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Enzymatic synthesis of capric acid-rich structured lipids and their effects on mice with high-fat diet-induced obesity

Julián Paul Martínez-Galán [a,*], Catalina María Ontibón-Echeverri [a], Mariana Campos Costa [b], Alexander Batista-Duharte [b], Vinicius Guerso Batista [c], Victoria Mesa [a], Rubens Monti [c], Ariela Veloso de Paula [c,*,1], Amanda Martins Baviera [b,*,1]

[a] School of Nutrition and Dietetic, University of Antioquia (UdeA), Calle 70 No. 52-21, Medellín, Colombia
[b] School of Pharmaceutical Sciences, Department of Clinical Analysis, São Paulo State University (Unesp), Araraquara, São Paulo, Brazil
[c] School of Pharmaceutical Sciences, Department of Bioprocess Engineering and Biotechnology, São Paulo State University (Unesp), Araraquara, São Paulo, Brazil

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ABSTRACT

The objective of this study was to produce structured lipids (SLs) by enzymatic acidolysis using *Rhizopus oryzae* lipase covalently immobilized in a low-cost material. Grape seed oil was used to synthesize SLs containing the medium-chain fatty acid (C10:0) capric acid. SL synthesis led to 38.8% medium-chain fatty acid incorporation with 5 reuses of the enzymatic derivative. The reaction conditions for the synthesis of MLM-TAGs (triacylglycerols with one long- and two medium-chain acyl residues) were at a molar ratio of fatty acid:oil of 3:1, performed at 40 °C and lipase immobilized load of 5% (w/w). The *in vivo* effects of SLs were studied in Swiss mice fed premade diets: control (C) diet, high-fat diet (HFD) with 100% lipid content as lard, HFD with 50% lipid content as grape seed oil (HG) or HFD with 50% lipid content as capric acid-containing SLs produced from grape seed oil (HG-MCT). Mice from HG and HG-MCT groups had decreases in body weight gain and reductions in the weights of white adipose tissues. In addition, HG and HG-MCT mice had low plasma levels of glucose and total cholesterol, and improvements in the glucose tolerance. HG and HG-MCT diets have remarkable antioxidant properties, since low plasma levels of TBARS (thiobarbituric acid reactive substances, biomarkers of lipid peroxidation) were found in mice fed these diets. Interestingly, TBARS levels in HG-MCT mice were further decreased than values of HG mice. Mice fed HG and HG-MCT diets also showed preservation in the activity of the antioxidant enzyme paraoxonase 1. Both HG and HG-MCT diets promoted reduction of IL-6 and IL-10 production by splenocytes. The capric acid-containing SLs produced from grape seed oil emerges as a functional oil capable to mitigate obesity complications resulting from oxidative stress and inflammation.

1. Introduction

Obesity is a leading cause of morbidity and premature mortality worldwide. It represents a risk factor for several comorbidities and noncommunicable diseases, including diabetes mellitus, dyslipidemia, cardiovascular disorders, several types of cancer among others, which have detrimental effects on the quality of life (An, Ji, & Zhang, 2018; Chooi, Ding, & Magkos, 2019). Obesity is defined as excessive fat accumulation resulting from an energy imbalance between energy intake and energy expenditure. Globalization is associated with lifestyle changes that contribute with the rise of obesity, having a major impact on food systems that led to the nutrition transition, characterized by the increased consumption of energy-dense foods, high in fats and sweeteners (Hawkes, 2006; Kearney, 2010). Furthermore, there is evidence that sedentary behaviour is also involved in the onset of obesity (Heinonen et al., 2013; Mitchell et al., 2009).

The treatment of obesity focuses not only in the body weight reduction, but also in the management of comorbidities and complications, improving the quality of life. Changes in the eating behavior have been proposed to treat obesity, including the decrease in the ingestion of energy density of foods and drinks and in the size of food portions, change in the macronutrient proportion of diets (low fat, low

* Corresponding authors.
E-mail addresses: julian.martinez@udea.edu.co (J.P. Martínez-Galán), ariela.veloso@unesp.br (A. Veloso de Paula), amanda.baviera@unesp.br (A. Martins Baviera).
[1] These authors have contributed equally to this study.

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carbohydrate or high protein), and the preferential consumption of vegetables, beans, legumes, grain, cereals, and fibers (Yumuk et al., 2015). Although main dietary approaches to weight loss include low-fat and very low-fat diets (Makris & Foster, 2011), it should be noted that some fats have potential health benefits, including the functional lipids (Alabdulkarim, Bakeet, & Arzoo, 2012). Both medium-chain fatty acids (MCFAs) and medium-chain triglycerides (MCTs) are cited as functional lipids. MCFAs are fatty acids consisting of 6–10 or 6–12 carbons, while MCTs are MCFA esters of glycerol. The beneficial effects of MCFAs and MCTs against metabolic disorders, including obesity and associated complications, have been reported (Nagao & Yanagita, 2010; Rial, Karelis, Bergeron, & Mounier, 2016; St-Onge & Jones, 2002; Taketa Moreira, Ract, Ribeiro, & Macedo, 2017).

The interest in the nutritional and functional effects of foods has increased, as well as the market for reduced calorie foods and fat substitutes (Kanjilal et al., 2013), which has stimulated the production of healthy triglycerides, also called dietary or structured triglycerides, through the modification of triglycerides in oils and fats (Abed et al., 2017), where the physicochemical, nutritional and functional properties of these are strongly dependent on the composition of their fatty acids and their distribution in the glycerol structure (Xie & Hu, 2016).

Structured lipids (SLs) or dietary triglycerides are defined as restructured or modified triglycerides through chemical or enzymatic processes, with the aim of changing the composition of fatty acids and/or their positional distribution in the glycerol molecule. They are synthesized in order to obtain nutraceutical or functional lipids providing health benefits, including the prevention of obesity (Kanjilal et al., 2013; Taketa Moreira, Ract, Ribeiro, & Macedo, 2017; D'Agostini and Gioielli, 2002). To act as an ideal substitute, the product must contain unsaturated, essential fatty acids, and have no side effects or be toxic. SLs of greatest interest are those of MLM-type (triglyceride(s) containing MCFA at positions 1 and 3 (M) and a long chain fatty acid (L) at position 2 (Cao et al., 2013). Due to their hypocaloric capacity, they provide a quick source of energy, more complete at nutritional level and suitable for individuals with disturbances on nutrient absorption and/or lipid metabolism (Morales-Medina, Munio, Guadix, & Guadix, 2017; Nunes et al., 2010). Furthermore, the fatty acid found in the *sn*-2 position is generally long-chain and polyunsaturated, increasing its bioavailability (Kanjilal et al., 2013; Taketa Moreira, Ract, Ribeiro, & Macedo, 2017; D'Agostini and Gioielli, 2002). SLs are considered of low caloric value, since they contain MCFA in the *sn*-1 and *sn*-3 positions, and a long chain essential fatty acid in *sn*-2 in the glycerol molecule.

SLs can be synthesized by acidolysis, hydrolysis, esterification, and transesterification reactions using *sn*-1,3-selective catalysts. These processes are expensive (Kim et al., 2010; Nagata et al., 2003), but have advantages over the chemical processes, such as to achieve more natural and healthier fats with fewer by-products with side effects, which is not an option for chemical catalysts. Likewise, if the catalyst is immobilized on a support, its recovery is easy and allows its reuse (Nunes, Pires-Cabral, & Ferreira-Dias, 2011; Palla, Pacheco, & Carrín, 2012), which reduces costs, increases the stability and activity of the biocatalysts, avoiding the generation of toxic products and using a solvent-free system, which is safer for food and friendly to the environment (Abed et al., 2017).

The objective of the present study was to synthesize and characterize low-calorie SLs formulated from grape seed oil and capric acid by lipase-catalyzed acidolysis using a batch stirred-tank reactor. Grape seed oil was selected for its high nutritional value, it has a high content of unsaturated fatty acids, such as essential linoleic acid (58–78%), high amount of antioxidant compounds, such as vitamin E, antioxidants and essential oils, especially linoleic oil (C18:2), and oleic (C18:1), containing traces of linolenic (C18:3) and palmitoleic (C16: 1), being linoleic and linolenic essential for the proper functioning of various organs and because they are not produced by the body must be present in the diet (Choi et al., 2012; De Souza et al., 2018). Reaction conditions of five-step reuse enzymatic process, such as substrate mole ratio, temperature and reaction time, were investigated. Furthermore, we investigated the antiobesogenic effects of these SLs, monitoring the changes in the body weight gain and in the levels of biomarkers related to metabolic disturbances, oxidative damage, and antioxidant defenses in obese mice.

2. Materials and methods

2.1. Material

2.1.1. Raw material

Grape seed (*Vitis vinifera* L.) oil was obtained by cold pressing and filtration by Distriol® (São Paulo, Brazil). Capric acid was purchased from Merck (Darmstadt, Germany).

2.1.2. Biocatalyst

The enzyme used as biocatilizer was a *Rhizopus oryzae* food grade lipase, kindly donated by the company PROZYN® (São Paulo, Brazil). For its immobilization, corn cob powder donated by the Rasul company (São Paulo, Brazil) was used. Porcine pancreatic lipase was purchased from Sigma-Aldrich (Sigma Aldrich, St. Louis, USA).

3. Methods

3.1. Characterization of grape seed oil

Grape ssed oil was characterized for their composition in fatty acids by gas chromatography, according to the American Oil Chemists' Society Method Ce 2–66, acid and peroxide values were obtained according to the methods AOCS Ca 5a-40 and AOCS Cd 8b-90, respectively (AOCS, 2011).

3.2. Preparation and physicochemical characterization of pretreated corn cob powder (CCP)

The functionalization of CCP was carried out according to the methodology described by Bassan et al. (2016). The organic support CCP was pretreated to remove lignin and hemicellulose, increasing internal area and contact surface. Then, also treated with glycidol, ethylenediamine and glutaraldehyde for getting a link between the amino group of the enzyme to the aldehyde group of the glutaraldehyde.

3.3. Enzymatic immobilization

After the functionalization of support, the enzymatic immobilization was realized. For each gram of activated support, 10 mL of 50 mmol/L sodium phosphate buffer (pH 7.0) containing enzymatic solution (25 mg/mL) was added. The suspension was submitted to agitation for 24 h at 25 °C in a roll bed (J. C. Bassan et al., 2016). Covalent immobilization of enzymes by glutaraldehyde chemistry is one of the most widely used technologies for enzyme immobilization. In practice this is produced through a two step mechanism: in a first step the adsorption of the protein on the support is produced and in a second step the covalent reaction occurs. Glutaraldehyde supports are produced by the addition of this reagent to a activated support with the amine groups, in this case epoxy or aldehyde with ethylenediamine (EDA) (Bezbradica, Mateo, & Guisan, 2014; Lalonde & Margolin, 2008).

The protein quantification was measured by the Bradford method (Bradford, 1976) on the supernatant before starting the contact time and at the end to calculate the immobilization percentage of the enzyme on the support.

3.4. Determination of hydrolytic activity of immobilized Rhizopus oryzae lipase

The hydrolytic activity of the biocatalyst was evaluated by the

titration method using an olive oil emulsion (1:1, w/v) with triton like emulsifier (Paula, 2012). One unit of activity (U) was defined as the amount of enzyme that released 1 μmol of fatty acid per minute under the assay conditions.

4. Acidolysis reactions

Acidolysis reactions were performed batchwise in a jacketed cylindrical glass reactor (internal diameter of 3 cm, height of 6 cm) operating in a batch regime. The reactions occurred at a tem- perature of 40 °C, with constant agitation of 400 rpm, molar ratio ratio (MR) fatty acid: oil of 3:1 and lipase immobilized in CCP load of 5% (w/w) (relative to the total mass of medium). This molar ratio corresponds to the stoichiometric value needed for the esterification of the free fatty acids at *sn*-1 and *sn*-3 positions by *sn*-1,3-selective lipases. Samples (0.05 mL) were collected at 0 time (oil without modification) and after 24 h reaction from the reaction medium to evaluate acidity index, peroxide, saponification and iodine values, fatty acid composition at the *sn*-2 position and the fatty acid profile (Bassan et al., 2019).

4.1. Triglyceride separation and methylation

After 24 h of reaction, tryglyceride separation and methylation was realized to remove the free fatty acids produced during the reaction according to the methodology described by Bassan et al. (2019).

4.2. Determination of the fatty acid composition of SLs and incorporation degree (ID, %)

The fatty acid composition of the structured lipids obtained was determined by gas chromatography, according to the American Oil Chemists' Society Method Ce 2–66 method (AOCS, 2011). It was carried out using a mixture of fatty acid ester standards (FAME's). The tests were carried out in a gas chromatograph (PerkinElmer) with a Split injector, flame ionization detector (FID) and a Supelcowax column (L × I.D. 30 m × 0.32 mm, df 0.50 μm). The injector and detector temperature was 250 °C, being a Split injection mode (1:10), using nitrogen as a stripping gas with a flow of 0.5 mL / min. The heating ramp was established as follows: initial temperature from 60 °C to 210 °C at a heating rate of 20 °C / min; the temperature of 210 °C was maintained for 7 min, and a heating rate of 20 °C / min, to achieve a final temperature of 250 °C, this was maintained for 25 min (Bassan et al., 2019). The incorporation degree (ID) was estimated according to the following equation (Casas-Godoy, Marty, Sandoval, & Ferreira-Dias, 2013):

$$ID\% = \frac{MFA}{MT} * 100 \quad (1)$$

Where MFA is the number of moles of medium chain fatty acids (C10:0) in TAGs and MT is the number of total moles of fatty acids in TAGs.

4.3. Fatty acid at the sn-2 position in triacylglycerols

The fatty acid composition at position *sn*-2 of the triacylglycerols was analyzed following the porcine pancreatic lipase hydrolysis procedure according to Caballero et al. (2014) and modified by Bassan et al. (2019).

5. Experimental design

Swiss male mice (*Mus musculus*; 7-week-old) were obtained from the Central Animal Facilities of School of Pharmaceutical Sciences, Unesp, Araraquara, Brazil. Mice were housed (two animals per cage) under a controlled environment of temperature (23 ± 1 °C) and humidity (55 ± 5%) with 12 h light/dark cycle and with food and water *ad libitum*. During 2 weeks of acclimation, the animals were fed a standard chow diet (Presence Nutrição Animal, Paulínia, Brazil). After this period, mice were fed a control diet (C; 3.85 kcal/g; 4% lipids; Table 1) or high-fat diets (HFD; 5.40 kcal/g; 35% lipids; Table 1). Two groups received experimental HFD composed of a mixture of lard and grape seed oil (HG) or lard and grape seed oil containing capric acid as MCT (HG-MCT); lard was replaced by grape seed oil or modified grape seed oil rich in MCT by 50% proportion (Table 1). The premade diets were purchased from PragSoluções Biociências Serviços e Comércio Ltda (Jaú, Brazil). Mice were randomly separated into four groups (10 animals/group):

(a) mice fed a control diet (C);
(b) mice fed a HFD (H);
(c) mice fed a HFD containing grape seed oil (HG);
(d) mice fed a HFD containing grape seed oil rich in MCT (HG-MCT)

Animals were maintained in these diets during 8 weeks. Body weight and food intake were assessed weekly. Oral glucose tolerance test (OGTT) was performed at week 7. At the end of the experiment (week 8), animals were fasted for 6 h, anesthetized (16 mg/kg xylazine, 90 mg/kg ketamine), blood samples were collected by cardiac puncture in heparinized tubes and immediately centrifuged at 700 g for 10 min at 25 °C to obtain plasma samples that were stored at −80 °C for further analysis. Epididymal and retroperitoneal white adipose tissues were removed and weighed. All procedures were previously approved by the Committee for Ethics in Animal Experimentation of the School of Pharmaceutical Sciences, Unesp, Araraquara (CEUA/FCF/CAr n° 11/2017).

5.1. Glucose homeostasis assay

At week 7, mice were fasted for 12 h, and the OGTT was performed at 11:00 a.m. after gavage with glucose (1 g/kg). Glycemia were measured before (0 min) and after (15, 30, 60, 90, and 120 min) the glucose overload. A drop of blood was collected from the tip of the mouse tail to determine the glycemia levels measured by glucometer (Abbott Diabetes Care Ltd, Brazil).

Table 1
Compositions and energy content of standard, control diet (C), high-fat diet (HFD), HDF containing grape seed oil (HG), and HDF containing grape seed oil rich in MCT (HG-MCT).

Ingredients	Control diet (C) (g/100 g)	High-fat diet (HFD) (g/100 g)	HFD + grape seed oil (HG) (g/100 g)	HFD + grape seed oil rich in MCT (HG-MCT) (g/100 g)
Starch	42.75	14.95	14.95	14.95
Casein	20	20	20	20
Dextrin	13.2	10	10	10
Sucrose	10	10	10	10
Soy oil	4	4	4	4
Lard	–	31	15.5	15.5
Grape seed oil	–	–	15.5	–
Grape seed oil rich in MCT	–	–	–	15.5
Cellulose	5	5	5	5
Mix of minerals *	3.5	3.5	3.5	3.5
Mix of vitamins **	1	1	1	1
L-cystine	0.30	0.30	0.30	0.30
Choline bitartrate	0.25	0.25	0.25	0.25
Total	100	100	100	100
Energy (kcal/ 100 g)	385	540	540	540

* Mix of minerals: calcium, phosphorus, potassium, magnesium, iron, manganese, selenium, zinc, chromium, nickel, lithium, sulfur, copper, iodine, molybdenum, silicon, chloride, fluoride, boron, vanadium.

** Mix of vitamins: nicotinic acid, calcium pantothenate, pyridoxine, thiamine, riboflavin, folic acid, vitamin K, D-biotin, vitamin B12, vitamin A, vitamin D3, vitamin E, choline.

5.2. Biochemical analyses

Glucose, triglycerides, total cholesterol, high-density lipoprotein-cholesterol (HDL-cholesterol), alanine aminotransferase (ALT), creatinine, and urea levels were measured in plasma using commercial kits (Labtest Diagnostica SA, Lagoa Santa, Brazil). The levels of products related to lipid peroxidation were measured in deproteinized plasma samples using the thiobarbituric acid assay. Thiobarbituric acid reactive substances (TBARS) were quantified spectrofluorometrically at excitation and emission wavelengths of 510 nm and 553 nm, respectively (Kohn & Liversedge, 1944). 1,1,3,3-tetramethoxypropane (Sigma Aldrich) was used as standard. The activity of the antioxidant enzyme paraoxonase 1 (PON 1) was measured in plasma by the hydrolysis of paraoxon and the release of *p*-nitrophenol (Assis et al., 2017). The activity was monitored by measuring the absorbance at 405 nm over a period of 5 min. The results were calculated using the molar extinction coefficient of *p*-nitrophenol (18,000 $M^{-1}.cm^{-1}$).

5.3. Preparation of splenocytes and measurement of the ex vivo release of cytokines

Splenocyte preparations and cytokine measurement were performed as previously described (Batista-Duharte et al., 2016). Spleens were aseptically removed and passed through a 100 μm cell strainer into a Petri dish containing 2 mL of PBS with the aid of a syringe plunger. For red cell lysis, the resulting suspension was mixed with 6 mL of a 0.17 M ammonium chloride solution and then incubated on ice for 5 min. The splenocytes were then separated from the supernatant by centrifugation at 300 g for 5 min at 4 °C, washed once with 3 mL of cell culture medium RPMI 1640 (Sigma Aldrich) (pH 7.2) supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml 2-mecaptoethanol) and then resuspended in 1 mL of the same medium. Cell concentration and viability were determined by microscopy using the Trypan blue exclusion test and then the viable splenocytes were adjusted to 5×10^6 cells/mL in RPMI complete medium. For the measurement of the *ex vivo* release of IL-6 and IL-10, splenocytes were cultured for 24 h at 37 °C and 5% CO_2 on flat bottom 48-well tissue culture plates in the presence concanavalin A (0.25 μg/mL in RPMI complete medium) for T lymphocytes stimulation and RPMI alone were used as negative control. Cytokines were measured by ELISA kits (eBioscience), according to the manufacturer's instructions.

6. Statistical analysis

Data are expressed as mean ± standard error of mean. One-way analysis of variance followed by the Student-Newman-Keuls test was used to compare intergroup differences. Data were considered statistically significant at $p < 0.05$. Statistical analyses were performed using the program GraphPad Prism 5.01 (GraphPad Software, San Diego, California, USA).

7. Results

7.1. Physico-chemical characterization of grape seed oil

The physico-chemical characterization of grape seed oil showed an acid value of 0.225 ± 0.021 mg KOH/g of oil, peroxide value of 0.905 ± 0.148 mEq O_2/kg of oil, iodine value of 128.100 ± 0.141 g I_2/100 g and aponification value of 191.505 ± 0.091 mg KOH/g. The results indicated that the raw material is within the specified standards (FAO, 1999): 0.6 mg KOH/g of oil for the acid value and 10 mEq O_2/kg of oil for the peroxide value.

7.2. Hydrolytic activity of lipase

Transesterification reactions of oils and fats, including acidolysis, involve, sequential reactions of hydrolysis and esterification (re-synthesis) of the triacylglycerols (Paula, Nunes, De Castro, & Santos, 2015). The hydrolytic activity of *Rhizopus oryzae* lipase was 62 ± 4.45 U/g after the glutaraldehyde treatment, which corresponds to an immobilization yield of 58.13 ± 0.94%.

A new immobilization, on the best selected loading and with a new batch of lipase, was performed. In this way, the whole process of functionalization of the support was performed again. The results obtained then are presented in table 2.

We can observe that the immobilization yield was 58.13%, with the immobilized derivative having hydrolytic activity of 62 ± 4.45 U/g. The higher immobilization yield in this case can be justified by the batch change of the enzyme used.

7.3. Fatty acid profil of grape seed oil and dietary triacylglycerols (MLM) synthesized

The oil has high amounts of unsaturated fatty acids (78%). Essential linoleic acid (C18:2) is the major fatty acid (46.2%), followed by oleic acid (C18:1; 26.6%), palmitic (C16:0; 11.1%) and stearic acids (C18:0; 6.72%). The highly polyunsaturated fatty acids C18:3 and C20:3 are present in low amounts (3.46 and 2.11%, respectively). In the structured lipid, we found a lower amount of unsaturated fatty acids (51%), with a trend to be similar to that of the oil of origin (C18:2; 33.76%, C18:1; 13.6%, C16:0; 5.48% and C18:0; 1.60%). Highly polyunsaturated fatty acids C18:3 are present also in relative low amounts (3.49%). Fatty acid C20:3 was not identified (Table 3).

7.4. Incorporation degree of capric acid and reuse of the enzymatic derivative

From the fatty acid composition, the values of incorporation degree of capric acid in the grape seed oil after each reuse were calculated using the enzymatic derivative. The results are shown in Fig. 1. Incorporation degree obtained varied from 23.18 to 51%, highlighting that for the first reuse an incorporation degree of 46% was obtained. Otherwise, 5 reuses of the enzymatic derivative were carried out in order to collect the sufficient volume for the elaboration of experimental diets. Collecting and mixing all the volumes of the reuses, an average of the incorporation degree of 37% was obtained. Finally, it was observed that the acidolysis reaction where a higher degree of incorporation was obtained was in the reuse 2 (51%), possibly due to the stability acquired by the immobilization material to the enzyme.

7.5. Changes in body weight gain, fat depots, and food/energy intake in obese mice fed with HG or HG-MCT diets

Throughout the experimental period, mice fed a HFD (H group) exhibited minor food intake (Fig. 2A) but increased energy intake (Fig. 2B) in comparison to mice fed a control diet (C group). Consequently, H mice underwent a significant increase in the body weight gain, mostly from the 3rd week (Fig. 3A), and the total body weight gain was 2.2-fold higher in H mice than in C mice (Fig. 3B). Fat mass

Table 2
Comparison between protein concentration in the free enzyme and in the supernatant after immobilization of the new batch of enzyme and loading of 25 mg/ml.

Yield of immobilization (%)	
Free lipase	100
Supernant (t = 0.5 h)	97.61 ± 2.28
Supernant (t = 1 h)	83.40 ± 0.38
Supernant (t = 2 h)	67.83 ± 0.20
Supernant (t = 24 h)	41.87 ± 0.79
Total yield of immobilization	58.13 ± 0.94

Table 3
The profile of fatty acid composition of grape seed oil and grape seed oil rich in MCT after 24 h of acidolysis catalyzed by lipase (*Rhizopus oryzae*).

Fatty acid	Grape seed oil (%)	Grape seed oil rich in MCT (%)
C10:0	–	42.07
C16:0	11.10	5.48
C18:0	6.72	1.60
C18:1	26.57	13.60
C18:2	46.25	33.76
C18:3	3.46	3.49
C20:3	2.11	–
C22:0	1.16	–
UN*	2.63	–
Total	100	100

* UN = unidentified

accretion was also observed in epididymal (Fig. 4A) and retroperitoneal (Fig. 4B) white adipose tissues of H mice.

Mice fed HFD containing grape seed oil (HG group) or modified grape seed oil rich in MCT (HG-MCT group) had values of food intake (Fig. 2A) and energy intake (Fig. 2B) similar to those of H mice. However, body weight values were reduced in HG and HG-MCT mice (Fig. 3A), leading to significant decreases in the total body weight gain when compared to H mice (Fig. 3B). Mice from HG group had decreases in the weight of epididymal adipose tissue (Fig. 4A), while mice from HG-MCT group had decreases in the weights of both epididymal (Fig. 4A) and retroperitoneal (Fig. 4B) adipose tissues, in comparison with H mice.

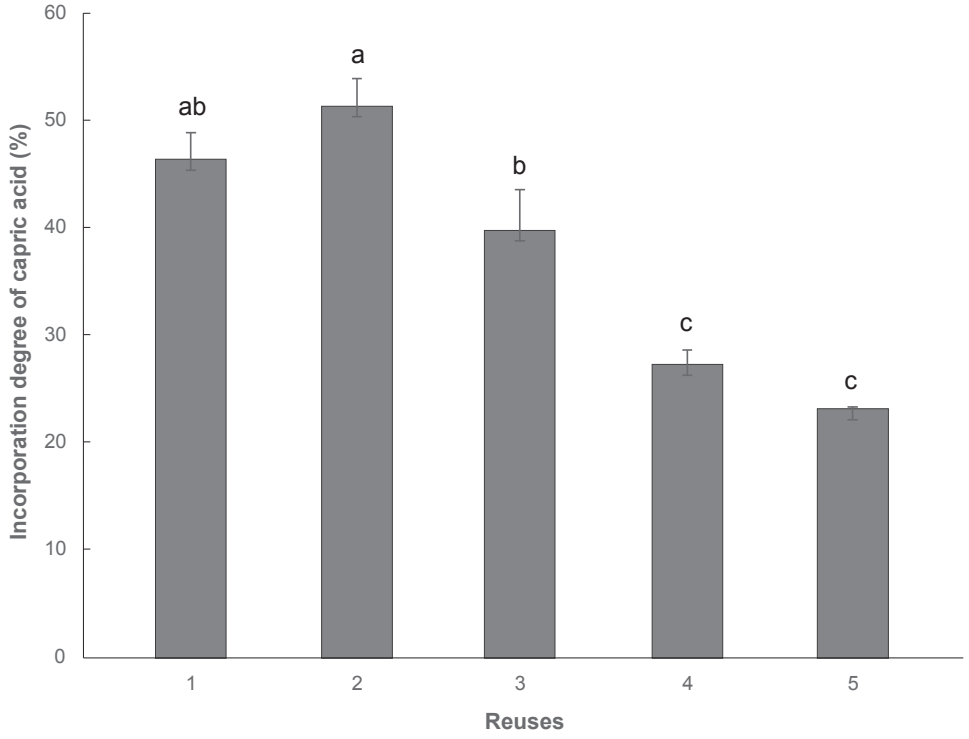

Fig. 1. Incorporation degree of capric acid in grape seed oil after 5 reuses of enzymatic derivative (reaction time of reuse: 24 h). Values are expressed in terms of mean ± SEM, n = 3. Differences between groups were considered significant at $p < 0.05$ and were analyzed using one-way ANOVA followed by the Tukey's mean grouping test and same letter denotes means belonging to the same group.

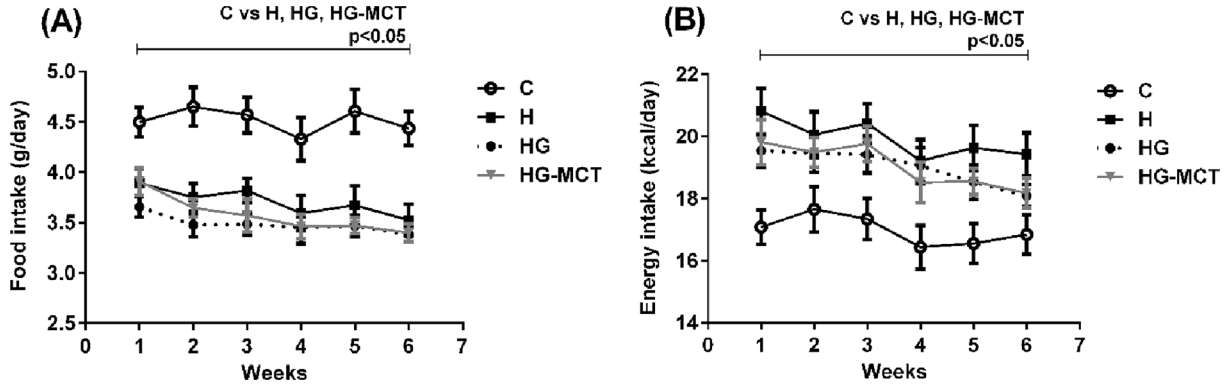

Fig. 2. Food intake (A) and energy intake (B) of mice fed a high-fat diet (HFD) containing modified grape seed oil rich in MCT. Values are expressed in terms of mean ± SEM, n = 10. C: mice fed control diet; H: mice fed HFD; HG: mice fed HFD containing grape seed oil; HG-MCT: mice fed HFD containing grape seed oil rich in MCT. Differences between groups were considered significant at $p < 0.05$ and were analyzed using one-way ANOVA followed by the Student-Newman-Keuls test.

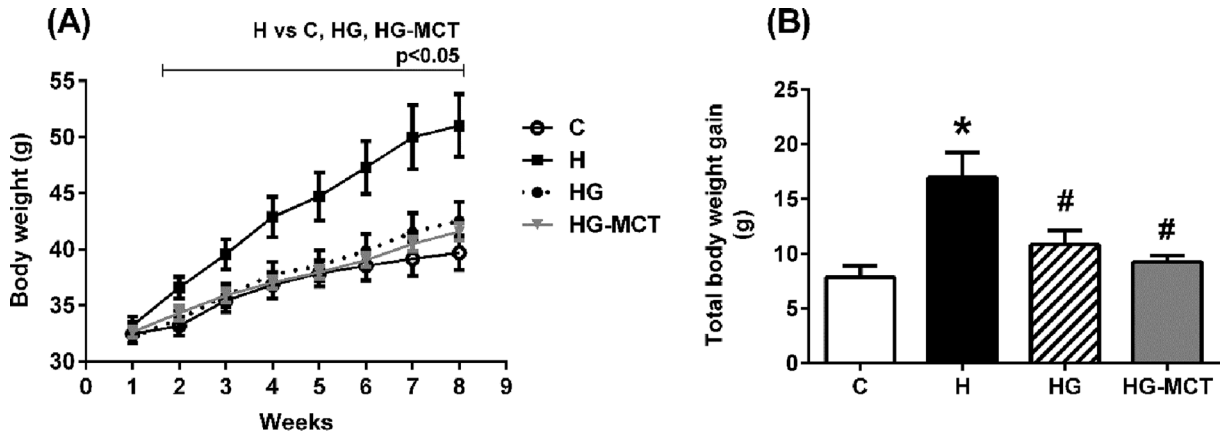

Fig. 3. Body weight (A) and total body weight gain (B) of mice fed a high-fat diet (HFD) containing grape seed oil rich in MCT. Values are expressed in terms of mean ± SEM, n = 10. C: mice fed control diet; H: mice fed HFD; HG: mice fed HFD containing grape seed oil; HG-MCT: mice fed HFD containing grape seed oil rich in MCT. Differences between groups were considered significant at $p < 0.05$ and were analyzed using one-way ANOVA followed by the Student-Newman-Keuls test. *, differences with C; #, differences with H.

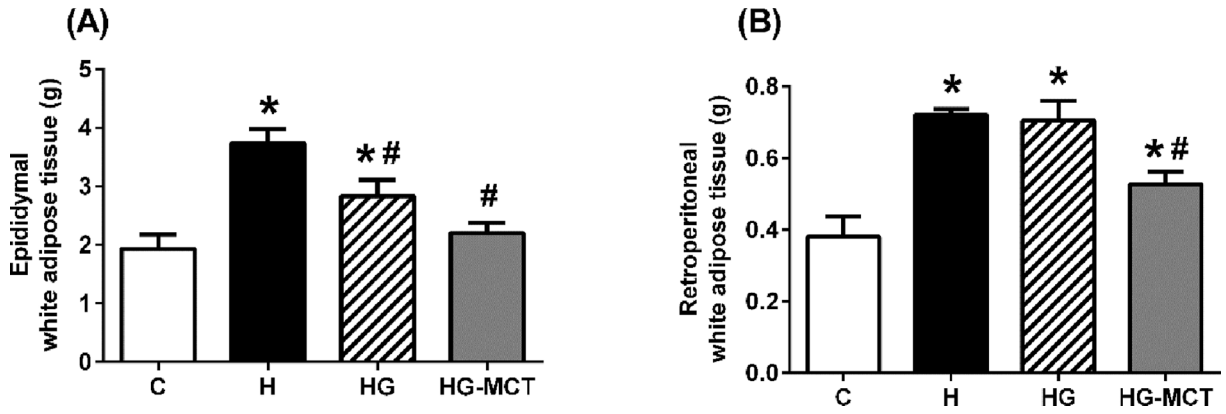

Fig. 4. Weights of epididymal (A) and retroperitoneal (B) adipose tissues of mice fed a high-fat diet (HFD) containing grape seed oil rich in MCT. Values are expressed in terms of mean ± SEM, n = 10. C: mice fed control diet; H: mice fed HFD; HG: mice fed HFD containing grape seed oil; HG-MCT: mice fed HFD containing grape seed oil rich in MCT. Differences between groups were considered significant at $p < 0.05$ and were analyzed using one-way ANOVA followed by the Student-Newman-Keuls test. *, differences with C; #, differences with H.

7.6. Changes in glycemia, lipid profile, and biomarkers of hepatic damage and renal function in obese mice fed with HG or HG-MCT diets

Mice fed HFD (H group) for 8 weeks exhibited higher fasting plasma levels of glucose (72%), total cholesterol (25%), and HDL-cholesterol (17%) than values of mice fed C diet (Table 3). No changes were observed in plasma levels of triglycerides, creatinine, urea, and AST in H mice. Plasma levels of ALT were 44% increased in H mice, suggesting hepatic damage (Table 4).

Mice fed HFD containing grape seed oil or grape seed oil rich in MCT had improvements in various biochemical parameters, with decreases in the plasma levels of glucose, total cholesterol levels and ALT (Table 4). The levels of HDL-cholesterol in HG and HG-MCT mice were similar from those of H mice. No changes were observed in plasma levels of triglycerides, AST, and biomarkers of kidney function (creatinine, urea) of HG and HG-MCT mice in comparison to C and H groups (Table 4).

7.7. Changes in glucose tolerance in obese mice fed with HG or HG-MCT diets

In the glucose tolerance assay, the hyperglycemia peak developed by the H group, 30 min after the glucose overload, was 58% higher than that by the C group. After 120 min, H mice did not correct the glycemia (376 ± 22.4 mg/dL) with the same efficiency as C mice (191 ± 10.2 mg/

Table 4
Plasma biochemical parameters of mice fed a high-fat diet (HFD) containing grape seed oil rich in MCT.

Plasma parameters	C	H	HG	HG-MCT
Glucose (mg/dL)	139 ± 9.86	239 ± 22.56 *	167 ± 7.38 *,#	181 ± 7.33 *,#
Triglycerides (mg/dL)	97 ± 15.99	98 ± 14.28	100 ± 17.01	100 ± 15.93
Total cholesterol (mg/dL)	136 ± 3.32	170 ± 6.00 *	154 ± 4.89 *,#	148 ± 4.19 *,#
HDL-cholesterol (mg/dL)	114 ± 2.81	133 ± 2.77 *	127 ± 2.05[b]	128 ± 2.04[b]
ALT (U/L)	34 ± 1.25	51 ± 3.34 *	41 ± 1.28 *,#	40 ± 0.83 *,#
AST (U/L)	81 ± 7.03	80 ± 9.77	81 ± 9.25	78 ± 6.19
Creatinine (mg/dL)	0.110 ± 0.007	0.115 ± 0.008	0.111 ± 0.007	0.115 ± 0.007
Urea (mg/dL)	42 ± 1.78	41 ± 1.84	42 ± 0.74	42 ± 1.13

Values are expressed in terms of mean ± SEM, n = 9. C: mice fed control diet; H: mice fed HFD; HG: mice fed HFD containing grape seed oil; HG-MCT: mice fed HFD containing grape seed oil rich in MCT. ALT: alanine aminotransferase; AST: aspartate aminotransferase. Differences between groups were considered significant at $p < 0.05$ and were analyzed using one-way ANOVA followed by the Student-Newman-Keuls test.

[*] Differences with C;
[#] Differences with H.

dL) (Fig. 5A). As a result, glucose tolerance was significantly impaired in H mice in comparison to C mice (Fig. 5B).

Mice from HG or HG-MCT groups showed minor values of hyperglycemia peak, 30 min after the glucose overload: glycemia levels were decreased in HG and HG-MCT mice (27% and 26%, respectively) in comparison to H mice, reaching values similar to those of C group. The glycemia correction after 120 min was improved in mice from HG (289 ± 19.9 mg/dL) and HG-MCT (300 ± 30.7 mg/dL) groups (Fig. 5A). Therefore, mice fed a HFD containing grape seed oil or modified grape seed oil rich in MCT had improvements in the glucose tolerance (Fig. 5B).

7.8. Changes in biomarkers of lipid oxidative damage and antioxidant defenses in obese mice fed with HG or HG-MCT diets

Oxidative stress was observed in mice fed a HFD for 8 weeks, since there was an increase of 77% in the plasma levels of TBARS (Fig. 6A). Furthermore, the activity of the antioxidant enzyme PON 1 was 13% decreased in plasma from H mice, in comparison with the activity of this enzyme in C mice (Fig. 6B).

The TBARS levels were decreased in plasma of HG (11%) and HG-MCT (24%) mice compared to the corresponding values in H mice (Fig. 6A), suggesting a decrease in the oxidative stress. In addition, increases in the activity of PON 1 were observed in plasma of HG and HG-MCT mice, whose values were similar from those of C mice (Fig. 6B).

7.9. Ex vivo cytokine production by splenocytes from obese mice fed with HG or HG-MCT diets

Splenocytes of C mice that were not stimulated with LPS released small amounts of IL-6 (pro-inflammatory cytokine) and IL-10 (anti-inflammatory cytokine), but H mice showed a higher production of these cytokines than the other groups ($p < 0.05$). When splenocytes were stimulated with LPS, a high production of IL-6 and IL-10 was observed, however, H mice showed the strongest production, while C mice showed the lowest levels of both IL-6 and IL-10 compared with the other groups ($p < 0.05$) (Fig. 7).

For IL-6, a significant decrease ($p < 0.05$) was observed in the production of this cytokine by splenocytes from HG and HG-MCT mice, when compared to the corresponding values of H mice, in both stimulated and unstimulated conditions. In addition, differences were also observed between stimulated HG-MCT group *versus* unstimulated HG and HG-MCT groups. For IL-10, it was observed that the production of this cytokine was decreased by stimulated splenocytes of HG and HG-MCT mice, in comparison to H group. The IL-10 production by splenocytes of HG-MCT mice were not different from that of C mice, in both unstimulated and stimulated conditions (Fig. 7).

8. Discussion

The determination of acid and peroxide values in oils are of great importance, from these parameters an oil can be considered suitable to be used in food or as a material for the generation of other products. The acidity index shows the amount of free fatty acids in the oil. Pardo et al. (2009) reported an acid value of 0.37 mg KO/g, which was close to the observed in the present study. The determination of the peroxide index assesses how much the unsaturated fatty acids are with their broken double bonds. Dalmolin et al. (2010), studying the behavior of grape seed oil in the presence of dioxide, carbon and ethanol, they found a peroxide index of 5 mEq O_2/kg of oil, a result higher than the obtained in the present study. The results of Dalmolin et al. (2010) are within the specified by current legislation, showing that grape seed oil has good oxidative stability; this oil is rich in antioxidant compounds, including tocopherol and tocotrienol (Dalmolin et al., 2010; Ismail, Salem, & Eassawy, 2016; Lutterodt et al., 2011). The quantification of iodine index can indicate the amount of unsaturated fatty acids; the higher this index, the greater the amount of fatty acids with double bonds in the molecule. Haro et al. (2016) studied the production of epoxidized grape seed oil, obtained an iodine index of 141.52 g I_2/100 g, a result slightly higher than that found in the present study. Lastly, the saponification index determination evaluates the amount of low molecular weight fatty acids present in fat oils. Yousefi et al. (2013) performed oil characterization of grape species, obtaining a saponification index of 187.50 mg KOH/g in the cultivar oil of *Khalili Shahrodi*, a result close to that obtained in the present study.

In the study, the covalent immobilization of the lipase was achieved in a low-cost material with 5 effective reuses, which generates low costs in the process of dietary triglycerides synthesis, thus favoring its possible industrial application. An average capric acid incorporation of 38.8% was obtained once the volume of reuses was collected, which compared to other studies show promising results. *Rhizomucor miehei* lipase immobilized on an anion exchange resin, *Thermomyces lanuginose* lipase immobilized in a silica granulate, *Candida antarctica* lipase immobilized on an acrylic resin with macropores, recombinant lipase from *C. antarctica* expressed in *Aspergillus niger* and immobilized in Lewatit have been reported, indicating incorporations of caprylic acid of 20.14 ± 0.8%, 17.08 ± 0.84%, 12.43 ± 0.75% and 11.97 ± 0.9%, respectively (Abed et al., 2017). Other studies reported 48.7% for lipase from

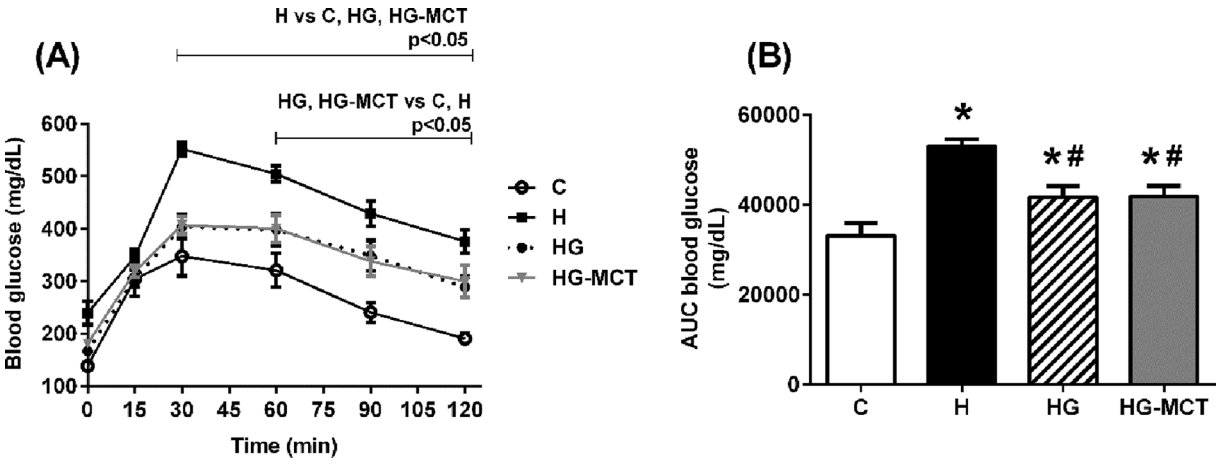

Fig. 5. Oral glucose tolerance test (OGTT) (A) and area under the curve (AUC) of OGTT (B) of mice fed a high-fat diet (HFD) containing grape seed oil rich in MCT. Values are expressed in terms of mean ± SEM, n = 10. C: mice fed control diet; H: mice fed HFD; HG: mice fed HFD containing grape seed oil; HG-MCT: mice fed HFD containing grape seed oil rich in MCT. Differences between groups were considered significant at $p < 0.05$ and were analyzed using one-way ANOVA followed by the Student-Newman-Keuls test. *, differences with C; #, differences with H.

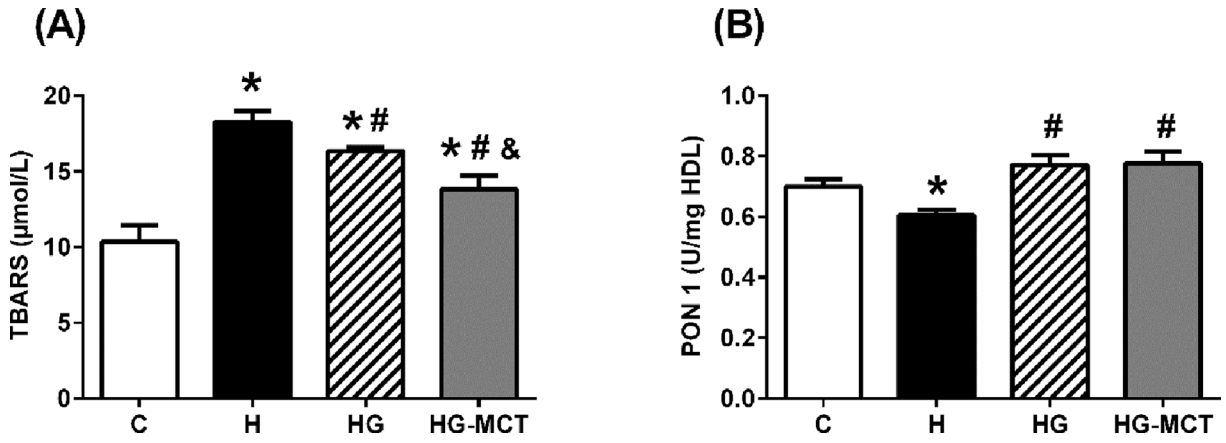

Fig. 6. Levels of TBARS (A) and activity of PON 1 (B) in plasma of mice fed a high-fat diet (HFD) containing grape seed oil rich in MCT. Values are expressed in terms of mean ± SEM, n = 10. C: mice fed control diet; H: mice fed HFD; HG: mice fed HFD containing grape seed oil; HG-MCT: mice fed HFD containing grape seed oil rich in MCT. Differences between groups were considered significant at $p < 0.05$ and were analyzed using one-way ANOVA followed by the Student-Newman-Keuls test. *, differences with C; #, differences with H; &, differences with HG.

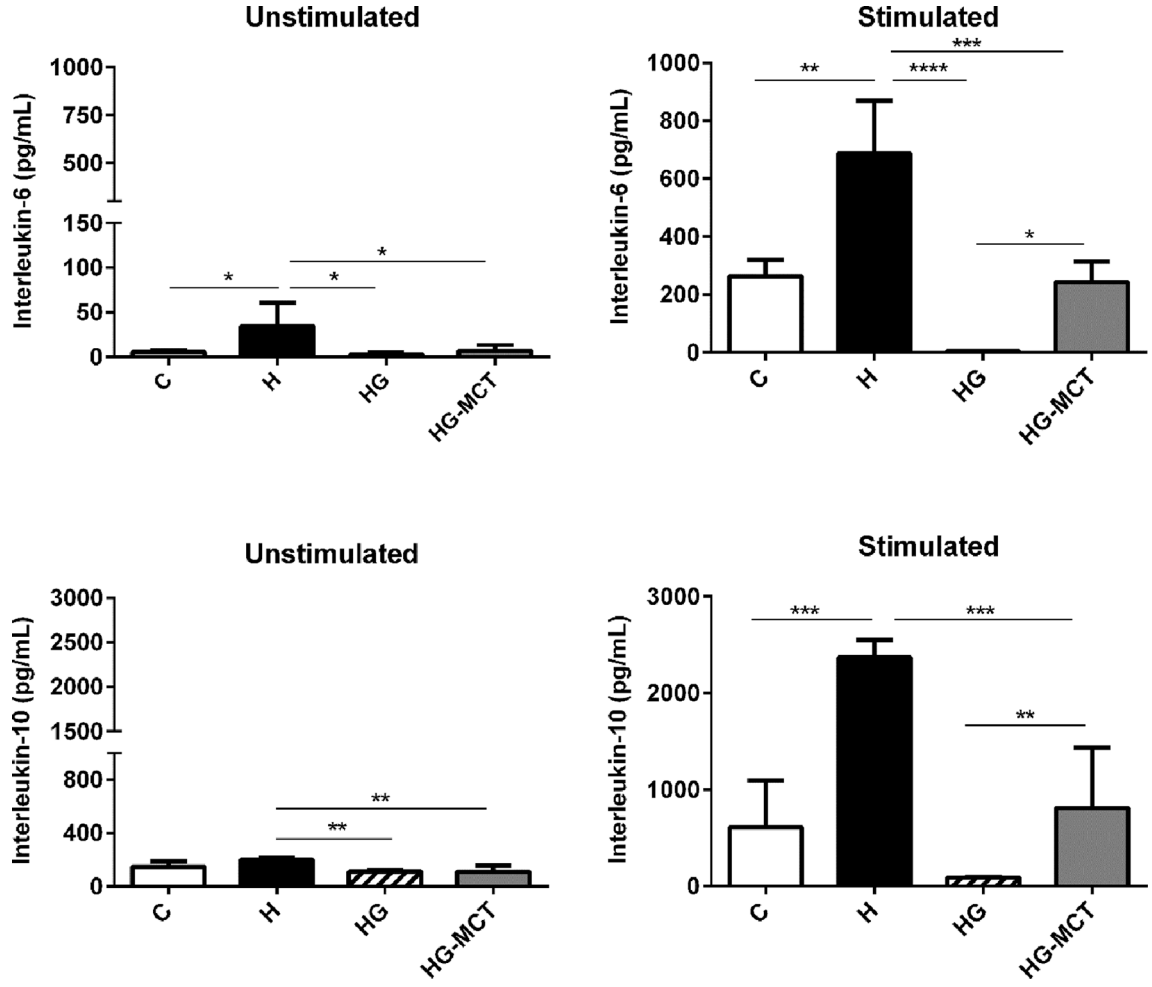

Fig. 7. Levels of IL-6 and IL-10 in unstimulated and LPS-stimulated splenocytes of mice fed a high-fat diet (HFD) containing grape seed oil rich in MCT.Values are expressed in terms of mean ± SEM, n = 10. C: mice fed control diet; H: mice fed HFD; HG: mice fed HFD containing grape seed oil; HG-MCT: mice fed HFD containing grape seed oil rich in MCT. Differences between groups were analyzed using one-way ANOVA followed by the Student-Newman-Keuls test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

R. miehei and 47.5% for lipase from *Pichia lynferdii* (Kim et al., 2010). In the case of *Rhizopus oryzae* lipase, incorporation of 31.4% was reported (Nunes et al., 2011).

According to the results of chemical characterization, the acidolysis reaction promoted changes in the compositional profile of fatty acids. Comparing the fatty acid composition of oil and acidified products, it

was observed decreases in the percentage of palmitic (C16:0), stearic (C18:0), oleic (C18:1) and linoleic (C18:2) acids. These decreases are related to the incorporation of medium chain acids (C10:0), since this fatty acid was quantified only in the product, not being present in the raw materials. Regarding other fatty acids, little or no variation was observed. Likewise, some fatty acids were not identified. In addition, the oil showed high amounts of unsaturated fatty acids, such as oleic acid (C18:1), and the high amounts of essential linoleic acid (C18:2). Due to the presence of high amounts of these fatty acids and also due to the high amount of tocopherol, the use of grape seed oil has been applied in different industrial sectors, including food and cosmetics industries. In addition to having attractive chemical characteristics, this oil has a smooth taste and pleasant aroma, making its applicability even more effective (Bail et al., 2008; Mohamed et al., 2016; Glampedaki & Dutschk, 2014; Malićanin et al., 2014; Passos, Silva, Da Silva, Coimbra, & Silva, 2010).

Studies have reported that the amounts of oleic acid in grape seed oil can vary from 10 to 22% and linoleic acid from 60 to 75% (Irandoost, Ebrahimi-Mameghani, & Pirouzpanah, 2013; Mironeasa et al., 2010; Rombaut et al., 2014). According to our results, the grape seed oil used in this study presented values of oleic acid (26.57%) and linoleic (46.25%) close to those reported in the literature. This variation can be explained by several factors, including the type of plant cultivar, climate change, cultivated region, soil, among others (Duba & Fiori, 2015; Fiori et al., 2014). In addition, the oil used in this study was commercially purchased in food grade.

Decreases were observed in the percentage of long-chain unsaturated fatty acids (51%), which were related to the incorporation of medium chain fatty acids (C10:0), having 42.07% of capric acid in the *sn*-2 position. This result was expected due to the catalyst used, which was *sn*-1,3 regioselective, showing that lipase from *Rhizopus oryzae* immobilized on the low-cost support is a suitable biocatalyst for acidolysis synthesis of triglycerides with MLM configuration.

For grape seed oil, incorporation degree of capric acid ranging from 23.38 to 54%. Collecting all volumes of reuse, an average ID of 38.8% was obtained. This value was higher than those described (28.8% and 30.4%) by Nunes et al. (2010). It should be noted that the enzyme immobilized in this study was reused up to 5 times. The ID of the capric acid (C10) is possibly due to the fact that it has a longer "carbon" chain when compared to low molecular weights of fatty acids. The literature reports that the specificity of this lipase is directly related to the size of the fatty acid chain, and the lipase has a preference for fatty acids with a high carbon chain (Caballero, Soto, Olivares, & Altamirano, 2014; Rodrigues & Fernandez-Lafuente, 2010b, 2010a).

The offering of HFD to mice for 8 weeks promoted obesity; H mice had increases in the body weight gain and weights of adiposes tissues. H mice also had other changes typical of metabolic syndrome, including high plasma levels of glucose and cholesterol, and glucose intolerance. It is well known that white adipose tissue expansion leads to macrophages infiltration and overproduction of proinflammatory cytokines (Han & Levings, 2013). Adipose tissue inflammation exerts a major role in the development of insulin resistance during obesity, which may explain, at least partially, the glucose intolerance in H mice. The increased plasma levels of ALT in H mice suggested hepatic damage. Obesity is a risk factor for various hepatic diseases, including non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, and cirrhosis (Sun & Karin, 2012). Lastly, H mice exhibited changes related to the onset of oxidative stress, including the increase in plasma levels of lipid peroxidation biomarkers (TBARS) and decreases in PON 1 activity, as previously observed in obese mice (Costa et al., 2020). PON 1 is a key functional constituent of HDL, being responsible for the antioxidant activity of this lipoprotein. PON 1 has the ability to hydrolyze lipid peroxides into lipoproteins, mainly low-density lipoprotein (LDL), delaying LDL oxidation and the development of atherosclerosis (Levy, Reichert, & Bydlowski, 2019). Reductions in PON 1 activity are described in various metabolic dysfunctions, including obesity (Cervellati et al., 2018). Glycation of PON 1 has been associated with decreases in its activity (Yu et al., 2017); thus, the increased plasma levels of glucose found in H mice may explain, at least partially, the fall in PON 1 activity.

One main finding of this study was the antiobesogenic effects of HFD with half the lipid content consisting of grape seed oil (HG) or capric acid-containing SLs produced from grape seed oil (HG-MCT). Mice from HG and HG-MCT groups had decreases in body weight gain, which were accompanied by reductions in the weights of epididymal (HG and HG-MCT groups) and retroperitoneal (HG-MCT group) adipose tissues. Interestingly, the minor values of body weight gain and minor body fat accumulation in mice fed HG or HG-MCT diets were not accompanied by changes in food or energy intakes, whose values were similar between H, HG and HG-MCT groups. In agreement with our findings, (Zhou et al., 2017) found that HFD containing SLs with various contents (10%, 20%, 30%; w/w) of medium-chain fatty acids (C8:0 and C10:0) caused reductions in the body weight gain of mice without changes in food consumption. Therefore, it can be suggested that these oils in HG or HG-MCT diets may have stimulating the whole body energy expenditure. Zhang et al. (2015) found that obese mice fed HFD containing 2% medium-chain triglyceride (mainly C8:0 and C10:0) had activation of thermogenesis in brown adipose tissue (BAT), leading to the reduction in body weight and weights of white adipose tissues. It can be inferred that the minor body weight values of mice fed HFD rich in capric acid-containing SLs (HG-MCT group) may have been a consequence, at least partially, of BAT thermogenesis activation. However, it can not be discharged the possibility that other components of grape seed oil may have contributing to the antiobesogenic effects, since HG mice also had minor values of body weight gain and adipose tissues. Grape seed oil is rich in unsaturated fatty acids, mainly linoleic (C18:2) and oleic (C18:1) acids. Studies have shown that linoleic acid-rich fats do not reduce the body weight gain in mice under experimental models of metabolic disturbances (Sato et al., 2005; Vaughan et al., 2015). On the other hand, it has been observed reductions in the body weight gain of mice fed diets containing oleic acid-rich oils (Deol et al., 2017; Oliveira et al., 2015).

Mice fed HG and HG-MCT diets had improvements in glucose tolerance, which may be attributed, at least partially, to the effects of these experimental diets on decreasing the body weight gain. However, in addition to the antiobesogenic effects, the improvements in the glucose tolerance may also be related to other mechanisms related to components found in these oils. Geng et al. (2016) observed that mice fed a HFD containing MCT (composed by caprylic and capric acids) had low levels of fasting glycemia and improvements in glucose tolerance and insulin sensitivity. Authors attributed these benefits to the anti-inflammatory effects of MCTs, since they downregulated the expression of IL-6 and other biomarkers of inflammation. These findings are consistent with our data on HG-MCT mice, since they had low IL-6 production by splenocytes. Glucose tolerance was also improved in mice fed HG diet. There is evidence that the consumption of grape seed oil improves glucose homeostasis in obese mice (Mahanna et al., 2019) and reduces inflammation and insulin resistance in obese women (Irandoost et al., 2013); these benefits have been attributed to the presence of phenolic compounds. Lastly, in addition to improve glucose tolerance, HG and HG-MCT diets also decreased the plasma levels of cholesterol. The hypocholesterolemic effects of HFD containing MCT have been previously observed (Sengupta & Ghosh, 2011; Zhou et al., 2017).

HG and HG-MCT diets also have remarkable antioxidant properties. The consumption of these diets decreased the plasma levels of TBARS, biomarkers of lipid peroxidation. The antioxidant properties of grape seed oils can be attributed to the presence of tocopherols, tocotrienols (Garavaglia, Markoski, Oliveira, & Marcadenti, 2016) and phenolic compounds (Shinagawa, de Santana, Torres, & Mancini-Filho, 2015), which may be helpful to explain the low TBARS levels in mice fed HG diet. However, it is interesting to note that the levels of TBARS in mice fed HG-MCT diet were further decreased in comparison to mice fed HG diet. There is evidence that supplementation with capric acid (Lee &

Kang, 2017) or capric acid-enriched oil (Sengupta & Ghosh, 2011) alleviated oxidative stress *in vivo* by increasing the expression of antioxidant enzymes. Thus, the synergistic antioxidant effect of HG-MCT diet may be related to the following combination: (*i*) the antioxidant compounds found in grape seed oil, and (*ii*) the ability of capric acid to stimulate the endogenous antioxidant defenses, thus promoting a further decrease in TBARS levels. Furthermore, HG and HG-MCT diets mitigate oxidative stress in mice by increasing PON 1 activity. It can be proposed that the low glycemia levels in HG and HG-MCT mice may have reduced the glycation of PON 1, restoring its activity, which can be contributing to decrease the TBARS levels. To the best of our knowledge, this is the first study to provide evidence about PON 1 activation in obese mice after the consumption of diets enriched with grape seed oil or capric acid-containing SLs. Lastly, since HG-MCT diet restored PON 1 activity and significantly decreased the plasma levels of TBARS and cholesterol, it can be proposed that the consumption of capric acid-containing SLs produced from grape seed oil appears as an interesting dietary option against obesity complications, especially cardiovascular diseases.

Low-intensity chronic inflammatory process is observed in obesity. Local inflammation around dying adipocytes exhibit a microenvironment for macrophage proliferation and release of inflammatory mediators, which affects systemic metabolic processes where IL-6 is a key component (Braune et al., 2017). Despite the main source of inflammatory mediators are arisen in adipose tissues, the state of systemic inflammation affects a complex network where different components of the immune system (Ellulu et al., 2017; Rodríguez, González, Aguilar-Salinas, & Nájera-Medina, 2018). Direct associations between different measures of obesity and plasma IL-6 levels have been described (Fontana et al., 2007; Straub et al., 2000). On the other hand, IL-10 is an anti-inflammatory cytokine being a major inhibitor of cytokine synthesis by suppressing macrophage function and inhibits the production of proinflammatory cytokines (Minton, 2017). Circulating levels of IL-10 are elevated in obese women while low IL −10 levels are associated with metabolic syndrome (Esposito et al., 2003).

In the present study, the H diet promoted a high production of IL-6 in both unstimulated and LPS-stimulated splenocytes, but the levels of IL-10 were only elevated in stimulated splenocytes. However, in mice fed HG or HG-MCT diets, a significant reduction of both IL-6 and IL-10 was observed, as clear evidence of their effects on reducing the inflammatory state associated with obesity, although the anti-inflammatory effect was stronger in mice fed HG diet than those fed HG-MCT diet. If there is no inflammatory reaction, the compensatory mechanisms mediated by IL-10 and other suppressor mechanisms are not stimulated (Minton, 2017). Accordingly, the results observed in the IL-6/IL-10 balance reinforce the hypothesis of the beneficial effects of HG-MCT diet in the control and reduction of obesity-associated inflammation.

Lastly, the possibility that the benefits found in mice from HG and HG-MCT groups are ocuring due to the 50% decrease in the lard content cannot be ruled out. In order to verify the direct benefits of grape seed oil or grape seed oil rich in MCTs, it would be important to investigate their impacts on mice fed low-fat diets, which becomes a future perspective of this study. In addition, evidence indicate that the *in vivo* effects of structured lipids can be different when offered on low-fat or high-fat diets. Investigating the antiobesogenic effects of a structured lipid with behenic acid obtained by interesterification of a mixture of oils, Moreira and collaborators (Taketa Moreira, Ract, Ribeiro, & Macedo, 2017) fed C57B/l6 mice with a low-fat diet rich in structured lipids (replacing 100% soybean oil) or HFD rich in structured lipids (replacing 100% lard). The authors found that the impacts of the structured lipid in the lipid profile were diferent in animals fed a low-fat diet *versus* HFD; while mice fed a HFD rich in structured lipids had decreased plasma levels of total and LDL-cholesterol and increased HDL-cholesterol levels, mice fed a low-fat diet rich in structured lipids had increased plasma levels of LDL-cholesterol and low HDL-cholesterol levels.

9. Conclusion

In summary, it was possible to obtain by enzymatic acidolysis SLs of the MLM-type based on grape seed oil and capric acid using an immobilized enzyme in a low-cost material with 5 reuses of the enzymatic derivative and a degree of incorporation with promising results. In addition, the consumption of HFD enriched with capric acid-containing SLs produced by enzymatic acidolysis from grape seed oil had anti-obesogenic effects, improved glucose tolerance and decreased dyslipidemia. Furthermore, the consumption of diet enriched with capric acid-containing SLs decreased biomarkers of lipid peroxidation and increased PON 1 activity, thus emerging as a functional oil capable to mitigate the obesity complications resulting from oxidative stress and inflammation.

Declaration of Competing Interest

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

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