# Medium-chain triglycerides and conjugated linolenic acids in functional yogurts: impact of GIT and potential biological activities

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**Abstract**

In recent years, bioactive lipids particularly medium-chain triglycerides and conjugated linolenic fatty acids have obtained more attention due to their possible applicability in obesity metabolism modulation. These compounds are capable to increase thermogenesis and reduce weight gain through the modulation of key neurohormones such as leptin and adiponectin. The purpose of this work was to develop functional yogurts through the addition of coconut (rich in medium-chain fatty acids) or pomegranate oils (rich in conjugated linolenic acids). The presence of these oils led to a significant alteration in the nutritional value of yogurts, showing a capacity to reduce the accumulation of lipids in hepatocytes and increase the release of triglycerides in adipocytes. These results demonstrate that functional yogurts can be a valuable strategy for obesity prevention.

**Keywords:** lipid metabolism, obesity, functional foods, coconut oil, pomegranate oil

## Introduction

Currently, obesity is one of the most important public health problems. According to the World Health Organization, 1.9 billion adults and 38.2 million children are estimated to be overweight or obese [1]. The leading causes of obesity are a lack of physical activity and overconsumption of high-energy foods [2]. Common strategies to prevent and treat obesity and related disorders are ineffective in the long term. For this reason, functional foods and nutraceuticals have appeared as a complement to the classic therapeutic approach [3]. In this context, bioactive lipids can play an important role in the modulation of obesity-related metabolism. One of the most studied cases are the medium-chain triglycerides (MCTs) present in coconut oil, palm oil, and dairy fat. Medium-chain triglycerides-rich oils have been positively associated with reduced triglycerides levels, and short-term increases in fatty acid oxidation and thermogenesis[4,5]. Another important class of compounds with anti-obesity effects are conjugated linolenic acids isomers (CLNAs), particularly, punicic acid (C18:3 *c*9*t*11*c*13) present in pomegranate seed oil). This fatty acid can reduce the percentage of weight gain through the modulation of leptin and adiponectin secretion [6]. Moreover, punicic acid is capable to regulate the fatty acids storage in adipose tissue and glucose metabolism by peroxisome proliferator-activated receptor γ (PPARγ) inhibition [6].

Despite the proven important benefits, the incorporation of bioactive oils in functional foods has several challenges, including oxidative stability. Bioactive lipids, particularly oils rich in polyunsaturated fatty acids are susceptible to oxidation, which can result not only in a decrease in oils nutritional value but also in the formation of oxidative primary and secondary metabolites that may have a negative impact on human health [7–9]. One of the major strategies to solve this problem is the use of biphasic semisolid delivery systems  In fact, over the recent years bigels have appeared as promising formulation technologies for the food industry due to their capacity to deliver sensitive compounds such as bioactive lipids and probiotics [10]. To the best of our knowledge, the application of this delivery strategy for pomegranate oil or coconut oil

incorporation in functional dairy foods has not yet been explored in the available literature. Considering the above, this work aimed to develop functional yogurts with a source of MCTs (coconut oil) and a source of CLNAs (pomegranate oil) and evaluate their ability to modulate the obesity-related metabolism. For this purpose, yogurts were prepared with the oils in free and encapsulated form (as bigels) to prevent possible oxidation during the yogurt production process. After submission to simulated gastrointestinal tract conditions, the yogurt digests were evaluated for their potential bioactivity through the hepatic lipid accumulation and adipolysis assays.

## Material and methods

### Bigel preparation

Melted geleol (Gatefossé, France) at 60 °C in a hotplate, was mixed with pre-heated tween 80 (1:1 w/w) (Sigma, USA) and carboxymethylcellulose (2% w/v) (Merck, USA) under continuous mixing for 2 min. Pomegranate (All Organic Treasure, Germany) and coconut oils (OrigensBio, Portugal) were added. The resulting mixture was homogenized at 18000 rpm for 1 min in an ultra-turrax (IKA T 25 digital, Janke and Kunkel IKA-Labortechnik, Germany) and then sonicated (Sonics Vibra-Cell™ VCX 130) at 60% amplitude for 1 min. The resulting mixture was then cooled and stored at room temperature until use.

### Yogurt Production

To produce the yogurts, commercially available powdered skimmed milk was ressuspended in water (13 % w/v; Nestlé, Portugal), heated to 85 °C in a water-bath, homogenized at 200 bar, and then pasteurized at 90 °C for 1 min. The pasteurized milk samples were then transferred into a water bath at 30 °C to slowly cool down before storage at 4 °C, where they were kept until the fermentation step. The addition of the vegetable oils to the milk was done as follows: the

free pomegranate and coconut oils (1.5% w/w) were added to the reconstituted milk before heating to 85 °C to allow for better sample homogenization whereas the encapsulated oils (3% w/w) were added at the end of the pasteurization step to avoid heat/pressure-mediated bigel destruction. Plain reconstituted milk was used as a control. Before fermentation, stored samples were heated to 85 °C, cooled to 45 °C, and inoculated with 3% (w/w) natural yogurt (Mimosa Natural, Lactogal, Portugal). The resulting mixtures were incubated at 43 °C, in sealed plastic yogurt containers, until a pH of 4.6 was reached, and then stored at 4 °C. A total of 110 yogurts were prepared distributed over twenty-two yogurts of each yogurt type, namely, plain yogurt control (CT), coconut oil fortified yogurt (COY), coconut oil bigel fortified yogurt (CBY), pomegranate oil fortified yogurt (POY), and pomegranate oil bigel fortified yogurt (PBY).

**Physicochemical analysis**

The yogurts' pH value was measured using a pH meter (Micro pH 2002, Crison, Barcelona, Spain). The protein content was evaluated by the Bradford method [11]. Sugar content was performed by DNS method adapted for microplate reader [12]. Syneresis was measured according to Lopes et al. (2019) [13]. The physical properties of yogurt samples were assessed through rheological parameters. Rheology was performed in a rotational rheometer (Bohlin Instruments, United Kingdom), which was 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 degrees angle, was used and samples were analyzed at 12 °C. For homogenization, samples were gently stirred and, afterwards, were 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 linear viscoelastic region (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*).

127

128 **Fatty acids profile**

129 The determination of yogurts fatty acids profile was carried as previously described by Sousa et
130 al. 2022 [14]. Briefly, 150 mg of sample were added to 200 µL of tritridecanoin (Larodan, Solna,
131 Sweden), followed by (in order) 2.26 mL of methanol (VWR, USA), 800 µL of hexane (VWR, USA),
132 and 240 µL of sodium methoxide (5.4 M) (Acros Organics, USA). Samples were vortexed and
133 incubated at 80 ˚C for 10 min. After cooling in ice, 1.25 mL of DMF (VWR, USA) and 1.25 mL of
134 sulfuric acid (Sigma, USA) (3 M) in methanol were added, the samples vortexed and incubated
135 at 60˚C for 30 min. After cooling, samples were vortexed and centrifuged (1250 g; 18˚C; 5 min).
136 The upper layer containing fatty acids methyl esters was collected and analyzed in a gas
137 chromatograph HP6890A (Hewlett-Packard, Avondale, PA, USA), equipped with a flame
138 ionization detector and a BPX70 capillary column (60 m x 0.25 mm x 0.25 µm; SGE Europe Ltd,
139 Courtaboeuf, France). Analysis conditions were as follows: injector (split 25:1; injection volume
140 1 µL), injector and detector temperatures were 250˚C and 275˚C, respectively; hydrogen was
141 used as a carrier gas at flow rate of 1 mL/min. The oven temperature was initially at 60˚C and
142 then increased to a final temperature of 225˚C. Supelco 37 (Sigma, USA) and CRM-164 (Sigma,
143 USA) were used for the identification of fatty acids.

144 Furthermore, atherogenic index (AI), thrombogenic index (TI), and
145 hypocholesterolemic/hypercholesterolemic ratio (HH), concerning lipid nutritional quality were
146 calculated according to Alba et al. 2019 [15] using the following equations:

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

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

149

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

***In vitro* simulation of the gastrointestinal tract**

The *in vitro* simulation of the gastrointestinal tract was performed according to the INFOGEST method [16], in triplicate.. In brief, to mimic the oral phase, 5 g of yogurt was mixed with simulated salivary fluid (1:1 w/w), 75 U/ mL of α-amylase (Sigma, USA) was added, and the mixture was incubated for 2 min at 37ºC under continuous shaking. In the gastric phase, samples were added to simulated gastric fluid (1:1 v/v) and the pH was set to 3 using HCL (1M), the enzymes pepsin (Sigma, USA) and lipase (Lipolytech, France) were added (2000 U/mL and 60 U/mL, respectively) and the mixture was incubated 120 min at 37ºC under continuous shaking. Finally, to mimic the intestinal phase, the pH was set to 7 using NaOH (1M) and simulated intestinal fluid was added (1:1 v/v). Porcine pancreatin and bile salts (Sigma, USA) were added to the digestion to achieve 100 U/mL and 10mM, respectively. The mixture was incubated for another 120 min at 37ºC under continuous shaking. 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 the GIT, digested samples were centrifuged at 14000 rpm for 30 min, and the supernatant was collected for cell-based assays.

**Bioactive Properties**

**Cell lines growth conditions**

Human caucasian colon carcinoma epithelial cells, Caco-2 (ECACC 86010202), were obtained from the European Collection of Authenticated Cell Cultures. Human hepatocellular carcinoma cells, Hep G2 (ATCC HB-8065), and mouse pre-adipocytes 3T3-L1 (ATCC CL-173) were acquired

from American Type Culture Collection. Caco-2 and Hep G2 cells were cultured, as monolayers using Dulbecco's Modified Eagle's Medium (DMEM with 4.5 g/L glucose, L-glutamine without pyruvate (Gibco, Thermo Scientific, USA) supplemented with 10% (v/v) of fetal bovine serum (FBS, Biowest, France) and 1% (v/v) of Penicillin-Streptomycin-Fungizone (Lonza, Belgium). Caco-2 cells' media was also supplemented with 1% (v/v) non-essential amino acids (Gibco, Thermo Scientific, USA). Pre-adipocytes were cultured in DMEM with 10% (v/v) of Calf Bovine Serum, Iron Fortified (ATCC, USA) and 1% (v/v) of Penicillin-Streptomycin-Fungizone (Lonza, Belgium). All cell incubations were carried out 37 °C in a humidified atmosphere of 95% air and 5% $CO_2$

**Cytotoxicity**

In order to assess eventual changes on mammalian cell viability of the yogurt digests (*in vitro* cytotoxicity), the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed according to ISO 10993-5 (International Standard Organization (ISO), 2009), with slight modifications. Briefly, cells were seeded at 1.0 x $10^5$ cells/mL into wells of 96-well tissue culture plates and allowed to adhere for 24 h. Afterwards, the medium was replaced by 100 µL of digested samples diluted 1:10 (v/v) and 1:20 (v/v) in culture media. Medium with dimethyl sulfoxide (DMSO) was used as a negative control and fresh cell culture medium as a positive control (cells in normal growth conditions capable of high redox potential). After 24 h of exposure, 100 µL of MTT (Sigma, USA) solution (0.5 mg/mL) were added to each well and the plates were incubated at 37 °C, in the dark. After 2 h, the MTT solution was removed and 100 µL of DMSO (Sigma, USA) were added. The plates were shaken protected from light for 10 min and absorbance was read at 570 nm in a microplate reader (Synergy H1, Biotek Instruments, Winooski, VT, USA).

**Hepatic lipid accumulation**

Hepatic lipid accumulation assay was performed using Hepatic Lipid Accumulation Kit (Abcam ab133131) according to the manufacturer's instructions. Briefly, hepatocytes, seeded at $10^4$ cells/mL in a 96-well plate, were allowed to adhere. After 24 h the medium was replaced by fresh medium with digested samples diluted 1:10 or chloroquine (25 µM steatosis induction control). After 72 h of exposure, cells were stained with Oil Red O and the absorbance at 490 nm was measured using a microplate reader (Synergy H1, Biotek Instruments, Winooski, VT, USA). To evaluate the possible effect of fatty acids on hepatic steatosis, a second assay was conducted, in which test samples were diluted in medium with 25 µM chloroquine. All samples were analyzed in quintuplicate.

**Adipolysis**

The yogurt samples' effect upon adipolysis was performed using the Adipolysis Assay Kit from Abcam (Abcam ab133115) according to the manufacturer's instructions. Briefly, 3T3-L1 cells were seeded at $3.0x10^4$ cells/mL in 96 microwell plates. Two-day post confluence the medium was replaced by differentiation induction medium. Three days after induction, the medium was replaced by insulin medium. Every 3 d the medium was replaced by fresh insulin medium until more than 80% of the cells differentiated. Afterwards, the medium was replaced by medium with digested samples (diluted 1:10) and incubated for 24 h. Isoproterenol solution (10 µM) was used as a positive control, and plain medium as a negative control. The glycerol concentration was measured by adding glycerol-free reagent to 25 µL of cell supernatants or 25 µL of glycerol standards (concentrations ranged between 0 and 125 µg/mL), incubating the mixture for 15 min at room temperature and measuring the absorbance at 540 nm in a microplate reader (Synergy H1, Biotek Instruments, Winooski, VT, USA). All samples were analyzed in quintuplicate.

**Statistical analysis**

Minitab 17 (Minitab, LCC, USA) was used to carry out statistical analysis. All data were 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 analysis of variance (ANOVA), followed by a multiple comparisons test (Tukey) to identify statistically significant differences between samples.

## Results and Discussion

### Physicochemical properties

The physicochemical characterization of yogurts is presented in table 1. Regarding the pH values, significant differences ($p < 0.05$) were verified between fortified yogurts and the control yogurt. The presence of oil (in free or bigel form) led to a decrease in pH values, which can be probably a consequence of their free fatty acid indices. Similar results have been reported by other authors, for yogurts fortified with flaxseed and fish oils [17,18]. Syneresis is defined as the separation of whey from the gel matrix after its shrinkage. This parameter is very important and has a direct effect on the consumer's acceptance. Significant differences ($p < 0.05$) were verified between yogurts with bigels and yogurts with oil in free form and the control. These results are consistent with those published by other authors for yogurts with omega-3 rich oils [18–20]. The highest syneresis value for OBY and PBY samples can be explained by the presence of carboxymethylcellulose as it, and other anionic polymers, can react with the positive charges of casein micelles. This interaction may disorganize the structure of casein micelles, which is associated with the low pH values, and consequently can increase syneresis [21,22]. This problem can be solved using different polymers in bigel production, such as xanthan or carrageenan gums which are reported as responsible for a decrease in the syneresis percentages in yogurts [21]. Regarding the protein content, significant differences ($p < 0.05$) were verified between all yogurts. These differences could be related to the presence of tween 80 in the bigel formulation.

249 Studies have previously reported that the presence of this surfactant can increase the
250 absorbance in the Coomassie blue dye assay, by the alterations in the micelle's formation. This
251 can lead to the introduction of errors in the presence of high concentrations of the tween, thus
252 giving false readings [23–25].

253 Despite this, the protein values were similar to those published by other authors [26,27].
254 Concerning the sugars, no significant differences ($p > 0.05$) between the control and yogurts
255 with the oil in free from. However, significant ($p< 0.05$) differences were observed in yogurts
256 with bigels, this can be related to the CMC used in bigel preparation. Globally, the presence of
257 these vegetable oils in free or bigels form did not negatively affect the chemical composition of
258 yogurts.

259 Rheological analyses results are presented in Figure 1 and showed no relevant differences
260 between the different yogurt formulations. Elastic (or storage) modulus (G') is related to the
261 stress contribution from the materials elastic structure and is a measure of the energy stored,
262 while the viscous (or loss) modulus (G'') is related to the stress contribution from the viscous
263 elements and is a measure of the energy dissipated. According to the difference between these
264 moduli, a material can be classified as presenting a solid-like behavior, when G' > G'', or liquid-
265 like behavior, when G' < G''. Results from rheological analyses of the different yogurts showed
266 that all yogurts presented a solid-like behavior, as the elastic modulus (Figure 1A) prevailed over
267 the corresponding viscous modulus (Figure 1B). Similar results were obtained for yogurts with
268 flaxseed and walnut oils.[16,23] Moreover, a slight increase in both moduli was registered with the
269 frequency increase, in all yogurt types. Regarding viscosity (specifically, complex viscosity - η*;
270 Figure 1C), results showed in all yogurts a decrease with increasing frequency, which translates
271 an increase in fluidity consequent of the oscillatory frequency increase. In comparison with the
272 control yogurts, incorporation of pomegranate or coconut oils (in free or bigel form) did not
273 exert any quantifiable effect in the assessed physical properties.

**Fatty acids profile**

The presence of coconut oil enhanced the saturated fatty acids (SFAs) content, particularly in lauric acid (C12) (Table 2). Significant differences ($p < 0.05$) in total fatty acids amount were verified between COY and CBY yogurts (Table 2), the total amount was 2.8 times higher in CBY samples than the control. This difference can be explained by the high oil retention and protection capacities of the bigel during yogurt production. The strategy adopted for the bigels incorporation can also be responsible for that difference. The significant increase in some of the SFAs in coconut yogurts lead to a decrease in their nutritional quality, due to their potential relationship with the risk of cardiovascular diseases. The nutritional quality can be assessed by the calculation of the atherogenic index (AI), thrombogenic index (TI), and hypocholesterolemic/hypercholesterolemic ratio (HH). By definition, AI is a ratio of specific saturated and unsaturated fatty acids [15] which, in the case of coconut oil yogurts, was 2.1 and 2.9 times (for COY and CBY, respectively) higher than the control. The AI values for control yogurt samples are in agreement with those reported in the literature for this dairy product [28–31]. The TI assesses the tendency to form blood clots in blood vessels associating thrombogenic SFAs with anti-thrombogenic monounsaturated fatty acids (MUFAs) and, omega-3 and omega-6 polyunsaturated fatty acids (PUFAs) [15,30]. In coconut oil fortified yogurts, TI values were higher (6.29±0.57 and 7.95±0.53 for COY and CBY, respectively) than the control yogurts (5.05±0.93), due to the higher SFA content. The HH ratio characterizes the relationship between hypocholesterolemic fatty acids and hypercholesterolemic fatty acids. It is well established that unsaturated fatty acids (MUFAs and PUFAs) decrease serum cholesterol, while some SFA, namely, lauric (C12:0), myristic (C14:0) and palmitic acids raise serum cholesterol [15]. Contrary to the other indexes, higher HH values indicate a high sample capacity to reduce cholesterol levels. Due to the presence of the high amount of SFAs the HH values of both coconut oil fortified

299 samples were low: 0.39±0.03, 0.17±0.02, and 0.31±0.13 for COY, CBY and CT yogurts,
300 respectively. Despite this, the results were similar to those obtained for whole cow milk yogurts
301 [28,30].

302 In contrast, the addition of pomegranate oil increased the amount of PUFAs (Table 2),
303 particularly in the case of PBY yogurts, due to the oil retention capacity of bigels. The enhanced
304 amount of PUFAs was linked to an improvement in nutritional value, due to the presence of
305 essential fatty acids (linoleic and alfa-linolenic) and conjugated fatty acids. The AI values of PBY
306 (0.15±0.00) were 24 times lower than the control CT (3.62±1.07), and 5 times lower than POY
307 (0.71±0.19) yogurts. The differences in AI between PBY and POY yogurts were related to the high
308 pomegranate oil retention in bigel fortified yogurts. Moreover, the addition of bigel added after
309 the first pasteurization during yogurt production can be also responsible for this difference.
310 Similar AI values have been reported for flaxseed oil fortified yogurts [28]. Similarly to AI, the TI
311 values were lower than those reported for whole cow milk yogurts and omega-3 or olive oil
312 fortified yogurts [28,30,31]. Pomegranate oil bigel yogurt presented the lowest TI value (0.01±0.00),
313 due to the high omega-3 and omega-6 fatty acids content. As expected, these yogurts showed a
314 significantly higher ($p < 0.05$) HH ratio than the control yogurt, which makes them a potential
315 functional food for cholesterol control.

316

317 **Impact of the gastrointestinal tract (GIT) on fatty acids profile**

318 The type of dietary lipids present in a matrix can affect how they are digested and subsequently
319 absorbed. The fatty acid chain length, degree of unsaturation, and positional distribution may
320 affect the lipolysis rate [32]. Moreover, the concentration of bile salts, the presence of calcium ions
321 and oxygen can also affect the digestion process [33]. Coconut oil is composed mainly of MCTs,
322 and these compounds are absorbed directly from the epithelium and enter the portal vein, and
323 may be immediately used as energy sources in the liver [5,34]. On the other hand, pomegranate oil

is mainly composed of long-chain fatty acids. In this case, after ingestion, they undergo lipolysis by pancreatic lipase that split them into monoacylglycerols and free fatty acids. These decomposition products form micelles with phospholipids, cholesterol, and bile acids and are absorbed into enterocytes. Their triglycerides are reconstructed to form chylomicrons with cholesterol and proteins. These chylomicrons are released from the basal side of enterocytes and move to the lymph system [34].

In coconut oil yogurts (COY) (Table 3) the caprylic and capric acid contents were significantly ($p < 0.05$) affected by oral digestion; after this step caprylic acid was no longer detected, and capric acid content was reduced by 62%. Conversely, in coconut bigel yogurts (CBY) although during the initial GIT phases caprylic and capric acid contents underwent a reduction of around 45 and 55%, respectively, it was still possible to quantify them in the intestinal phase. This result can be explained by the enhanced retention capacity of coconut oil in the bigel formulation, and its ability to protect the oil during the GIT. No significant differences ($p > 0.05$) were verified in the amount of lauric (reduction around 30%), myristic (30% of reduction for COY and around 10% for CBY), palmitic, and stearic acids during the GIT in both formulations. Regarding the POY and PBY samples (Table 4), the amount of saturated fatty acids was not significantly ($p > 0.05$) affected during the GIT. However, the amount of PUFAS was significantly reduced, particularly in the case of CLNAs. In POY samples it was not possible to quantify linolenic acid and CLNAs after the oral phase and, the amount of linoleic acid suffered a 67% reduction upon the oral phase. In contrast, the delivery via bigels allowed CLNAs to reach the intestine although at very low concentrations (approximately twenty times lower than the initial amount). In PBY samples, the amount of linoleic acid had a lower reduction (approximately 33%) than the POY samples. The differences between POY and PBY demonstrated that bigels can be an interesting strategy to protect pomegranate oil during the GIT, and as mentioned above, in this formulation CLNAs remain quantifiable after the intestinal step. Despite the lack of GIT studies with this bioactive

oil, many studies demonstrated that encapsulation is one of the most important strategies to protect bioactive molecules during the GIT [32,33,35–38].

## Bioactive Properties

### Cytotoxicity

The cytotoxicity was evaluated in Caco-2, Hep G2, and 3T3-L1 cells using the MTT cell viability assay (Figure 2). The results demonstrated that the COY and CBY digested samples did not exert any inhibitory effect upon the cellular metabolism of all tested cell lines. In the case of POY and PBY digested samples at 1:10, these had a significant inhibitory effect, particularly on HepG2 and Caco-2 cells. With these results, it was possible to choose the best dilution to normalize the fatty acids content of the yogurts produced with the same oil. Therefore, for COY and CBY oils the total amount of fatty acids was set at 0.5 mg/mL and in POY and PBY at 0.2 mg/mL.

### Effect on hepatic lipid accumulation

Non-alcoholic fatty liver disease (NAFLD) has been recognized as one of the leading causes of liver dysfunction. This disease results from an imbalanced energy expenditure and intake and it is associated with visceral obesity and insulin resistance [39].

Concerning the impact of coconut oil in yogurts, HepG2 cells treated with COY and CBY digested samples showed high percentages of lipid accumulation (Figure 3A). However, when comparing COY and CBY yogurts with the control yogurt formulation it was possible to verify that the lipid accumulation was 1.46 and 1.28 times lower in coconut formulations (COY and CBY) than in the control yogurt, although such differences were not statistically significant ($p > 0.05$). A similar trend was observed for the same yogurts in cells subjected to chloroquine-induced steatosis (impaired lipolysis); the presence of COY and CBY digested yogurt samples appeared to reduce hepatic lipid accumulation in comparison to the control digested yogurt sample, although not to

375 a significant extent ($p>0.05$). This can be related to the presence of MCTs (C8, C10, and C12).
376 These fatty acids, particularly C8, can reduce the fatty acid synthase (FAS) activity in HepG2 cells,
377 and the expression of acetyl-CoA carboxylase (ACC). Moreover, the presence of MCTs may
378 promote the breakdown of triglycerides and their accumulation through the up-regulation of
379 adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) [39]. Another possible
380 mechanism of action of MCTs is the down-regulation of sterol regulatory element-binding
381 protein c1 (SREBP-1c) and the inhibition of anti-liver x receptor (LXR) expression [39].
382 Regarding POY and PBY, a similar effect was observed. High percentages of lipid accumulation
383 in Hep G2 cells were verified, in both pomegranate oil yogurts. However, the hepatic lipid
384 accumulation of these yogurts was 1.49 and 1.46 (POY and PBY respectively) times lower when
385 compared with the control yogurts. In steatosis-induced cells, the presence of POY and PBY
386 digested samples could reduce the lipid accumulation, however, no significant ($p > 0.05$)
387 differences were observed between the samples. An animal study demonstrated that a diet-rich
388 in punicic acid (the main fatty acid found in pomegranate oil) reduced the accumulation of
389 triglycerides in liver and the lipid droplets size were also reduced [40]. In this case, the action
390 mechanism can be related to omega-3 fatty acids' ability to increase hepatic fatty acid β-
391 oxidation through the activation of PPARα, inhibit SREBP-1c and carbohydrate response
392 element-binding protein (ChREBP) [41,42].
393
394 **Effect on adipolysis**
395 The adipolysis process is related to the degradation of triglycerides in differentiated adipocytes
396 [43,44]. The effect of yogurt samples is significantly different ($p < 0.05$) between the oils used (Figure
397 3B). The yogurts with pomegranate oil slightly increase glycerol release from 3T3-L1 cells.
398 Considerable inhibitions of triglycerides' accumulation on 3T3-L1 have been reported by other
399 authors for PUFAS [45,46]. The possible mechanism of action can be related to the activation of
400 PPAR-γ and PPAR-α. Studies demonstrated that punicic acid (and other CLNAs) can increase the

activity of these two peroxisomes receptors [47–49]. The low effect of PYO and PBY on adipolysis compared with the published data can be related to the poor amount of PUFAs, particularly the CLNAs after GIT. After GIT, in POY samples it was not possible to quantify CLNAs, and the amount of unsaturated fatty acids was very reduced.

Coconut yogurts (COY and CBY) promoted a lower adipolysis rate than POY and PBY samples. The effect of these yogurts may be explained by the reduction of lipoprotein lipase, promoted by the presence of MCTs, and the inhibition of stearoyl-CoA desaturase (SDC) [39]. Moreover, a study with 3T3-L1 cells demonstrated an increase in fatty acids β-oxidation, in the presence of caprylic and caproic acids [50].

## Conclusion

The fortification of yogurts with MCTs and CLNAs demonstrated to be a possible strategy to obtain functional foods for obesity modulation, principally by the reduction of triglycerides degradation in adipocytes. The yogurt's fatty acids profile was highly affected by the GIT. However, the strategy used for bigels, allowed the main bioactive compounds of coconut oil (lauric acid) and pomegranate oil (punicic acid) to reach the intestine. Despite the apparent decrease in the nutritional quality of coconut yogurts, reflected in the increase in AI and TI, these yogurts showed potential for reducing lipid accumulation in liver cells and triglycerides degradation in adipocytes. Although the pomegranate oil is very affected by the GIT passage conditions a positive effect was achieved, with the digested sample being capable of modulating obesity-related cellular metabolites in an *in vitro* setting.

## Author Contributions

Manuela Machado: investigation, formal analysis, writing - original Draft. Sérgio Sousa: investigation, writing - review & editing. Pilar Morais: conceptualization. Arménio Miranda:

conceptualization. Luís M. Rodriguez-Alcalá: writing - review & editing. Ana M. Gomes: conceptualization, writing - review & editing, supervision. Manuela Pintado: conceptualization, writing - review & editing, supervision, funding acquisition.

**Declaration of interests**

The authors declare no conflict of interest.

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Table 1: Physicochemical characterization of fortified yogurts and control.

| | pH | Syneresis (%) | Protein g/100g | Sugars g/100g |
|---|---|---|---|---|
| **CT** | $4.64 \pm 0.03^{a}$ | $51.07 \pm 1.18^{b}$ | $2.54 \pm 0.21^{bc}$ | $1.84 \pm 0.02^{b}$ |
| **COY** | $4.46 \pm 0.04^{b}$ | $48.40 \pm 1.23^{b}$ | $2.92 \pm 0.88^{c}$ | $1.09 \pm 0.05^{b}$ |
| **CBY** | $4.47 \pm 0.01^{b}$ | $59.21 \pm 0.84^{a}$ | $3.19 \pm 0.19^{ab}$ | $1.87 \pm 0.19^{a}$ |
| **POY** | $4.49 \pm 0.07^{b}$ | $50.73 \pm 3.17^{b}$ | $3.86 \pm 0.38^{a}$ | $0.84 \pm 0.03^{b}$ |
| **PBY** | $4.52 \pm 0.03^{b}$ | $57.63 \pm 1.87^{a}$ | $3.12 \pm 0.10^{abc}$ | $2.05 \pm 0.10^{a}$ |

Results are the means of three determinations ± standard deviation. Values with different letters in the same column are significantly different, as determined by one-way ANOVA test ($p < 0.05$).

CT – control yogurt; COY – yogurt with free coconut oil; CBY – yogurt with coconut oil bigel; POY yogurt with free pomegranate oil; PBY – yogurt with pomegranate oil bigel

Table 2: Fatty acids profile of coconut oil, pomegranate oil and control yogurts and their nutritional quality indexes.

| Fatty acid | COY | CBY | CT |
|---|---|---|---|
| **C8:0** | 0.08 ±0.01$^{b}$ | 0.33±0.03$^{a}$ | n.d. |
| **C10:0** | 0.08±0.03$^{b}$ | 0.29±0.03$^{a}$ | n.d. |
| **C12:0** | 0.78±0.04$^{b}$ | 2.90±0.26$^{a}$ | n.d. |
| **C14:0** | 0.57±0.07$^{b}$ | 1.55±0.13$^{a}$ | 0.16±0.04$^{c}$ |
| **C16:0** | 0.71±0.11$^{b}$ | 1.42±0.10$^{a}$ | 0.42±0.07$^{c}$ |
| **C18:0** | 0.27±0.05$^{b}$ | 0.96±0.03$^{a}$ | 0.16±0.01$^{c}$ |
| **C18:1 *c*9** | 0.49±0.03$^{b}$ | 0.99±0.10$^{a}$ | 0.30±0.04$^{c}$ |
| **Total** | 2.97+0.28$^{b}$ | 8.44±0.67$^{a}$ | 1.04±0.08$^{c}$ |
| **AI** | 7.61±0.04$^{b}$ | 10.60±0.26$^{a}$ | 3.62±1.07$^{c}$ |
| **TI** | 6.29±0.57$^{b}$ | 7.95±0.53$^{a}$ | 5.05±0.93$^{b}$ |
| **HH** | 0.39±0.03$^{ab}$ | 0.17±0.03$^{b}$ | 0.31±0.13$^{a}$ |

| Fatty acid | POY | PBY | CT |
|---|---|---|---|
| **C14:0** | 0.18±0.03$^{a}$ | 0.14±0.04$^{a}$ | 0.16±0.04$^{a}$ |
| **C16:0** | 0.71±0.17$^{b}$ | 1.57±0.44$^{a}$ | 0.42±0.07$^{b}$ |
| **C18:0** | 0.32±0.07$^{b}$ | 1.49±0.43$^{a}$ | 0.16±0.01$^{b}$ |
| **C18:1 *c*9** | 0.65±0.13$^{b}$ | 1.71±0.45$^{a}$ | 0.30±0.04$^{b}$ |
| **C18:1 *c*11** | 0.06±0.01$^{b}$ | 0.13±0.04$^{a}$ | n.d. |
| **C18:2 *c*9*c*12** | 0.28±0.02$^{b}$ | 0.95±0.24$^{a}$ | n.d. |
| **C18:3 *c*9*c*12*c*15** | n.d. | 0.12±0.03 | n.d. |
| **C20:0** | n.d. | 0.15±0.05 | n.d. |
| **C18:3 c9t11c13** | 2.06±0.29$^{b}$ | 9.81±2.53$^{a}$ | n.d. |
| **C18:3 t9t11c13** | 0.59±0.12$^{b}$ | 1.14±0.31$^{a}$ | n.d. |
| **Total** | 5.07±0.34$^{b}$ | 17.21±4.56$^{a}$ | 1.04±0.08$^{b}$ |
| **AI** | 0.71±0.19$^{b}$ | 0.15±0.00$^{b}$ | 3.62±1.07$^{a}$ |
| **TI** | 0.59±0.14$^{b}$ | 0.01±0.00$^{b}$ | 5.05±0.93$^{a}$ |
| **HH** | 3.54±0.68$^{b}$ | 8.17±0.32$^{a}$ | 0.31±0.13$^{c}$ |

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 one-way ANOVA test ($p < 0.05$).

CT – control yogurt; COY – yogurt with free coconut oil; CBY – yogurt with coconut oil bigel; POY - yogurt with free pomegranate oil; PBY – yogurt with pomegranate oil bigel

n.d. not detected

C8 – caprylic acid; 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; C18:3 *c*9*c*12*c*15 – linolenic acid; C20 – Arachidic acid; AI atherogenic index; TI thrombogenic index; HH hypocholesterolemic/hypercholesterolemic ratio.

Table 3: Effect of *in vitro* GIT conditions on yogurts fatty acids profile

| | | | | |
|---|---|---|---|---|
| | | **COY** | | |
| | ***Sample*** | ***Oral*** | ***Gastric*** | ***Intestinal*** |
| **C8:0** | 0.08±0.01$^{b}$ | n.d. | n.d. | n.d. |
| **C10:0** | 0.08±0.03$^{b}$ | 0.05±0.02$^{b}$ | n.d. | n.d. |
| **C12:0** | 0.78±0.04$^{ab}$ | 0.57±0.38$^{ab}$ | 0.50±0.04$^{ab}$ | 0.55±0.11$^{ab}$ |
| **C14:0** | 0.57±0.07$^{ab}$ | 0.32±0.10$^{ab}$ | 0.43±0.03$^{ab}$ | 0.41±0.07$^{ab}$ |
| **C16:0** | 0.71±0.11$^{a}$ | 0.49±0.17$^{a}$ | 0.67±0.01$^{a}$ | 1.85±0.14$^{a}$ |
| **C18:0** | 0.27±0.05$^{ab}$ | 0.24±0.08$^{ab}$ | 0.28±0.03$^{ab}$ | 1.19±0.12$^{ab}$ |
| **C18:1 *c*9** | 0.49±0.03$^{ab}$ | 0.32±0.15$^{ab}$ | 0.40±0.06$^{ab}$ | 1.08±0.10$^{ab}$ |
| | | **CBY** | | |
| | ***Sample*** | ***Oral*** | ***Gastric*** | ***Intestinal*** |
| **C8:0** | 0.33±0.03$^{b}$ | 0.10±0.00$^{b}$ | 0.15±0.07$^{b}$ | 0.37±0.01$^{b}$ |
| **C10:0** | 0.29±0.03$^{b}$ | 0.11±0.01$^{b}$ | 0.16±0.03$^{b}$ | 0.31±0.02$^{b}$ |
| **C12:0** | 2.90±0.26$^{a}$ | 1.11±0.04$^{a}$ | 2.14±0.05$^{a}$ | 2.99±0.24$^{a}$ |
| **C14:0** | 1.55±0.13$^{ab}$ | 0.68±0.03$^{ab}$ | 1.31±0.07$^{ab}$ | 1.46±0.08$^{ab}$ |
| **C16:0** | 1.42±0.10$^{ab}$ | 0.70±0.04$^{ab}$ | 1.43±0.04$^{ab}$ | 3.04±0.29$^{ab}$ |
| **C18:0** | 0.96±0.03$^{ab}$ | 0.56±0.01$^{ab}$ | 1.14±0.13$^{ab}$ | 2.19±0.16$^{ab}$ |
| **C18:1 *c*9** | 0.99±0.10$^{ab}$ | 0.36±0.00$^{ab}$ | 0.89±0.11$^{ab}$ | 1.73±0.15$^{ab}$ |
| | | **CT** | | |
| | ***Sample*** | ***Oral*** | ***Gastric*** | ***Intestinal*** |
| **C14:0** | 0.16±0.04$^{a}$ | 0.15±0.15$^{a}$ | 0.29±0.06$^{a}$ | 0.18±0.03$^{a}$ |
| **C16:0** | 0.42±0.07$^{a}$ | 0.14±0.02$^{a}$ | 0.19±0.07$^{a}$ | 1.97±0.31$^{a}$ |
| **C18:0** | 0.16±0.01$^{a}$ | n.d. | 0.80±0.43$^{a}$ | 0.71±0.01$^{a}$ |
| **C18:1 *c*9** | 0.30±0.04$^{a}$ | n.d. | n.d. | n.d. |

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 one-way ANOVA test ($p < 0.05$).
n.d. – not detected;
CT – control yogurt; COY – yogurt with coconut oil free form; CBY – yogurt with coconut oil bigel
C8 – caprylic acid; C10 – capric acid; C12 – lauric acid; C14 – myristic acid; C16 – palmitic acid; C18 – stearic acid; C18:1 *c*9 – oleic acid

Table 4: Effect of *in vitro* GIT conditions on pomegranate oils yogurts fatty acids profile

| | | | | |
|---|---|---|---|---|
| | | **POY** | | |
| | ***Sample*** | ***Oral*** | ***Gastric*** | ***Intestinal*** |
| **C14:0** | 0.18±0.03[a] | 0.09±0.01[a] | 0.14±0.01[a] | 0.13±0.01[a] |
| **C16:0** | 0.71±0.17[a] | 0.36±0.00[a] | 0.54±0.03[a] | 1.02±0.40[a] |
| **C18:0** | 0.32±0.07[a] | 0.25±0.01[a] | 0.39±0.02[a] | 0.67±0.30[a] |
| **C18:1 *c*9** | 0.65±0.13[a] | 0.29±0.07[a] | 0.43±0.12[a] | 0.52±0.25[a] |
| **C18:1 *c*11** | 0.06±0.01[a] | n.d. | n.d. | n.d. |
| **C18:2 *c*9*c*12** | 0.28±0.02[a] | 0.09±0.03[a] | 0.13±0.03[a] | 0.40±0.19[a] |
| **C18:3 c9t11c13** | 2.06±0.90[a] | n.d. | n.d. | n.d. |
| **C18:3 t9t11c13** | 0.59±0.12[a] | n.d. | n.d. | n.d. |
| | | **PBY** | | |
| | ***Sample*** | ***Oral*** | ***Gastric*** | ***Intestinal*** |
| **C14:0** | 0.14±0.04[a] | 0.12±0.02[a] | 0.17±0.04[a] | 0.11±0.04[a] |
| **C16:0** | 1.57±0.44[a] | 1.45±0.46[a] | 1.76±0.46[a] | 1.67±0.69[a] |
| **C18:0** | 1.49±0.43[a] | 1.64±0.74[a] | 2.19±0.8[a] | 1.52±0.63[a] |
| **C18:1 *c*9** | 1.71±0.45[a] | 1.54±0.57[a] | 1.96±0.59[a] | 1.08±0.15[a] |
| **C18:1 *c*11** | 0.13±0.04[a] | 0.14±0.15[a] | 0.21±0.01[a] | 0.55±0.07[a] |
| **C18:2 *c*9*c*12** | 0.95±0.24[a] | 0.71±0.02[a] | 0.90±0.06[a] | 0.64±0.05[a] |
| **C18:3 *c*9*c*12*c*15** | 0.12±0.03[a] | 0.08±0.11[a] | 0.18±0.05[a] | n.d. |
| **C20:0** | 0.15±0.05[a] | 0.14±0.16[a] | 0.19±0.04[a] | n.d. |
| **C18:3 c9t11c13** | 9.81±2.00[a] | 0.93±0.09[a] | 0.44±0.06[a] | 0.20±0.01[a] |
| **C18:3 t9t11c13** | 1.14±0.31[a] | 0.70±0.04[a] | 0.88±0.05[a] | 0.35±0.09[a] |

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 one-way ANOVA test ($p < 0.05$).
n.d. – not detected;
POY - yogurt with pomegranate oil free form; PBY – yogurt with pomegranate oil bigel
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; C18:3 *c*9*c*12*c*15 – linoleic acid; C20 – Arachidic acid

Figure 1: Rheological parameters: elastic modulus; viscous modulus and complex viscosity of yogurts control and fortified yogurts. CT – control yogurt; COY – yogurt with coconut oil free form; CBY – yogurt with coconut oil bigel; POY - yogurt with pomegranate oil free form; PBY – yogurt with pomegranate oil bigel

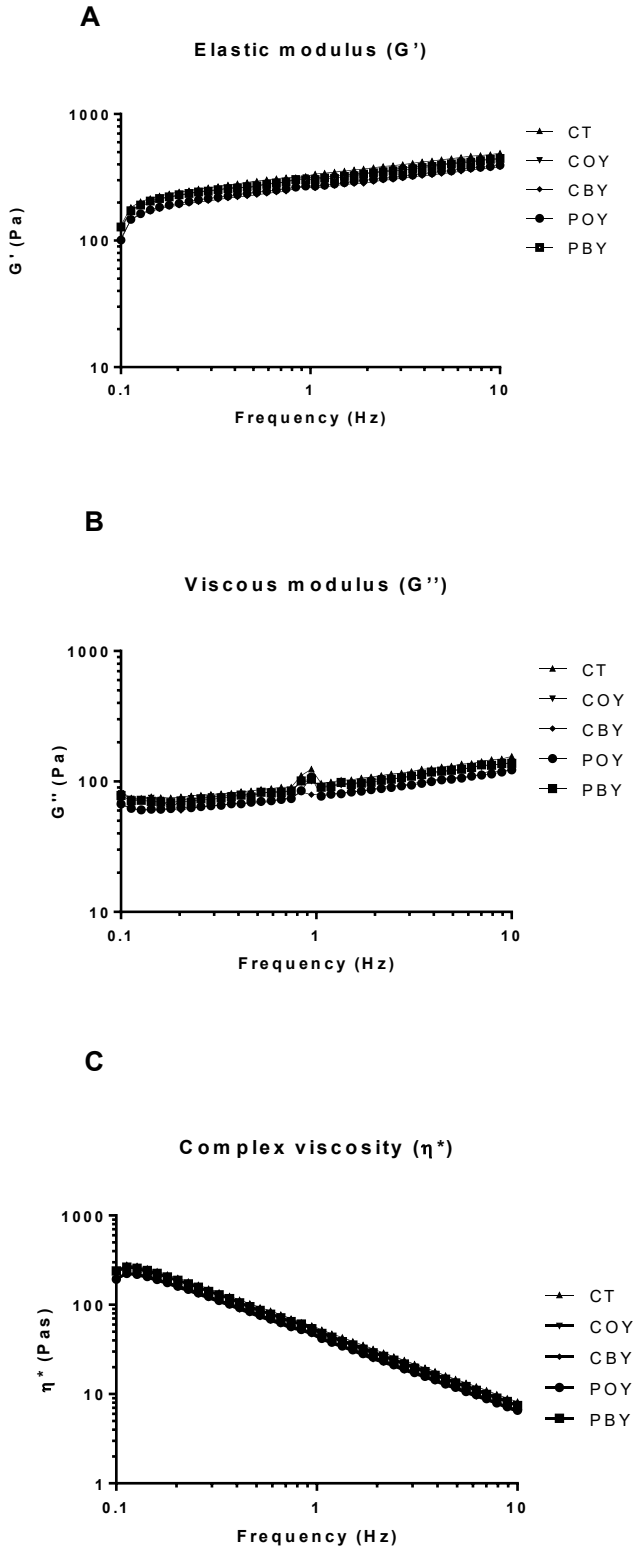

Figure 2: Samples cytotoxicity towards the target cells lines at 1:10 and 1:20 dilutions. The dotted line represents the 30% cytotoxicity limit as defined by [51].

Different letters mean significant differences as determined by one-way ANOVA test ($p < 0.05$).

CT – control yogurt; COY – yogurt with free coconut oil; CBY – yogurt with coconut oil bigel; POY yogurt with free pomegranate oil; PBY – yogurt with pomegranate oil bigel

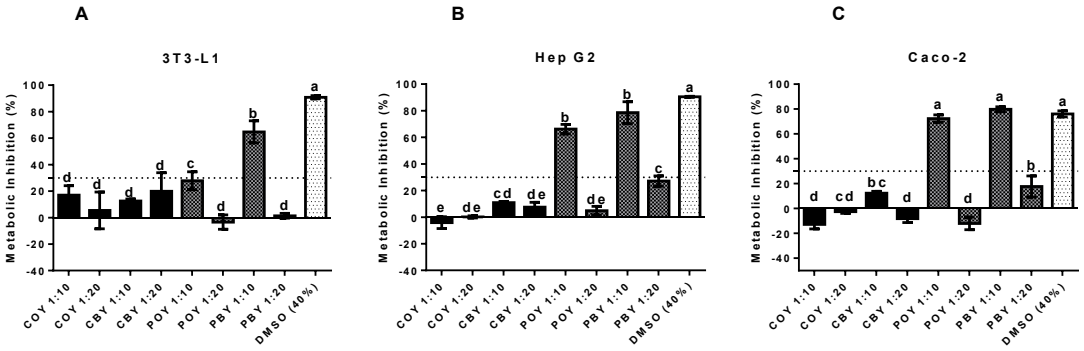

Figure 3: Biological assays results for the different samples: A) Hepatic lipid accumulation results for the different samples with and without induced steatosis. B) Adipolysis results for the different samples with Isoproteranol at 10 µM used as positive control.

In all assays different letters mean significant differences as determined by one-way ANOVA test ($p < 0.05$). CT – control yogurt; COY – yogurt with free coconut oil; CBY – yogurt with coconut oil bigel; POY yogurt with free pomegranate oil; PBY – yogurt with pomegranate oil bigel