocopherol is hydrophobic, it should be primarily entrapped into mixed micelles to be solubilized (Yang et al., 2015). This mechanism is mainly mediated by biliary salts and depends on several factors, such as the release of the bioactive compound from the nanoemulsion core, its



		D [3,2] (μm)		pH	
	Young adult	Old adult	*p* value	Young adult	Old adult
Yogurt G0	41.5 ± 2.8	43.7 ± 1.3	0.221 (ns)		
t (1/4)	5.2 ± 7.5	0.2 ± 0.01	0.262 (ns)	3.30	3.95
t (1/2)	0.7 ± 0.4	0.2 ± 0.03	0.014 (*)	2.42	3.06
t (3/4)	6.1 ± 6.5	0.5 ± 0.2	0.123 (ns)	2.02	2.40

Fig. 2. Particle size distribution stability during the gastric phase of the *in vitro* digestion using the young adult and the old adult model. Samples were diluted in SDS to avoid aggregation effects. Statistically significant differences are indicated by * ($p < 0.05$).

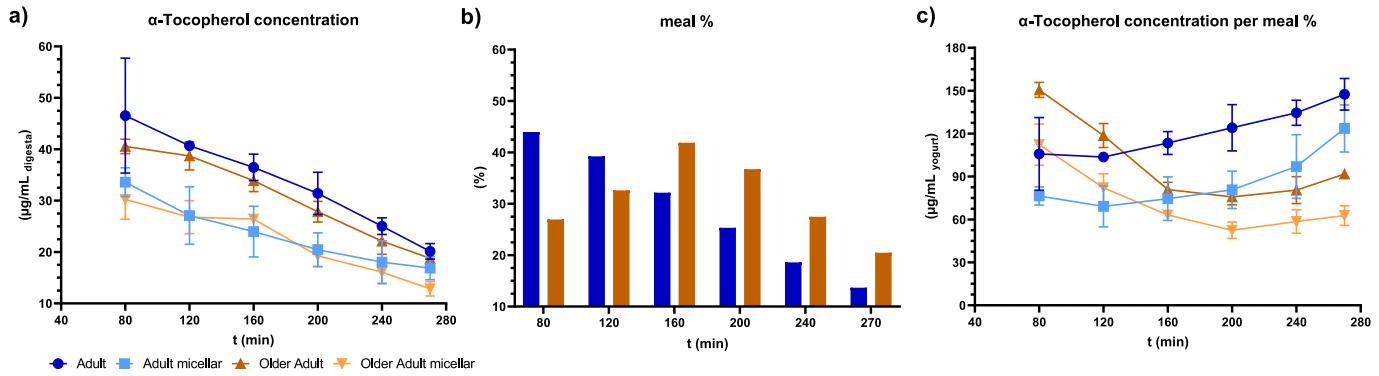


Fig. 3. α-Tocopherol release kinetics during the intestinal phase of the *in vitro* digestion using young adult and old adult models. Concentration values presented in c) were determined considering the amount of meal available in the intestinal reactor at each sampling point (b).

stability within the digesta medium, the availability of biliary salts, phospholipids and products of lipolysis, among others (Macierzanka et al., 2019). Comparing α-tocopherol concentrations throughout the intestinal phase (**Fig. 3**b), digestions performed using the young adult model achieved statistically significant (p *value* = 0.044) higher levels in all digesta sampling points; the area under the curve (AUC) of α-tocopherol concentrations for the young and old adult model were 6450 ± 266.5 and 5882 ± 133.7, respectively. These results reflect the higher enzymatic activity in the young adult model, leading to a faster proteolysis of the food matrix and a higher release of α-tocopherol. In fact, these results were expected and are easily related to physiological parameters found in the target population. Literature suggests that malnutrition in older adults may be both a cause and an effect of age-related physiological changes, such as changes in body composition, in the gastrointestinal tract, in sensory function and in fluid and electrolyte regulation, which lead to a loss of appetite and a reduction of the metabolic rate and nutrient absorption (Fernandes et al., 2021). However, when comparing the results of the micellar samples, the α-tocopherol concentration obtained was superior in the old adult model at 160 min (23.98 ± 4.9 and 26.42 ± 0.90 $\mu g/mL_{digesta}$ in the young and old adult models, respectively). At first this result appears to be an outlier, but in fact it can be explained by the differences in gastric emptying between the protocols (i.e., young adult $t_{(1/2)}$ gastric emptying was 70 min, whereas in the old adult it was 98 min). Gastric emptying is also a main parameter explaining differences of concentration in the intestinal reactor between the young and old adult, that affects the meal percentage in the intestine at a given time (**Fig. 3**b). At 160 min in the young adult model, due to different gastric emptying rates, the gastric content was completely emptied into the intestinal compartment, whereas it was not totally emptied in the old adult model. In addition, micellization of α-tocopherol may have been more effective in the final stages of the old adult model because its gastric emptying was slower, which consequently may have resulted in higher availability of bile salts, phospholipids and free fatty acids compared to the young adult model (Yang et al., 2015).

To further discuss this hypothesis and better understand the kinetics of α-tocopherol release during the intestinal phase, the data obtained were normalized considering the amount of fortified yogurt at each sampling time point (presented in **Fig. 3**c). This calculation allowed the comparison of the α-tocopherol release between young and old adult digestions. α-tocopherol concentration after 80 min in the digesta and micellar phases obtained using the old adult model were higher (150.51 ± 5.25 and 112.32 ± 14.35 $\mu g/mL_{yogurt}$ in the digesta and micellar phase) than that measured using the young adult model (105.86 ± 25.44 and 76.41 ± 6.36 $\mu g/mL_{yogurt}$ in the digesta and micellar phase). Likewise, at 120 min α-tocopherol concentration was higher in the old adult model (118.66 ± 8.42 and 82.13 ± 9.82 $\mu g/mL_{yogurt}$ in the digesta and micellar phase) than in the young adult model (103.68 ± 0.69 and 69.09 ± 14.27 $\mu g/mL_{yogurt}$ in the digesta and micellar phase) Interestingly, α-tocopherol concentration decreased in the old adult model up to 200 min of the intestinal phase. However, as mentioned above, gastric emptying was slower in the old adult model, meaning that less food was available in the intestinal reactor in the early stages, thus influencing the results obtained for the old adult digestion. Moreover, as the gastric phase of the old adult model was less intense (i.e., lower proteolytic enzymes activities and slower acidification), the initial gastric samples delivered into the intestinal reactor should have presented a different degradation extent compared to the samples delivered using the young adult model. As such, samples delivered from the gastric phase using the young adult model must have had a higher content of peptides that may have protected nanoemulsions and hindered α-tocopherol release. It has been shown in the literature that milk and whey peptides are effective emulsifiers in nanoemulsions because of their amphiphilic nature, and due to their smaller dimensions compared to nanoemulsions droplets, they are able to form and stabilize nanoemulsions systems alone or in combination with other emulsifiers (Adjonu et al., 2014). In fact, in the young adult model α-tocopherol concentrations increased throughout the intestinal phase duration, suggesting a gradual proteolysis, lipolysis and destabilization of the nanoemulsions. Although an opposite result was observed in the old adult model, the same possibility could justify the results obtained: since yogurt digestion in the gastric phase was delayed, higher concentrations of α-tocopherol were released at initial stages of the intestinal phase. As the gastric emptying progressed, higher amounts of peptides were delivered to the intestinal phase, hindering the destabilization of the nanoemulsions and reducing the release of α-tocopherol. This hypothesis is in accordance with other studies, since it has been suggested that the degree of intestinal proteolysis and lipolysis depends on the amount of substrate or hydrolytic products accumulated during intestinal digestion, which is modulated by the gastric emptying parameters established (Mella et al., 2021).

As discussed, α-tocopherol release kinetics were greatly affected by the application of different digestion parameters, altering the extent and profile of its release. Moreover, it can be suggested that the duration of the gastric phase and the by-products obtained from gastric digestion affect the subsequent intestinal degradation and with the release of the bioactive compound of interest. Therefore, applying the appropriate digestive parameters in *in vitro* digestion systems is very important to properly tailor the digestion and release profiles of food products for specific populations.

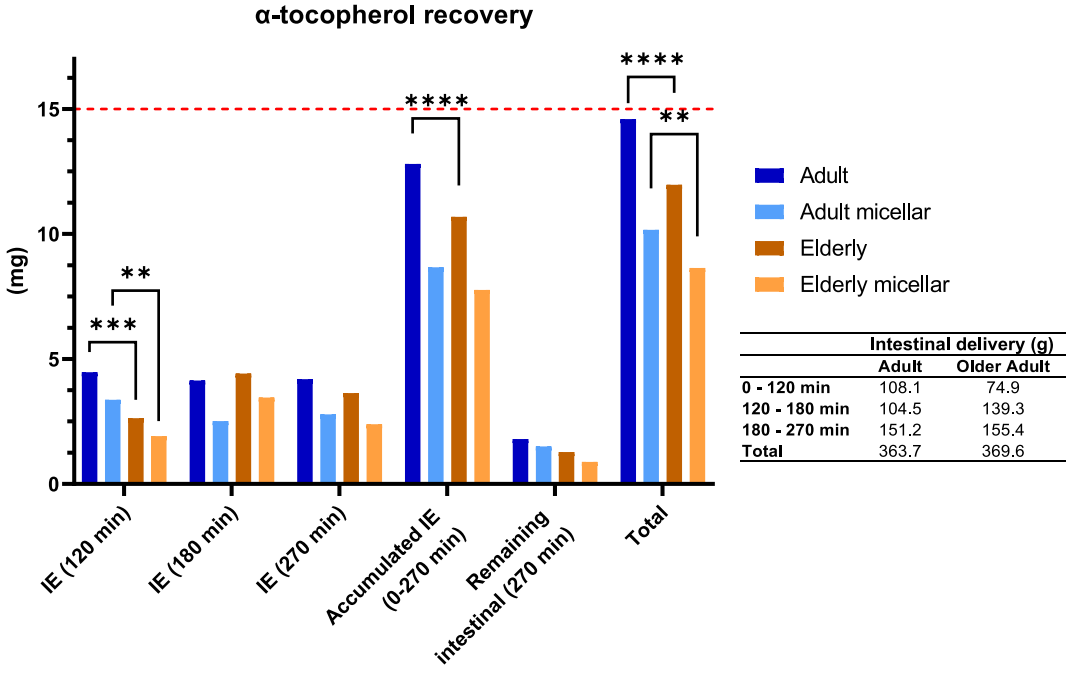
3.3. *α-Tocopherol recovery, bioaccessibility and estimated bioavailability*

α-Tocopherol recovery, bioaccessibility and estimated bioavailability were calculated as described in section 2.3. These calculations were done using samples from the intestinal emptied fractions of the *in vitro* digestions, because it is at this stage of the digestion that the micellization/solubilization and absorption of liposoluble vitamins

takes place (Reboul, 2017). Fig. 4 shows the amount of α-tocopherol quantified in different fractions after intestinal digestion. For the young adult model, α-tocopherol mass release was constant during the selected sampling points of the intestinal phase (i.e., 4.47 ± 0.23, 4.14 ± 0.19 and 4.19 ± 0.33 mg, between 0 and 120, 120–180 and 180–270 min, respectively). However, as the sampling intervals had different durations, the α-tocopherol release rate varied, being much faster between 120 and 180 min (i.e., 2.23, 4.14 and 2.79 mg/h, between 0 and 120, 120–180 and 180–270 min, respectively).

The higher release rate between 120 and 180 min are correlated with established digestion parameters, such as the gastric emptying. In the young adult model, the gastric $t_{(1/2)}$ was 70 min, therefore the entire gastric content was set to be totally delivered between 120 and 180 min, thus all the α-tocopherol available had been delivered to the intestinal reactor. On the contrary, for the old adult model, gastric $t_{(1/2)}$ was 98 min, so the gastric content was set to be fully delivered between 180 and 270 min. In fact, the young adult model had a significantly higher ($p < 0.05$) α-tocopherol release between 0 and 120 min than the old adult model, and although there was no statistical significance (p *value* > 0.05), the opposite was observed between 120 and 180 min, with superior α-tocopherol release in the old adult model. Because α-tocopherol release in the young adult model was significantly higher at initial stages, the accumulated recovery of α-tocopherol was also statistically significantly higher in the young adult model. The total amount of α-tocopherol recovered (obtained by the sum of the accumulated intestinal delivery and the remaining intestinal quantity) was higher in the young adult model (14.60 ± 0.89 mg of α-tocopherol was obtained out of the 15 mg added into the yogurt), whereas this was not the case in the old adult model only 11.96 ± 0.78 mg of α-tocopherol was recovered). As such, the percentage of α-tocopherol recovery (determined by comparing amount recovered until 270 min to the amount ingested) was 97.30 ± 5.91 and 79.76 ± 5.21 % for the young and old adult model, respectively. As the digestion was stopped at 270 min, further α-tocopherol release may have occurred latter in the digestion process, especially in the old adult model. Thus, this result demonstrates that food digestion and bioactive compound release may be delayed in old young adults. It has been suggested that aged-related changes in metabolic expenditure, within the gastrointestinal motor function and in endogenous enzyme production may lead to reduced postprandial estimated bioavailability of amino acids, minerals and vitamins (Fernandes et al., 2021; Rémond et al., 2015). It is also important to note that α-tocopherol stability could be affected by the digestion outcome, being possible the occurrence of oxidation or hydrolysis during the digestive process (Desmarchelier et al., 2013; Maldonado et al., 2023). Additionally, release and recovery of the encapsulated bioactive compound is correlated with the composition of the food formulation and it has been suggested that the oil type, emulsifier type and food matrix all play an important role on stability of the compound of interest (Molet-Rodríguez et al., 2023; Zhang et al., 2015; Zou et al., 2016). For instance, the stability of α-tocopherol was significantly increased by incorporating the nanoemulsion into yogurts, since authors have reported that α-tocopherol stability in similarly produced O/W nanoemulsions and pickering emulsions incorporated into gelatine or milk was ca. between 50 and 63, 49 and 63 %, respectively (Ribeiro et al., 2022). From our result we can conclude that the kinetics of α-tocopherol release were fundamentally changed in the different models, and such differences should be mainly attributed to the differences in gastric emptying. By increasing the gastric emptying in the old adult model, yogurt transit from the stomach to the small intestine was delayed, resulting in a slower α-tocopherol release and subsequent solubilization/micellization.

Nonetheless, and despite the reduced recovery and slower release obtained for the old adult model, the proportion between α-tocopherol in the IE sample and in the soluble fraction (i.e., micellar phase obtained after centrifugation) was similar across all sampling points of the intestinal digestion on both models (Fig. 3c and 4). This result indicates that the digested sample would have similar characteristics, and that the encapsulation of α-tocopherol had a similar outcome on the bioaccessibility of both models. Fig. 5a shows the α-tocopherol bioaccessibility of the samples at the intestinal emptying level. The bioaccessibility ranged between 60.54 ± 7.38 and 78.90 ± 8.88 %, in agreement with the results obtained for the bioaccessibility of α-tocopherol when encapsulated into O/W nanoemulsions (Fernandes



	Intestinal delivery (g)	
	Adult	Older Adult
0 - 120 min	108.1	74.9
120 - 180 min	104.5	139.3
180 - 270 min	151.2	155.4
Total	363.7	369.6

Fig. 4. α-Tocopherol recovery from the digesta and micellar phase of samples from the intestinal emptying (IE). Accumulated IE represents the sum of intestinal emptying from 0 to 270 min. Remaining intestinal represents the amount of α-tocopherol that remained inside the intestinal compartment at 270 min. Total represents the sum of IE fractions and the remaining intestinal. Red dotted line indicates the total amount of α-tocopherol added into the fortified yogurts. Statistically significant differences are indicated by ** ($p < 0.01$), *** ($p < 0.001$) and **** ($p < 0.0001$).

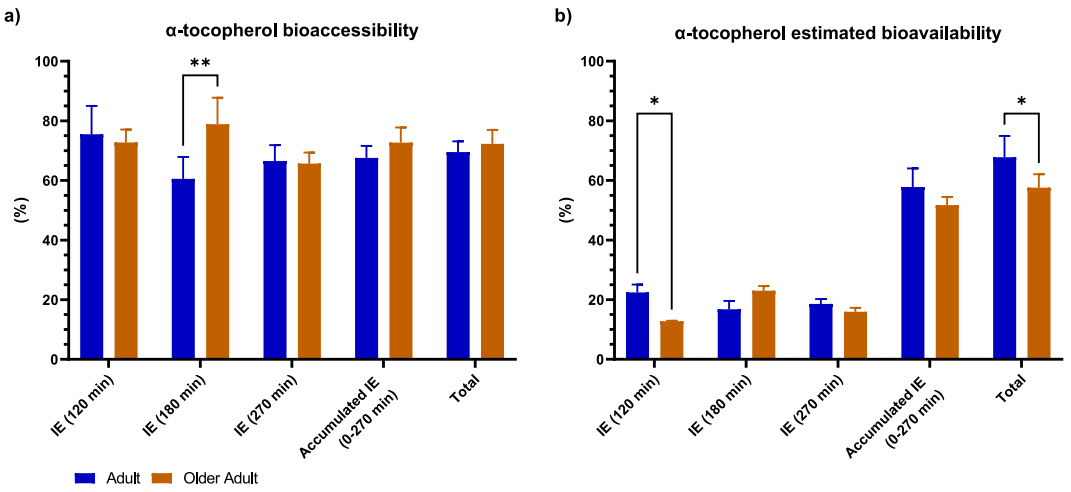


Fig. 5. α-Tocopherol bioaccessibility (a) and estimated bioavailability (b) during the intestinal phase of the *in vitro* digestion. Accumulated IE represents the sum of intestinal emptying from o to 270 min. Total represents the sum of IE fractions and the remaining intestinal. Statistically significant differences are indicated by * ($p < 0.05$) and ** ($p < 0.01$).

et al., 2024; Lv et al., 2019; Maldonado et al., 2023). Statistically significant differences between models were observed at 180 min, which can be explained by higher meal percentage available in the intestinal reactor in the old adult model (Fig. 3b). Consequently, a higher intestinal delivery mass (Fig. 4) was obtained in the old adult model (i.e., 139.3 and 104.5 g were emptied from the intestinal reactor between 120 and 180 min in the old and young adult model, respectively). In addition, the delayed α-tocopherol release and the lower percentage delivered from the intestinal reactor at 120 min may have contributed to an improved micellization at latter stages, as found in the old adult model (Yang et al., 2015).

However, because digestion is a continuous process, the release profile and its extent can have a significant impact on the absorption of dietary compounds. This phenomenon is observed when analysing the estimated bioavailability, presented in Fig. 5b. It is important to mention that the estimated bioavailability is dependent on several physiological factors and is modulated by different cell types and microorganisms present in the intestinal epithelium and, therefore, this result presents an estimation based on the available bioactive compound at a given sampling point that had been solubilized into mixed micelles (Kiyose, 2021). Once more we see statistically significant (p *value* < 0.05) differences at IE 120 min that could be justified by differences in the gastric emptying time between protocols, suggesting that the young adult model would lead to a higher absorption of the bioactive compound at initial stages of the digestion. Through the digestion there were no significant differences (p *value* > 0.05) between the estimated bioavailability on both protocols. However, a statistical significantly (p *value* < 0.05) higher estimated bioavailability was on overall obtained in the young adult model. Albeit this result is indicative, it can be suggested that the digestive conditions proposed for young adults contemplate a faster and superior absorption of bioactive compounds compared to older adults. Such outcome is important and must be considered when proposing targeted food products (or even medicines) for the elderly population.

4. Conclusions

This study highlights the critical role of age-related digestive physiology in modulating the release and bioavailability of α-tocopherol-fortified yogurts as a model system. Through the application of a dynamic *in vitro* gastrointestinal system adapted to simulate young and old adult digestion, clear differences emerged in the function of O/W nanoemulsions across physiological stages. The slower gastric emptying rate and reduced enzymatic activity characteristic of the old adult model significantly altered the α-tocopherol release profile, resulting in earlier but less sustained release compared to the young adult model. Although initial release rates in the old adult model were higher, likely due to reduced proteolytic degradation of the yogurt matrix, this did not result in improved α-tocopherol recovery or increased estimated bioavailability. In contrast, the young adult model, with its more rapid gastric emptying and higher enzyme activity, granted higher protection and delayed the release of nanoemulsions, ultimately resulting in more efficient delivery of the active compound.

These findings suggest that the interaction between digestion kinetics, food matrix structure, and nanoemulsion stability is strongly dependent on the physiological stage, with younger digestive conditions offering a more favorable environment for the controlled release and absorption of encapsulated bioactives. The use of liquid matrices or modified release systems designed to adapt to the slower gastric dynamics of older adults may help mitigate these limitations and improve nutrient delivery in this demographic. Overall, the study underscores the necessity of using physiologically relevant, age-specific *in vitro* models to evaluate the performance of functional foods, enabling more precise nutritional strategies tailored to aging populations and advancing the field of personalized nutrition.

CRediT authorship contribution statement

Jean Michel Fernandes: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Olivia Ménard:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Marie-Françoise Cochet:** Software, Resources, Investigation, Formal analysis. **Jordane Ossemond:** Software, Resources, Methodology, Investigation, Formal analysis. **António A. Vicente:** Writing – review & editing, Validation, Supervision, Funding acquisition. **Ana C. Pinheiro:** Writing – review & editing, Validation, Supervision. **Didier Dupont:** Writing – review & editing, Validation, Supervision, Software, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

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

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Data availability

Data will be made available on request.

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