Food Research International 173 (2023) 113364

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Anti-obesity potential of a yogurt functionalized with a CLNA-rich pomegranate oil

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ARTICLE INFO

Keywords:
Lipid metabolism
Immune response
Conjugated linolenic acids
Dairy products

ABSTRACT

Pomegranate oil is rich in conjugated linolenic acids, compounds which have attracted attention due to their potential applicability in obesity management as they are capable of modulating leptin and adiponectin secretion and regulate fatty acids storage and glucose metabolism. Among the possible bioactive foodstuffs capable of delivering these bioactive compounds yogurts have shown potential. Thus, the purpose of this work was to develop functional yogurts through the addition of pomegranate oil either in its free or encapsulated (used as a protective strategy against oxidation and gastrointestinal tract passage) forms. To that end, the pomegranate oil (free and encapsulated) was incorporated in yogurt and the functional yogurt capacity to modulate hepatic lipid accumulation, adipocyte metabolism (in terms of lipolysis, and adipokines secretion) and immune response was evaluated. The results obtained showed that the pomegranate oil's incorporation led to an improvement in the yogurts' nutritional values, with a reduction in its atherogenic and thrombogenic indexes (more than 78% for atherogenic and 76% for thrombogenic index) and an enhancement of its hypocholesterolemic/hypercholesterolemic ratio (more than 62%) when compared to the control yogurt. Furthermore, data also showed for the first time how these functional yogurts promoted modulation of metabolic processes post GIT as they were capable of reducing by 40% triglycerides accumulation in steatosis-induced Hep G2 cells and by 30 % in differentiated adipocytes. Moreover, samples also showed a capacity to modulate the leptin and adiponectin secretion (56 % of increase in adiponectin) and reduce the IL-6 secretion (*ca* 44%) and TNF-α (*ca* 12%) in LPS-stimulated cells. Thus, the CLNA-rich yogurt here developed showed potential as a viable nutraceutical alternative for obesity management.

1. Introduction

Pomegranate oil (PO), in addition to being rich in other unsaturated fatty acids, tocopherols, and phytosterols, is the major source of the highly valued punicic acid in food systems (Paul & Radhakrishnan, 2020). Its unique chemical composition is translated into important benefits for human health such as modulation of the immune system, obesity risk reduction through regulation of lipid metabolism, and reduction of cardiovascular disease factors risk (de O. Silva et al., 2019; Paul & Radhakrishnan, 2020). These biological properties have led to an increased interest from the food industry for the application of this oil as an antimicrobial agent in packaging, as an ingredient in animal feed to improve the fatty acids profile of meat and dairy products or as a functional ingredient in foodstuffs (Paul & Radhakrishnan, 2020). Currently, the applicability of PO as a functional ingredient is linked to its anti-obesity properties, particularly through with its capacity to reduce weight gain via modulation of leptin and adiponectin secretion (Aruna et al., 2016). Additionally, this bioactive oil has also been shown to be capable of regulating the fatty acids storage in adipose tissue and glucose metabolism (Aruna et al., 2016).

Despite these benefits, the incorporation of PO into functional foods faces several challenges due to its susceptibility to oxidation which leads to the formation of off-flavors and a reduction of its nutritional value. Moreover, these compounds are also degraded in digestion during the passage through the gastrointestinal tract which decreases their bioavailability (Machado, Rodriguez-Alcalá, et al., 2022). Encapsulation is one of the main strategies employed to solve this problem as it can improve the handling, stability, and bioavailability of lipids (Akhavan

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https://doi.org/10.1016/j.foodres.2023.113364
Received 14 January 2023; Received in revised form 3 August 2023; Accepted 5 August 2023
Available online 7 August 2023

et al., 2018). Over the last years, PO has been successfully encapsulated in microparticles, nanoparticles, nanostructured carries, and nanoemulsions (Comunian et al., 2020; Costa et al., 2020; Gaikwad et al., 2021; Sahafi et al., 2021; Soleimanian et al., 2018; Yekdane & Goli, 2019) and its potential for the development of food products has been hinted at. For example, PO gold nanoparticles were incorporated into yogurts and proved to enhance their antioxidant capacity (Esther Lydia et al., 2020). However, most of these works were limited in scope and lacked biological assays. Taking the available information into consideration, this work aimed to develop functional yogurts with PO (added either in free form or loaded into chitosan microparticles), to show that the developed functionalized foodstuff possesses more interesting nutritional characteristics than non-supplemented yogurt and, for the first time, evaluate the functional yogurt post-digestion (upon passage through simulated GIT conditions) capacity to modulate obesity-related metabolism and immune response. To that end *in vitro* cellular models were used to evaluate the capacity to modulate distinct processes associated with obesity: lipid accumulation, lipolysis, adipokines secretion, and inflammation.

2. Materials and methods

2.1. Materials and chemicals

Pomegranate oil was supplied by All Organic Treasure (Germany). Chitosan 95/100 was obtained from Heppe Medical Chitosan (Germany), acetic acid from Merk (Missouri, USA), Sodium Tripolyphosphate (TPP), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sulfuric acid (97%), dimethyl sulfoxide (DMSO), glutaraldehyde, hexamethyldisilazane were obtained from Sigma (Missouri, USA). Dulbecco's Modified Eagle's Medium (DMEM) and, non-essential amino acids (NEAA) obtained from Gibco, (Thermo Scientific, USA) Fetal Bovine Serum from (FBS; Biowest, France), Penicillin-Streptomycin-Fungizone from Lonza, (Belgium), and Calf Bovine Serum (CBS), Iron Fortified from ATCC (USA), lipopolysaccharides from Escherichia coli O111:B4 (LPS) from Invitrogen (USA). Adipolysis Assay Kit from Abcam (Abcam ab133115), Mouse Leptin ELISA kit (Abcam, ab199082), Mouse Adiponectin ELISA kit (Abcam, ab18785, Hepatic Lipid Accumulation Kit (Abcam ab133131). Legend Max Mouse ELISA Kit IL-6 and the Legend Max Mouse ELISA Kit TNF-α were obtained from BioLegend (San Diego, USA) and BCA Pierce Assay Kit from Thermo-Scientific (Massachusetts, USA).

2.2. Chitosan microparticles production

PO-loaded chitosan microparticles were produced using low molecular weight (100 kDa) chitosan with 95% degree of deacetylation (2% w/v) dissolved in acetic acid (1% v/v) and the pH value was adjusted to 5.0 using NaOH 2 M. Firstly, 2 mL of chitosan solution was mixed in 3 mL of ultra-pure water and stirred for 5 min. Then, 1 mL of PO and 2 mL of Tween 80 were added with continuous stirring. Finally, 1 mL of TPP (1% v/v) was added, and the mixture was sonicated (Sonics Vibra-Cell™ VCX 130) at 60% amplitude for 5 min (pulses 30/30 s). Afterward, the mixture was cooled and stored at room temperature until use.

2.3. Microparticles characterization

2.3.1. Size and zeta potential determination

The microparticle suspensions were analyzed concerning their physical properties by dynamic light scattering (DLS) using a Malvern Instruments NanoZSP (UK). The measured parameters were particle size (PS), polydispersity index (PdI), and zeta potential (ZP). All assays were performed using a disposable folded capillary cell (Malvern, UK), with a 90° laser angle and at room temperature (25 °C). All assays were performed in quadruplicate.

2.3.2. Determination of entrapment efficiency

PO-loaded chitosan microparticles were centrifuged for 10 min at 3075*g*. Then, 100 μL of supernatant and pellet fractions were collected and their fatty acids profile was determined as subsequently described. The fatty acid profile was evaluated by gas chromatography after transesterification, according to the method described by Machado et al. (2022b). Fatty acid methyl esters were analyzed in a gas chromatograph Agilent 8860 (Agilent, USA), equipped with a flame ionization detector and a BPX70 capillary column (60 m × 0.25 mm × 0.25 μm; SGE Europe Ltd, France). Analysis conditions were as follows: injector (split 25:1; injection volume 1 μL), injector, and detector temperatures were 250 ℃ and 275 ℃, respectively; hydrogen was used as a carrier gas at a flow rate of 1 mL/min. The oven temperature was initially at 60 ℃ and then increased to a final temperature of 225 ℃. Supelco 37 was used for the identification of fatty acids. Each sample was analyzed in triplicate.

2.3.3. FTIR-ATR analysis

The spectra of chitosan, chitosan particles, and PO-loaded chitosan particles were obtained with a FTIR (Spectrum 100 Perkin Elmer, USA), with a horizontal attenuated total reflectance (ATR) accessory. All spectra were acquired from 4000−400 cm^{-1} at a resolution of 4 cm^{-1} and an average of 32 scans was reported. These spectra were subtracted from the background spectrum. Three replicates were collected for each sample. All spectra were normalized through dividing by standard deviation.

2.4. Yogurt production

To produce the pomegranate oil fortified yogurts, commercially available powdered skimmed milk was dissolved in water (13% (w/v); Nestlé, Portugal). PO and pomegranate microparticles suspension were added to the milk (to achieve 1.5% w/w of oil) and then pasteurized (85 °C, 5 min) in a water bath. Yogurt prepared with plain reconstituted skim milk was used as a control. Before fermentation, samples were cooled to 45 °C and inoculated at 3% (w/w) with natural yogurt (Mimosa Natural, Lactogal, Portugal). The resulting mixtures were incubated at 43 °C, in plastic yogurt containers, until a final pH of 4.6 was reached. The finalized yogurts were cooled down and subsequently stored at 4 °C. Fifteen yogurts were prepared per sample: plain yogurt control (CT), pomegranate oil fortified yogurt (POY), and pomegranate oil microparticles fortified yogurt (PMY).

2.5. Microbiological quality control

Yogurt samples were analyzed according to international guideline ISO 6887-5 for milk and milk products (ISO 6887-5, 2010). Briefly, 10 g of sample were diluted in 90 mL of buffered peptone water and homogenized in a stomacher (Seward, UK) for 5 min at 240 rpm. The presence of *Enterobacteriaceae* was evaluated according to ISO 21528-2, and molds and yeasts according to ISO 21527 (ISO (2152)7-1, 2008, ISO (2152)8-2, 2004). The viable counts of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* were performed according to ISO 7889 (ISO 7889, 2003). The viable counts obtained were expressed as log CFU/mL.

2.6. Fatty acid profile

The yogurts' pH value was measured using a pH meter (Micro pH 2002, Crison, Spain). The protein content was evaluated by the Bradford method (Kielkopf et al., 2020). Sugar content was performed by the DNS method adapted for microplate reader (Gonçalves et al., 2010). Syneresis was measured according to Lopes *et al.* (Lopes et al., 2019). Briefly, 10 g of yogurt were centrifuged at 7870*g* for 10 min at 4 ℃. The supernatant was then removed and weighed. The fatty acids profile was evaluated as described above. Additionally, the nutritional quality indexes atherogenic index (AI), thrombogenic acid (TI), hypo/

hypercholesterolemic ratio (HH) and health promoting index (HPI) were calculated according to the Eqs. (1) to (4).

$$AI = \frac{[C12:0 + 4 \times (C14:0) + C16:0]}{(\sum MUFA + \sum PUFA\,n6 + \sum PUFA\,n3)} \quad (1)$$

$$TI = \frac{(C14:0 + C16:0 + C18:0)}{\left[0.5 \times \sum MUFA + 0.5 \times \sum PUFA\,n6 + 3 \times \sum PUFA\,n3 + \left(\frac{\sum PUFA\,n3}{\sum PUFA\,n6}\right)\right]} \quad (2)$$

$$HH = \frac{(C18:1n9 + C18:2n6 + C18:3n3 + C20:4n6 + C20:5n3)}{(C14:0 + C16:0)} \quad (3)$$

$$HPI = \frac{\sum UFA}{[C12:0 + (4 \times C14) + C16]} \quad (4)$$

2.7. Rheological analysis

The physical properties of yogurt samples were assessed through rheological analysis performed in a rotational rheometer (Bohlin Instruments, United Kingdom), coupled with a Peltier unit for temperature control of the measurement plate in which samples were placed. A cone-and-plate geometry probe, with 40 mm diameter and 4° angle, was used and samples were analyzed at 12 °C. For homogenization, samples were gently stirred and, subsequently, placed on the measurement plate. Oscillatory assays were performed, namely, frequency sweeps from 0.1 to 10 Hz, which were conducted using a strain (0.005) within the LVER of the yogurts, which was previously determined (data not shown) through amplitude sweeps (strain varied from 0.001 to 1) at a constant frequency (1 Hz). The parameters determined to compare the different yogurt samples were elastic modulus (G'), viscous modulus (G''), and complex viscosity (η*).

2.8. In vitro simulation of the gastrointestinal tract (GIT)

The *in vitro* simulation of GIT was performed according to the INFOGEST method (Brodkorb et al., 2019), in triplicate, using 5 g of each yogurt type. To screen the impact of GIT on fatty acids profile, yogurt samples were collected at different stages (after oral, gastric, and intestinal steps). The fatty acids profile was evaluated as described above using 500 μL of the sample. After *in vitro* digestion, samples were centrifuged at 7000*g* for 30 min, and the supernatant was collected for cell-based assays.

2.9. Biological properties

2.9.1. Cell lines

Four different cell lines were used in the current work: Human Caucasian colon carcinoma epithelial cells (Caco-2, ECACC 86010202) were acquired from the European Collection of Authenticated Cell Cultures. The Human hepatocellular carcinoma (Hep G2, ATCC HB-8065), mouse macrophages Raw 264.7 (ATCC TIB-71) and mouse pre-adipocytes 3T3-L1 (ATCC CL-173) were acquired from American Type Culture Collection. Human cell lines and mouse macrophages were cultured using DMEM supplemented with 10% (v/v) FBS and 1% (v/v) of Penicillin-Streptomycin-Fungizone. Caco-2's media was also supplemented with 1% (v/v) NEAA. Pre-adipocytes were cultured in DMEM with 10% (v/v) of iron-fortified CBS and 1% (v/v) of Penicillin-Streptomycin-Fungizone. All cell lines were incubated at 37 °C under a humidified atmosphere comprised of 5% CO_2 and 95% air.

2.9.2. Cytotoxicity

Cytotoxicity assays were carried out using MTT viable die as previously described by Machado et al. (2022b). Briefly, cells were seeded ($1.0x10^4$ cells/well) into 96-well tissue culture plates (Thermo Scientific, Denmark) and incubated. After 24 h, the media was carefully replaced with 100 μL of digested samples (0.08 mg/mL of fatty acids), plain media (positive control), and media containing 40% (v/v) DMSO and incubated again for 24 h. After exposure, 100 μL of MTT solution (0.5 mg/mL) were added to each well, and the plates were incubated once more. After 2 h, the wells' content was carefully aspirated, 100 μL of DMSO were added and the plates were shaken (protected from light) to ensure the stain dissolution. After 10 min, the absorbance was read (570 nm) using a microplate reader (Synergy H1, Biotek Instruments, USA).

2.9.3. Hepatic lipid accumulation

Hepatic Lipid Accumulation was performed according to the kit manufacturer's instructions. Hep G2 (1.0×10^3 cells/well) were seeded in 96-well tissue culture plates and incubated to allow cells to adhere. After 24 h, the media was carefully removed and replaced with digested samples (0.08 mg/mL of fatty acids), plain media (negative control), or 25 μM chloroquine (positive control). After 72 h exposure, media was removed, lipid droplets were stained with Oil Red O, and then dissolved using the dye extraction buffer included in the kit. After gentle shaking, the absorbance at 490 nm was read using a microplate reader (Synergy H1, Biotek Instruments, USA). To evaluate the possible effects of fatty acids on hepatic steatosis, another assay was conducted in which digested samples were diluted in media with 25 μM of chloroquine.

2.9.4. Adipolysis

Adipolysis assays were carried out according to the method described by Machado et al. (2022). Briefly, 3T3-L1 cells were seeded (3.0×10^3 cells/well) in 96-well tissue culture plates and grown until confluent. Two days after confluence, differentiation was induced using differentiation media, and subsequent media renewals were conducted every 3 d using insulin media. When 80% differentiation was reached, cells were exposed to digested samples (0.08 mg/mL of fatty acids) or exposed to 10 μM isoproterenol (positive control). After 24 h incubation, the glycerol concentration in the media was measured by adding a glycerol-free reagent to 25 μL of cell supernatants. This mixture was incubated for 15 min at room temperature and the absorbance at 520 nm was read using a microplate reader (Synergy H1, Biotek Instruments, USA).

2.9.5. SEM of adipocytes

Pre-adipocytes (3T3-L1 cells) were seeded at 2.0×10^4 cells/mL in a 24 wells microplate with coverslips (Corning, USA) and differentiated and treated as described above. At the end of the assay fixation of the adhered cells was performed after media removal, using 2.5% (v/v) glutaraldehyde solution for at least 1 h. Next, glutaraldehyde solution was removed, cells were washed by immersing the coverslips in deionized water (for 10 min), and were then dehydrated by a gradient ethanol series (30, 50, 70, 80, 90, and 100% (v/v)) through immersion in each solution for 10 min. After the 100% ethanol solution, a couple of drops of hexamethyldisilazane were placed over the cells and immediately evaporated under a gentle stream of nitrogen. Coverslips were placed on top of observation pins (covered with a double-sided adhesive carbon tape (NEM tape; Nisshin, Japan)), and sputter coated with gold/palladium. The observation was performed in a Scanning Electron Microscope, Phenom XL G2 by Thermo Fischer Scientific – FEI (Netherlands) with a secondary electron detector (SED).

2.9.6. Adipokines secretion

Pre-adipocytes (3T3-L1 cells) were seeded at 2.0×10^4 cells/mL and differentiated as described for adipolysis (section 2.9.4). When cells reached 80% of differentiation the medium was replaced with a medium with diested samples (0.08 mg/mL of fatty acids) and incubated for 24 h. For basal activity, plain media was used as a control. At the end of the assay, supernatants were collected and centrifuged to remove debris. Adiponectin and leptin detection was performed by enzyme-linked

immunosorbent assay (ELISA) using Abcam's Mouse Leptin ELISA kit and Mouse Adiponectin ELISA kit according to the manufacturer's instructions. The protein content of samples was determined using the BCA Pierce Assay Kit. Leptin values were obtained in pg/μg of protein in the sample and adiponectin in ng/ng of protein.

2.9.7. Immunomodulation in Raw 264.7

Raw 264.7 cells were seeded (2×10^5 cells/mL) into 24-well tissue culture plates. After 24 h the culture media was carefully replaced with digested samples (0.08 mg/mL of fatty acids), plain culture media (basal expression control), or 1 μg/mL of lipopolysaccharides from *Escherichia coli* O111:B4 (LPS, Invitrogen) (inflammation control), and the plates were incubated. After 24 h of exposure, the supernatants were collected, centrifuged to remove cellular debris, aliquoted, and stored at −80 °C until analysis. Cytokine quantification was carried out using commercially available kits as per the manufacturer's instructions. Interleukin 6 (IL-6) was determined using Biolegend's LEGEND MAX Mouse IL-6 ELISA Kit and Tumor Necrosis Factor α (TNF-α) was determined using BioLegend's LEGEND MAX Mouse TNF-α ELISA Kit. Total protein (in the supernatant) was determined using BCA Pierce Assay Kit and the cytokine values expressed in pg cytokine/μg of protein.

2.10. Statistical analysis

Minitab 17 was used to carry out statistical analysis. All data was reported as mean ± standard deviation. Shapiro-Wilk's test was used to confirm the normality of data distribution. The results obtained were tested at a 0.05 significance level using a one-way ANOVA, followed by a multiple comparisons test (Tukey) to identify statistically significant differences between samples.

3. Results and discussion

3.1. Pomegranate oil-loaded chitosan microparticles characterization

The produced particles showed an average size of 188.33 ± 1.94 nm with a polydispersity index of 0.163 ± 0.01 fitting within what has been described in legislation for microparticles size and characteristics (Regulation (EC) No 258/97 of the European Parliament and of the Council and Commission Regulation (EC) No 1852/2001 (Text with EEA Relevance), 1169). The ZP was of −1.41 ± 0.15 mV, suggesting that the microparticles produced were negatively charged. The PO entrapment percentage was of 83.45 ± 0.10%. The encapsulation efficiency obtained was similar to those obtained by other authors for PO-loaded-zein particles (Tavakolipour et al., 2015). In this work, the authors produced particles with 394–541 nm with 87% of encapsulation efficiency (Tavakolipour et al., 2015).

The results obtained by FTIR characterization can be seen in Fig. 1. The void microparticles showed the characteristic peaks of chitosan and TPP. Chitosan microparticle's characteristic peaks were observed at 3345 cm^{-1} which corresponds to –OH stretching, and 1624 cm^{-1} which corresponds to amide I stretching (Chang et al., 2021; Costa et al., 2017). In the region between 1000 and 1200 cm^{-1} characteristic chitosan and TPP absorption peaks, namely, the peak around 1150 cm^{-1} which indicates the P=O stretching, and the peak at 1078 cm^{-1} which corresponds to C–O–C stretching. The absorption peaks at 638 cm^{-1} indicate C–H deformation (Chang et al., 2021; Costa et al., 2021). The PO-loaded particles showed all the characteristic peaks of chitosan particles and some new absorption peaks reflected the presence of the oil, namely, the peak at 1726 cm^{-1} indicates the presence of C=O stretching vibration of the ester group of PO (Amit et al., 2020). The signals between 2848 and 2928 cm^{-1} represented the stretching vibration of C–H and HC=CH bonds and correspond to the presence of unsaturated and saturated fatty acyl chains (Zhu et al., 2021).

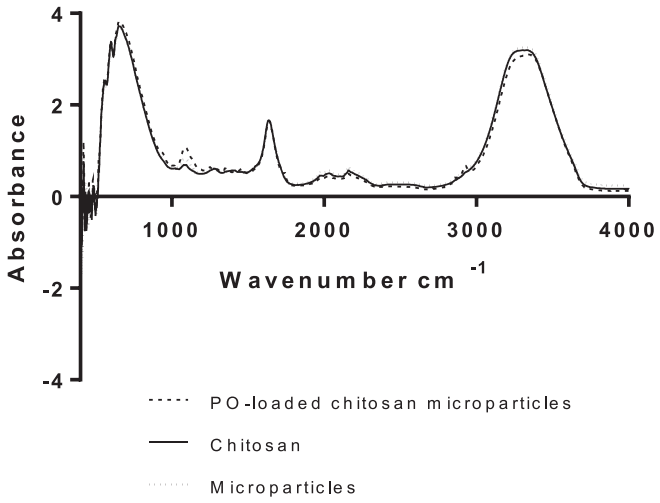

Fig. 1. FTIR spectra of PO-loaded chitosan microparticles, chitosan and microparticles.

3.2. Microbiological quality control

Regarding the microbiological quality of the produced samples, the yeasts, molds and *Enterobacteriaceae* viable counts were not detected in any of the yogurts throughout the 28 storage days. This indicates the yogurts' safety, as these microbial groups are considered important indicators of food safety (Bakry et al., 2019). Regarding the lactic acid bacteria, the Codex Alimentarius specification states that their viable counts should be above 1.0×10^7 CFU/g and should remain viable during the yogurt shelf-life, a value which was achieved in all yogurts tested. In fact, the total viable counts varied between 5.9×10^7 and 1.1×10^8 CFU/g for POY samples, between 6.6×10^7 to 4.8×10^8 CFU/g in PMY samples and between 4.3×10^7 to 1.14×10^8 CFU/g for control sample.

3.3. Fatty acid profile

The saturated profile of traditional dairy products limits their consumption, for this reason, the addition of pomegranate oil (high unsaturated oil, Table 1) can be an interesting strategy to balance the nutritional profile of yogurts. Concerning the fatty acids profile (Table 2), the first major takeaway is that oil encapsulation affected the yogurt's overall characteristics with particular emphasis in the threefold lower CLNAs content present (Table 2). Th difference between POY and PMY may be related to the impact of thermal treatment in the chitosan microparticles. The addition of PO, independently of its added form (free or encapsulated) increased (>94%) the amount of PUFAs available, which has been linked to an improvement in the yogurt's nutritional value (Gumus & Gharibzahedi, 2021; Van Nieuwenhove et al., 2019). The fatty acids' nutritional quality can be assessed by the calculation of atherogenic index AI, TI, and HH. The AI is a ratio of specific SFAs (C12, C14, and C16) which are considered to be pro-atherogenic, and the sum of unsaturated fatty acids (anti-atherogenic fatty acids) (de Alba et al., 2019). The consumption of foods with a lower AI can reduce the levels of total cholesterol (Chen & Liu, 2020). The AI values of PMY were 8.8 times lower than those of the control (3.52 ± 0.07) and 1.8 times lower than the of the POY yogurts. These significant differences ($p < 0.05$) were linked to the fatty acids profile of the samples, in particular with the high amount of mono- and poly-unsaturated fatty acids (MUFAs and PUFAs) present in the functional yogurts. Moreover, the differences between PMY and POY yogurts can be related to the higher quantities of oleic acid (C18:1 *c*9) present in the PMY sample. The same trend was verified for the TI index, which assesses the tendency for blood clots formation in blood vessels through

Table 1
Fatty acid profile of commercial pomegranate oil.

Fatty Acid	
C16	38.80 ± 12.32
C18	35.19 ± 10.80
C18:1 *t*9	2.53 ± 0.62
C18:1 *t*10	4.30 ± 0.64
C18:1 *t*11	5.14 ± 1.39
C18:1 *t*12	4.00 ± 0.93
C18:1	96.08 ± 3.75
C18:1 *c*11	6.72 ± 2.10
C18:1 *c*12	1.36 ± 0.32
C18:1 *c*13	1.20 ± 0.33
C18:2 *t*9*t*12	1.37 ± 0.41
C18:1 *c*4/*t*15	5.04 ± 1.40
C18:2	90.76 ± 3.27
C18:3 *c*6*c*9*c*13	1.24 ± 0.42
C18:3 *c*9*c*12*c*15	10.38 ± 0.60
C20	5.83 ± 1.61
C18:2 *c*9*t*11	6.59 ± 1.84
C20:1	12.00 ± 3.32
C20:4	2.11 ± 0.67
C18:3 *c*9*t*11*c*13	858.16 ± 11.03
C18:3 c9*t*11*t*13	10.67 ± 0.66
C18:3 *t*9*t*11*c*13	36.29 ± 5.27
C18:3 *t*9*t*11*t*13	7.59 ± 2.23
C24	1.51 ± 0.15
∑ Fatty acids	1237.36 ± 42.68

Results are expressed in mg/g and are the means of three determinations ± standard deviation. C16 palmitic acid; C18 stearic acid; C18:1 *t*9 elaidic acid, C18:1 *t*10 *trans*-10-octadecenoic aid; C18:1 *t*11 *trans*-vaccenic acid; C18:1 *t*12 *trans*-12-elaidic acid; C18:1 *c*9 oleic acid; C18:1 *c*11 *cis*-vaccenic acid; C18:1 *c*12, C18:1 *c*13 *cis* isomers of oleic acid; C18:2 *c*9*c*12 linoleic acid; C18:3 *c*6*c*9*c*12 γ-Linolenic acid; C18:3 *c*9*c*12*c*15 linolenic acid; C20 Arachidic acid; C18:2 *c*9*t*11 rumenic acid; C20:1 *cis*-gondoic acid; C20:4 Arachidonic acid; C18:3 *c*9*t*11*c*13 punicic acid; C18:3 *c*9*t*11*t*13 α-eleostearic acid; C18:3 *t*9*t*11*c*13 catalpic acid; C18:3 *t*9*t*11*t*13 β-eleostearic acid.; C24 Lignoceric acid.

the association of thrombogenic SFA with MUFAs and antithrombogenic omega-3 and omega-6 PUFAs (Chen & Liu, 2020; de Alba et al., 2019). The PMY yogurts showed the lowest TI value due to the lower amount in thrombogenic fatty acids (C14, C16, C18). The values reported for the functional yogurts were lower than those previously reported for omega-3 and olive oil fortified yogurts due to the high amount of PUFAs present in the developed yogurts (Ahmad et al., 2020; Ribeiro et al., 2021).

Functional pomegranate oil yogurts showed a higher HH ratio than the control. HH ratio describes the relationship between hypo and hypercholesterolemic fatty acids. In the case of POY, the HH ratio is 2.3 times higher than of the control, and 1.4 times higher than the one obtained for the PMY samples, a result obtained due to the higher amount of PUFAs (hypocholesterolemic fatty acids) found. Compared to the available literature, the functional yogurts HH values were 6 or 4 times higher (for POY and PMY, respectively) than the values previously reported for cow milk yogurt (Chen & Liu, 2020). In relation to HPI, pomegranate oil-fortified yogurts showed significant ($p < 0.05$) higher values than the control (12 times higher for POY and 8 times higher for PMY). Overall, PMY seemed to be the most promising formulation due to the lower values of AI and TI. The results for the nutritional quality indexes showed the potential of the development of a functional yogurts-based approach for cholesterol management and cardiovascular disease prevention.

3.4. Rheological properties

The results from the rheological analysis are presented in Fig. 2. The elastic modulus (G') prevailed over the corresponding viscous modulus (G'') which means that the yogurts showed a solid-like behavior. A similar result was previously reported for yogurts with flaxseed and walnut oils (Baba et al., 2018; Bakry et al., 2019). Moreover, a slight increase, for all yogurts, in both moduli was registered with a frequency increase. In particular, the elastic and viscous moduli of yogurts with PO were higher than those of the control, with no differences observed between the POY and PMY yogurts. Regarding viscosity (specifically, complex viscosity - η*), the results obtained showed, in all yogurts, a decrease with increasing frequency. Like before, the samples presented higher values than the control. In comparison with the control, incorporation of PO impacted rheological parameters (G', G'' and η*), by increasing them significantly, which can indicate a yogurt with lower fluidity.

Table 2
Fatty acids profile of control and pomegranate oil- fortified yogurts and their nutritional quality indexes.

	CT	POY	PMY
C10	0.02 ± 0.01	n.d.	n.d
C12	0.03 ± 0.02	n.d	n.d
C14	0.11 ± 0.07^{a}	0.06 ± 0.00^{a}	0.04 ± 0.01^{a}
C16	0.36 ± 0.22^{a}	0.24 ± 0.02^{a}	0.18 ± 0.03^{a}
C18	0.12 ± 0.07^{a}	0.12 ± 0.02^{a}	0.07 ± 0.01^{a}
C18:1 *c*9	0.24 ± 0.14^{a}	0.24 ± 0.05^{a}	0.46 ± 0.09^{a}
C18:1 *c*11	n.d	0.02 ± 0.00^{a}	0.01 ± 0.00^{b}
C18:2 *c*9*c*12	0.03 ± 0.01^{a}	0.15 ± 0.05^{b}	0.06 ± 0.01^{a}
C20:1	n.d	0.01 ± 0.00^{a}	0.02 ± 0.00^{a}
C18:3 *c*9*c*12*c*15	n.d	0.02 ± 0.01^{a}	0.01 ± 0.00^{a}
C18:3 *c*9*t*11*c*13	n.d	1.34 ± 0.60^{a}	0.36 ± 0.02^{b}
C18:3 *c*9*t*11*t*13	n.d	0.12 ± 0.06^{a}	0.05 ± 0.01^{a}
C18:3 *t*9*t*11*c*13	n.d	0.04 ± 0.02^{a}	0.04 ± 0.01^{a}
C18: 3 *t*9*t*11*t*13	n.d	0.09 ± 0.06^{a}	0.02 ± 0.01^{a}
∑ Fatty acids	0.91 ± 0.53^{b}	2.44 ± 0.85^{a}	1.32 ± 0.19^{ab}
∑ CLNAs	–	1.59 ± 0.70^{a}	0.47 ± 0.05^{a}
Nutritional Quality Indexes			
AI	3.52 ± 0.07^{a}	0.72 ± 0.15^{b}	0.40 ± 0.03^{c}
TI	4.37 ± 0.12^{a}	1.04 ± 0.11^{b}	0.56 ± 0.07^{c}
HPI	0.33 ± 0.01^{b}	4.25 ± 1.59^{a}	2.94 ± 0.20^{a}
HH	2.82 ± 0.13^{b}	6.59 ± 2.28^{a}	4.59 ± 0.26^{ab}

Results are expressed in mg/g and are the means of three determinations ± standard deviation. Values with different letters in the same line are significantly different, as determined by the one-way ANOVA test ($p < 0.05$). CT control yogurt; POY yogurt with pomegranate oil-free form; PMY yogurt with pomegranate loaded-chitosan microparticles.
C10 capric acid; C12 lauric acid; C14 myristic acid; C16 palmitic acid; C18 stearic acid; C18:1 *c*9 oleic acid; C18:1 *c*11 *cis*-vaccenic acid; C18: 2 *c*9*c*12 linoleic acid; C20:1 *cis*-gondoic acid; C18:3 *c*9*c*12*c*15 linolenic acid; C18:3 *c*9*t*11*c*13 punicic acid; C18:3 *c*9*t*11*t*13 α-eleostearic acid; C18:3 *t*9*t*11*c*13 catalpic acid; C18:3 *t*9*t*11*t*13 β- eleostearic acid. AI atherogenic index; TI thrombogenic index; HPI health promoting index; HH hyper/hypocholesterolemic ratio.

3.5. Impact of GIT on fatty acids profile

The type of dietary lipids present in a matrix can affect how they are digested and subsequently absorbed. Several intrinsic and extrinsic factors influence lipolysis rate, with fatty acids chain length, unsaturation degree, and positional distribution being examples of the former and bile salts concentration and calcium ions and oxygen presence being examples of the latter (Tullberg et al., 2019; Ye et al., 2019). PO lipolysis is mainly performed by pancreatic lipase, due to its high long-chain fatty acids content, with monoacylglycerols and free fatty acids being released (Machado et al., 2022a). These compounds will then be absorbed into enterocytes, through the formation of micelles with phospholipids, free and esterified cholesterol, and bile acids, with triglycerides being reconstructed inside the cell and originating chylomicrons in conjunction with cholesterol and proteins. These chylomicrons will then be released from the basal side of enterocytes and move to the lymph system bypassing the hepatic first-pass metabolism (Kataoka et al., 2020; Mansbach & Siddiqi, 2009).

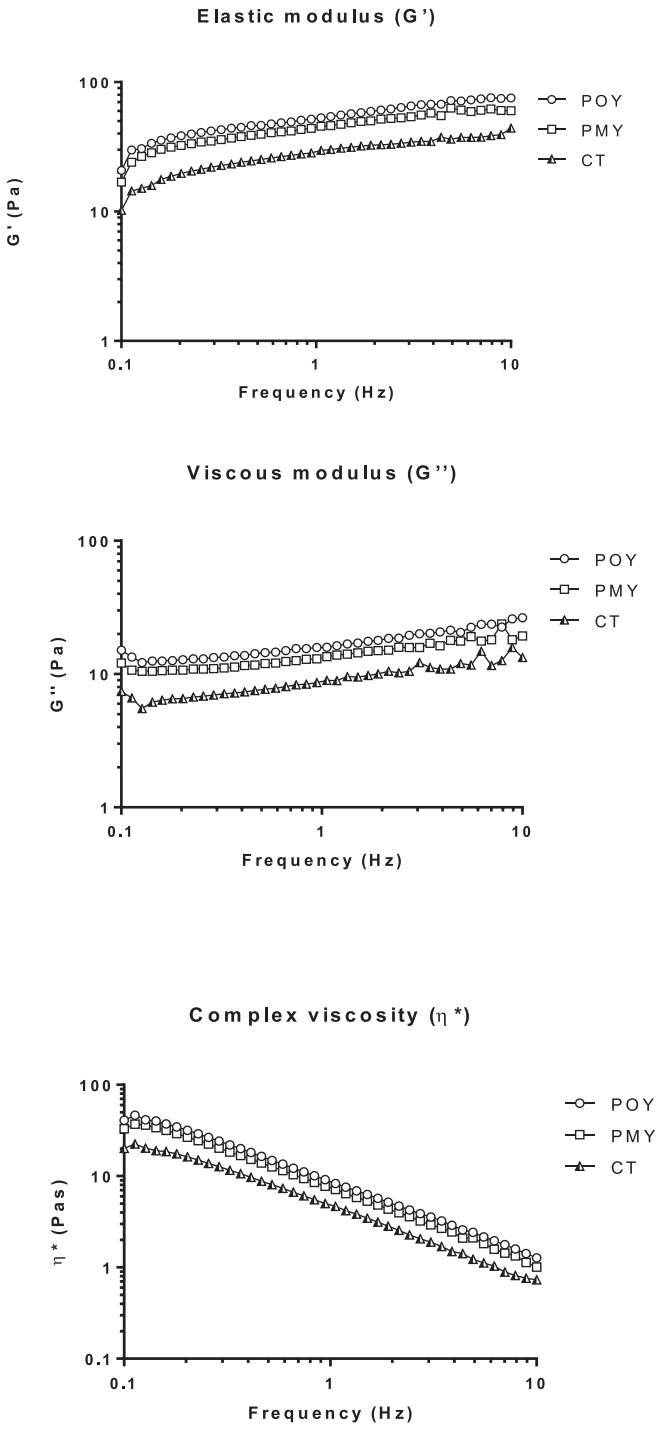

Fig. 2. Rheological parameters: elastic modulus (G'); viscous modulus (G'') and complex viscosity (η*) of yogurts control and fortified yogurts. CT control yogurt; POY yogurt with pomegranate oil-free form; PMY yogurt with pomegranate loaded-chitosan microparticles.

The fatty acids profile of functional yogurts was significantly ($p < 0.05$) altered during the passage through the simulated GIT conditions (Fig. 3), with particular emphasis to the alterations suffered by the CLNAs fraction, which is responsible for PO bioactivity. In the POY samples, the overall CLNAs content was reduced by 57% with the main differences relatively to the undigested sample being detected for punicic (C18:3 *c*9*t*11*c*13) and β-eleostearic (C18:3 *t*9*t*11*t*13) acids, with reductions of 68% and 73%, respectively, being observed. For PMY samples, the CLNA fraction degradation reached 54%, with catalpic acid (C18:3 *t*9*t*11*c*13; 75% reduction) being the more affected CLNA, followed by α-eleostearic (C18:3 *c*9*t*11*t*13; 69% reduction), β-eleostearic (C18:3 *t*9*t*11*t*13; 69% reduction) and punicic acid (C18:3 *c*9*t*11*c*13; 50% reduction). Overall, the total amount of fatty acids was reduced by 28% in POY samples and by 52% in PMY samples upon *in vitro* digestion. Despite the significant reductions in total percentages observed for the encapsulated PMY yogurts, encapsulation of PO offered a protective effect against CLNAs loss and a higher stability during *in vitro* digestion. However, some modifications of the encapsulation process must be made to increase CLNAs' retention. Nonetheless, this study reports for the first time the impact of GIT on the lipid profile of yogurts functionalized with pomegranate oil-loaded chitosan microparticles.

3.6. Biological potential

3.6.1. Cytotoxicity

The cytotoxicity of the digested yogurt samples was evaluated against four different cell types, which were then used for the subsequent biological properties' evaluation. As can be seen in Fig. 4, the functional yogurts and control did not exert any inhibitory activity upon the cellular metabolism of all tested cell lines at the tested concentrations, as all values were below the 30% threshold (dotted line) as defined by the international guideline ISO 10993-5 (ISO 10993-5 Biological Evaluation of Medical Devices — Part 5: Tests for in Vitro Cytotoxicity, 2009). Moreover, when considering the impact of yogurt samples upon Caco-2 metabolism an apparent metabolic stimulation has been found for POY (metabolism inhibition negative values), which represents a metabolic activity higher than that of the growth control.

3.6.2. Effect on hepatic lipid accumulation

NAFLD is characterized by steatosis and may be the first step toward the development of more serious hepatic pathologies, such as hepatocarcinoma (Zeng et al., 2020). Two of the main risk factors for NAFLD development are excess weight and obesity, due to the excessive intake of fat and carbohydrates that may affect lipid metabolism (Scorletti & Byrne, 2018). For this reason, modifications of dietary fatty acids can be a relevant strategy to prevent the development of NAFLD. Hep G2 cells treated with digested yogurt samples showed high percentages of lipids accumulation with no significant differences ($p > 0.05$) observed between the functional yogurts and the control (Fig. 5). On the other hand, in the steatosis-induced cells, the presence of all yogurt samples reduced lipid accumulation. Significant differences ($p < 0.05$) were observed between all yogurts, with POY showing the lowest lipid accumulation percentage ($59.59 \pm 11.49\%$). The differences found between functional yogurts can be related to the different amounts of total CLNAs and omega-3 fatty acids present in each one, as the POY digested sample had 40% more CLNAs and omega-3 fatty acids than the PMY samples, and these compounds were probably responsible for the positive effects on hepatic lipid accumulation (Raffaele et al., 2020; Yuan et al., 2021). The reductions observed are in agreement with previous works. First, an *in vitro* study showed that PO could reduce by 30% triglycerides accumulation in Hep G2 cells (Machado, Costa, et al., 2022). Secondly, the same positive effect has been reported by an animal study, which demonstrated that punicic acid could reduce lipid accumulation in the liver and reduced lipid droplet size (Yuan et al., 2021). The action mechanism of these bioactive compounds is not yet completely understood, as according to the available literature the possible mechanism can be related to the omega-3 fatty acid's ability to increase the β-oxidation of hepatic fatty acids through the activation of PPAR-α. Furthermore, these compounds are also responsible for the inhibition of SREBP-1c, an important regulator of fatty acids synthesis (Ipsen et al., 2018; Masterton et al., 2010; Scorletti & Byrne, 2013). Another possible mechanism could be related to the inhibition of glycolysis by suppressing the ChREBP, and consequently inhibiting the L-pyruvate kinase (Dentin et al., 2005; Masterton et al., 2010).

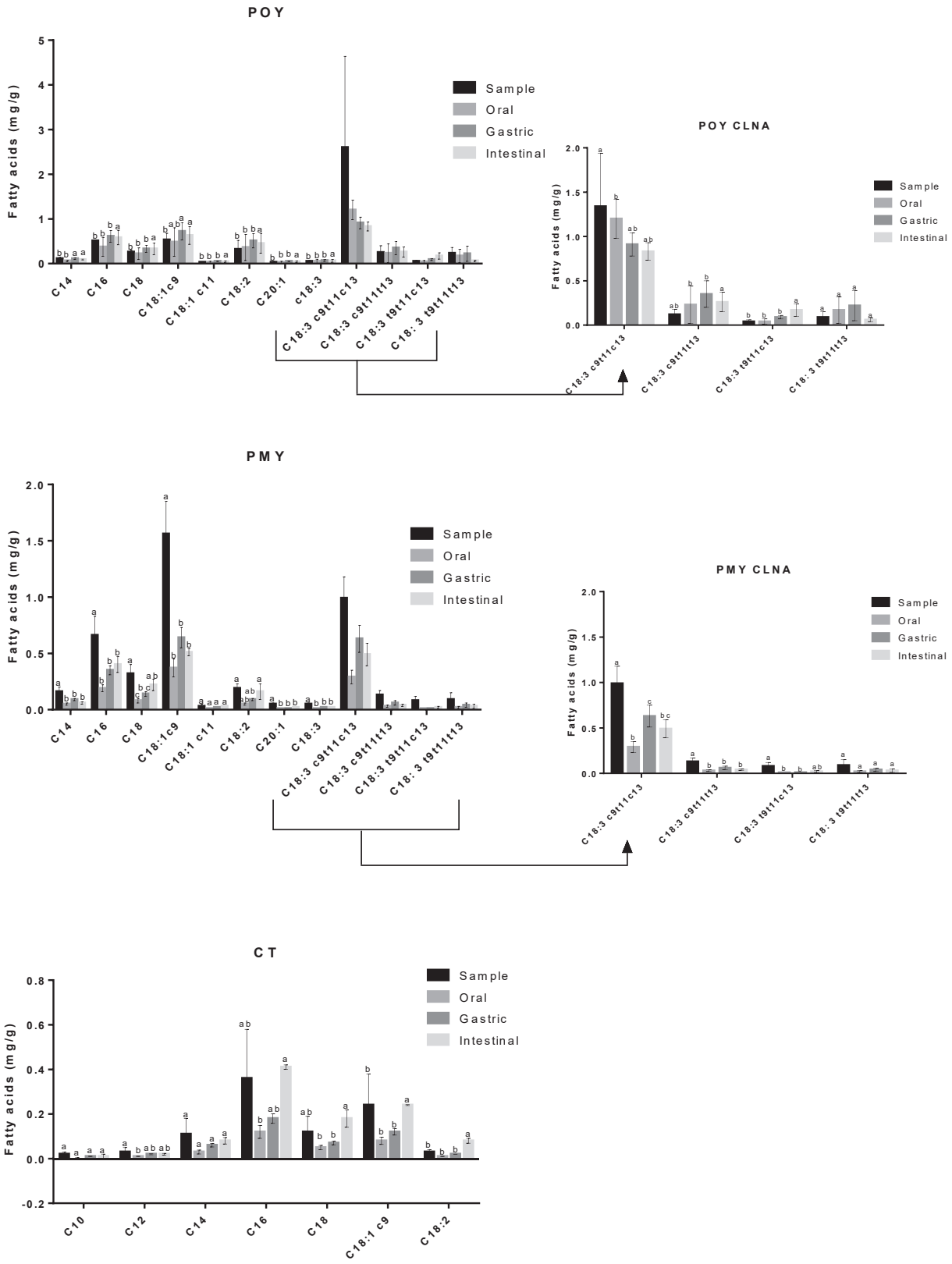

Fig. 3. Effect of *in vitro* GIT on fatty acids quantitative profile of functional and control yogurts. CT control yogurt; POY yogurt with pomegranate oil-free form; PMY yogurt with pomegranate loaded-chitosan microparticles.

3.6.3. Effect on adipolysis, 3T3-L1 cells morphology, and adipokines secretion

Adipolysis (or lipolysis) is a process related to the degradation of triglycerides in differentiated adipocytes (Schweiger et al., 2014). As can be seen in Fig. 6 for the results obtained no significant differences ($p > 0.05$) were observed between fortified yogurts and controls. Despite this, PMY showed 1.3 times more glycerol release than the positive control (isoproterenol), which indicated that it could reduce the

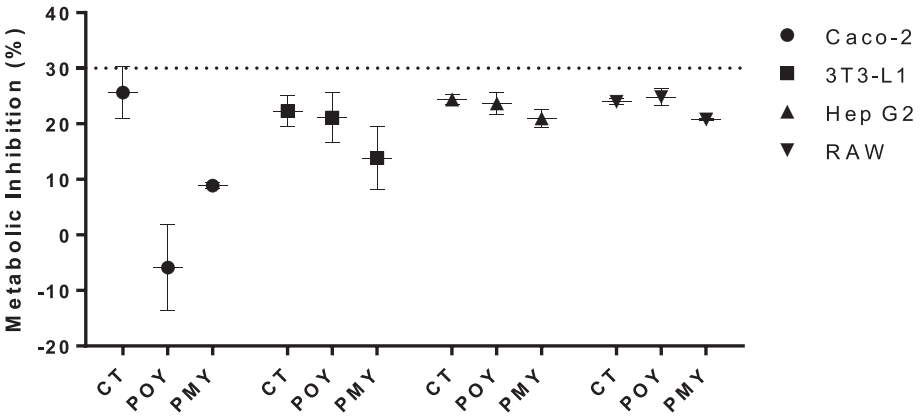

Fig. 4. Cytotoxicity of Functional and control yogurts towards the target cell lines. The dotted line represents the 30% cytotoxicity limit as defined by the (International Standard Organization, 2009). CT control yogurt; POY yogurt with pomegranate oil-free form; PMY yogurt with pomegranate loaded-chitosan microparticles.

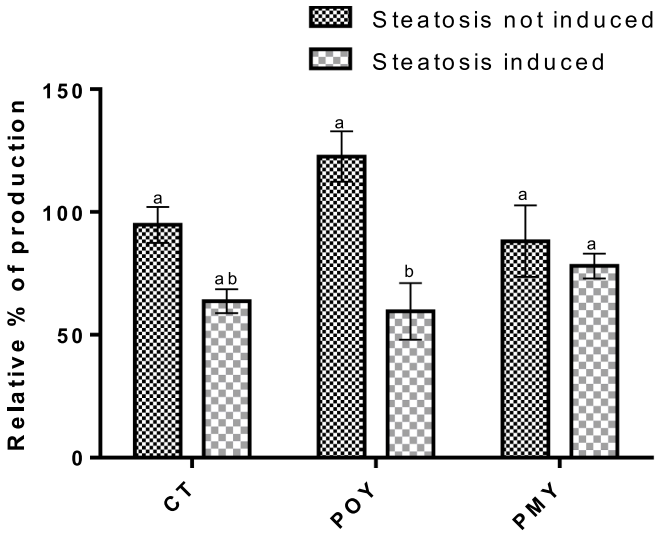

Fig. 5. Hepatic lipid accumulation results for the different yogurts with and without induced steatosis. Different letters mean significant differences as determined by a one-way ANOVA test ($p < 0.05$). CT control yogurt; POY yogurt with pomegranate oil-free form; PMY yogurt with pomegranate loaded-chitosan microparticles.

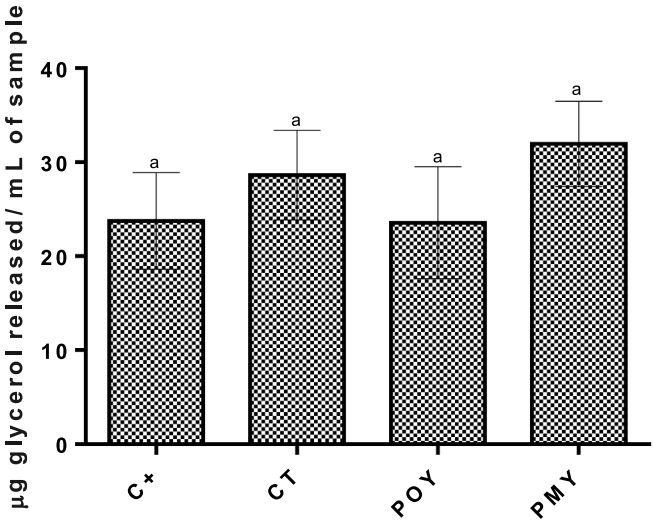

Fig. 6. Adipolysis results for the different yogurts with Isoproteranol at 10 µM used as the positive control. C+ - Positive control, CT control yogurt; POY yogurt with pomegranate oil-free form; PMY yogurt with pomegranate loaded-chitosan microparticles.

triglycerides accumulation in 3T3-L1 differentiated adipocytes. This effect may possibly be related to the presence of PUFAs, particularly CLNAs as previous studies have shown that 3T3-L1 cells treated with these bioactive fatty acids presented considerable inhibitions of triglycerides accumulation (Anusree et al., 2014; Barber et al., 2013; Li et al., 2017; Machado, Costa, et al., 2022). From a mechanistic standpoint, the inhibitory effect observed could be related to the activation of PPAR-γ and PPAR-α, which are responsible for adipogenesis/lipogenesis and fatty acids oxidation, respectively (Hontecillas et al., 2009; Miranda et al., 2011; Yuan et al., 2015).

In addition to the ability to increase lipolysis rate, the presence of PUFAs has been previously described as being capable of leading to cell apoptosis (Colitti & Grasso, 2014; Rayalam et al., 2008). The results obtained showed that (Fig. 7) the presence of POY and PMY led to alterations in adipocyte morphology (Fig. 7C and D) when compared to the control differentiated adipocytes (Fig. 7A). In the presence of all yogurt samples (Fig. 7B, C, D), the adipocytes lost their typical spherical aspect and acquired characteristics typical of apoptotic morphology.

When considering the data obtained for the adipokines secretion, all yogurt samples led to significant reductions ($p < 0.05$) in leptin secretion relatively to the basal control (Fig. 8A). For yogurts with PO, no statistically significant ($p > 0.05$) differences were found between samples, and reductions observed varied between 77 and 85% for PMY and POY, respectively. These results could be related to the caused by the presence of PUFAs (particularly CLNAs) which have been described as being capable of up-regulation of PPAR-γ expression (Al-Muammar & Khan, 2012; Fan et al., 2011; McFarlin et al., 2009; Verardo et al., 2014). The up-regulation of PPAR-γ could also be responsible for the increase in adiponectin levels observed in this study (Fig. 8B) (Fan et al., 2011). POY and PMY samples were responsible for a significant increase ($p < 0.05$) relative to the basal activity and the blank yogurt, with an increase of more than 50% in adiponectin secretion being observed. Similar results have been previously obtained by other authors in a study with punicic acid (Anusree et al., 2014). The increase in adiponectin secretion can be particularly important in the context of obesity and metabolic syndrome due to their anti-inflammatory and anti-diabetic potential, particularly linked to promote the insulin sensitivity (Wang & Scherer, 2016). Moreover, adiponectin is also associated with the maintenance of body energy homeostasis.

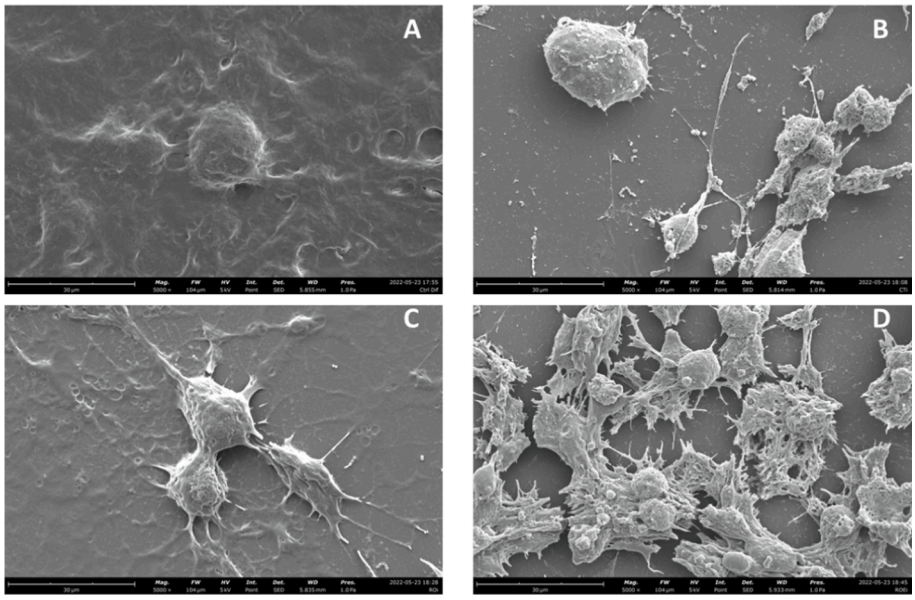

Fig. 7. SEM images of differentiated 3 T3-L1 adipocytes treated with yogurts digested samples. A) 3 T3-L1 differentiated cells; B) cells treated with control yogurt (CT); C) cells treated with yogurt with pomegranate oil-free form (POY); D) cells treated with yogurt with pomegranate loaded-chitosan microparticles (PMY).

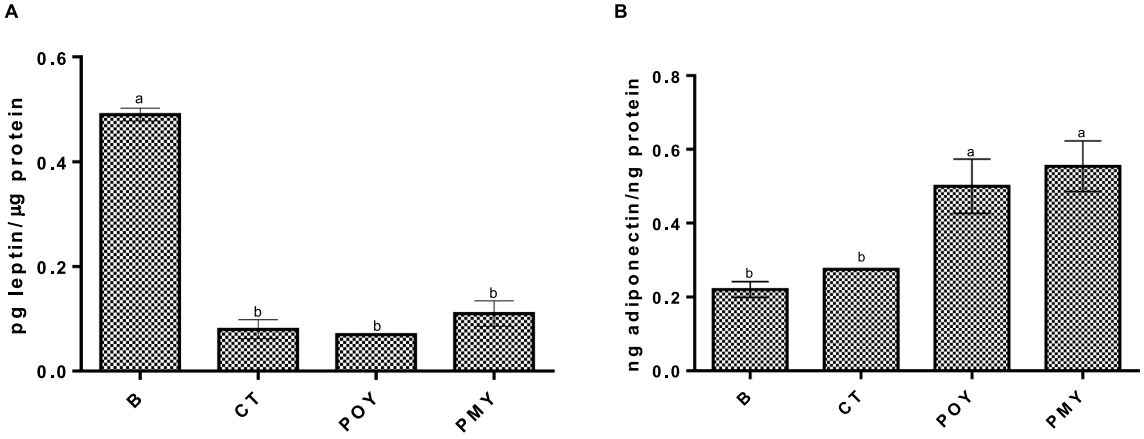

Fig. 8. Effect of yogurts samples on adipokines secretion in differentiated 3T3-L1 cells: A) leptin, B) adiponectin. Different letters mean significant differences as determined by a one-way ANOVA test ($p < 0.05$). B – basal activity; CT control yogurt; POY yogurt with pomegranate oil-free form; PMY yogurt with pomegranate loaded-chitosan microparticles.

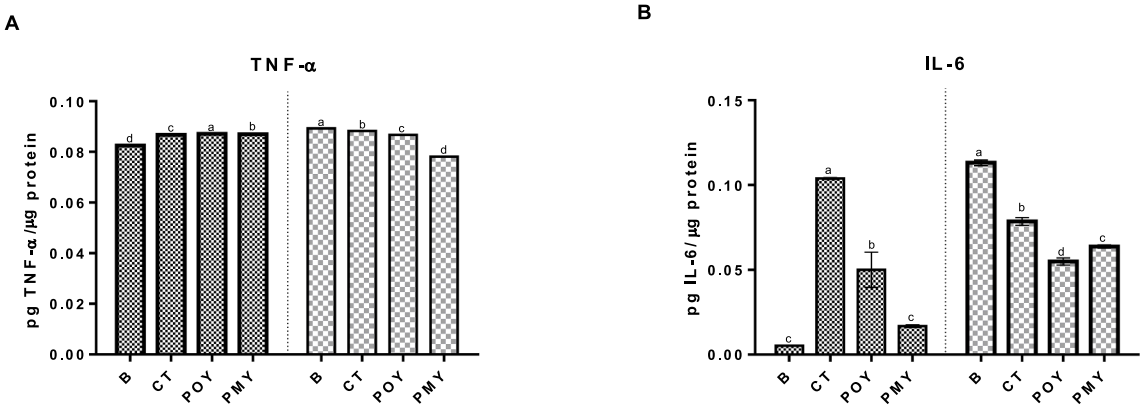

Fig. 9. Modulation of inflammatory response in RAW 264.7 cells yogurts A) TNF-α; B) IL-6. The left part of all graphs corresponds to the non-stimulated cell's response, and the right is related to the anti-inflammatory effect. Different letters mean significant differences as determined by a one-way ANOVA test ($p < 0.05$). B basal activity; CT control yogurt; POY yogurt with pomegranate oil-free form; PMY yogurt with pomegranate loaded-chitosan microparticles.

3.6.4. Immunomodulation capacity

The immunomodulatory capacity of functional yogurts was evaluated in Raw 264.7 cells. The results obtained showed significant differences ($p < 0.05$) for TNF-α secretion in basal and LPS-stimulated cells (Fig. 9A). In the non-stimulated assay, all yogurt samples presented a slight pro-inflammatory effect, with *ca.* 5% increase in TNF-α secretion being observed for the functionalized samples. In contrast, for LPS-stimulated cells, all yogurt samples exerted a slight anti-inflammatory effect with both functional yogurts presenting significantly ($p < 0.05$) lower TNF-α levels than the blank yogurt. On a sample per sample analysis, PMY yogurts showed a significantly ($p < 0.05$) lower amount of TNF-α than POY samples, with a 12% reduction in comparison to the basal activity. Relatively to IL-6 secretion, the data obtained showed significant differences ($p < 0.05$) between all yogurts and the basal activity control (Fig. 9B). When considering the non-stimulated assay, the control yogurt (CT) showed a high pro-inflammatory effect in the basal condition. The same effect was observed for POY samples. However, for PMY samples no significant differences ($p > 0.05$) were observed when compared to the basal activity, which means that no significant pro-inflammatory effect was observed. Regarding the LPS-stimulated cells, significant differences ($p < 0.05$) were observed between the yogurts and basal control. In this case, POY had a high anti-inflammatory effect with a 50% reduction in IL-6 secretion while PMY led to 44% reduction. The anti-inflammatory potential of the PUFAs present in PO has been previously demonstrated by *in vitro* and *in vivo* studies, and several potential mechanisms of action have been proposed, such as the down-regulation of eicosanoids production, up-regulation of PPAR, regulation of NF-kB and the reduction of the expression of pro-inflammatory proteins such as TNF-α, IL-6, and IL-1β (Hennessy et al., 2011). A previous study in Raw 264.7 cells showed that omega-3 fatty acids (particularly α-linolenic acid, the precursor of CLNAs) can inhibit the nuclear translocation of p65 protein and the LPS-induced activation of the NF-kB signaling pathway. Moreover, α-linolenic acid can also inhibit the phosphorylation of mitogen-activated protein kinase. The blocking of NF-kB activation was responsible for a reduction in TNF-α (Ren & Chung, 2007). The same inhibitory effect of NF-kB activation was reported by Calder and coworkers for long-chain fatty acids including punicic acid. In this study, the author suggested that these fatty acids are also responsible for the inhibition of PPARα and PPAR-γ activation (Aruna et al., 2016; Calder, 2013). Furthermore, punicic acid has been shown to be capable of inhibiting TNF-α-induced priming of NADPH oxidase by targeting the p38MAPKinase/Ser345-p47phox-axis and myeloperoxidase release (Aruna et al., 2016; Boussetta et al., 2009). The capacity of the bioactive fatty acids present in the functional yogurts to modulate the immune response, especially in the case of IL-6 can be particularly important in the obesity context, as this cytokine plays an important role in the energy homeostasis and it is capable of suppressing the lipoprotein lipase activity and control the appetite and energy intake at hypothalamic level (Rodríguez-Hernández et al., 2013; Stenlöf et al., 2003).

4. Conclusions

The PO-loaded chitosan particles showed high loading capacity, however, their incorporation into yogurts did not offer an improvement in their nutritional profile compared with PO in free form. The results obtained support the rationale that yogurts' functionalization with free PO can be a possible strategy to attain functional foods targeting obesity management and that chitosan-related encapsulation does not provide any functional or biological advantage in this scenario. These yogurts were capable of diminishing lipid accumulation in adipocytes and steatosis-induced hepatocytes. Moreover, their capacity to modulate adipokines secretion can be a valuable advantage for the management of insulin resistance. Finally, their capacity to reduce IL-6 secretion may also be an important benefit for energy intake management due to the role of this interleukin in inflammation and its connection with obesity. Despite the positive results for PO in free form, the major limitation of this work was the reduced recovery percentage of CLNAs. Moreover, despite the chitosan being considered GRAS, the incorporation of particles into foods requires, a more detailed characterization of particles and their possible toxicological effects according to EFSA guidelines. Overall, despite the drawbacks this work showed for the first time the potential of a functional pomegranate functional yogurt as a viable solution for a low cost, wide range and easy access solution for obesity management.

CRediT authorship contribution statement

Manuela Machado: Investigation, Formal analysis, Writing – original draft. **Sérgio Sousa:** Investigation, Writing – review & editing. **Luís M. Rodriguez-Alcalá:** Writing – review & editing. **Ana Maria Gomes:** Conceptualization, Writing – review & editing, Supervision. **Manuela Pintado:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

Acknowledgments

This work was supported by National Funds from FCT – Fundação para a Ciência e a Tecnologia through project UIDB/50016/2020. Manuela Machado is grateful for the financial support from FCT through the Doctoral grant reference SFRH/BD/136701/2018.

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