Assessment of the bioaccessibility and bioavailability prediction of omega 3 and conjugated fatty acids by *in vitro* standardized digestion model (INFOGEST) and cell model

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Abstract

Omega 3 EPA and DHA are polyunsaturated fatty acids with relevant health benefits. Conjugated linoleic and linolenic acids are known for their anti-carcinogenic effect, anti-inflammatory properties and body weight reduction. To achieve therapeutical doses, high amounts of these fatty acids' food sources must be consumed. Thus, the intake of enriched oils with a high concentration of these fatty acids is often used. But several factors influence their bioavailability. Here, by using the INFOGEST static *in vitro* protocol of gastrointestinal tract digestion it was studied the bioaccessibility of these fatty acids in different matrixes: Pomegranate and Fish oil and omega 3, CLA and CLNA soft-gel enriched capsules. After digestion, the Recovery Index for the major bioactive PUFAs are very low: Pomegranate oil is 2%, Fish oil 11-13%, CLNA 17%, CLA 6% and Omega 3 capsules 3%. Higher initial concentrations of these PUFAs seem to be related to higher degrees of oxidation. In Pomegranate oil, CLNA and Omega 3 capsules, the digestion process negatively influenced the antioxidant potential. The opposite was verified for the Fish oil and CLA capsules. Importantly, bioaccessibility studies of similar matrixes are very scarce and intestinal permeability is absent in most of the studies. Intestinal permeability studies were performed using a Caco-2/HT29-MTX co-culture: there is significative incorporation of the bioactive fatty acids into the intestinal cells, which may affect their permeability performance. Interestingly, most fatty acids remain in the non-bioaccessible fraction which may be relevant when designing oral routes of administration and in gut microbiota modulation.

Keywords

Fish oil; Pomegranate oil; INFOGEST digestion method; Bioaccessibility; Intestinal Permeability; Bioactive fatty acids

Abbreviations

α-linolenic acid (ALA); 2,2'-Azobis(2-methyl-propionamidine) dihydrochloride (AAPH); 2-azinobis-(3-ethylbenzothiazoline-6 sulfonic acid) diammonium salt (ABTS); 2,2-diphenyl-1- picrylhydrazyl (DPPH); Apparent permeability (Papp); Bioaccessibility index (BI); Conjugated fatty acids (CFAs); Conjugated linoleic acid isomers (CLA); Conjugated linolenic acid (CLNA); Dimethylformamide (DMF); Docosahexaenoic acid (DHA); Docosapentaenoic acid (DPA); Dulbecco's Modified Eagle Medium (DMEM); Eicosapentaenoic acid (EPA); European Food and Safety Authority (EFSA); Fatty acids (FAs); Free fatty acids (FFAs); Gastrointestinal tract (GIT); Linoleic (LA); Peroxisome proliferator-activated receptor (PPAR); Polyunsaturated fatty acids (PUFAs); Punicic acid (PUA); Randomly methylated β-cyclodextrin (RMCD); Rumenic acid (RA); Recovery index (RI); Reactive oxygen species (ROS); Transepithelial electrical resistance (TEER); Triglycerides (TGs); Triacylglycerol (TAG); Trolox Equivalent (TE).

1. Introduction

The importance of lipids in our health is now well-established (Salsinha et al., 2023). Among the main important lipid molecules are triglycerides (TGs) and their metabolites (mono- and diglycerides and fatty acids (FAs)) (Valenzuela, 2012). Regarding FAs, polyunsaturated fatty acids (PUFAs) are the ones most widely recognized and studied due to their proven health benefits. The omega 3 PUFAs eicosapentaenoic acid (EPA, C20:5 *c*5*c*8*c*11*c*14*c*17) and docosahexaenoic acid (DHA, C22:6 *c*4*c*7*c*10*c*13*c*16*c*19) are considered essential nutrients found in fatty fish such as mackerel, herring and salmon (Ander et al., 2003). They have been associated with the reduction of TGs and cholesterol levels, normalization of blood pressure and the consequent promotion of cardiovascular health (Rangel-Huerta & Gil, 2018). Some studies have also reported potential antioxidant properties, which may be relevant in the treatment of Sickle cell disease (Kotue et al., 2019) or type-2 diabetes (Hajianfar et al., 2013). Other PUFAs have been gaining attention due to their role in health promotion. For instance, conjugated linoleic acid isomers (CLA) are a group of linoleic acid derivatives that can be found at low levels in ruminant fats (such as beef tallow and milk fat) and dairy products from the bovine, ovine and caprine origin (Pariza et al., 2001). The most studied isomers are C18:2 *c*9 *t*11 (rumenic acid (RA)) and C18:2 *t*10*c*12 (Nornberg, 2016); RA content represents about 73 to 94% (w/w) of the total CLA in milk, dairy products, meat and processed meat products (Park, 2009). CLA has been shown as an agonist of several peroxisome proliferator-activated receptor (PPAR) isoforms, being thus, responsible for the reduction of inflammatory responses (Salsinha et al., 2021). Additionally, conjugated linolenic acid (CLNA) isomers are naturally found in vegetable oils; for instance, punicic acid (PUA) (C18:3 *c*9*t*11*c*13), the most studied CLNA isomer, is mostly found in Pomegranate (*Punica granatum*) seed oil, with approximately 70 g of PUA per 100 g of fat (Fontes et al., 2017). A positive effect of PUA on body weight has been reported (Cao et al., 2007; Saha, Chakraborty, et al., 2012; Sengupta et al., 2015) and its effects on peripheral tissues, are widely recognized (Miranda et al., 2011).

European Food Safety Authority (EFSA) has established that 250 to 500 mg/day of EPA and DHA are the dietary recommended daily dose for European adults, based on cardiovascular risk considerations (Opinion, 2012). Regarding CFAs, namely CLA – specifically RA and C18:2 *t*10*c*12 - and CLNA – specifically PUA – the effective doses have been described as 3 g/day (Ip et al., 1994) and 2-3 g/day (Shinohara et al., 2012), respectively. Consequently, one of the biggest concern is that considerably high amounts of Fish, Pomegranate seed oil, CLA, or CLNA food products would have to be consumed to achieve an adequate intake of their respective bioactive FAs to attain their therapeutic benefits. Thus, the adopted strategy has been raising PUFA's plasma concentration by supplementation of enriched oils containing high concentrations of the mentioned bioactive FAs in the form of soft gel or emulsions (Domoto et al., 2013). Indeed, oral supplementation has been a preferable way of delivering such compounds. Nevertheless, several factors such as the type and processing of food or even the presence of a certain antinutritional constituent, influence the bioavailability of a given FA (Gomes et al., 2019). This justifies the interest in FA composition and FA bioaccessibility and the relevance of our study: it aims to assess the bioaccessibility of bioactive FAs, omega 3 EPA and DHA, CLNA isomer PUA and CLA isomers RA and

C18:2 *t*10*c*12, using a standardized static *in vitro* protocol (INFOGEST 2.0) mimicking the gastrointestinal tract (GIT) digestion. Importantly, the GIT has been described as an important player in the release of bioactive compounds, being responsible for both positive and negative effects on bioactive compounds' bioaccessibility. This perception justifies the importance of GIT simulation, to fully comprehend the potential of bioactive ingredients present in a certain matrix after oral consumption. Considering that *in vivo* studies are very time-demanding, require large resources and present some analytical and ethical constraints (Cardoso et al., 2015), *in vitro* models have been used for many decades to simulate the digestion of food. But as far as we are concerned there are only a few studies on lipid bioaccessibility in general and even fewer using similar matrixes as the ones studied here. 7 studies were found applying *in vitro* GIT models to access FAs or lipid bioaccessibility, 2 studies (Domoto et al., 2013; Gervais et al., 2009) used a dynamic GIT model (TIM system) and 5 studies (Costa et al., 2015; Floros et al., 2022; Gomes et al., 2019; Tan, Zhang, Liu, et al., 2020) used a static *in vitro* model, only 3 (Floros et al., 2022; Tan, Zhang, Liu, et al., 2020; Tan, Zhang, Zhou, et al., 2020) used INFOGEST protocol. INFOGEST is a recent standardized protocol developed in 2014 and optimized to an improved digestion method (INFOGEST 2.0) in 2019 (Brodkorb et al., 2019). Such protocol was created since *in vitro* simulations have used a wide range of different conditions that often have very little physiological relevance, and this prevents the meaningful comparison of results. It was observed considerable discrepancies between different digestion models, which were attributed to variations in the experimental parameters, *e.g.* chyme transit and enzyme concentration (Lin et al., 2021). Thus, the importance of using a standardized method to compare the bioaccessibility results obtained is of foremost importance. In addition, in most experiments aiming to study the bioaccessibility of FAs these studies do not ultimately assess intestinal permeability lacking an adequate comprehension of bioavailability. Since there are very few studies addressing the bioaccessibility of FAs on bioactive oil matrixes and even fewer were performed using INFOGEST protocol, it was performed a deep study that intends to validate the most relevant PUFAs' bioaccessibility. So, this study intended to assess the impact of *in vitro* simulation of GIT on FAs composition and on the bioactive properties, specifically on antioxidant activity. Moreover, to study the bioavailability of these FAs, it was evaluated the absorption and intestinal permeability after digestion.

2. Materials and Methods

2.1. Chemicals and reagents

For the *in vitro* GIT tract digestion α-amylase from human saliva (A1031-5KU), bile salts (bile extract porcine – B8631) and pancreatin from porcine pancreas (P7545) were purchased from Sigma-Aldrich (Missouri, USA). Rabbit gastric extract (RGE 15) was obtained from Lipolytech (Marseille, France). The dialysis 3.5 kDa membranes (Pre-wetted RC Tubbing, Spectra/Por®6 Dialysis Membrane; 734-0652) were purchased from VWR Chemicals (Pennsylvania, USA).

For the FAs profile analysis, hexane, methanol, dimethylformamide (DMF) and acetonitrile were HPLC grade and purchased from VWR Chemicals. Sulphuric acid was obtained from Honeywell (North Carolina, USA). Sodium methoxide was from Acros Organics (Geel, Belgium). Tritridecanoin (33-1300-13) internal standard was from Larodan Research Grade Lipids (Solna, Sweden).

Regarding the antioxidant activity assessment, the DPPH reagent (2,2-diphenyl-1-picrylhydrazyl free radical, 44150) was obtained from Alfa Aesar (Kandel, Germany). Methyl-β-cyclodextrin (332615), fluorescein (F6377), 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, A1888), (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox, 238813) and 2,2'-Azobis(2-methyl-propionamidine) dihydrochloride (AAPH, 440914) were purchased from Sigma-Aldrich. Ethyl acetate (E/0906/17) was HPLC grade from Thermo Fisher Scientific (Massachusetts, USA). Ethanol absolute (4146052) was from Carlo Erba reagents (Val de Reuil, France) and Acetone from Honeywell.

2.2. FAs Sources

Fish oil from Menhaden (F8020) was purchased from Sigma-Aldrich. Pomegranate Kernel Oil cold pressed, as a PUA source, was supplied by All Organic Treasure (Germany). CLA, CLNA and Omega 3capsules were purchased from commercially available sources from two relevant Portuguese sports nutrition brands. The use of the capsules is intended to assess the relevance of different matrixes in the bioactive FAs' bioaccessibility. Indeed, using both oils and capsules the two main relevant sources of commercially available options for bioactive FAs oral supplementation, were covered. CLA capsules are MEGACLA A95 from Gold Nutrition, which presents as the main ingredient CLARINOL® A-95 Amber capsules with a 50:50 ratio of *c*9*t*11 and *t*10*c*12 isomers. Omega 3 Fully concentrated EPA&DHA capsules (here defined as Omega 3 capsules) were purchased in Prozis and have 360 mg of EPA and 240 mg of DHA. As a CLNA isomer source, specifically PUA, Xanthigen® capsules (here defined as CLNA capsules) purchased in Cellulase were used, with 100 mg of Pomegranate oil extract/capsule. The capsule content was extracted and further analyzed. The objective of this study was not to assess the gel capsules as a vehicle to deliver omega 3 FAs or conjugated FAs, but instead to determine how these FAs behave throughout the GIT tract in different formulations and concentrations.

2.3. *In vitro* GIT tract digestion

For the simulation of the GIT tract digestion, the standardized static digestion model INFOGEST 2.0 protocol (Brodkorb et al., 2019) was followed. The experimental procedure is divided into the oral, gastric and intestinal phases with the correspondent fluids to better simulate the *in vivo* conditions. Shortly, mouth digestion includes the dilution of food 1:1 (w/w) with simulated salivary fluid complemented with salivary α-amylase from human saliva. The mastication of the food is simulated using an Orbital Shaker MaxQ 6000 at 200 rpm and 37 °C for 2 min. As described in the INFOGEST protocol, the oral bolus is then diluted 1:1 (v/v) with simulated gastric fluid and gastric enzymes – pepsin and lipase from rabbit gastric extract – and agitated in the mentioned orbital shaker at 130 rpm and 37 °C, at pH 3.0 for 2 h. The resultant gastric chyme is subsequently diluted 1:1 (v/v) with simulated intestinal fluid, bile salts and pancreatic enzymes (pancreatin from the porcine pancreas) and incubated at pH 7.0 for 2 h, at 45 rpm and 37 °C in the orbital shaker. For each sample (3 g/sample) two replicas were performed and a negative control without a sample, using distilled water, was used.

To screen the action of GIT tract digestion conditions in the lipid profile of the samples, aliquots were collected at the different stages of digestion (oral, gastric and intestinal phases).

2.4. Bioaccessibility assessment using dialysis membranes

After intestinal digestion a segment of dialysis tubing (3.5 kDa molecular weight cut-off) was filled with the digested samples and placed inside a recipient filled with distilled water and further incubated overnight in the orbital shaker, at 37 °C and 50 rpm, mimicking peristaltic movements. The dialysis process had the goal to simulate the passage of the digested samples by duodenum and jejunum. At the end of the process, the solution left outside (OUT) represented the sample that is available for absorption (serum-available) and the solution that was left inside (IN) the dialysis membrane represented the non-absorbable sample (colon-available) (Gullon et al., 2015; Ribeiro et al., 2020).

After, the dialysis submitted samples, both retentate (IN) and permeate (OUT) were lyophilized, and stored for analysis of the compounds

2.5. Stability and bioaccessibility of compounds through *in vitro* GIT tract

2.5.1. FAs profile

2.5.1.1. Sample preparation

The FAs profile of the samples and the action of *in vitro* GIT tract in the different stages of the digestion were assessed through GC-FID and compared with the original samples which were not subjected to the GIT tract conditions. 250 µL of digested samples and 50-100 mg of lyophilized dialyzed solutions were prepared according to previous studies (Fontes et al., 2018; Pimentel et al., 2015). Briefly, for quantification purposes, 200 µL of tritridecanoin acid (1.5 mg/mL) was added before the derivatization process as an internal standard. Afterward, 2.26 mL of methanol, 1 mL of hexane and 240 µL of sodium methoxide (5M) were added. Samples were vortexed and incubated at 80°C for 10 min. After cooling in ice, 1.25 mL of DMF and 1.25 mL of sulphuric acid (3M) were added. Again, samples were vortexed and then incubated at 60 °C for 30 min. After

cooling, 800 µL of hexane was added. The samples were vortexed and centrifuged (1250xg, 18°C for 5 mins). The upper layer containing methyl esters (FAME) was collected for gas chromatography analysis.

2.5.1.2. FAs content analysis with gas chromatography

FAME extracts were analyzed using a gas chromatograph HP6890A (Hewlett-Packard, Avondaale, PA, USA) equipped with a flame-ionization detector (GC-FID) and a BPX70 capillary column (SGE Europe Ltd, Coutaboeuf, France). As previously described (Fontes et al., 2018), the injector temperature was 250 °C, split 25:1, the injection volume was 1 µL, and the detector (FID) temperature was 275 °C. Moreover, hydrogen was the carrier gas at 20.5 psi. The oven temperature program was as follows: starting at 60 °C (held for 5 min), then raised at 15 °C/min to 165 °C (held for 1 min) and finally at 2 °C/min to 225 °C (held 2 min). GLC-Nestlé36 was assayed for calculation of response factors and detection and quantification limits (LOD: 0.79ng FA/mL and LOQ:2.64ng FA/mL)

2.5.2. Recovery and bioaccessibility index

The recovery index (RI %, Equation 1) and bioaccessibility index (BI %, Equation 2) were calculated to determine the GIT digestion effect on the different studied matrixes based on previous studies (Gullon et al., 2015; Lucas-Gonzalez et al., 2016; Ribeiro et al., 2021). The values of the bioactive FAs in the original samples before digestion were assumed as 100%. Accordingly, the RI calculation allows the determination of the amount of a given main component - bioactive FAs, specifically -, in the tested matrix after digestion (in the oral, gastric and intestinal phases). So, RI is calculated as follows:

$$\text{RI (\%)} = \frac{\text{Bioactive content in the digested sample } (BC_{DF})}{\text{Bioactive content quantified in the test matrix } (BC_{TF})} \times 100 \quad (1)$$

Bioaccessibility is referred to the amount of a given compound that is released from its matrix in the digestive tract and could potentially become available for blood-stream absorption. The bioaccessibility index is defined as the percentage of the bioactive compound that is solubilized after intestinal dialysis. Such concept, here determined by using the *in vitro* GIT tract simulation and intestinal dialysis, is important for determining the bioavailability of a given compound, which corresponds to the fraction of an ingested bioactive compound or nutrient that reaches the systemic circulation and performs its bioactive functions (Ribeiro et al., 2020). Consequently, bioavailability includes the term bioaccessibility. Thus, the BI corresponds to the percentage of the bioactive compound that passes the dialysis membrane and consequently, this index defines the proportion of the bioactive compound that could, potentially, become available for absorption in the systemic circulation. The BI index is calculated as:

$$\text{BI (\%)} = \frac{\text{Bioactive content in the digested sample after the dialysis step } (BC_S)}{\text{Total bioactive content in the digested sample after the dialysis step } (BC_{DF})} \times 100 \quad (2)$$

Where BC_S corresponds to the OUT fraction of the dialysis step, the absorbable fraction. And BC_{DF} to the total bioactive content, IN (retentate/ non-absorbable) + OUT (permeate/absorbable) fractions.

Importantly, most studies refer to RI as BI, without taking into consideration that bioaccessibility is a term that refers not only to the effect of GIT in a given bioactive

molecule of a food matrix but also to the quantity of the bioactive compound that can be absorbed and potentially reach the systemic circulation.

2.5.3. Effect of *in vitro* GIT digestion on antioxidant activity

The antioxidant activity of the fully digested and lyophilized samples was determined using ABTS and DPPH methods as described in the following sections and using a multi-detection plate reader (Synergy H1, Vermont, USA). The activity of the lyophilized digested samples was compared to the non-digested original samples. All analyses were performed in triplicate and expressed in µmol of Trolox equivalent (TE)/g of sample. Both ABTS and DPPH assays were performed in 96 wells UV Flat Bottom Microtiter® Plates (8404, Thermo Fisher Scientific).

2.5.3.1. DPPH

The DPPH method followed was based on the method from (Brand-Williams et al., 1995) and (Schaich et al., 2015), with slight modifications: the DPPH stock solution is dissolved in ethyl acetate. After, the DPPH stock solution is diluted with ethyl acetate to obtain a solution with an absorbance of 0.600±0.100 at 515 nm. This working solution was prepared daily. A Trolox standard curve was prepared in ethyl acetate. Samples were prepared at concentrations of 60 and 100 mg/mL in ethyl acetate and further diluted in the same solvent. Ethyl acetate was successfully used as a solvent for lipidic samples in a previous study (Falcão et al., 2017). It was the solvent chosen since no other tested solvent – ethanol, methanol, hexane and acetone - was suitable for the total dissolution of the samples (results not shown).

2.5.3.2. ABTS

The ABTS method followed was based on the method from (Gonçalves et al., 2009) and (Sánchez-Moreno, 2002) with slight modifications: the ABTS and the potassium persulfate were dissolved in ultrapure water and stirred overnight. The ABTS stock solution was then filtered with a 0.45µm syringe filter and diluted with ethanol to an absorbance of 0.70 (±0.02) at 734 nm. The radical working solution was freshly prepared. A Trolox standard curve was prepared in ethyl acetate. Samples were prepared at concentrations of 60, 100 and 200 mg/mL in ethyl acetate and further diluted in the same solvent.

2.6. Assessment of intestinal permeability effect on digested samples: human intestinal epithelium Caco-2/HT29-MTX cell lines co-culture

2.6.1. Cell preparation

Caco-2 (Caucasian colon adenocarcinoma) cell line is established for the study of intestinal permeation of bioactive molecules. Nevertheless, a monoculture of Caco-2 does not represent accurately duodenum epithelia due to the presence of tight junctions typical of the colon but not of the small intestine, leading to limited absorption of hydrophilic molecules (Castro et al., 2019). Moreover, Caco-2 monoculture is exclusively composed of enterocytes and overexpress efflux transporters, which characterizes an excretory rather than absorptive epithelia. Thus, HT29-MTX (Caucasian colon adenocarcinoma grade II) cell line is used in co-culture with Caco-2

cells. Importantly, HT29-MTX possesses mucus-producing ability, mimicking what happens in the duodenum mucosa.

HT29-MTX E12 (ECACC 12040401) and Caco-2 (ECACC 86010202) cell lines were grown separately in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) L-glutamine, 1% (v/v) penicillin and streptomycin and 1% (v/v) of non-essential amino acids. Cells were maintained at 37 °C under a 5% CO_2 water-saturated atmosphere. Upon 70-80% confluence, cells were collected using trypsin. Co-culture seeding in Transwells (Milicell hanging cell culture insert, PET 0.4µm, 12-well, 48/pk, Merck) was performed in a 9:1 ratio of Caco-2 (3×10^5 cells/well) and HT29 (3×10^5 cells/well) cells, respectively (Castro et al., 2019).

2.6.2. Cell layer integrity

Transepithelial electrical resistance (TEER) was used to assess the cell layer integrity of the Caco-2/HT29 co-culture. TEER (supplementary material table 6) was determined at the different time points during the permeability assay, to assess the cell growth rate and cell viability after contacting with tested samples. For TEER measurement, it was used a Millicell® ERS-2 Voltohmmeter (Merck, Germany) (Araujo & Sarmento, 2013). During permeability experiments, TEER values were always above 250 $\Omega\cdot cm^2$, indicating that the cells were viable along the assay and the cell layer integrity was not compromised (Pan et al., 2015).

2.6.3. FAs transepithelial diffusion across intestinal (Caco-2/HT29-MTX) cell layers

Permeability assay was assessed in the mentioned Transwell inserts, using 12-well plates. Caco-2/HT29-MTX co-culture was seeded into the inserts to mimic the absorptive epithelia of the human intestine. For culture medium replacement, the medium was removed from the wells and 0.5 and 1.5 mL of fresh culture medium were added to the apical and basolateral sides, respectively. The incubation was performed for 21 days. On the day of the study, the culture medium was removed. Medium in the basolateral side (receptor part) was replaced with 1.5 mL of fresh medium. The medium on the apical side (donor part) was replaced with the samples. Briefly, 100µL of digested samples(already diluted in 1:10) was added to 900µL of the medium, fully dissolved by vortexing and further diluted at 1:100 in culture medium. This concentration was determined based on previous tests on cell cytotoxicity (results not shown). 500 µL of each sample solution was added in duplicate to the plate. DMSO (30% v/v) was used as the negative control and culture medium as the positive control. 500µL of samples were withdrawn from the basolateral side at 0, 1, 3 and 6 h. After 6 h the apical content was completely removed, and cells were collected using 500 µL of NaOH 0.1M solution.

2.6.3.1. FAs content analysis with gas chromatography

To calculate the apparent permeability (Papp, Equation 3), the analysis of FAs content in both basolateral and apical sides, and cells was assessed through gas-chromatography. As described in section 2.5.1.1 the upper layer containing FAs methyl esters was collected and further analyzed in a gas chromatograph Agilent 8860 (Agilent, USA), equipped with a flame ionization detector and a BPX70 capillary column (60 m x

0.25 mm x 0.25 µm; SGE Europe Ltd, Courtaboeuf, France). Analysis conditions were as follows: injector (split 25:1; injection volume 1 µL), injector, and detector temperatures were 250˚C and 275˚C, respectively; hydrogen was used as a carrier gas at a flow rate of 1 mL/min. The oven temperature was initially at 60˚C and then increased to a final temperature of 225˚C. Supelco 37 was used for the identification of FAs.

The Papp is calculated as follows (Dima et al., 2020):

$$Papp = \frac{1}{A}\frac{dC}{dt}\frac{Vr}{C_0} \quad (3)$$

Where A is the area of the cell monolayers, *Vr* is the compound concentration in the receptor chamber (basolateral side), C_0 is the initial compound concentration in the donor chamber (apical side) *dC/dt* is the slope of the function C=f(t).

2.7. Statistical analysis

Results are reported as mean values ± standard deviation. Analyses were performed using IBM SPSS Statistics 28 (SPSS Inc., IBM Corporation, NY, USA). First, data were analyzed for normal distribution using the Shapiro-Wilk test (n<50). Levene's test was applied to verify the homogeneity of the variances. When comparing the means of two groups a t-student test was applied and one-way ANOVA was for three or more groups. The Bonferroni *post hoc* test was used to determine differences among groups. When data didn't follow a normal distribution, it was transformed using the log base 10 function. When the transformation failed to employ normality, Kruskal-Wallis' test was used as a non-parametric test to compare the means of the groups. The level of significance was set at 0.05.

3. Results and Discussion

3.1. Stability and bioaccessibility of bioactive lipids through in vitro GIT tract

3.1.1. FAs profile and recovery index calculation

Four classes of lipids are usually found in vegetable oils: triacylglycerols (TAGs), diacylglycerols, polar lipids, and FFAs (Demir & Demir, 2018). Pomegranate seed oil, here simply mentioned as Pomegranate oil, is reported to yield oil contents ranging from 12.2 to 24.69% (Amri et al., 2017; Loukhmas et al., 2021). The most prevalent forms of lipids were glycolipids (23.9%) and phospholipids (24.35%) (Amri et al., 2017). High contents of PUFAs have been reported ranging from 46.44-89% (Amri et al., 2017; Loukhmas et al., 2021). TAGs are reported as important consituents of Pomegranate oil being the punicic acid-punicic acid-punicic acid, the punicic acid–punicic acid–catalpic acid (Topkafa et al., 2015) and the Stearic-Punicic-Punicic acid, some of the most reported TAGs (Kola et al., 2021). Regarding Fish oil, the primary chemical constituent has been reported to be TAGs, it also contain variable amounts of phospholipids and glycerol ethers (Mgbechidinma et al., 2023; Zhang, Shen, et al., 2018). These oils are rich in omega 3 PUFAs, typically containing between 20 and 30%, and DHA and EPA account for more than 80% of these total omega 3 PUFAs (Zhang, Zhao, et al., 2018). The omega 3 capsules contain Fish oil in their composition as a source of omega 3 FAs, so similarly to Fish oil the primary chemical consituint is TAGs. The CLA capsules used in this study present as the main ingredient CLARINOL® A-95 and as described by the manufacturer (Stepan Lipid Nutrition, USA) it is a FFA mixture of the CLA isomers. The CLNA capsules are Xanthigen capsules which are described by the manufacturer as a source of punicic acid derived from Pomegranate seed oil and brown seaweed, respectively.

The effect of INFOGEST GIT digestion on total FA profile is described in figure 1 (and in supplementary material table 2, 3 and 4), where the most relevant PUFAs for each sample are emphasized. In the different GIT tract phases (oral, gastric and intestinal) significant variations in FAs profile were observed ($p < 0.05$). As previously reported, fat digestion occurs mainly in the intestine, where 80% of the lipolysis reaction occurs (Ribeiro et al., 2021). Here, it was observed that indeed in Fish and Pomegranate oil and CLNA capsules' samples (figure 1) the major variation is observed in the intestinal phase where the RI values are lower: between 4 and 14% in Pomegranate oil, 11 and 18% in Fish oil and around 20% in CLNA capsules. Nevertheless, oral and gastric digestion presents an important action in the facilitation of lipid intestinal digestion (Ribeiro et al., 2021; Ye et al., 2019). Other studies have been reporting the negative effects of mastication in the mouth and the acidic pH of the stomach in fat-rich foods, resulting in lipid peroxidation (Kanner & Lapidot, 2001) concerning the lipid profile. RI between 20 and 40% in the oral and gastric phases have been reported in a previous study with fat-rich matrixes (olive pomace) (Ribeiro et al., 2021). These RI values indicated that a strong degradation effect on lipid fraction is occurring. In this study for Pomegranate oil the RI is slightly higher than the reported values for olive pomace, with values between ≈65-30%. Nevertheless, the RI values for Fish oil and CLNA capsules agreed with the reported values: ≈ 30-40% and 28-38%, respectively (Ribeiro et al., 2021). Regarding CLA (figure 1 and supplementary material table 3) and Omega 3 (figure 1 and supplementary material table 4) capsules, the observations are slightly

different: there is a high degradation of general FAs content in oral phase and a slight increase in the intestinal phase. The RI is extremely low in both oral and gastric phase, in CLA capsules it is situated in 5-8% and in omega 3capsules in 9-15%. The RI values increase slightly in intestine to 6-28% and 7-8% in CLA and Omega 3capsules, respectively. A factor that was recently stated as a potential explanation to the extensive loss of FAs, was the presence of oxygen during all the steps of INFOGEST GIT tract digestion. Thus, it has been suggested and reinforced in this study, that the use of N_2 gas at the start of each digestion step of fat-rich foods should be applied in order to reduce fat oxidation (Ribeiro et al., 2021; Tullberg et al., 2019). Considering this, such step should be mentioned or included in further INFOGEST based protocols. In fact, oxidation of omega 3 food supplements, such as capsules as used here, are an important issue that has been reported in early studies (Albert et al., 2015). Importantly, it is worth mentioning that the GIT negative effect was applied in the same degree for all FAs. It is important to mention again that in this study the capsules were not used in the GIT digestion, instead their content was removed and used directly for the digestion procedures. Although, a FA oxidation can be a plausible explanation for the degradation observed it is important to mentioned that all the capsules, for instance, present antioxidants in their composition, namely tocopherols. Importantly, besides oxidation due to the presence of oxygen in all the steps of the digestion, after consumption FAs are prone to oxidation. Indeed, they interact with the pro-oxidative environment of the digestive tract, often leading to limited bioavailability. Such processes highly depend on the physicochemical properties of the initial food, as well as the characteristics of the consumer (Floros et al., 2022). Recently, a study aiming to assess the bioaccessibility of omega 3 FAs in different matrixes (including soft capsules with concentrated Fish oil and antioxidants) and the oxidation effects throughout GIT, showed that regardless of raw material used there was a high degree of oxidation by the end of the digestion processes which ultimately resulted in the decreased bioaccessibility of omega 3 FAs. The authors described that primary oxidation takes place in the gastric phase and secondary oxidation mainly takes place in the intestinal phase. Interestingly, the authors described that antioxidants presence, either natural or added, was not sufficient to protect PUFAs from the pro-oxidative conditions in the GIT (Floros et al., 2022); the same was possibly observed here in the CLA and Omega 3 capsules. Moreover, the differences in RI observed between the Pomegranate and Fish oil and CLA and Omega 3 capsules seem to not be related to the initial concentration of PUFAs in these matrixes. Some studies have reported that the rate and degree of oxidation, for instance, were found to be strongly correlated with the initial concentration of the bioactive FAs (Floros et al., 2022). Here, considering in general the MUFA/ PUFA content we couldn't verify any correlation between the initial concentration and the RI calculated after the digestion process.

Interestingly, MUFAs and PUFAs presented a higher RI when compared to SFAs in the studied oils (Pomegranate and Fish oil), but not in CLNA capsules, where the values are identical. And regarding the bioactive FAs in these matrixes, in Pomegranate oil, PUA presented a RI of 50% in the oral phase, and 31% in the gastric phase and it was highly degraded in the intestinal phase since the RI lowered to 2%. Interestingly, smaller RIs were observed for both the oral (36.07%) and gastric phase (29.38%), but a higher RI (17%) was observed for PUA in CLNA capsules in the intestinal phase. The initial

concentration of PUA was higher in Pomegranate oil (577.33 µg/µL of the sample) when compared to the CLNA capsules (205.84 µg/µL of the sample). Regarding Fish oil, the RI of the main bioactive components were observed at the same degree for all of them. EPA presented a RI of 40% in the oral phase, 29% in the gastric phase and 13% in the intestinal phase; DPA showed a RI of 39%, 28% and 11% in the oral, gastric and intestinal phases, respectively. Lastly, DHA presented a RI of 39% in oral, 28% in gastric and 12% in intestinal phases. These values were slightly higher than the ones presented for the omega 3capsules: EPA – 12.29%, 13.36% and 3.04%; DPA – 7.79%, 11.66% and 3.11%; DHA – 14.11%, 14.92% and 2.94%, in oral, gastric and intestinal phases, respectively. The initial concentrations of the mentioned FAs were higher in the Omega 3 capsules (supplementary material table 4). In agreement with what was discussed for PUA, higher concentrations of the bioactive FAs result in lower RIs after digestion. Interestingly, in Fish oil as previously reported (Gomes et al., 2019) the RI of EPA (12.59%) and DHA (11.46%) was lower than that of oleic acid (14.13%) and total MUFA (15.49%). Moreover, an early study assessing salmon FAs bioaccessibility using an *in vitro* GIT model (not INFOGEST based), demonstrated that higher levels of unsaturation decreased RI (described in the study as bioaccessibility, BI%). The authors reported higher RI values (higher than 50%) for free fatty acids (FFAs). These different results are explained by the different matrixes: salmon slices in the mentioned study here compared to the Fish oil. The differences in RI values can be explained by the gradual release of FAs from the matrix and by the intrinsic antioxidant mechanisms present in the muscle tissue of fish, which may protect the FAs from such effects (Floros et al., 2022). Interestingly, in the same study, the authors described that RI values are enhanced by chain length but reduced with higher levels of FA unsaturation. Nevertheless, a study assessing the *in vitro* bioaccessibility of milk FAs and the C18:2 *c*9*t*11 CLA isomer using a TIM GIT *in vitro* model, reported that the degree of absorption of saturated FA decreased with chain length (Gervais et al., 2009). It was observed that the RI shows a positive correlation (r=0.9826) regarding chain length in SFAs in Pomegranate oil. But a negative correlation (r=-0.9332) was verified with Fish oil (figure 2). In this study, no statistically significant correlation was observed for the degree of unsaturation. The differences in the studies may be related to the GIT model since different models were used in all the mentioned studies. Indeed, in a work using two different GIT models – TIM-1 and an *in vitro* method adapted from the INFOGEST standardized static model, discrepancies in the RI of individual FAs were observed. The authors stated that this can be attributed to variations in the experimental parameters, such as chyme transit and enzyme concentrations (Lin et al., 2021). In the mentioned study using enriched CLA isomers milk, the reported RIs (described in the study as bioaccessibility BI%) for both C18:2 *c*9*t*11 and C18:2 *t*10*c*12 CLA isomers were much higher than the ones reported here for the CLA capsules: ≈86% (Gervais et al., 2009). In our study, we obtained similar RIs for both C18:2 *c*9*t*11 and C18:2 *t*10*c*12 CLA isomers (≈6%). Such differences between our results and the reported ones by the mentioned study, may, again, be explained by the naturally present antioxidants in milk and their possible release during GIT digestion. Nevertheless and besides the antioxidant content of milk, other important aspects have to be considered such as the chemical composition of the capsules: as mentioned the CLA content in the capsules is presented as FFAs while in milk FAs can be found esterified to the different compounds in fat majorly as TAGs (Rodríguez-Alcalá et al., 2017). Although FAs can be found in milk as FFAs, as

well, their major form is as TAGs and such difference may explain their distinct stability during GIT.

Interestingly, regarding the initial concentration for the mentioned bioactive FAs, it was observed that, possibly, higher concentrations in the original matrix, before digestion, seem to be related to lower recovery indexes. In fact, regarding omega 3 FAs, it was observed that the initial concentration of EPA in omega 3 capsules was higher (411.95±0.03 µg of FA/µL of the sample) than in Fish oil (84.07±0.11 µg of FA/µL of the sample), resulting in a lower RI (3.04%) for omega 3 capsules when comparing to Fish oil (12.59%). The same was observed for DHA: omega 3 presented a higher initial concentration (220.13 µg of FA/µL of the sample), resulting in lower RI (2.94%) than Fish oil (49.54 µg of FA/µL of the sample and RI=11.46%). Similar observations were detected for PUA in Pomegranate oil and CLNA capsules. The Pomegranate oil presented a higher initial concentration (577.33 µg of FA/µL of the sample) when compared to CLNA capsules (205.84 µg of FA/µL of the sample), which resulted in lower RI: 1.90% vs 17.03%, respectively. Importantly, a recent study has reported relevant results: they found a strong correlation between the initial concentration and oxidation rate. In fact, they observed that the higher the initial concentration of a specific PUFA was, the higher the degree of oxidation was. And this correlation was observed regardless of the length of the chain and degree of unsaturation (Floros et al., 2022). Such observations may explain the mentioned results obtained in this study.

3.1.2. Bioaccessibility assessment using dialysis membranes

Bioavailability is a term that can be referred to the speed of absorption and the quantity of the substance (molecule) absorbed. Traditionally, bioavailability can also be considered the amount of a substance that reaches systemic circulation. *In vivo*, the absorption of a bioactive molecule occurs in the GIT only to a certain extent, and such process depends on many factors (Cholewski et al., 2018). Considering the potential health benefits of these bioactive FAs, bioavailability determination is extremely important to understand if the studied matrixes can have an impact on human health through oral supplementation. As mentioned, bioavailability includes the term bioaccessibility. So, the caculation of the BI, of a certain bioactive compound, is relevant for the determination of its bioavailability.

Regarding the BI, shown in table 4, it was possible to observe that most FAs were not detected in the permeate (OUT) phase but instead in the retentate (IN) after the dialysis process. The BI for most of the FAs was thus, null, or very low 0.02-0.91%. Only the observed smaller chain FAs, such as myristic (C14), palmitic (C16) and palmitoleic (C16:1*c*9) acids presented higher BI values. Similar values for BI were also observed in a recent study using the same 3.5 kDa dialysis membranes for many of the same FAs (Ribeiro et al., 2021). In fact, the bioavailability of omega-3 FAs, for instance, varies depending on the type of chemical binding (lipid structure) (Schuchardt & Hahn, 2013). Moreover, bioaccessibility is also limited by the moderate to poor solubility of the FAs (Dima et al., 2020). Interestingly, the capsule samples (CLA, CLNA and omega 3) presented, in general, a higher BI compared to the oil samples (Pomegranate and Fish oil). Interestingly, chain length has been considered to have an impact on FAs' bioavailability. In a study aiming to determine the intestinal absorption of FAs from milk, especially CLA, short-to-medium chain saturated FAs (up to C12) were described to be absorbed more efficiently than long-chain saturated FAs C14, C16 and stearic acid C18 (Jedidi et al., 2014). Here, we observed that in the capsules, lower chain-length FAs (C14 and C16) presented considerable higher BIs than longer chain-legth FAs. These results may suggest that the FAs concentration and matrix composition may affect the specific FAs response to the GIT conditions and consequently the BI after the dialysis process.

Importantly, bioavailability is a complex term and when referring to nutrients the definition is still poorly defined and needs to be better standardized (Ghasemifard et al., 2014). As mentioned in this study, it is important to define that the calculation of the BI for each of the FAs present in the studied matrixes is relevant for the prediction of their bioavailability, but it must be carefully considered since it is only a prediction of the same measure. It has been reported that the chemical form of the omega 3 PUFAs affects their bioavailability, but there is still a lack of strong and consisted evidence to quantify the extent of the differences. However, it has been reported that bioavailability of EPA and DHA in ethyl esters form was significantly lower than that in TAG form. In addition, the FFA form seems to be the more bioavailable structure. Indeed, there are different factors that could affect the *in vivo* bioavailability of different forms of omega 3 PUFAs, including lymphatic recovery of FAs, the position of FAs in the glycerol backbone of TG, the position of double bond from carboxyl-end, the molecular weight of omega 3 PUFA formulations, and the actual matrix in which the omega 3 PUFA

were provided. Other important aspect to consider is the pancreatic lipase activity, which again justifies the importance of using GIT standardized *in vitro* methods (Ghasemifard et al., 2014). Here only CLA capsules presented a different formulation of the bioactive PUFAS (as FFA) comparing to the other matrixes (FAs mainly as TAGs), but there were no observable differences considering their BI.

There are very few studies aiming to determine the BI of a certain fatty acid where permeability assays, such as the ones presented here, are mentioned. But we believe such studies are a relevant prediction tool to consider in this kind of static *in vitro* tests and must be used as screening tools.

Since these results indicated that most FAs were retained in the non-bioaccessible fraction (retentate, IN), this could be interesting for further studies regarding their effects on gut health modulation.

3.1.3. Antioxidant potential

Human studies have shown that PUFAs in general improve metabolic and anti-inflammatory/antioxidant capacity of several organs. In heart it was demonstrated that PPARγ activation is a mechanism by which Fish oil n-3 PUFAs enhance mitochondrial FA oxidation and antioxidant capacity in human atrial myocardium (Anderson et al., 2014). Besides, they have been mentioned as enhancer factors in antioxidant defense against reactive oxygen species (ROS) (Heshmati et al., 2019). These identical results have been demonstrated in *in vivo* studies with rats for the ovarian ischemia/reperfusion. Interestingly, a low-dose Pomegranate seed oil extract showed anti-inflammatory potential by reducing TNF-α levels and significantly increased antioxidant activity (measured by superoxide dismutase activity and glutathione levels) (Yayla et al., 2018). The scavenging potential of these FAs and FAs' sources was also demonstrated *in vitro,* as it is going to be discussed in this section.

The antioxidant activity of the studied samples – CLA, CLNA and Omega 3 capsules and Fish and Pomegranate oil – before and after digestion was determined by two different methods: DPPH and ABTS (figure 3 and in supplementary material table 7). DPPH and ABTS are examples of electron transfer methods (Ribeiro et al., 2021). In this study, the ABTS method showed higher antioxidant values compared to DPPH. Such result was not expected since DPPH has been described as more efficient to measure the antioxidant activity of less polar compounds, due to its solubilization only in organic solvents. Nevertheless, both DPPH and ABTS assays confirmed the presence of free radical scavenging activity in the studied samples.

The antioxidant effect of the samples was concordant in both ABTS and DPPH methods: CLA capsules presented a higher antioxidant potential after the digestion process in both methods (ABTS, DPPH) and Fish oil presented the same result in ABTS, while no statistically significant difference was observed for DPPH. The antioxidant potential of Omega 3 and CLNA capsules and Pomegranate oil was negatively influenced by GIT tract digestion since they present lower antioxidant activity after the digestion process. Pomegranate juices are known to possess significant antioxidant activity due to its phenolics content (Badr et al., 2020). Indeed, a significant correlation was found between the antioxidant activity of Pomegranate seed oil and the

total content of tocopherols, suggesting the contribution of tocopherols to the antioxidant properties of this oil (de Melo et al., 2016). Moreover, CLNA isomers such as PUA (Saha, Patra, et al., 2012) and even, PUFAs (Richard et al., 2008) in general are known for their antioxidant capacity *in vivo*. Although other compounds in Pomegranate oil have important antioxidant potential, it was expected that since a reduction of these FAs is observed after GIT digestion the overall antioxidant potential was also affected. Importantly, similar values for the antioxidant activity of Pomegranate seed oil were reported elsewhere: 171.44 mg TE/100 g of sample (≈6.85µmol TE/g sample) (Taner Bozkurt & Zeynep Ergun, 2021). Significantly, DPPH was described as the most successful method to determine the antioxidant activity of CLA isomers (Elfalleh et al., 2011). CLNA capsules showed lower antioxidant activity in DPPH when compared to Pomegranate oil, but higher antioxidant activity with ABTS. Such results may be related to the fact that DPPH, due to its lipophilic nature, is better suited for samples with high lipidic contents such as oils. In addition, a study has reported that high-pigmented and hydrophilic antioxidants are better reflected by ABTS than DPPH (Floegel et al., 2011). Besides, it is also important to mention that ABTS allows the measure of antioxidant activity of both hydrophylic and lipophilic compounds, whereas DPPH, by its low solubility in aqueous compounds is appropriate for analysis of lipophilic compounds, which is an important limitation to consider when interpreting the role of hydrophilic antioxidants (Ribeiro et al., 2021). CLNA capsules (Xanthigen) present pigments and have a lower percentage of Pomegranate oil in their composition. In addition, it is relevant to mention that antioxidants are incorporated in the CLNA capsules. In addition there is also the presence of the polyphenols naturally present in the Pomegranate seed oil, as discussed. Thus, the antioxidant activity measured is affected by all these compounds and not only by their FA content.

We were only able to identify one *in vitro* study that specifically assessed the Fish oil antioxidant potential. In this study Fish oil was described as presenting a good free scavenging activity due to the presence of omega 3 FAs: $DPPH^{\bullet}$ inhibition values were around 97% (in smaller concentrations of 4 mg/mL of Fish oil) and 90% for $ABTS^{\bullet}$ (4 mg/mL of Fish oil) (Liyana Hannah Binti Izham Akmal & Roy, 2017). Some natural and synthetic antioxidants have been reported to be added to Fish oil to prevent lipid oxidation processes (Śpitalniak-Bajerska et al., 2018) and this must be considered for its antioxidant activity potential. Nevertheless, omega 3 EPA and DHA have been reported to have antioxidant activity (Kotue et al., 2019) or promote antioxidant cellular responses (Sakai et al., 2017) in some *in vitro* studies using cell lines. Here it was obtained significative values (4.11µmol TE/g sample), comparable to the Pomegranate oil (5.95 µmol TE/g sample). Lower values were obtained for the ABTS method, which can be explained by the high lipidic content of the sample, as already discussed. Slightly higher results were obtained for omega 3 capsules, which can be explained by the added tocopherol content (10 mg α-TE) in the capsules.

The antioxidant activity of CLA isomers has been reported as a possible mechanism behind its anticarcinogenic and antiatherogenic effects. Nevertheless, pro-oxidant properties have been attributed to them (Fagali & Catalá, 2008; Flintoff-Dye & Omaye, 2005) and thus, controversial reports have been made regarding their antioxidant potential. It has been reported that C18:2 *c*9*t*11 and C18:2 *t*10*c*12 CLA isomers reacted and quenched $DPPH^{\bullet}$ at several concentrations (Ali et al., 2012; Fagali & Catalá, 2008).

It is important to mention, again that an important antioxidant activity is due to the added tocopherol content (3 mg/g). Here, it is showed that indeed CLA capsules present some antioxidant activity, although the lowest among the tested samples in both ABTS and DPPH methods.

3.2. Assessment of intestinal permeability effect on digested samples: human intestinal epithelium Caco-2/HT29-MTX cell lines co-culture

To predict the bioavailability of the studied samples after GIT digestion, specifically of their main bioactive FAs, besides the use of dialysis membranes (as discussed in section 3.1.2) we performed a permeability assay using a co-culture of Caco-2/HT29-MTX cell lines. It was not possible to quantify any FAs on the basolateral side (receptor side) meaning that permeability was affected. Thus, the Papp was not possible to be calculated. To determine if the FAs were incorporated in the cells, the cells after the experimental time were collected. In figure 4 it is depicted the FAs profile of the original samples, cells and apical side, after normalization with control to eliminate the FFAs naturally present in the culture medium and cells. The RI was calculated in relation to the original sample added to the apical side (donor side). It was observed that in CLNA capsules and Pomegranate oil ≈10% of PUA is incorporated into the cells as well as 12-24% of other CLNA isomers, such as α-eleostearic acid (C18:3 *c*9*t*11*t*13), catalpic acid (C18:3 *t*9*t*11*c*13) and β-eleostearic acid (C18:3 *t*9*t*11*t*13). An early study aiming to investigate the uptake of CLNA isomers (α-eleostearic acid (C18:3 *c*9*t*11*t*13), catalpic acid (C18:3 *t*9*t*11*c*13) and β-eleostearic acid (C18:3 *t*9*t*11*t*13)) and their metabolism into CLA in Caco-2 cells, showed that a 24 h incubation with these isomers showed to induce an accumulation of these FAs, while the control cells were totally free of these FAs. Interestingly, the authors also demonstrated that these FAs can be converted in CLA (A.-C. Schneider et al., 2012; A. C. Schneider et al., 2013).

Regarding CLA capsules, C18:2 *c*9*t*11 and C18:2 *t*10*c*12 isomers were detected in cells with a RI of 21 and 27%, respectively. Nevertheless, a considerable RI was calculated on the apical side: 12 and 9%, for the same isomers, respectively. The C18:2 *c*9*t*11 and C18:2 *t*10*c*12 were found to alter the distribution of tight junction proteins occluding and ZO-1 in a Caco-2 cell model increasing the paracellular permeability. Such observations were associated with the incorporation of CLA isomers in the cellular membrane (Jewell et al., 2005; Roche et al., 2001). Thus, these results may explain the low permeability that was observed in our study since a considerable amount of CLA isomers were retained on the apical side.

Regarding Fish oil, 11% of EPA was detected in cells and 13% in the sample collected from the apical side after the experimental time. In addition, 8% of DHA was detected in the cells and 9% on the apical side. Indeed, one mechanism that is linked to the several regulatory properties attributed to omega 3 PUFAs is precisely the alteration and modulation of membrane FAs composition and this has been demonstrated in several cell types including intestinal cells. As reviewed by Durkin et al., 2021 (Durkin et al., 2021) it has been demonstrated that EPA supplementation of Caco-2 cells increased their membrane content of EPA and long incubations (96 h) with EPA and DHA increased their respective amounts in these cells. Interestingly, it has been reported that EPA accumulates mostly in the phospholipid fraction and DHA and ALA in the neutral

lipid pool. Moreover, bioconversion of exogenous omega 3PUFAs can also occur in epithelial cells. One explanation for the decreased permeability observed here and the considerable content of such FAs still found in the apical side, besides FAs incorporation in cells, may be related to the fact that EPA and DHA can improve barrier integrity in *in vitro* studies in caco-2 cells. In those studies, 24 h EPA supplementation reduced the permeability of horseradish peroxidase (Rosella et al., 2000) and incubation with EPA for 96 h was able to attenuate increased permeability in heat stress-impaired caco-2 monolayers (Xiao et al., 2013).

The incorporation of omega 3 FAs and CFAs in intestinal cells presents great interest since these FAs may develop important functions in gut health. A recent study reviewed the positive effects of omega 3 FAs, specifically EPA, DHA and ALA. Omega 3 is known to influence the gut microbiota community, since these FAs showed to exert beneficial effects by decreasing the growth of *Enterobacteria* and increasing the growth of *Bifidobacteria*, inhibiting the inflammatory response associated with endotoxemia. Moreover, omega 3 may also reduce gut inflammation by increasing Treg differentiation and decreasing IL-17 production. Interestingly, these FAs can also reduce intestinal epithelial cell damage caused by inflammatory factors, which may be highly relevant in some intestinal inflammatory diseases (Fu et al., 2021). Moreover, as discussed CLNA isomers, PUA and eleostearic acid are known to activate PPARγ to suppress inflammatory responses. In fact, PUA and eleostearic acid have been demonstrated to modulate mucosal immune responses by a PPARγ-dependent mechanism, ameliorating colitis (Viladomiu et al., 2013). Considering the ability of CLA isomers to activate PPARs, it is not surprising that they have been described for their anti-inflammatory potential, as well. Indeed, in mouse models of inflammatory bowel disease, CLA isomers are shown to have anti-inflammatory properties and other studies showed that CLA possesses anti-carcinogenic activity in the rat colon (McIntosh et al., 2009).

It was also possible to observe that there is high incorporation and possible synthesis (RI>100%) of palmitic acid (C16), stearic acid (C18) and oleic acid (C18:1 *c*9) in the cells after 6 h. Palmitic acid was found to be incorporated in cellular phospholipids in a great proportion that oleic acid (van Greevenbroek et al., 1995). The same results were observed here for all the samples studied, palmitic acid presented higher RI values than oleic acid (figure 4). Another study has reported that only 7% of palmitic acid was able to cross the Caco-2 cells monolayer, and 90% of it was incorporated into the cells (Puyol et al., 1995). Importantly, once again some FAs appear to alter intestinal permeability: palmitic acid was demonstrated to affect the intestinal permeability without inducing cytotoxicity or oxidative stress; it seems to cause a functional change in tight junctions and adherens junction barrier (Gori et al., 2020).

The same experience was not possible to be performed for Omega 3 capsules, since the TEER values obtained after 6h of incubation indicated that the membrane integrity and cell viability were compromised.

4. Conclusion

This work assesses the bioaccessibility of bioactive PUFAs, mostly PUA, present in Pomegranate oil and CLNA capsules (Xanthigen), EPA, DHA and DPA from Fish oil and omega 3 capsules and RA and C18:2 *t*10*c*12 CLA isomer from CLA capsules. By using an *in vitro* static protocol the effect of the GIT digestion was assessed and it was demonstrated that significant variations in FAs profile occur in the different GIT phases: for the Pomegranate and Fish oil and CLNA capsules a major variation is observed in the intestine since lower RI (%) values were obtained here. In CLA and omega 3 capsules it was observed a high degradation of general FA content right after the oral phase. Indeed, this observation may be explained by the oxidative process of PUFAs after digestion, which are demonstrated not to be protected by the presence of both natural and added antioxidants. After the instestinal digestion process the RI value for PUA in Pomegranate oil is 2% and 17% for CLNA capsules. In Fish oil the RI values for EPA, DPA and DHA are between 11-13% and in omega 3 around 3%. In CLA capsules the RI for RA and C18:2 *t*10*c*12 CLA isomer is 6%. In addition to the low RI obtained in our study, higher initial concentration seem to be correlated with higher oxidation rates of PUFAs. All these observations – composition of matrixes, PUFAs concentration in the matrix, oxidation processes and the low RI -, supported from previous studies, are important considerations when aiming to design an oral route of administration of the bioactive PUFAs studied here.

Moreover, using 3.5 kDa dialysis membranes it was detected that the BIs (%) were very low or null for most of the major bioactive FAs of all the samples. These results indicated that most FAs were retained in the non-bioaccessible fraction. In addition, intestinal permeability was assessed using a Caco-2/HT29-MTX co-culture. Reinforcing the results obtained with the dialysis membrane, it was observed a significative incorporation of the bioactive FAs into the intestinal cells. This membrane incorporation negatively affects intestinal cells permeability capacity. Indeed, one mechanism that is linked to the several regulatory properties attributed to omega 3 PUFAs is precisely the alteration and modulation of membrane FAs composition and this has been demonstrated in several cell types including intestinal cells. Such evidence increases the importance of our study: first it is important to consider the low bioavailability potential of these PUFAs when aiming to understand their potential health benefits providing them as oral supplements. Thus, such results are relevant for future considerations of bioavailability studies of these FAs reinforcing the need to look for further mechanisms of target-delivering. Secondly, considering their low permeability it is highly relevant to study and understand their potential in microbiota modulation and their role in gut dysbiosis, specifically, considering their anti-inflammatory potential, in inflammatory diseases such as Inflammatory bowel disease, for example.

Concluding, this work provides a full bioaccessibility assessment of different bioactive FAs using distinct matrixes, with diverse formulations (commercial soft-gel capsules and enriched oils), compositions and bioactive PUFAs concentrations. The major strengths of this work rely on it being a complete study using a standardized *in vitro* static protocol (INFOGEST 2.0 protocol) and providing some insights on intestinal permeability and absorption. The use of a standardized protocol is an important point in

this study since it allows the accurate comparison of inter-laboratory results. Nevertheless, there are some limitations in this study that are important to identify. It is important to mention, that although *in vitro* protocols are a valuable and easily accessible tool, when considering the simulation of GIT, this type of protocols only provide a predictive potential. Indeed, static digestion models are described as simplifications of a dynamic physiological process (Baptista et al., 2020). Thus, further studies using semi-dynamic or dynamic models, which include the physicochemical and mechanical processes as well as the gradual changes that occur in the GIT digestion *in vivo* and ultimately *in vivo* studies, are required to fully characterize the bioaccessibility and bioavailability potential of these PUFAs. Other important aspect that needs to be considered regarding the bioavailability prediction made in this study is the lack of concordance with *in vivo* data. Several *in vivo* (human and animal studies) have reported higher bioavailability levels when considering, specifically, omega 3 FAs, EPA and DHA. Nevertheless, important considerations need to be made regarding this observation. First, as mention the reader must always consider that *in vitro* protocols present only a predictive potential and in this study as mention, the bioavailability is just a prediction from the determined BIs (%). Secondly, as reviewed in a study by Ghasemifard and collaborators (Ghasemifard et al., 2014) there are still several limitations presented in these *in vivo* studies which difficults the comparison between them and a correct prediction: the lack of definition and proper characterization of the "bioavailability" term, the lack of standardization of analytical methodology, differences in the methodology of bioavailability measure, failure to provide equal amounts of omega 3 FAs failure to measure fatty acid control and lack of control of fat intake. Indeed, it is important to point out that in this *in vitro* study the amount of bioactive FAs provided are equivalent to just one intake, with a single dose of 3g. This dose is lower than the ones used in most of the *in vivo* studies presented in the mentioned review article. It is also important to consider that in most *in vivo* studies the FAs intake was provided more than once during different studies duration. Additionally, the omega 3 FAs were incorporated with high fat meals, which will impact the bioavailability measured. Besides, in most studies there was no information or control of the dietary fat intake. In addition, it is relevant to mention that in *in vivo* studies these supplementations are part of a normal daily diet which will, as expected, have an impact on the bioavailability. These kind of methodology is not possible to be completely translated in a *in vitro* "isolated" study as the one presented here. Importantly, in one example of the importance of fat intake in bioavailability measures, the authors pointed out that high fat breakfast, for instance, containing omega 3 PUFAs, followed by a high fat lunch (devoided of omega 3 PUFAs) can lead to a second peak in the appearance of omega 3 PUFAs in plasma lipids. In fact, it was suggested that some of the PUFAs from the breakfast were stored in the enterocytes and released after the high fat lunch. All these aspects may explain the differences observed between this *in vitro* study and the *in vivo* reports regarding PUFAs bioavailability.

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6. Conflicts of interest

The authors declare no conflict of interest.

7. References

Albert, B. B., Derraik, J. G. B., Cameron-Smith, D., Hofman, P. L., Tumanov, S., Villas-Boas, S. G., Garg, M. L., & Cutfield, W. S. (2015). Fish oil supplements in New Zealand are highly oxidised and do not meet label content of n-3 PUFA. *Scientific Reports*, *5*, 7928. https://doi.org/10.1038/srep07928

Ali, Y. M., Kadir, A. A., Ahmad, Z., Yaakub, H., Zakaria, Z. A., & Hakim Abdullah, M. N. (2012). Free radical scavenging activity of conjugated linoleic acid as single or mixed isomers. *Pharmaceutical Biology*, *50*(6), 712–719. https://doi.org/10.3109/13880209.2011.621714

Amri, Z., Lazreg-Aref, H., Mekni, M., El-Gharbi, S., Dabbaghi, O., Mechri, B., & Hammami, M. (2017). Oil Characterization and Lipids Class Composition of Pomegranate Seeds. *BioMed Research International*, *2017*, 2037341. https://doi.org/10.1155/2017/2037341

Ander, B. P., Dupasquier, C. M., Prociuk, M. A., & Pierce, G. N. (2003). Polyunsaturated fatty acids and their effects on cardiovascular disease. *Experimental and Clinical Cardiology*, *8*(4), 164–172. https://pubmed.ncbi.nlm.nih.gov/19649216

Anderson, E. J., Thayne, K. A., Harris, M., Shaikh, S. R., Darden, T. M., Lark, D. S., Williams, J. M., Chitwood, W. R., Kypson, A. P., & Rodriguez, E. (2014). Do fish oil omega-3 fatty acids enhance antioxidant capacity and mitochondrial fatty acid oxidation in human atrial myocardium via PPARγ activation? *Antioxidants & Redox Signaling*, *21*(8), 1156–1163. https://doi.org/10.1089/ars.2014.5888

Araujo, F., & Sarmento, B. (2013). Towards the characterization of an in vitro triple co-culture intestine cell model for permeability studies. *International Journal of Pharmaceutics*, *458*(1), 128–134. https://doi.org/10.1016/j.ijpharm.2013.10.003

Badr, A. N., Ali, H. S., Abdel-Razek, A. G., Shehata, M. G., & Albaridi, N. A. (2020). Bioactive components of pomegranate oil and their influence on mycotoxin secretion. *Toxins*, *12*(12), 1–18. https://doi.org/10.3390/toxins12120748

Baptista, D. P., Salgaço, M. K., Sivieri, K., & Gigante, M. L. (2020). Use of static and dynamic in vitro models to simulate Prato cheese gastrointestinal digestion: Effect of Lactobacillus helveticus LH-B02 addition on peptides bioaccessibility. *LWT*, *134*, 110229. https://doi.org/https://doi.org/10.1016/j.lwt.2020.110229

Brand-Williams, W., Cuvelier, M. E., & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *LWT - Food Science and Technology*, *28*(1), 25–30. https://doi.org/https://doi.org/10.1016/S0023-6438(95)80008-5

Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assunção, R., Ballance, S., Bohn, T., Bourlieu-lacanal, C., Boutrou, R., & Carrière, F. (2019). INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nature Protocols*, *14*, 991–1014. https://doi.org/10.1038/s41596-018-0119-1

Cao, Z. P., Wang, F., Xiang, X. S., Cao, R., Zhang, W. Bin, & Gao, S. Bin. (2007). Intracerebroventricular administration of conjugated linoleic acid (CLA) inhibits food intake by decreasing gene expression of NPY and AgRP. *Neuroscience Letters*, *418*(3), 217–221. https://doi.org/10.1016/j.neulet.2007.03.010

Cardoso, C., Afonso, C., Lourenço, H., Costa, S., & Nunes, M. L. (2015). Bioaccessibility assessment methodologies and their consequences for the risk-benefit evaluation of food. *Trends in Food Science and Technology*, *41*(1), 5–23. https://doi.org/10.1016/j.tifs.2014.08.008

Castro, P. M., Baptista, P., Zuccheri, G., Madureira, A. R., Sarmento, B., & Pintado, M. E. (2019). Film-nanoparticle composite for enhanced oral delivery of alpha-casozepine. *Colloids and Surfaces B: Biointerfaces*, *181*(February), 149–157. https://doi.org/10.1016/j.colsurfb.2019.05.029

Costa, S., Afonso, C., Cardoso, C., Batista, I., Chaveiro, N., Nunes, M. L., & Bandarra, N. M. (2015). Fatty acids, mercury, and methylmercury bioaccessibility in salmon (Salmo salar) using an in vitro model: Effect of culinary treatment. *Food Chemistry*, *185*, 268–276. https://doi.org/10.1016/j.foodchem.2015.03.141

de Melo, I. L. P., de Carvalho, E. B. T., e Silva, A. M. de O., Yoshime, L. T., Sattler, J. A. G., Pavan, R. T., & Mancini-Filho, J. (2016). Characterization of constituents, quality and stability of pomegranate seed oil (Punica granatum L.). *Food Science and Technology (Brazil)*, *36*(1), 132–139. https://doi.org/10.1590/1678-457X.0069

Demir, H., & Demir, B. B. (2018). Comparison of Chemical Composition of Pomegranate (Punica Granatum L .) and Grape (Vitis Vinifera L .) Seed Oils Obtained By Extraction. *Journal of Advancements in Food Technology*, *1*(2), 18–23.

Dima, C., Assadpour, E., Dima, S., & Jafari, S. M. (2020). Bioavailability and bioaccessibility of food bioactive compounds; overview and assessment by in vitro methods. *Comprehensive Reviews in Food Science and Food Safety*, *19*(6), 2862–2884. https://doi.org/https://doi.org/10.1111/1541-4337.12623

Domoto, N., Koenen, M. E., Havenaar, R., Mikajiri, A., & Chu, B. (2013). The bioaccessibility of eicosapentaenoic acid was higher from phospholipid food products than from mono- and triacylglycerol food products in a dynamic gastrointestinal model. *Food Science & Nutrition*, *1*(6), 409–415. https://doi.org/10.1002/fsn3.58

Durkin, L. A., Childs, C. E., & Calder, P. C. (2021). Omega-3 Polyunsaturated Fatty Acids and the Intestinal Epithelium-A Review. *Foods (Basel, Switzerland)*, *10*(1), 199. https://doi.org/10.3390/foods10010199

Elfalleh, W., Ying, M., Nasri, N., Sheng-Hua, H., Guasmi, F., & Ferchichi, A. (2011). Fatty acids from Tunisian and Chinese pomegranate (Punica granatum L.) seeds. *International Journal of Food Sciences and Nutrition*, *62*(3), 200–206. https://doi.org/10.3109/09637486.2010.526932

Fagali, N., & Catalá, A. (2008). Antioxidant activity of conjugated linoleic acid isomers, linoleic acid and its methyl ester determined by photoemission and DPPH techniques. *Biophysical Chemistry*, *137*(1), 56–62. https://doi.org/https://doi.org/10.1016/j.bpc.2008.07.001

Falcão, A. de O., Speranza, P., Ueta, T., Martins, I. M., Macedo, G. A., & Macedo, J. A. (2017). Antioxidant potential and modulatory effects of restructured lipids from the Amazonian palms on Liver cells. *Food Technology and Biotechnology*, *55*(4), 553–561. https://doi.org/10.17113/ftb.55.04.17.5157

924 Flintoff-Dye, N. L., & Omaye, S. T. (2005). Antioxidant effects of conjugated linoleic
925 acid isomers in isolated human low-density lipoproteins. *Nutrition Research*,
926 *25*(1), 1–12. https://doi.org/https://doi.org/10.1016/j.nutres.2004.10.003

927 Floegel, A., Kim, D.-O., Chung, S.-J., Koo, S. I., & Chun, O. K. (2011). Comparison of
928 ABTS/DPPH assays to measure antioxidant capacity in popular antioxidant-rich
929 US foods. *Journal of Food Composition and Analysis*, *24*(7), 1043–1048.
930 https://doi.org/https://doi.org/10.1016/j.jfca.2011.01.008

931 Floros, S., Toskas, A., Pasidi, E., & Vareltzis, P. (2022). Bioaccessibility and Oxidative
932 Stability of Omega-3 Fatty Acids in Supplements, Sardines and Enriched Eggs
933 Studied Using a Static In Vitro Gastrointestinal Model. *Molecules*, *27*(2).
934 https://doi.org/10.3390/molecules27020415

935 Fontes, A. L., Pimentel, L. L., Simoes, C. D., Gomes, A. M. P., & Rodriguez-Alcala, L.
936 M. (2017). Evidences and perspectives in the utilization of CLNA isomers as
937 bioactive compound in foods. *Critical Reviews in Food Science and Nutrition*,
938 *57*(12), 2611–2622. https://doi.org/10.1080/10408398.2015.1063478

939 Fontes, A. L., Pimentel, L., Rodríguez-Alcalá, L. M., & Gomes, A. (2018). Effect of
940 Pufa Substrates on Fatty Acid Profile of Bifidobacterium breve Ncimb 702258 and
941 CLA/CLNA Production in Commercial Semi-Skimmed Milk. *Scientific Reports*,
942 *8*(1), 15591. https://doi.org/10.1038/s41598-018-33970-2

943 Fu, Y., Wang, Y., Gao, H., Li, D., Jiang, R., Ge, L., Tong, C., & Xu, K. (2021).
944 Associations among Dietary Omega-3 Polyunsaturated Fatty Acids, the Gut
945 Microbiota, and Intestinal Immunity. *Mediators of Inflammation*, *2021*.
946 https://doi.org/10.1155/2021/8879227

947 Gervais, R., Gagnon, F., Kheadr, E. E., Van Calsteren, M. R., Farnworth, E. R., Fliss, I.,
948 & Chouinard, P. Y. (2009). Bioaccessibility of fatty acids from conjugated linoleic
949 acid-enriched milk and milk emulsions studied in a dynamic in vitro
950 gastrointestinal model. *International Dairy Journal*, *19*(10), 574–581.
951 https://doi.org/10.1016/j.idairyj.2009.05.001

952 Ghasemifard, S., Turchini, G. M., & Sinclair, A. J. (2014). Omega-3 long chain fatty
953 acid "bioavailability": A review of evidence and methodological considerations.
954 *Progress in Lipid Research*, *56*, 92–108.
955 https://doi.org/10.1016/j.plipres.2014.09.001

956 Gomes, R., Martins, S., Afonso, C., Bandarra, N. M., & Cardoso, C. (2019).
957 Comparison of fish and oil supplements for a better understanding of the role of fat
958 level and other food constituents in determining bioaccessibility. *Food Science and*
959 *Nutrition*, *7*(4), 1179–1189. https://doi.org/10.1002/fsn3.894

960 Gonçalves, B., Falco, V., Moutinho-Pereira, J., Bacelar, E., Peixoto, F., & Correia, C.
961 (2009). Effects of Elevated CO2 on Grapevine (Vitis vinifera L.): Volatile
962 Composition, Phenolic Content, and in Vitro Antioxidant Activity of Red Wine.
963 *Journal of Agricultural and Food Chemistry*, *57*(1), 265–273.
964 https://doi.org/10.1021/jf8020199

965 Gori, M., Altomare, A., Cocca, S., Solida, E., Ribolsi, M., Carotti, S., Rainer, A.,
966 Francesconi, M., Morini, S., Cicala, M., & Pier Luca Guarino, M. (2020). Palmitic
967 Acid Affects Intestinal Epithelial Barrier Integrity and Permeability In Vitro. In

Antioxidants (Vol. 9, Issue 5). https://doi.org/10.3390/antiox9050417

Gullon, B., Pintado, M. E., Fernández-López, J., Pérez-Álvarez, J. A., & Viuda-Martos, M. (2015). In vitro gastrointestinal digestion of pomegranate peel (Punica granatum) flour obtained from co-products: Changes in the antioxidant potential and bioactive compounds. *Journal of Functional Foods*, *19*, 617–628. https://doi.org/10.1016/j.jff.2015.09.056

Hajianfar, H., Paknahad, Z., & Bahonar, A. (2013). The effect of omega-3 supplements on antioxidant capacity in patients with type 2 diabetes. *International Journal of Preventive Medicine*, *4*(Suppl 2), S234-8.

Heshmati, J., Morvaridzadeh, M., Maroufizadeh, S., Akbari, A., Yavari, M., Amirinejad, A., Maleki-Hajiagha, A., & Sepidarkish, M. (2019). Omega-3 fatty acids supplementation and oxidative stress parameters: A systematic review and meta-analysis of clinical trials. *Pharmacological Research*, *149*, 104462. https://doi.org/https://doi.org/10.1016/j.phrs.2019.104462

Ip, C., Singh, M., Thompson, H. J., & Scimeca, J. A. (1994). Conjugated linoleic acid suppresses mammary carcinogenesis and proliferative activity of the mammary gland in the rat. *Cancer Research*, *54*(5), 1212–1215.

Jedidi, H., Kheadr, E., Gagnon, F., Van Calsteren, M. R., Farnworth, E., & Fliss, I. (2014). Invitro study of the bioavailability of fatty acids in cow milk containing conjugated linoleic acids produced invivo or added in synthetic form. *International Dairy Journal*, *36*(2), 95–100. https://doi.org/10.1016/j.idairyj.2014.01.009

Jewell, C., Cusack, S., & Cashman, K. D. (2005). The effect of conjugated linoleic acid on transepithelial calcium transport and mediators of paracellular permeability in human intestinal-like Caco-2 cells. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, *72*(3), 163–171. https://doi.org/https://doi.org/10.1016/j.plefa.2004.10.015

Kanner, J., & Lapidot, T. (2001). The stomach as a bioreactor: Dietary lipid peroxidation in the gastric fluid and the effects of plant-derived antioxidants. *Free Radical Biology and Medicine*, *31*(11), 1388–1395. https://doi.org/10.1016/S0891-5849(01)00718-3

Kola, O., Parildi, E., Akkaya, M. R., Özcan, B. D., Mamur, E., & Türker, B. (2021). Fatty acids, sterols and triglycerides composition of cold pressed oil from pomegranate seeds. *Rivista Italiana Delle Sostanze Grasse*, *98*(3), 197–204.

Kotue, T. C., Djote, W. N. B., Marlyne, M., Pieme, A. C., Kansci, G., & Fokou, E. (2019). Antisickling and Antioxidant Properties of Omega-3 Fatty Acids EPA/DHA. *Nutrition & Food Science International Journal*, *9*(1), 1–6. https://doi.org/10.19080/NFSIJ.2019.09.555752

Lin, X., Chen, P. X., Robinson, L. E., Rogers, M. A., & Wright, A. J. (2021). Lipid digestibility and bioaccessibility of a high dairy fat meal is altered when consumed with whole apples: Investigations using static and dynamic in vitro digestion models. *Food Structure*, *28*(November 2020), 100191. https://doi.org/10.1016/j.foostr.2021.100191

Liyana Hannah Binti Izham Akmal, N., & Roy, A. (2017). Free Radical Scavenging Activity of Fish Oil-an in-Vitro Study. *International Journal of Pharmaceutical*

Sciences and Research, *8*(9), 3872–3875. https://doi.org/10.13040/IJPSR.0975-8232.8(9).3872-75

Loukhmas, S., Kerak, E., Elgadi, S., Ettalibi, F., El Antari, A., & Harrak, H. (2021). Oil Content, Fatty Acid Composition, Physicochemical Properties, and Antioxidant Activity of Seed Oils of Ten Moroccan Pomegranate Cultivars. *Journal of Food Quality*, *2021*, 6617863. https://doi.org/10.1155/2021/6617863

Lucas-Gonzalez, R., Navarro-Coves, S., Pérez-Álvarez, J. A., Fernández-López, J., Muñoz, L. A., & Viuda-Martos, M. (2016). Assessment of polyphenolic profile stability and changes in the antioxidant potential of maqui berry (Aristotelia chilensis (Molina) Stuntz) during in vitro gastrointestinal digestion. *Industrial Crops and Products*, *94*, 774–782. https://doi.org/https://doi.org/10.1016/j.indcrop.2016.09.057

McIntosh, F. M., Shingfield, K. J., Devillard, E., Russell, W. R., & Wallace, R. J. (2009). Mechanism of conjugated linoleic acid and vaccenic acid formation in human faecal suspensions and pure cultures of intestinal bacteria. *Microbiology*, *155*(1), 285–294. https://doi.org/10.1099/mic.0.022921-0

Mgbechidinma, C. L., Zheng, G., Baguya, E. B., Zhou, H., Okon, S. U., & Zhang, C. (2023). Fatty acid composition and nutritional analysis of waste crude fish oil obtained by optimized milder extraction methods. *Environmental Engineering Research*, *28*(2), 220030–220034. https://doi.org/10.4491/eer.2022.034

Miranda, J., Lasa, A., Fernández-Quintela, A., García-Marzo, C., Ayo, J., Dentin, R., & Portillo, M. P. (2011). Cis-9,trans-11,cis-15 and cis-9,trans-13,cis-15 CLNA mixture activates PPARα in HEK293 and reduces triacylglycerols in 3T3-L1 cells. *Lipids*, *46*(11), 1005–1012. https://doi.org/10.1007/s11745-011-3615-4

Nornberg, G. F. J. L. (2016). Systematic evaluation on the effectiveness of conjugated linoleic acid in human health. *Critical Reviews in Food Science and Nutrition*, *September 2016*. https://doi.org/10.1080/10408398.2012.716800

Opinion, S. (2012). Scientific Opinion on the Tolerable Upper Intake Level of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and docosapentaenoic acid (DPA). *EFSA Journal*, *10*(7), 1–48. https://doi.org/10.2903/j.efsa.2012.2815

Pan, F., Han, L., Zhang, Y., Yu, Y., & Liu, J. (2015). Optimization of Caco-2 and HT29 co-culture in vitro cell models for permeability studies. *International Journal of Food Sciences and Nutrition*, *66*(6), 680–685. https://doi.org/10.3109/09637486.2015.1077792

Pariza, M. W., Park, Y., & Cook, M. E. (2001). The biologically active isomers of conjugated linoleic acid. *Progress in Lipid Research*, *40*(4), 283–298. https://doi.org/10.1016/S0163-7827(01)00008-X

Park, Y. (2009). Conjugated linoleic acid (CLA): Good or bad trans fat ? *Journal of Food Composition and Analysis*, *22S*, S4–S12. https://doi.org/10.1016/j.jfca.2008.12.002

Pimentel, L. L., Fontes, A. L., Gomes, A. M., & Rodríguez-Alcalá, L. M. (2015). Considerations about the in situ derivatization and fractionation of EFA and NEFA in biological and food samples. *MethodsX*, *2*, 475–484. https://doi.org/10.1016/j.mex.2015.11.006

Puyol, P., Perez, M. D., Sanchez, L., Ena, J. M., & Calvo, M. (1995). Uptake and passage of beta-lactoglobulin, palmitic acid and retinol across the Caco-2 monolayer. *Biochimica et Biophysica Acta*, *1236*(1), 149–154. https://doi.org/10.1016/0005-2736(95)00041-z

Rangel-Huerta, O. D., & Gil, A. (2018). Omega 3 fatty acids in cardiovascular disease risk factors: An updated systematic review of randomised clinical trials. *Clinical Nutrition*, *37*(1), 72–77. https://doi.org/10.1016/j.clnu.2017.05.015

Ribeiro, T. B., Campos, D., Oliveira, A., Nunes, J., Vicente, A. A., & Pintado, M. (2021). Study of olive pomace antioxidant dietary fibre powder throughout gastrointestinal tract as multisource of phenolics , fatty acids and dietary fibre. *Food Research International*, *142*(110032). https://doi.org/10.1016/j.foodres.2020.110032

Ribeiro, T. B., Oliveira, A., Campos, D., Nunes, J., Vicente, A. A., & Pintado, M. (2020). Simulated digestion of olive pomace water-soluble ingredient: Relationship between the compounds bioaccessibility and their potential health benefits. *Food & Function*, *11*(3), 2238–2254. https://doi.org/10.1039/C9FO03000J

Richard, D., Kefi, K., Barbe, U., Bausero, P., & Visioli, F. (2008). Polyunsaturated fatty acids as antioxidants. *Pharmacological Research*, *57*(6), 451–455. https://doi.org/https://doi.org/10.1016/j.phrs.2008.05.002

Roche, H. M., Terres, A. M., Black, I. B., Gibney, M. J., & Kelleher, D. (2001). Fatty acids and epithelial permeability: effect of conjugated linoleic acid in Caco-2 cells. *Gut*, *48*(6), 797–802. https://doi.org/10.1136/gut.48.6.797

Rodríguez-Alcalá, L. M. L. M., Castro-Gómez, M. P. P., Pimentel, L. L. L. L., & Fontecha, J. (2017). Milk fat components with potential anticancer activity — a review. *Bioscience Reports*, *37*(6), 1–18. https://doi.org/10.1042/BSR20170705

Rosella, O., Sinclair, A., & Gibson, P. R. (2000). Polyunsaturated fatty acids reduce non-receptor-mediated transcellular permeation of protein across a model of intestinal epithelium in vitro. *Journal of Gastroenterology and Hepatology*, *15*(6), 626–631. https://doi.org/10.1046/j.1440-1746.2000.02215.x

Saha, S. S., Chakraborty, A., Ghosh, S., & Ghosh, M. (2012). Comparative study of hypocholesterolemic and hypolipidemic effects of conjugated linolenic acid isomers against induced biochemical perturbations and aberration in erythrocyte membrane fluidity. *European Journal of Nutrition*, *51*(4), 483–495. https://doi.org/10.1007/s00394-011-0233-0

Saha, S. S., Patra, M., & Ghosh, M. (2012). In vitro antioxidant study of vegetable oils containing conjugated linolenic acid isomers. *LWT - Food Science and Technology*, *46*(1), 10–15. https://doi.org/https://doi.org/10.1016/j.lwt.2011.11.008

Sakai, C., Ishida, M., Ohba, H., Yamashita, H., Uchida, H., Yoshizumi, M., & Ishida, T. (2017). Fish oil omega-3 polyunsaturated fatty acids attenuate oxidative stress-induced DNA damage in vascular endothelial cells. *PLOS ONE*, *12*(11), e0187934. https://doi.org/10.1371/journal.pone.0187934

Salsinha, A. S., Machado, M., Rodríguez-Alcalá, L. M., Gomes, A. M., & Pintado, M. (2023). *Chapter 1 - Bioactive lipids: Chemistry, biochemistry, and biological properties* (M. Pintado, M. Machado, A. M. Gomes, A. S. Salsinha, & L. M. B. T.-

B. L. Rodríguez-Alcalá (eds.); pp. 1–35). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-824043-4.00014-2

Salsinha, A. S., Rodríguez-Alcalá, L. M., Relvas, J. B., & Pintado, M. E. (2021). Fatty acids role on obesity induced hypothalamus inflammation: From problem to solution – A review. *Trends in Food Science & Technology*, *112*(September 2020), 592–607. https://doi.org/10.1016/j.tifs.2021.03.042

Sánchez-Moreno, C. (2002). Review: Methods Used to Evaluate the Free Radical Scavenging Activity in Foods and Biological Systems. *Food Science and Technology International*, *8*(3), 121–137. https://doi.org/10.1106/108201302026770

Schaich, K. M., Tian, X., & Xie, J. (2015). Reprint of "Hurdles and pitfalls in measuring antioxidant efficacy: A critical evaluation of ABTS, DPPH, and ORAC assays." *Journal of Functional Foods*, *18*, 782–796.

Schneider, A.-C., Beguin, P., Bourez, S., Perfield II, J. W., Mignolet, E., Debier, C., Schneider, Y.-J., & Larondelle, Y. (2012). Conversion of t11t13 CLA into c9t11 CLA in Caco-2 Cells and Inhibition by Sterculic Oil. *PLOS ONE*, *7*(3), e32824. https://doi.org/10.1371/journal.pone.0032824

Schneider, A. C., Mignolet, E., Schneider, Y. J., & Larondelle, Y. (2013). Uptake of conjugated linolenic acids and conversion to cis-9, trans-11-or trans-9, trans-11-conjugated linoleic acids in Caco-2 cells. *British Journal of Nutrition*, *109*(1), 57–64. https://doi.org/10.1017/S0007114512000608

Schuchardt, J. P., & Hahn, A. (2013). Bioavailability of long-chain omega-3 fatty acids. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, *89*(1), 1–8. https://doi.org/https://doi.org/10.1016/j.plefa.2013.03.010

Sengupta, A., Gupta, S. Sen, Nandi, I., & Ghosh, M. (2015). Conjugated linolenic acid nanoparticles inhibit hypercholesterolemia induced by feeding a high-fat diet in male albino rats. *Journal of Food Science and Technology*, *52*(1), 458–464. https://doi.org/10.1007/s13197-013-0974-2

Shinohara, N., Tsuduki, T., Ito, J., Honma, T., Kijima, R., Sugawara, S., Arai, T., Yamasaki, M., Ikezaki, A., Yokoyama, M., Nishiyama, K., Nakagawa, K., Miyazawa, T., & Ikeda, I. (2012). Jacaric acid, a linolenic acid isomer with a conjugated triene system, has a strong antitumor effect in vitro and in vivo. *Biochimica et Biophysica Acta*, *1821*(7), 980–988. https://doi.org/10.1016/j.bbalip.2012.04.001

Śpitalniak-Bajerska, K., Szumny, A., Kucharska, A. Z., & Kupczyński, R. (2018). Effect of Natural Antioxidants on the Stability of Linseed Oil and Fish Stored under Anaerobic Conditions. *Journal of Chemistry*, *2018*, 9375085. https://doi.org/10.1155/2018/9375085

Tan, Y., Zhang, Z., Liu, J., Xiao, H., & McClements, D. J. (2020). Factors impacting lipid digestion and nutraceutical bioaccessibility assessed by standardized gastrointestinal model (INFOGEST): Oil. *Food and Function*, *11*(11), 9936–9946. https://doi.org/10.1039/d0fo01505a

Tan, Y., Zhang, Z., Zhou, H., Xiao, H., & McClements, D. J. (2020). Factors impacting lipid digestion and β-carotene bioaccessibility assessed by standardized

gastrointestinal model (INFOGEST): oil droplet concentration. *Food & Function*, *11*(8), 7126–7137. https://doi.org/https://doi.org/10.1039/D0FO01506G

Taner Bozkurt, & Zeynep Ergun. (2021). Fatty acid composition and antioxidant capacity of pomegranate seed oil. *GSC Biological and Pharmaceutical Sciences*, *015*(02), 103–110. https://doi.org/10.30574/gscbps.2021.15.2.0126

Topkafa, M., Kara, H., & Sherazi, S. T. H. (2015). Evaluation of the Triglyceride Composition of Pomegranate Seed Oil by RP-HPLC Followed by GC-MS. *Journal of the American Oil Chemists' Society*, *92*(6), 791–800. https://doi.org/10.1007/s11746-015-2652-1

Tullberg, C., Vegarud, G., & Undeland, I. (2019). Oxidation of marine oils during in vitro gastrointestinal digestion with human digestive fluids – Role of oil origin, added tocopherols and lipolytic activity. *Food Chemistry*, *270*(March 2021), 527–537. https://doi.org/10.1016/j.foodchem.2018.07.049

Valenzuela, R. V. A. (2012). Overview About Lipid Structure. In *Intech: Vol. i* (Issue tourism, p. 38). https://doi.org/http://dx.doi.org/10.5772/52306 Chapter 1 1.

van Greevenbroek, M. M., Voorhout, W. F., Erkelens, D. W., van Meer, G., & de Bruin, T. W. (1995). Palmitic acid and linoleic acid metabolism in Caco-2 cells: different triglyceride synthesis and lipoprotein secretion. *Journal of Lipid Research*, *36*(1), 13–24.

Viladomiu, M., Hontecillas, R., Yuan, L., Lu, P., & Bassaganya-Riera, J. (2013). Nutritional protective mechanisms against gut inflammation. *Journal of Nutritional Biochemistry*, *24*(6), 929–939. https://doi.org/10.1016/j.jnutbio.2013.01.006

Xiao, G., Tang, L., Yuan, F., Zhu, W., Zhang, S., Liu, Z., Geng, Y., Qiu, X., Zhang, Y., & Su, L. (2013). Eicosapentaenoic acid enhances heat stress-impaired intestinal epithelial barrier function in Caco-2 cells. *PloS One*, *8*(9), e73571. https://doi.org/10.1371/journal.pone.0073571

Yayla, M., Cetin, D., Adali, Y., Kilicle, P. A., & Toktay, E. (2018). Potential therapeutic effect of pomegranate seed oil on ovarian ischemia/reperfusion injury in rats. *Iranian Journal of Basic Medical Sciences*, *21*(12), 1262–1268. https://doi.org/10.22038/ijbms.2018.30149.7268

Ye, Z., Li, R., Cao, C., Xu, Y. J., Cao, P., Li, Q., & Liu, Y. (2019). Fatty acid profiles of typical dietary lipids after gastrointestinal digestion and absorbtion: A combination study between in-vitro and in-vivo. *Food Chemistry*, *280*(September 2018), 34–44. https://doi.org/10.1016/j.foodchem.2018.12.032

Zhang, H., Shen, Y., Zhang, Y., Li, L., & Wang, X. (2018). Regiospecific Analysis of Fatty Acids and Calculation of Triglyceride Molecular Species in Marine Fish Oils. *BioMed Research International*, *2018*, 9016840. https://doi.org/10.1155/2018/9016840

Zhang, H., Zhao, H., Zhang, Y., Shen, Y., Su, H., Jin, J., Jin, Q., & Wang, X. (2018). Characterization of Positional Distribution of Fatty Acids and Triacylglycerol Molecular Compositions of Marine Fish Oils Rich in Omega-3 Polyunsaturated Fatty Acids. *BioMed Research International*, *2018*, 3529682. https://doi.org/10.1155/2018/3529682

8. List of tables

9. List of figures

Table 1- Major FAs of samples submitted to the dialysis, with 3.5kDa membranes, process after in vitro INFOGEST GIT tract digestion. The IN corresponds to the retentate and the OUT samples to the permeate. The bioaccessibility index (BI%) was calculated for each sample. Values are expressed as mean±standard deviation of µg of FAs/ µL of sample.

µg of FAs/ mg of sample		CLA capsules			CLNA capsules (Xanthigen)			Omega 3			Pomegranate oil			Fish oil		
		Retentate (IN)	**Permeate (OUT)**	**BI (%)**	**Retentate (IN)**	**Permeate (OUT)**	**BI (%)**	**Retentate (IN)**	**Permeate (OUT)**	**BI (%)**	**Retentate (IN)**	**Permeate (OUT)**	**BI (%)**	**Retentate (IN)**	**Permeate (OUT)**	**BI (%)**
C14	Myristic acid	0.17±0.01	0.17±0.00	49.78	0.24±0	0.21±0.00	47.42	0.29±0.01	0.31±0.00	51.94	n.d.	n.d.		66.55±0.04	0.98±0	1.46
C16	Palmitic acid	7.4±0.02	0.86±0.01	10.44	89.61±0.27	1.27±0.04	1.4	3.64±0.02	1.88±0.01	34.07	29.59±0.03	2.04±0.03	6.44	176.14±0.21	1.83±0	1.03
C16:1*c*9	Palmitoleic acid	0.6±0.00	0.28±0.01	32.02	4.86±0.01	0.76±0.03	13.52	1.1±0.01	0.91±0.01	45.32	n.d.	n.d.		86.76±0.19	n.d.	
C18	Stearic acid	5.21±0.06	0.14±0.01	2.69	37.38±0.20			4.66±0.02	0.14±0	3.00	25.39±0.03	0.22±0.01	0.86	38.29±0.04	0.17±0.01	0.45
C18:1 *c*9	Oleic acid	42.06±0.06	0.42±0.03	0.99	460.79±1.6	0.36±0.02	0.08	7.88±0.03	0.04±0	0.5	56.57±0.17	1.23±0.03	2.13	70.21±0.08	0.03±0	0.05
C18:1 *c*11	cis-vaccenic acid	3.93±0.07	n.d.		16.34±0.11	n.d.		2.59±0.01	1.19±0.03	31.49	4.49±0.04	n.d.		30.98±0.05	1.32±0.00	4.09
C18:2 *c*9*c*12	Linoleic acid	7.52±0.01	n.d.		63.19±0.11	n.d.		1.8±0.01	0.05±0	2.86	51.13±0.04	0.1±0.01	0.2	11.0±0.02	n.d.	
C18:2 *c*9*t*11	Rumenic acid	186.27±1.56	n.d.		1.1±0.10	n.d.		0.2±0.01	n.d.		0.92±0.11	n.d.		0.36±0.00	n.d.	
C18:2 *t*10*c*12	CLA isomer	185.05±0.42	0.08±0.00	0.04	1.67±0.15	n.d.		10.25±0.05	n.d.		1.28±0.08	n.d.		13.12±0.07	n.d.	
C18:3 *c*9*c*12*c*15	α-linolenic acid	21.87±0.21	n.d.		6.19±0.07	n.d.		2.45±0.01	n.d.		7.92±0.06	n.d.		11.7±0.03	0.11±0.01	0.91
C18:2 t9t11		22.01±0.08	n.d.		5.94±0.04	n.d.		±	n.d.		7.84±1.39	n.d.		1.66±0.03	n.d.	
C20:3 *c*8*c*11*c*14	Dihomo-γ-linolenic acid	n.d.	n.d.			n.d.		7.72±0.03	n.d.		n.d.	n.d.		6.26±0.02	n.d.	
C20:4 n-6	Arachidonic acid	n.d.	n.d.			n.d.		10.83±0.13	n.d.		n.d.	n.d.		8.93±0.08	n.d.	
C20:5 n-3	EPA	n.d.	n.d.			n.d.		214.34±0.56	0.05±0.00	0.02	n.d.	n.d.		80.68±0.29	0.1±0.00	0.12
C18:3 *c*9*t*11*c*13	Punicic acid	n.d.	n.d.		109.09±0.83	n.d.		n.d.	n.d.		180.58±1.37	0.04±0.00	0.02	n.d.	n.d.	
C18:3 *c*9*t*11*t*13	α-eleostearic acid	n.d.	n.d.		5.58±0	n.d.		n.d.	n.d.		14.4±0.06	0.07±0.00	0.47	n.d.	n.d.	
C18:3 *t*9*t*11*c*13	catalpic acid	n.d.	n.d.		59.22±0.38	n.d.		0.3±0.02	n.d.		115.82±1.03	n.d.		n.d.	n.d.	

C18:3 *t*9*t*11*t*13	β-eleostearic acid	0.36±0.01	n.d.		70.32±0.13	n.d.		n.d.	n.d.		222.57±1.78	0.12±0.00	0.05	n.d.	n.d.	
C22:5n-3	DPA	n.d.	n.d.		n.d.	n.d.		32.21±0.03	n.d.		n.d.	n.d.		18.94±0.06	n.d.	
C22:6 n-3	DHA	n.d.	n.d.		n.d.	n.d.		118.29±0.31	n.d.		0.35±0.01	n.d.		47.32±0.25	n.d.	

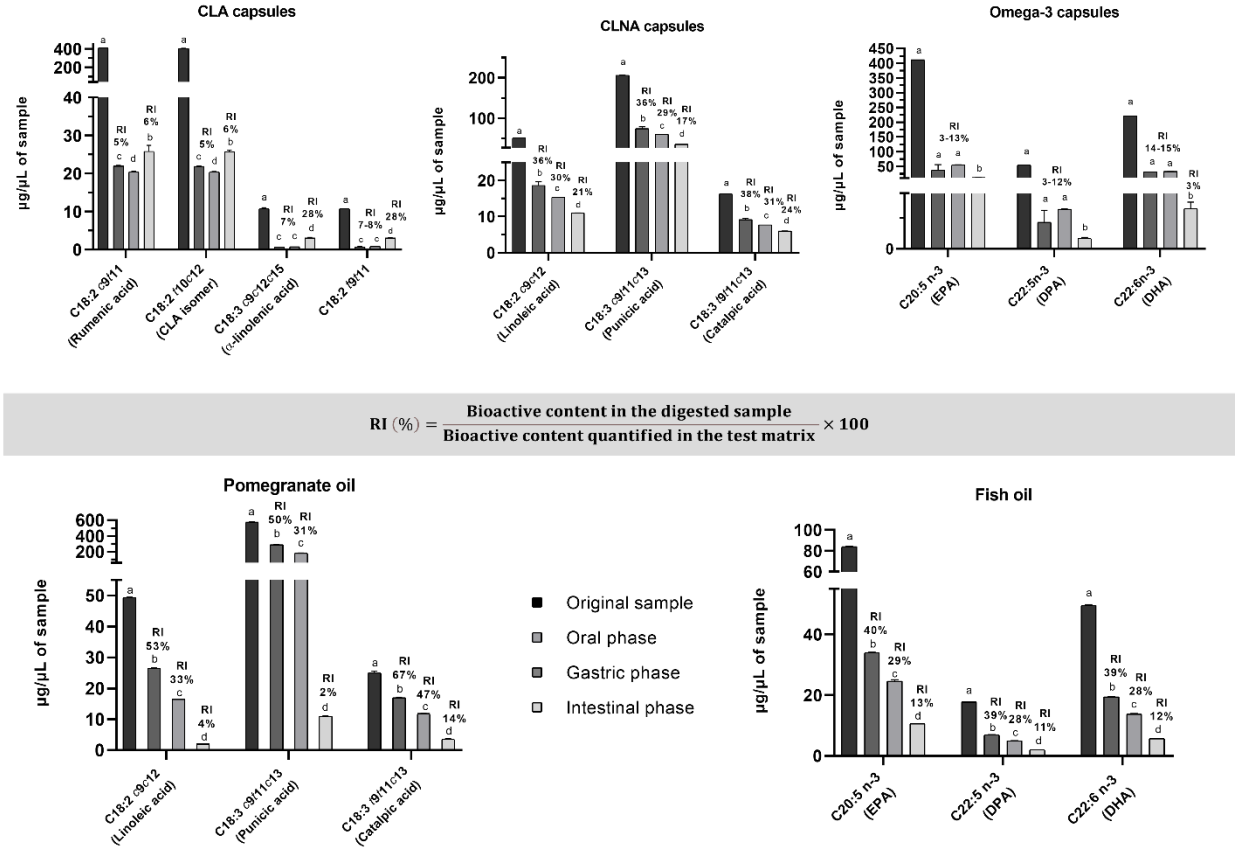


Figure 1- Major FAs content of each sample in the different steps (oral, gastric and intestinal phases) of GIT tract digestion. The recovery index (RI%) was calculated for each phase using the equation presented in the figure. Values are expressed as mean±standard deviation of µg of FAs/ µL of sample (n=2). ^{a,b,c,d}Different superscript letters for significant differences ($p < 0.05$) between the same FAs.

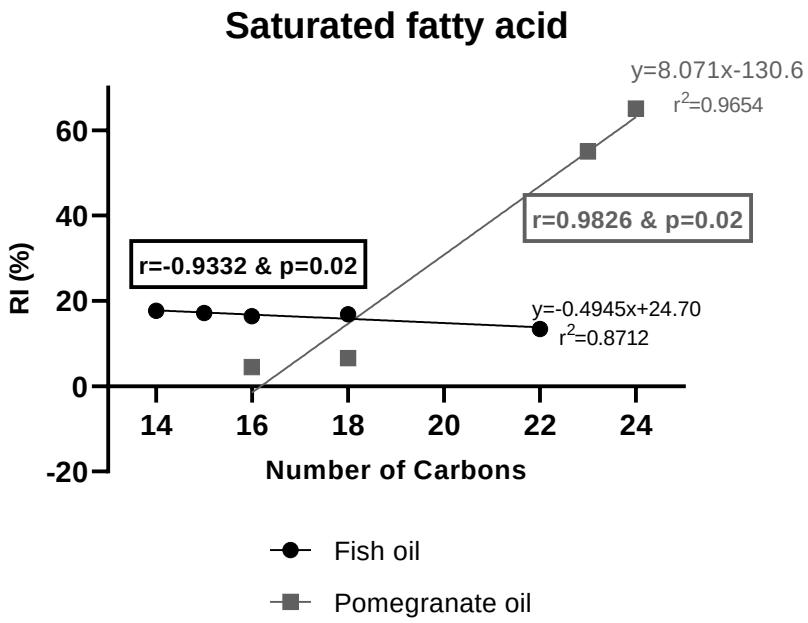


Figure 2 - Correlation between the number of carbons (chain length) and recovery index (RI%) for pomegranate and Fish oil. The p value concerns the slope parameter.

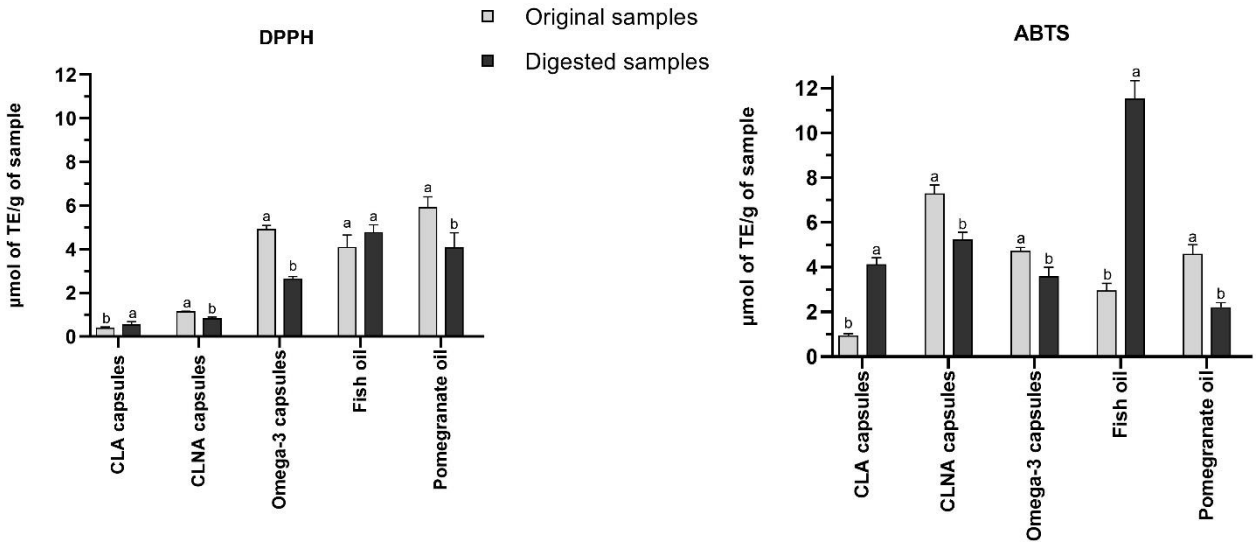
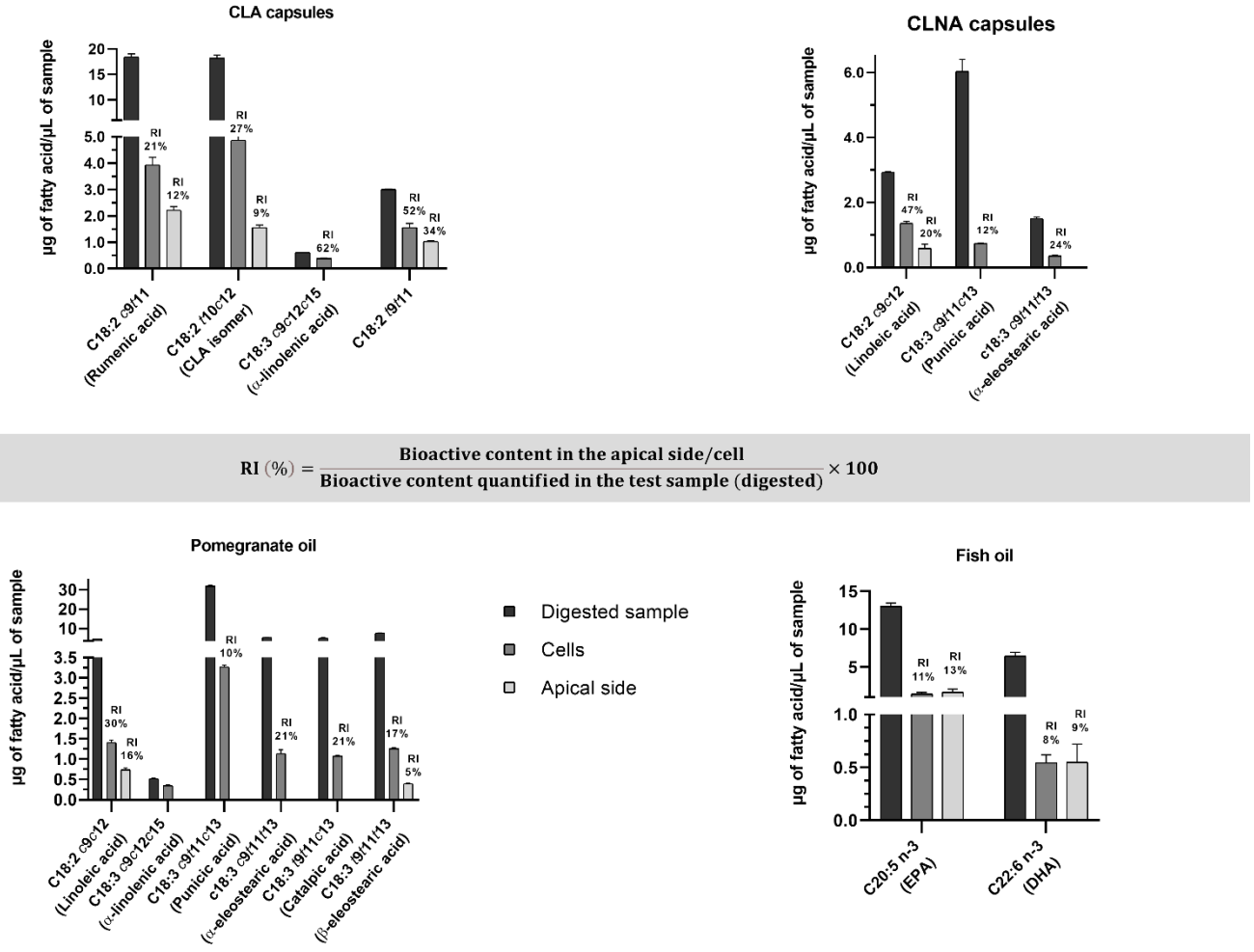


Figure 3 - Antioxidant potential assessment through DPPH and ABTS methods before and after GIT tract digestion of CLA, CLNA and Omega 3 capsules and Fish and Pomegranate oil. Values are expressed as mean±standard deviation of µmol of Trolox Equivalents/ g of sample (n=3). [a,b] Different superscript letters for significant differences ($p < 0.05$) between original and digested samples.



$$\mathbf{RI}\ (\%) = \frac{\textbf{Bioactive content in the apical side/cell}}{\textbf{Bioactive content quantified in the test sample (digested)}} \times 100$$

Figure 4 - Major FAs in the digested samples and their quantification in apical side and cells content after permeability assays using a Caco-2/HT29-MTX cell lines co-culture. The recovery index (RI %) was calculated using the equation presented in the image. Values are expressed as mean±standard deviation of µg of FAs/ µL of sample (n=4).

10. Supplementary material

Table 2 - FAs profile of original tested samples (Pomegranate and Fish oil) and samples after *in vitro* INFOGEST GIT tract digestion in the different phases of the process (oral, gastric and intestinal). The recovery index (RI%) was calculated for each phase. Values are expressed as mean±standard deviation of µg of FAs/ µL of sample. [a,b,c,d]Different superscript letters within a row for significant differences ($p < 0.05$) between the same sample (Pomegranate oil or Fish oil).

µg of FAs/ µL of sample		Pomegranate oil							Fish oil						
		Original	Oral phase	RI (%)	Gastric phase	RI (%)	Intestinal	RI (%)	Original	Oral phase	RI (%)	Gastric phase	RI (%)	Intestinal	RI (%)
Saturated FAs (SFAs)															
C14	Myristic acid	n.d.	n.d.		n.d.		n.d.		53.19±0.39[a]	21±0.02[b]	39.47	15.36±0.18[c]	28.88	9.41±0.03[d]	17.69
C15		n.d.	n.d.		n.d.		n.d.		3.35±0.03[a]	1.29±0.01[b]	38.56	0.94±0.00[c]	28.13	0.57±0.01[d]	17.13
C16	Palmitic acid	24.29±0.05[a]	12.94±0.03[b]	53.28	7.85±0.02[c]	32.32	1.1±0.11[d]	4.51	115.93±0.8[a]	44.48±0.13[b]	38.37	32.57±0.45[c]	28.09	19.01±0.03[d]	16.40
C18	Stearic acid	19.61±0.08[a]	10.82±0.12[b]	55.21	6.6±0.00[c]	33.67	1.3±0.05[d]	6.62	21.76±0.17[a]	8.17±0.05[b]	37.56	6.01±0.09[c]	27.64	3.67±0.01[d]	16.85
C22	Behenic acid	n.d.	n.d.		n.d.		n.d.		1.63±0.01[a]	0.61±0[b]	37.21	0.46±0.01[c]	28.11	0.22±0.00[d]	13.42
C23		1.80±0.03[a]	1.27±0.08[a,b]	70.55	1.23±0.00[a,b]	67.58	0.99±0.11[b]	55.00	n.d.	n.d.		n.d.		n.d.	
C24	Lignoceric acid	1.32±0.00[a]	1.11±0.07[a]	84.70	1.01±0.02[a]	77.07	0.86±0.07[a]	65.06	n.d.	n.d.		n.d.		n.d.	
TOTAL (SFAs)		**45.22**	**24.87**	**55**	**15.46**	**34.2**	**3.26**	**7.21**	**357.23**	**75.55**	**21.15**	**55.34**	**15.49**	**32.88**	**9.20**
Monounsaturated FAs (MUFAs)															
C16:1*c*9	Palmitoleic acid	n.d.	n.d.		n.d.		n.d.		69.82±0.41[a]	27.43±0.09[b]	39.28	20.29±0.27[c]	29.06	10.96±0.04[d]	15.7
C18:1 *t*9	Elaidic acid	n.d.	n.d.		n.d.		n.d.		0.84±0.00[a]	0.31±0.01[b]	36.88	0.22±0.02[c]	26.29	0.12±0.01[d]	14.48
C18:1 *t*11	Vaccenic acid	1.26±0.11[a]	1.31±0.14[a]	103.54	1.03±0.04[a]	82.04	0.33±0.02[b]	26.46	0.84±0.01[a]	0.35±0.01[b]	41.59	0.25±0.01[c]	30.23	0.20±0.01[d]	23.76
C18:1 *t*12		1.15±0.08[a]	1.45±0.12[a]	126.29	1.23±0.00[a]	107.08	0.43±0.02[b]	37.68							
C18:1 *c*9	Oleic acid	48.8±0.14[a]	26.28±0.14[b]	53.85	15.98±0.05[c]	32.75	2.18±0.08[d]	4.47	51.25±0.28[a]	19.66±0.13[b]	38.36	14.49±0.21[c]	28.27	7.24±0.05[d]	14.13
C18:1 *c*11	cis-vaccenic acid	3.79±0.01[a]	2.19±0.04[b]	57.84	1.43±0.02[c]	37.83	0.43±0.00[d]	11.23	22.18±0.15[a]	8.51±0.07[b]	38.35	6.29±0.11[c]	28.37	3.38±0.04[d]	15.23
C18:1 *c*4/ *t*6		2.76±0.09[a]	1.9±0.1[b]	68.76	1.3±0.00[c]	47.31	0.26±0.01[d]	9.57	0.76±0.01[a]	0.28±0.00[b]	37.70	0.21±0.01[c]	28.17	0.12±0.01[d]	15.89
C20:1 *c*11	Gondoic acid	n.d.	n.d.		n.d.		n.d.		4.17±0.01[a]	1.67±0.02[b]	39.97	1.23±0.02[c]	29.49	0.61±0.01[d]	14.68
C24:1 *c*15	Nervonic acid	0.6±0.01[a]	0.33±0.01[b]	55.26	0.19±0.00[c]	31.19	n.d.		n.d.	n.d.		n.d.		n.d.	
TOTAL (MUFAs)		**58.36**	**33.46**	**57.33**	**21.16**	**36.26**	**3.63**	**6.22**	**146.1**	**58.21**	**39.84**	**42.98**	**29.42**	**22.63**	**15.49**
[MUFA/SFA]		**0.77**	**1.34**		**1.37**		**1.11**		**0.41**	**0.77**		**0.78**		**0.69**	
Polyunsaturated FAs (PUFAs)															
C18:2 *c*9*c*12	Linoleic acid	49.34±0.12[a]	26.48±0.12[b]	53.67	16.49±0.03[c]	33.41	2.07±0.08[d]	4.19	7.94±0.05[a]	3.1±0.03[b]	39.01	2.3±0.04[c]	28.91	0.9±0.02[d]	11.39
C18:3 *c*6*c*9*c*13	γ-linolenic acid	0.4±0.00[a]	0.21±0.00[b]	53.87	0.15±0.00[c]	38.71	n.d.		4.92±0.02[a]	1.97±0.01[b]	39.97	1.45±0.01[c]	29.47	0.73±0.00[d]	14.88
C18:2 *c*9*t*11	Rumenic acid	1.51±0.13[a]	1.57±0.12[a]	104.53	1.27±0.00[a]	84.1	0.35±0.01[a]	23.21	0.36±0.01[a]	0.14±0.00[b]	37.82	0.11±0.01[c]	30.35	n.d.	n.d.
C18:2 *t*10*c*12	CLA isomer	1.65±0.07[a]	1.77±0.02[a]	107.39	1.32±0.00[a]	79.83	0.27±0.02[a]	16.12	13.36±0.01[a]	5.41±0.05[b]	40.45	3.94±0.06[c]	29.47	1.78±0[d]	13.31
C18:3 *c*9*c*12*c*15	α-linolenic acid	6.59±0.05[a]	0.23±0.02[b]	3.55	0.22±0.00[b]	3.35	n.d.		7.76±0.01[a]	2.88±0.04[b]	37.16	2.14±0.03[c]	27.54	1.08±0.01[d]	13.92

C18:2 t9t11		3.05±0.31[a]	3.39±0.25[a]	111.32	2.67±0.03[a]	87.47	0.65±0.03[b]	21.38	1.67±0.00[a]	0.69±0.02[b]	41.14	0.49±0.00[c]	29.41	0.23±0.00[d]	13.84
C20:3 *c*8c11*c*14	Dihomo-γ-linolenic acid	n.d.	n.d.		n.d.		n.d.		5.46±0.01[a]	2.13±0.03[b]	39.05	1.59±0.01[c]	29.03	0.95±0.04[d]	17.30
C20:3 *c*11c14*c*17		0.96±0.01[a]	0.52±0.00[a]	54.69	0.32±0.01[b]	33.19	n.d.		1.5±0.03[a]	0.65±0.05[b]	43.68	0.58±0.04[b]	39.05	0.4±0.02[d]	26.90
C20:4 n-6	Arachidonic acid	n.d.	n.d.		n.d.		n.d.		8.05±0.01[a]	3.26±0.02[b]	40.51	2.39±0.04[c]	29.67	1.1±0.01[d]	13.65
C20:5 n-3	EPA	n.d.	n.d.		n.d.		n.d.		84.07±0.11[a]	33.89±0.22[b]	40.31	24.58±0.48[c]	29.24	10.59±0.02[d]	12.59
C18:3 *c*9t11*c*13	Punicic acid	577.33±2.17[a]	290.12±2.73[b]	50.25	183.96±1.27[c]	31.86	10.97±0.13[d]	1.90	n.d.	n.d.		n.d.		n.d.	
C18:3 *c*9t11t13	α-eleostearic acid	8.62±0.43[a]	5.76±0.28[b]	66.91	4.41±0.21[b]	51.14	1.17±0.14[c]	13.57	n.d.	n.d.		n.d.		n.d.	
C18:3 *t*9t11*c*13	catalpic acid	24.99±0.64[a]	16.99±0.16[b]	67.96	11.83±0.11[c]	47.33	3.59±0.25[d]	14.35	n.d.	n.d.		n.d.		n.d.	
C18:3 *t*9t11t13	β-eleostearic acid	5.09±0.3[a]	4.76±0.09[a]	93.62	3.38±0.11[b]	66.42	2.34±0.08[c]	46.06	n.d.	n.d.		n.d.		n.d.	
C22:5n-3	DPA	n.d.	n.d.		n.d.		n.d.		17.71±0.00[a]	6.91±0.03[b]	39.03	4.97±0.03[c]	28.07	1.97±0.02[d]	11.10
C22:6n-3	DHA	0.29±0.00[a]	0.14±0.00[b]	49.01	0.08±0.00[c]	25.80			49.54±0.22[a]	19.44±0.01[b]	39.24	13.72±0.21[c]	27.69	5.68±0.05[d]	11.46
TOTAL (PUFAs)		**679.79**	**351.94**	**51.77**	**226.10**	**33.26**	**21.41**	**3.15**	**202.34**	**80.47**	**39.77**	**58.26**	**27.31**	**25.39**	**12.55**
[PUFA/SFA]		**15.03**	**14.15**		**14.62**		**6.57**		**0.57**	**1.06**		**1.05**		**0.77**	

11.

Table 3 - FAs profile of original tested samples (CLA capsules and CLNA capsules) and samples after *in vitro* INFOGEST GIT tract digestion in the different phases of the process (oral, gastric and intestinal). The recovery index (RI%) was calculated for each phase. Values are expressed as mean±standard deviation of µg of FAs/ µL of sample (n=2). [a,b,c,d]Different superscript letters within a row for significant differences ($p < 0.05$) between the same sample (CLA capsules or CLNA capsules).

µg of FAs/ µL of sample		CLA capsules							CLNA capsules						
		Original	**Oral phase**	**RI (%)**	**Gastric phase**	**RI (%)**	**Intestinal**	**RI (%)**	**Original**	**Oral phase**	**RI (%)**	**Gastric phase**	**RI (%)**	**Intestinal**	**RI (%)**
Saturated FAs (SFAs)															
C14	Myristic acid	0.11±0.00	n.d.		n.d.		n.d.		0.10±0.002	n.d.		n.d.		n.d.	
C15		0.02±0.00	n.d.		n.d.		n.d.		0.09±0.003^{a}	0.03±0.002^{b}	38.69	n.d.		n.d.	
C16	Palmitic acid	4.8±0.00^{a}	0.27±0.00^{b}	5.59	0.27±0.00^{b}	5.61	n.d.		65.92±0.14^{a}	22.82±1.13^{b}	34.61	18.81±0.07^{c}	28.53	14.00±0.04^{d}	21.24
C18	Stearic acid	2.76±0.04^{a}	0.34±0.00^{b}	12.15	0.42±0.01^{b}	15.39	n.d.		23.65±0.08^{a}	8.02±0.37^{b}	33.91	6.63±0.02^{c}	28.04	4.81±0.02^{d}	20.36
C24	Lignoceric acid	0.56±0.00	n.d.		n.d.		n.d.		0.66±1.5x10$^{-4\,a}$	0.21±0.005^{b}	31.60	0.17±0.004^{c}	26.45	n.d.	
TOTAL (SFAs)		**8.77**	**0.64**	**7.30**	**0.69**	**7.86**			**90.42**	**31.08**	**34.37**	**25.61**	**28.32**	**18.81**	**20.80**
Monounsaturated FAs (MUFAs)															
C16:1*c*9	Palmitoleic acid	0.52±0.00^{a}	0.03±0.00^{b}	5.60	n.d.		0.08±0.00^{c}	15.87	0.04±0.00	n.d.		n.d.		n.d.	
C18:1 *t*9	Elaidic acid	n.d.	n.d.		n.d.		n.d.		n.d.	n.d.		n.d.		n.d.	
C18:1 *t*11	Vaccenic acid	0.63±0.05^{a}	0.22±0.01^{c}	34.90	0.27±0.00^{c}	43.33	0.35±0.22^{b}	55.66	n.d.	n.d.		n.d.		n.d.	
C18:1 *t*12		6.48±0.25^{a}	0.77±0.04^{c}	11.93	0.92±0.02^{c}	14.22	1.9±0.74^{b}	29.37	n.d.	n.d.		n.d.		n.d.	
C18:1 *c*9	Oleic acid	35.94±0.04^{a}	2.14±0.01^{c}	5.96	2.12±0.01^{c}	5.91	4.58±0.06^{b}	12.76	357.69±1.14^{a}	123.35±6.03^{b}	34.49	100.97±0.02^{c}	28.23	66.79±0.20^{d}	18.68
C18:1 *c*11	cis-vaccenic acid	2.7±0.02^{a}	0.24±0.00^{b}	9.02	0.28±0.01^{b}	10.40	0.55±0.07^{b}	20.26	9.21±0.01^{a}	4.20±0.20^{b}	45.65	3.48±0.04^{c}	37.82	2.42±0.003^{d}	26.27
C18:1 *c*4*l*t6		2.45±0.01^{a}	0.13±0.00^{c}	5.25	0.13±0.00^{c}	5.33	0.35±0.01^{b}	14.37	1.03±0.01^{a}	0.40±0.01^{b}	38.67	0.37±0.00^{b}	35.74	0.29±0.00^{c}	28.62
C24:1 *c*15	Nervonic acid	n.d.	n.d.		n.d.		n.d.		0.07±0.00	n.d.		n.d.		n.d.	
TOTAL (MUFAs)		**48.2**	**3.50**	**7.26**	**3.72**	**7.72**	**7.73**	**16.04**	**368.04**	**127.95**	**34.76**	**104.82**	**28.71**	**69.50**	**18.88**
[MUFA/SFA]		**5.50**	**5.47**		**5.39**				**4.07**	**4.11**		**3.70**		**3.69**	
Polyunsaturated FAs (PUFAs)															
C18:2 *c*9*c*12	Linoleic acid	6.31±0.01^{a}	0.35±0.00^{b}	5.59	0.34±0.00^{b}	5.42	n.d.		51.71±0.16^{a}	18.63±0.97^{b}	36.03	15.39±0.01^{c}	29.76	11.00±0.02^{d}	21.27
C18:3 *c*6*c*9*c*13	γ-linolenic acid	n.d.	n.d.		n.d.		n.d.		4.28±0.03^{a}	1.40±0.06^{b}	32.78	1.17±0.00^{c}	27.24	0.68±0.03^{d}	15.85
C18:2 *c*9*t*11	Rumenic acid	407.99±0.12^{a}	21.97±0.17^{c}	5.39	20.49±0.11^{d}	5.02	25.85±1.56^{b}	6.34	0.12±0.02^{bc}	0.10±0.003^{c}	80.92	0.13±0.00^{bc}	107.56	0.18±0.00^{ab}	141.54
C18:2 *t*10*c*12	CLA isomer	403.54±0.39^{a}	21.88±0.18^{c}	5.42	20.43±0.11^{d}	5.06	25.68±0.42^{b}	6.36	0.26±0.00^{a}	0.11±0.00^{d}	44.05	0.12±0.00^{c}	48.18	0.13±0.002^{b}	51.28
C18:3 *c*9*c*12*c*15	α-linolenic acid	10.78±0.25^{a}	0.71±0.00^{c}	6.55	0.75±0.02^{c}	6.91	3.04±0.21^{b}	28.17	4.15±0.001^{a}	1.38±0.06^{b}	33.26	1.14±3.2x10$^{-4\,c}$	27.43	0.72±0.01^{d}	17.30
C18:2 t9t11		10.8±0.00^{a}	0.72±0.05^{c}	6.65	0.83±0.01^{c}	7.71	3.06±0.08^{b}	28.3	0.07±0.00^{a}	0.03±0.003^{b}	50.93	n.d.		n.d.	
C18:3 *c*9*t*11*c*13	Punicic acid	0.46±0.01	n.d.		n.d.		n.d.		205.84±0.38^{a}	74.24±4.33^{b}	36.07	60.48±0.02^{c}	29.38	35.05±0.07^{d}	17.03

C18:3 *c*9*t*11*t*13	α-eleostearic acid	0.17±0.01	n.d.		n.d.		n.d.		16.33±0.001[a]	9.15±0.40[b]	56.01	7.72±0.008[c]	47.28	5.99±0.03[d]	36.68
C18:3 *t*9*t*11*c*13	catalpic acid	0.44±0.00	n.d.		n.d.		n.d.		23.80±0.06[a]	9.00±0.46[b]	37.82	7.39±0.04[c]	31.08	5.82±0.12[d]	24.45
C18:3 *t*9*t*11*t*13	β-eleostearic acid	0.51±0.00	n.d.		n.d.		n.d.		5.04±0.00[a]	2.04±0.09[b]	40.53	1.82±0.003[b]	36.23	2.48±0.05[c]	49.21
C22:5n-3	DPA	n.d.	n.d.		n.d.		n.d.		0.22±0.00[a]	0.08±0.00[c]	36.11	0.09±0.00[b]	39.91	n.d.	
C22:6n-3	DHA	n.d.	n.d.		n.d.		n.d.		0.17±0.02	n.d.		n.d.		n.d.	
TOTAL (PUFAs)		**841.00**	**45.63**	**5.42**	**42.84**	**5.09**	**57.63**	**6.85**	**311.19**	**116.16**	**37.32**	**95.62**	**30.72**	**50.19**	**16.13**
[PUFA/SFA]		**95.90**	**71.29**		**62.09**				**3.44**	**3.74**		**3.73**		**2.67**	

13.

Table 4 - FAs profile of original tested samples (Omega 3capsules) and samples after *in vitro* INFOGEST GIT tract digestion in the different phases of the process (oral. gastric and intestinal). The recovery index (RI%) was calculated for each phase. Values are expressed as mean±standard deviation of µg of FAs/ µL of sample. [a,b,c,d]Different superscript letters within a row for significant differences ($p < 0.05$).

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µg of FAs/ µL of sample		Omega 3capsules						
		Original	**Oral phase**	**RI (%)**	**Gastric phase**	**RI (%)**	**Intestinal**	**RI (%)**
Saturated FAs (SFAs)								
C14	Myristic acid	0.73±0.01[a]	0.07±0.02[b]	9.58	0.1±0.01[b]	13.69	0.11±0.02[b]	15.06
C16	Palmitic acid	3.86±0.01[a]	0.31±0.07[b]	8.03	0.45±0.01[b]	11.65	2.61±0.34[a]	67.62
C18	Stearic acid	5.26±0.01[a]	0.39±0.04[b]	7.41	0.55±0.01[b]	10.45	2.20±0.35 [b]	41.82
C22	Behenic acid	1.02±0.02[a]	0.14±0.01[b]	13.72	0.14±0.00[b]	13.72	n.d.	
TOTAL (SFAs)		**10.87**	**0.91**	**8.37**	**1.24**	**11.41**	**4.92**	**45.26**
Monounsaturated FAs (MUFAs)								
C16:1*c*9	Palmitoleic acid	1.96±0.01[a]	0.17±0.06[b]	8.80	0.24±0.00[b]	12.24	0.23±0.05[b]	11.73
C18:1 *c*9	Oleic acid	10.75±0.00[a]	0.58±0.10[c]	5.39	1.22±0.01[b]	11.35	1.71±0.26[b]	15.90
C18:1 *c*11	cis-vaccenic acid	3.76±0.01[a]	0.29±0.08[b]	7.71	0.41±0.01[b]	10.90	0.33±0.06[b]	10.67
TOTAL (MUFAs)		**16.47**	**1.04**	**6.33**	**1.87**	**11.35**	**2.27**	**13.78**
[MUFA/SFA]		**1.51**	**1.14**		**1.51**		**0.46**	
Polyunsaturated FAs (PUFAs)								
C18:2 *c*9*c*12	Linoleic acid	1.50±0.00[a]	0.12±0.04[c]	8.00	0.19±0.00[c]	12.70	0.91±0.13[b]	40.67
C18:3 *c*6*c*9*c*13	γ-linolenic acid	1.89±0.00[a]	0.16±0.07[b]	8.47	0.24±0.00[b]	12.70	n.d.	
C18:2 *c*9*t*11	Rumenic acid	0.40±0.004[a]	0.07±0.01[b]	17.5	0.07±0.01[b]	17.50	n.d.	
C18:2 *t*10*c*12	CLA isomer	20.66±0.03[a]	2.72±0.02 [b]	13.36	2.92±0.02 [b]	14.13	1.15±0.52 [b]	5.57
C18:3 *c*9*c*12*c*15	α-linolenic acid	3.5±0.00[a]	0.23±0.03[b]	6.57	0.34±0.00[b]	9.71	0.21±0.06[b]	6.00
C18:2 t9t11		1.94±0.00[a]	0.18±0.07[b]	9.27	0.25±0.00[b]	12.88	n.d.	
C20:3 *c*8*c*11*c*14	Dihomo-γ-linolenic acid	12.87±0.00[a]	1.13±0.56[b]	8.78	1.73±0.02[b]	13.44	0.74±0.35[b]	5.75
C20:3 *c*11*c*14*c*17		0.83±0.02[a]	0.96±0.08[b]	115.67	0.85±0.01[b]	102.81	0.18±0.06[b]	21.69
C20:4 n-6	Arachidonic acid	18.17±0.01[a]	2.01±0.11[b]	11.08	2.34±0.08[b]	12.88	0.90±0.4[c]	4.95
C20:5 n-3	EPA	411.95±0.03[a]	50.64±0.47[a]	12.29	55.05±0.51[a]	13.36	12.53±1.43[b]	3.04
C22:5n-3	DPA	55.03±0.06[a]	4.29±1.93[a]	7.79	6.42±0.06[a]	11.66	1.71±0.14[b]	3.11
C22:6n-3	DHA	220.13±0.38[a]	31.06±0.39 [a]	14.11	32.84±0.19 [a]	14.92	6.48±1.08[b]	2.94
TOTAL (PUFAs)		**748.87**	**93.54**	**12.49**	**103.24**	**13.79**	**24.81**	**3.31**
[PUFA/SFA]		**68.89**	**102.79**		**83.26**		**5.04**	

Table 5- FAs composition of the original digested sample (Fish oil and Pomegranate oil) before the Caco-2/HT29-MTX permeability assays and of cells. apical side and basoletaral side collected after the end of permeability assays (6h) for each of the tested samples. Values are expressed as mean±standard deviation of µg of FAs/ µL of sample. The positive and negative control were used to normalize the values. The recovery index (RI%) was calculated for each phase. Values are expressed as mean±standard deviation of µg of FAs/ µL of sample. [a,b,c,d]Different superscript letters within a row for significant differences ($p < 0.05$).

µg of fatty acid/ µL of sample		CLA capsules					CLNA capsules (Xanthigen)					Fish oil					Pomegranate oil				
		Digested sample	Cells	RI (%)	Apical side	RI (%)	Digested sample	Cells	RI (%)	Apical side	RI (%)	Digested sample	Cells	RI (%)	Apical side	RI (%)	Digested sample	Cells	RI (%)	Apical side	RI (%)
C14	Myristic acid	n.d.	n.d.		n.d.		n.d.	n.d.		n.d.		8.81±0.02	2.41±0.22	27.41	4.02±0.05	45.61	n.d.	n.d.		n.d.	
C15	Pentadecylic acid	n.d.	n.d.		n.d.		n.d.	n.d.		n.d.		0.54±0.01	0.40±0.05	73.69	0.34±0.00	62.71	n.d.	n.d.		n.d.	
C16	Palmitic acid	0.42±0.01	4.80±0.23	1134.51	0.20±0.01	47.39	3.71±0.00	1.79±0.01	48.21	1.83±0.23	49.29	17.49±0.07	4.16±0.59	23.76	8.95±0.07	51.19	2.28±0.01	5.41±0.41	237.40	0.60±0.07	26.40
C16:1 *c*9	Palmitoleic acid	n.d.	n.d.		n.d.		n.d.	n.d.		n.d.		10.78±0.02	3.53±0.32	32.76	4.07±0.19	37.76	n.d.	n.d.		n.d.	
C18	Stearic acid	0.41±0.05	0.79±0.26	191.10	1.40±0.10	338.75	1.36±0.03	0.91±0.01	67.05	1.62±0.27	119.86	2.82±0.01	0.37±0.17	13.21	1.83±0.12	64.84	1.68±0.05	0.74±0.02	44.16	0.85±0.07	50.75
C18:1 *t*9	Elaidic acid	n.d.	n.d.		n.d.		n.d.	n.d.		n.d.		n.d.	n.d.		n.d.		0.22±0.08	0.05±0.05	22.48	0.31±0.02	143.02
C18:1 *t*11	Vaccenic acid	n.d.	n.d.		n.d.		n.d.	n.d.		n.d.		n.d.	n.d.		n.d.		0.27±0.09	0.43±0.06	155.93	n.d.	0
C18:1 *t*12		0.83±0.26	1.14±0.16	136.95	1.00±0.05	120.64	n.d.	n.d.		n.d.		n.d.	n.d.		n.d.		0.32±0.11	0.70±0.09	221.12	0.62±0.01	195.42
C18:1 *c*9	Oleic acid	1.81±0.03	1.02±0.26	56.54	0.75±0.05	41.48	17.54±0.09	8.15±0.13	46.43	4.83±0.82	27.53	7.29±0.06	1.76±0.39	24.12	3.03±0.11	41.54	4.22±0.09	5.82±0.34	137.87	0.86±0.09	20.49
C18:1 *c*11	cis-vaccenic acid	0.23±0.03	0.26±0.08	111.50	0.33±0.01	141.97	0.60±0.00	12.10±0.13	20007.09	n.d.	0.00	3.01±0.03	0.55±0.05	18.29	1.38±0.06	45.73	0.35±0.02	0.27±0.07	78.68	n.d.	
C18:2 *c*9*c*12	Linoleic acid	0.42±0.00	0.28±0.05	66.77	n.d.	0.00	2.93±0.03	1.36±0.04	46.50	0.59±0.13	19.96	n.d.	n.d.		n.d.		4.67±0.04	1.40±0.12	30.05	0.74±0.09	15.80
C18:2 *c*9*t*11	Rumenic acid	18.47±0.58	3.93±.29	21.29	2.22±0.23	12.01	n.d.	n.d.		n.d.		n.d.	n.d.		n.d.		0.31±0.08	0.41±0.03	133.74	n.d.	
C18:2 *t*10*c*12	CLA isomer	18.22±0.61	4.87±0.44	26.71	1.56±0.16	8.58	n.d.	n.d.		n.d.		2.31±0.04	0.55±0.05	23.87	0.49±0.06	21.38	n.d.	n.d.		n.d.	
C18:3 *c*9*c*12*c*15	α-linolenic acid	0.61±.000	0.38±0.02	62.38	n.d.		n.d.	n.d.		n.d.		1.18±0.01	0.41±0.03	34.61	0.42±0.05	35.79	0.52±0.02	0.35±0.04	67.69	n.d.	
C18:2 *t*9*t*11	CLA isomer	3.01±0.01	1.56±0.15	51.99	1.03±0.05	34.23	0.63±0.10	0.51±0.04	81.75	n.d.		n.d.	n.d.		n.d.		1.23±0.31	1.24±0.12	100.92	n.d.	

C20:5 n-3	EPA	n.d.	n.d.		n.d.		n.d.	n.d.		n.d.		13.01±0.43	1.43±0.20	11.03	1.64±0.43	12.59	n.d.	n.d.		n.d.	
C18:3 *c*9*t*11*c*13	Punicic acid	n.d.	n.d.		n.d.		6.03±0.37	0.74±0.02	12.23	n.d.		n.d.	n.d.		n.d.		31.88±0.72	3.27±0.09	10.25	n.d.	
c18:3 *c*9*t*11*t*13	α-eleostearic acid	n.d.	n.d.		n.d.		1.50±0.05	0.36±0.02	23.65	n.d.		n.d.	n.d.		n.d.		5.32±0.06	1.13±0.20	21.24		
C18:3 *t*9*t*11*c*13	Catalpic acid	n.d.	n.d.		n.d.		1.61±0.09	n.d.		n.d.		n.d.	n.d.		n.d.		5.19±0.12	1.08±0.04	20.72		
C18:3 *t*9*t*11*t*13	β-eleostearic acid	n.d.	n.d.		n.d.		n.d.	n.d.		n.d.		n.d.	n.d.		n.d.		7.62±0.16	1.26±0.05	16.53	0.39±0.03	5.16
C22:6 n-3	DHA	n.d.	n.d.		n.d.		n.d.	n.d.		n.d.		6.44±0.50	0.54±0.08	8.44	0.55±0.17	8.52	n.d.	n.d.		n.d.	

Table 6 - Transepithelial electrical resistance (TEER) measures ($\Omega \cdot cm^2$) used to assess the cell layer integrity of the Caco-2/HT29 co-culture.

Sample		0H	1H	3H	6H
Positive control		1110	1030	954	881
Negative control		370	169	180	184
CLA capsules	**1**	1012	890	830	774
	2	1090	950	877	864
CLNA capsules (Xanthigen)	**1**	884	789	671	700
	2	859	700	660	710
Omega 3capsules	**1**	1030	930	680	190
	2	1025	953	740	164
Fish oil	**1**	1142	1047	980	990
	2	1159	1070	1056	1055
Pomegranate oil	**1**	1035	745	570	555
	2	1061	630	515	555

Table 7 – Antioxidant acitivity of Pomegranate oil. Fish oil. CLA capsules. CLNA capsules (Xanthigen). Omega 3capsules using DPPH and ABTS radicals methods. The antioxidant activity is presented in mg of Trolox equivalente (TE)/ g of sample and µmol TE/g of sample. [a,b] Different superscript letters within a row for significant differences ($p < 0.05$).

Samples		DPPH		ABTS	
		mg TE/g of sample	**µmol TE/g of sample**	mg TE/g of sample	**µmol TE/g of sample**
Pomegranate oil	**Original**	1.48±0.12	**5.93±0.47[a]**	0.74±0.08	**4.60±0.40[a]**
	Digested	1.02±0.16	**4.09±0.66[b]**	2.89±0.20	**2.21±0.19[b]**
Fish oil	**Original**	1.03±0.14	**4.11±0.55[a]**	1.15±0.10	**2.97±0.31[b]**
	Digested	1.20±0.08	**4.79±0.33[a]**	0.55±0.05	**11.55±0.79[a]**
CLA capsules	**Original**	0.16±0.03	**0.42±0.02[b]**	0.23±0.02	**0.94±0.09[b]**
	Digested	0.14±0.03	**0.57±0.14[a]**	0.97±0.14	**4.13±0.29[a]**
CLNA capsules	**Original**	0.07±0.001	**1.15±0.015[a]**	1.83±0.09	**7.30±0.38[a]**
	Digested	0.05±0.004	**0.83±0.06[b]**	1.31±0.08	**5.24±0.10[b]**
Omega 3capsules	**Original**	1.23±0.04	**4.93±0.17[a]**	1.19±0.04	**4.74±0.14[a]**
	Digested	0.66±0.03	**2.65±0.10[b]**	0.90±0.10	**3.60±0.39[b]**