FLÁVIA GALVÃO CÂNDIDO

EFFECT OF COCONUT, OLIVE, AND SOYBEAN OIL ON ENDOTOXEMIA, INFLAMMATION, BODY COMPOSITION, AND METABOLIC STATUS RELATED TO OBESITY

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Ciência da Nutrição, para obtenção do título de *Doctor Scientiae*.

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Rita de Cássia Gonçalves Alfenas
(orientadora)

Ao Deus que tudo pode e tudo vê, NADA é impossível!

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ABREVIATURAS

ALP	Alkaline phosphatase
ALT	Alanine amino transferase
AST	Aspartate amino transferase
BMI	Body mass index
CB1	Cannabinoid receptor
CI	Conicity index
CLA	Conjugated linoleic acid
CRP	C-reactive protein
CVD	Cardiovascular diseases
DHA	Docosahexaenoic acid
DPPH:	1,1-Diphenyl-2-picrylhydrazyl
DEXA	Dual energy X-ray absorptiometry
ECs	Endocannabinoid system
EPA	Eicosapentaenoic acid
EVOO	Extra virgin olive oil
FIAF	Fasting Induced Adipocyte Factor
FID	Flame ionization detector
GC	Gas Chromatography
GLP	Glucagon-like peptide
GPR	G-protein-coupled receptors
GT	Glutamyltransferase
HDL-c	High-density-lipoprotein cholesterol
HOMA-IR	Homeostasis model assessment of insulin resistance
HPLC	High-performance liquid chromatography
IL	Interleukin
LCFA	Long chain fatty acids
LDL-c	Low-density lipoprotein cholesterol
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
MCFA	Medium chain fatty acids
MCT	Medium-chain triacylglycerols
MUFA	Monounsaturated fatty acids

PUFA	Polyunsaturated fatty acids
PYY	Peptide YY
SCFA	Short-chain fatty acids
SD	Standard deviation
SE	Standard error
SFA	Saturated fatty acids
TG	Triglycerides
TH1	T helper type 1
TLR4	Toll-like receptor-4
TNF	Tumor necrosis factor
UFA	Unsaturated fatty acids

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pressure (E) changes (9 week values – baseline values). Energy restricted nutritionally balanced diets (-2090 kJ/d) containing 25 mL of soybean oil (control group, n = 20) or extra-virgin olive oil – EVOO (EVOO group, n = 21) were prescribed. *Within-group significant differences (paired Student's t-test or Wilcoxon signed-rank test, $P < 0.05$). P Inter values indicated between-groups differences (Student's t-test or Mann-Whitney's test, §$P < 0.050$ was considered significant).

Fig. 3 Mean ± SE interleukin-12p70 - IL-12p70 (A), tumor necrosis factor-α - TNF-α (B), interleukin-10 - IL-10 (C), interleukin-6 – IL-6 (D), interleukin-1β - IL-1β (E), and interleukin-8 – IL-8 (F) concentrations changes (9 week values – baseline values). Energy restricted nutritionally balanced diets (-2090 kJ/d) containing 25 mL of soybean oil (control group, n = 17) or extra-virgin olive oil – EVOO (EVOO group, n = 19) were prescribed. *Within-group significant differences (paired Student's t-test, $P < 0.05$). **Trend to significant differences (paired Student's t-test, $P = 0.060$). P Inter values indicate between-groups differences (Student's t-test or Mann-Whitney's test). 77

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RESUMO

CÂNDIDO, Flávia Galvão, Dr. Universidade Federal de Viçosa, novembro de 2016. **Effect of coconut, olive, and soybean oil on endotoxemia, inflammation, body composition, and metabolic status related to obesity.** Orientador: Rita de Cássia Gonçalves Alfenas. Coorientadores: Maria do Carmo Gouveia Peluzio, Hércia Stampini Duarte Martino, Hilário Cuquetto Mantovani e Ana Paula Boroni Moreira.

Objetivo: Avaliar o efeito da ingestão de diferentes fontes lipídicas sobre a translocação de lipopolissacarídeos (LPS), a inflamação subclínica, a composição corporal e o estado metabólico em mulheres com excesso de peso corporal. Materiais e métodos. Artigo original 1: trata-se de um estudo randomizado de braços paralelos, que envolveu 78 mulheres com excesso de gordura corporal (idade entre 20 a 41 anos, média ± erro padrão de 47,23 ± 0,48% de gordura corporal) as quais foram alocadas em grupos distintos e consumiram uma bebida contendo 25mL de um dos três tipos de lipídeos testados: óleo de coco (OC, n = 23), azeite de oliva extra virgem (AOEV, n = 31) ou óleo de soja (OS, n = 24). As mulheres chegaram ao laboratório em jejum (12h) e amostras sanguíneas foram coletadas no nível basal e após duas e quatro horas do início do consumo das bebidas. Foram feitas análises das concentrações de triglicerídeos, LPS a das citocinas IL-8, IL-1β, IL-6, IL-10, TNF-α e IL-12p70. Artigo original 2: trata-se de um estudo randomizado, duplo-cego, controlado por placebo, no qual 41 mulheres adultas com excesso de peso corporal (19 a 40 anos, 46,8 ± 0,6% de gordura corporal) receberam diariamente em laboratório desjejuns contendo 25mL de OS (grupo controle, n = 20) ou AOEV (n = 21) durante nove semanas consecutivas. Detas hipocalóricas (-2090kJ, ~32%E) foram prescritas. As avaliações antropométricas, de composição corporal e sanguíneas foram feitas no primeiro e no último dia de intervenção em jejum. Resultados. Artigo original 1: as concentrações de LPS não foram afetadas durante a intervenção. OS aumentou mais a trigliceridemia e IL-8 do que o AOEV. O OC foi o único que aumentou a razão IL-1β/IL-10 e as alterações nas concentrações de LPS se associaram positivamente com um perfil inflamatório somente no grupo OC. Artigo original 2: AOEV reduziu a pressão arterial diastólica e aumentou a perda de gordura corporal em ~80% em relação ao grupo controle. Houve redução do colesterol HDL e aumento de IL-10 no grupo controle, enquanto o grupo AOEV aumentou a creatinina sérica e reduziu a fosfatase alcalina. Conclusões: O consumo de doses usuais de diferentes lipídeos influencia a inflamação pós-prandial sem alterar as concentrações sanguíneas de LPS. Alterações prejudiciais foram observadas após o consumo do OS e do OC, sendo que as análises de correlação apontaram a existência de um mecanismo sinérgico entre o

consumo do óleo de coco e a manifestação de inflamação sistêmica induzida pelo LPS. Uma vez que o AOEV aumentou a perda de gordura e reduziu a pressão sanguínea, seu consumo deve ser estimulado em programas de emagrecimento.

ABSTRACT

CÂNDIDO, Flávia Galvão, PhD. Universidade Federal de Viçosa, November, 2016. **Effect of coconut, olive, and soybean oil on endotoxemia, inflammation, body composition, and metabolic status related to obesity.** Adviser: Rita de Cássia Gonçalves Alfenas. Co-advisers: Maria do Carmo Gouveia Peluzio, Hércia Stampini Duarte Martino, Hilário Cuquetto Mantovani and Ana Paula Boroni Moreira.

Objectives: To evaluate the effects of different fat types on lipopolysaccharides (LPS) translocation, systemic low-grade inflammation, body composition, and metabolic status in woman with excess body fat. Materials and methods. Original article 1: This is a randomized parallel arm study in which 78 excess body fat woman (aged 20 to 41y, mean ± standard error of 47.23 ± 0.48% of total body fat) were allocated to receive a drink containing 25mL of one of the three tested oils: coconut oil (CO, n = 23), extra-virgin olive oil (EVOO, n = 31), or soybean oil (SO, n = 24). Participants reported to the laboratory in a fasting state (12h). Blood samples were taken at baseline and 2 and 4h after starting one of the drinks. Triglycerides, LPS, and the citokines IL-8, IL-1β, IL-6, IL-10, TNF-α, and IL-12p70 concentrations were assessed. Original article 2: This is a randomized, double-blind, placebo-controlled clinical trial in which 41 excess body fat woman (aged 19 to 40y, 46.8 ± 0.6% of total body fat) consumed breakfasts containing 25mL of SO (control group, n = 20) or EVOO (n = 21) daily in the laboratory during nine consecutive weeks. Energy-restricted diets (-2090kJ, ~32%E) were prescribed. Anthropometric, body composition, blood pressure, and biochemical assessments were conducted in fasting state in the first and last day of experiment. Results. Original article 1: LPS concentrations were not affected by intervention. SO increased more triglicerydes and IL-8 than EVOO. CO was the only group that presented increase in IL-1β/IL-10 ratio. Changes in LPS were positively associated with pro-inflammatory profile only in CO. Original article 2: EVOO reduced diastolic blood pressure and increased total body fat loss in ~80% when compared to control group. There were a decrease in HDL-c and an increase in IL-10 in control group, while EVOO increased serum creatinine and reduced alkaline phosphatase (P between-groups > 0.050). Conclusions: The consumption of reasonable doses of distinct dietary fats influences postprandial systemic inflammation without changing plasma LPS concentrations. Detrimental changes were observed after consumption of SO and CO and correlation analyses suggested a synergic mechanism between CO and LPS-induced inflammation. Since EVOO contributed to improve fat loss and blood pressure, EVOO consumption should be stimulated in weight-loss programs.

SUMÁRIO

1. INTRODUCTION

Obesity is one of the most prevalent non-communicable diseases worldwide (HEREDIA et al., 2012). It represents a serious public health concern due to its association with arterial hypertension (DORRESTEIJN et al., 2012), type 2 diabetes (ABDULLAH et al., 2010), cardiovascular diseases (ABBASI et al., 2013), and certain types of cancer (HARVEY et al., 2011). Obesity has an inflammatory component which is considered the link between obesity and the aforementioned associated diseases (HARVEY et al., 2011; WANG; NAKAYAMA, 2010; DONATH; SHOELSON, 2011). Thus, there is great scientific interest on the identification of strategies capable to control inflammation (TABAS; GLASS, 2013).

It has been suggested that gut microbiota plays a role in obesity pathogenesis (TURNBAUGH, 2006; LEY, 2005; LEY, 2006; TURNBAUGH, 2009), in part, by favoring systemic low grade inflammation (BLAUT; KLAUS, 2012). The contribution of bacteria from different phyla was assessed by a metagenomic study (TURNBAUGH, 2009). Results showed that 75% of obesity-related genes belonged to the phylum *Actinobatéria* and 25% to *Firmicutes*, while 42% thinness-related genes belonged to *Bacteroidetes*. Although there is no consensus about the dynamics of microbial in the feces of obese versus eutrophic individuals (as well explored in a recent review TAGLIABUE; ELLI et al., 2013), it is recognized that gut microbiota present in obese individuals may favor intestinal permeability increase and, therefore, contribute to increased systemic endotoxins concentrations (BRUN et al., 2007). Lipopolysaccharides - LPS are the main representative of endotoxins. They derive from the outer cellular membrane of gram-negative bacteria present in the intestinal lumen, and are usually present in low concentrations in the blood of healthy individuals. When their concentration increase, they cause low-grade inflammation, insulin resistance, adipocyte hyperplasia, and impaired beta-pancreatic cell function. These changes in obesity characterize a phenomenon called metabolic endotoxemia (KRAJMALNIK-BROWN et al., 2012).

Diet composition can change gut microbiota composition and metabolic endotoxemia (FLINT, 2012; CLAESSON et al., 2012). The role of dietary fat content and types has been gaining prominence in the scientific community because they may induct obesity by affecting gut microbiota (HILDEBRANDT et al., 2009; LAUGERETTE et al., 2011; MOREIRA et al., 2012; SHI et al., 2006; SUGANAMI et al., 2007a, b). In addition to modifying the gut microbiota composition, dietary fats can alter the rates of LPS uptake

during their absorption process and certain types of fat may decrease systemic inflammation directly or by their antioxidant compounds (SENEVIRATNE; DISSANAYAKE, 2008; MARINA et al., 2009; PARFENE et al., 2013; HUANG et al., 2011; SADO-KAMDEM et al., 2008; SADO-KAMDEM et al., 2009; NOBMANN et al., 2009).

Vegetable oils are sources of fatty acids that exhibit high antimicrobial activity (PARFENE et al., 2013). Coconut oil and extra-virgin olive oil (EVOO) are recommended for the control of obesity and associated diseases. However, these oils have a very different composition (ASSUNÇÃO et al., 2009; PÉREZ-MARTÍNEZ et al., 2011). Coconut oil has about 90% saturated fatty acids and more than 60% of its content of medium chain fatty acids (MARINA et al., 2009). The presence of phenolic compounds and medium chain fatty acids are associated with a good blood lipid profile, an increase in fat oxidation rate, and a decrease in fat deposition in adipose tissue especially the abdominal one (ST-ONGE et al. 2003; ASSUNÇÃO et al., 2009). In turn, EVOO is recognized for its high content of long-chain monounsaturated fatty acids and phenolic antioxidant compounds (FRANKEL, 2011). Its properties are mainly linked to the improvement of lipid profile, inflammatory and oxidative processes, and endothelial injury (LÓPEZ-MIRANDA et al., 2008; PÉREZ-MARTÍNEZ et al., 2011).

The antimicrobial activities of coconut oil and EVOO have been evaluated in studies aimed the elimination of specific pathogens present in foods or the increase in shelf life of food products (PARFENE et al., 2013; HUANG et al., 2011; SADO-KAMDEM et al., 2008; SADO-KAMDEM et al., 2009; NOBMANN et al., 2009). Nevertheless, to the best of our knowledge, their role over obesity-induced dysbiosis has not been investigated yet. Furthermore, the need of studies assessing the role of dietary fat types on metabolic endotoxemia and obesity was recently highlighted (BUCKLAND; GONZALEZ, 2015; MICHALSKI et al., 2016).

2.OBJECTIVES

2.1 General objective

To evaluate the effects of different fat types on LPS translocation, systemic low-grade inflammation, body composition, and metabolic status in women with excess body fat.

2.2 Specific objectives

- To perform a critical review of the current literature about the effects of dietary lipid consumption on the composition/activity of the gut microbiota, metabolic endotoxemia, and obesity;
- To evaluate the effects of acute consumption of coconut oil, EVOO, and soybean oil on plasma LPS and cytokines concentrations in excess body fat women;
- To evaluate the effects of chronic consumption of EVOO on anthropometry, body composition, and metabolic markers in excess body fat women.

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4. REVIEW ARTICLE – Impact of dietary fat on gut microbiota and low-grade systemic inflammation: mechanisms and clinical implications on obesity

Artigo a ser submetido à revista *Critical Reviews in Food Science and Nutrition*

4.1 ABSTRACT

Background/Objective: Dietary fat strongly affects human health by modulating gut microbiota composition and systemic inflammation. However, this relationship has been neglected. In this manuscript, we highlight the most important recent advances linking high-fat diets and different fatty acids versus changes in gut microbiota and obesity, explore possible mechanisms for these effects, and examine the implications of probiotics administration in reversing high-fat diet dysbiosis.

Methods: Studies published from over the past 10 years exploring human and animal data regarding the effects of fat consumption on obese-induced dysbiosis and low-grade systemic inflammation.

Results: High-fat diets have been implicated in reduced gut microbiota richness, increased *Firmicutes* to *Bacteroidetes* ratio, and several changes at family, genus, and species levels. Saturated (SFA), monounsaturated (MUFA), polyunsaturades (PUFA), and conjungated linolenic fatty acids share important pathways of immune system activation/inhibition with gut microbes, modulating obesogenic and pro-inflammatory profile. Mechanisms that link dietary fat, gut microbiota and obesity are mediated by increased intestinal permeability, systemic endotoxemia, and the activity of endocannabinoid system. Although the probiotic therapy could be a complementary strategy to improve gut microbiota composition, it did not show permanent effects to treat fat-induced dysbiosis.

Conclusion: Based upon evidence to date, we believe that high-fat diets and SFA consumption should be avoided and MUFA and omega-3 PUFA intake should be encouraged in order to regulate gut microbiota and inflammation, promoting body weight/fat control.

Keywords: high-fat diets; metabolic endotoxemia; lipopolysaccharide; monounsaturated fatty acid; polyunsaturated fatty acids; probiotics.

4.2 INTRODUCTION

Obesity is the most prevalent non-communicable disorder worldwide and a major concern for public health [1] This concern is partially attributed to its association with hypertension [2], type 2 diabetes [3], cardiovascular disease [4], and some types of cancers [5]. Obesity, as well as associated disorders, has an inflammatory component that is considered a link between these illnesses. Thus, there is a great scientific interest in identifying strategies to control the inflammation [6].

It has been suggested that gut microbiota plays a role on obesity pathogenesis [7–11] by mechanisms that involve, in part, its action on systemic inflammation [12]. Higher number of gram-negative bacteria and increased intestinal permeability in obese microbiota favor the occurrence of metabolic endotoxemia characterized by a high concentration of lipopolysaccharide (LPS) in the bloodstream [13]. Metabolic endotoxemia leads to low-grade inflammation, insulin resistance, adipocyte hyperplasia, and reduction of pancreatic beta-cells function [13, 14].

Although the most studied dietary factor associated with gut microbiota changes has been prebiotic soluble fibers and probiotics [15, 16], the amount of dietary fat as well as its fatty acid composition can affect gut microbiota. However, the effect of dietary fatty acids on the relationship between obesity and gut microbiota has been neglected. Antimicrobial activity of fatty acids is more explored as a way to increase the shelf-life of food and not to induce changes in gut microbiota [17]. Furthermore, high-fat diets have been implicated in reduction of gut microbiota richness [18, 19], increase LPS translocation [20], intestinal permeability [21], systemic inflammation [22], and disruption of the immune system [23–26]. Therefore, there is a growing interest in assessing the role of fat content and type in obesity induction mediated by gut microbiota [23–25, 27–29].

Thus, the aim of this review is to critically analyze human and animal studies in which the roles of dietary fats on gut microbiota, obesity, and low-grade systemic inflammation were investigated. It is intended, therefore, to clarify important issues on this topic and to provide scientists and clinicians a whole and realistic update about the subject.

4.3 METHODS

Medline/Pubmed, Science Direct, and Lilacs databases were searched for studies published from 2006 to 2016 about the topic of interest. Studies published before this

period was also included when its relevance justified the inclusion. Main terms used alone or in combination for search were: gut microbiota; inflammation; obesity; metabolic endotoxemia; dietary fat; fatty acids; probiotics; high-fat diet. All articles were selected if they were related to obesity, dietary fats, and gut microbiota interactions. Each selected article was critically read and clustered according to their thematic and scientific relevance. In order to describe our findings, we presented the following sections in this article: "Gut microbiota in obesity", " Dysbiosis, weight gain, and low-grade inflammation", "Role of dietary fats on obese dysbiosis and low-grade inflammation", and "Role of probiotic/synbiotic in reversing high-fat diet induced dysbiosis".

4.4 GUT MICROBIOTA IN OBESITY

Excessive energy consumption is certainly an environmental factor associated with obesity and metabolic diseases. However, when people from the same population consume excess of energy, some subjects exhibit lower susceptibility to weight gain and metabolic changes [30]. This fact suggests involvement of gut microbiome, in addition to human genome, on the onset of obesity [31].

Most of bacteria that inhabit human and mice gastrointestinal tract (99%) belong to four major phyla: *Firmicutes*, *Bacteroidetes*, *Actinobacteria,* and *Proteobacteria* (Figure 1). Jeffrey Gordon was the first to suggest that changes in gut microbiota may contribute to obesity development [7–9, 32]. Conventional mice showed a 42% increase in body fat compared to germ-free mice, although their food intake was lower. Germ-free mice gut colonization with gut microbiota from conventional mice, in turn, had 60% increase in their body fat and presented insulin resistance [32]. Environmental effects on gut microbiota and our ability to manipulate it in a controlled manner are under increasing scrutiny. Recent research has suggested the use of fecal/gut microbiome transplantation, in which feces are transferred from a healthy donor to a recipiente. This practice is increasingly drawing attention as a potential treatment for obesity [33].

There is still no consensus among researchers regarding the dynamics of bacterial phyla, genera and species in fecal microbiota of obese and overweight compared with those of normal-weight subjects [34]. However, obese dysbiosis have been consistently correlated with an increased ratio of two dominant microbial groups, *Firmicutes* and *Bacteroidetes*, both in rodents [8, 35] and humans [9]. In addition, obesity is associated with lower bacterial diversity [10].

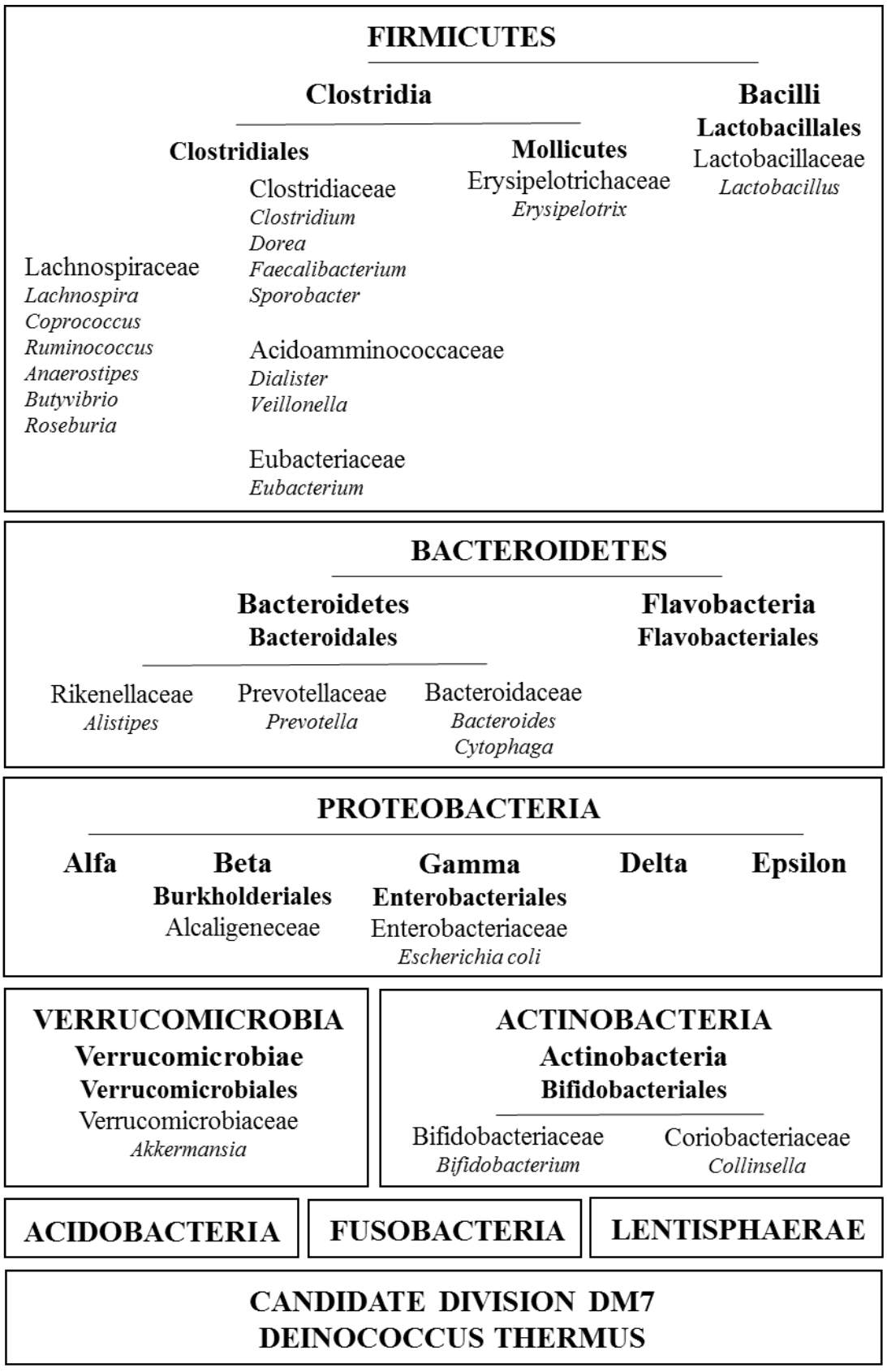

Fig. 1 Bacterial hierarchy in gut microbiota. Reprinted from "The role of gut microbiota in human obesity: Recent findings and future perspectives, volume 23" [31], Copyright number: 3906660903551 (2016), with permission from Elsevier.

4.5 DYSBIOSIS, WEIGHT GAIN, AND LOW-GRADE INFLAMMATION

Reduced bacterial richness seems to play an important role on the onset of excessive weight gain. Le Chatellier and colleagues [36] demonstrated that individuals with a low bacterial richness are characterized by more marked adiposity, insulin resistance, dyslipidemia, and systematic inflammation when compared to individuals with high bacterial richness. Dietary changes can restore gut microbiota richness resulting in bacterial equilibrium and more favorable metabolic profile [37].

The exact mechanism by which gut microbiome influences obesity remains obscure. However, it has been proposed that detrimental changes in gut microbiota could promote weight gain by increasing energy supply to body and lipogenesis [7, 9, 32, 38–40]. Dysbiosis-induced weight gain could also promote inflammation *per se*, since adipocyte hypertrophy favor macrophages recruitment in adipose tissue [29] and ectopic deposition of triglycerides in liver and muscles promotes pro-inflammatory factors secretion by macrophages [41]. Furthermore, dysbiosis could induce low-grade systemic inflammation by raising intestinal permeability to LPS and endocannabinoid system (ECs) activity [42, 43].

Increased energy supply by intestinal microbiome is due to short-chain fatty acids (SCFA) production, which can be oxidized by host providing extra calories [29]. It is estimated that more than 10% of total energy requirements can be supplied by dietary fiber fermentation [44]. Many biological effects seem to be mediated by these bacterial metabolites. SCFA, especially acetate, propionate and butyrate, can exert indirect effects in gene expression regulation by binding to G-protein-coupled receptors GPR41 and GPR43 [45]. Signaling through these receptors is associated with increased expression of glucagon-like peptide 1 (GLP-1, mechanism involving GPR43) and peptide YY (PYY, GPR41 pathway), both in the gut [46]. While both peptides are related to reduced hunger and appetite, PYY also decreases intestinal transit and may increases nutrients absorption including SCFA [12], favoring weight gain. Bacterial fermentation of CHO and proteins produces SCFA that emerge as mediators in linking nutrition, gut microbiota, physiology and pathology. The amount and relative abundance of SCFA need to be further investigated [47].

In addition, gut microbiota may favor fat gain by increasing adipocyte lipogenesis [38, 39]. Gut microbiota could suppress Fasting Induced Adipocyte Factor (FIAF) expression by interacting with entero-endocrine cell surface molecules, such as Toll-like receptors [40, 48]. FIAF is a peptide which is potent inhibitor of circulating lipoprotein lipase (LPL) [40]. Although FIAF suppression occurs only in intestinal epithelium, and

not in liver and adipose tissue where this factor is also produced, it increases LPL activity in adipocytes favoring triglycerides deposition [32]. Further, it could promote fat gain by changing fat absorption and turnover. FIAF-/- mice exhibited higher intestinal fat uptake and lower fat excretion leading to obese phenotype [49].

Great emphasis has been given to the role of changes in gut microbiota composition on metabolic endotoxemia [26]. The LPS and other compounds from gut microbiota, such as lipoteic acid, peptidoglycan, flagellin, and bacterial DNA can stimulate immune system and induce inflammation. The LPS however is considered a main inflammation inducer [43] through interaction with toll-like receptors-4 (TLR4). That inflammation inhibits the appropriate insulin signaling and leads to insulin resistance [27]. Under normal conditions, only small concentrations of LPS exceed intestinal epithelium and reach bloodstream of healthy subjects [28]. In obesity state, microbial dysbiosis can modulate the distribution of the tight junctions proteins, such as zonula occludens-1 (ZO-1) and occludin, increasing intestinal permeability and the passage of molecules like LPS into bloodstream, leading to systemic inflammation [26]. On the other hand, inflammation could increase intestinal permeability by reducing intestinal mucous layer thickness and increasing severity of inflammation [50], resulting in a vicious cycle of obesity, increased intestinal permeability, and inflammation.

Obesity is characterized by increased ECs activity. ECs is an important target in the context of obesity and inflammation. It has been demonstrated that ECs was involved in the control of glucose and energy metabolism, and ECs activity can be tuned up or down by specific gut microbes (e.g. *Akkermansia muciniphila*) [51]. Intestinal microbiome and ECs relationship is crucial for adipogenesis regulation [42]. While gut microbiota modulates ECs, it in turn regulates intestinal permeability and plasma LPS concentrations [26, 52]. Muccioli and colleagues [42] demonstrated that specific changes in gut microbiota could modify ECs activity in colon and adipose tissue. Blockage of the cannabinoid receptor CB1 reduced intestinal permeability by improving distribution and location of tight junction proteins in obese mice, whereas CB1 activation increased permeability markers *in vivo* and *in vitro* [42]. In addition, changes in gut microbiota and ECs activity regulate expression of adipose tissue hormones (e.g. apelin), which could aggravate low-grade inflammation [53].

Some dietary components, such as fat, has been shown to modulate gut microbiota and consequently influence all the mechanisms showed above. Therefore, both quantity and quality of dietary fat are related to obesity induction mediated by gut microbiota. This will be discuss in the next topics and summarized in Figures 2 and 3.

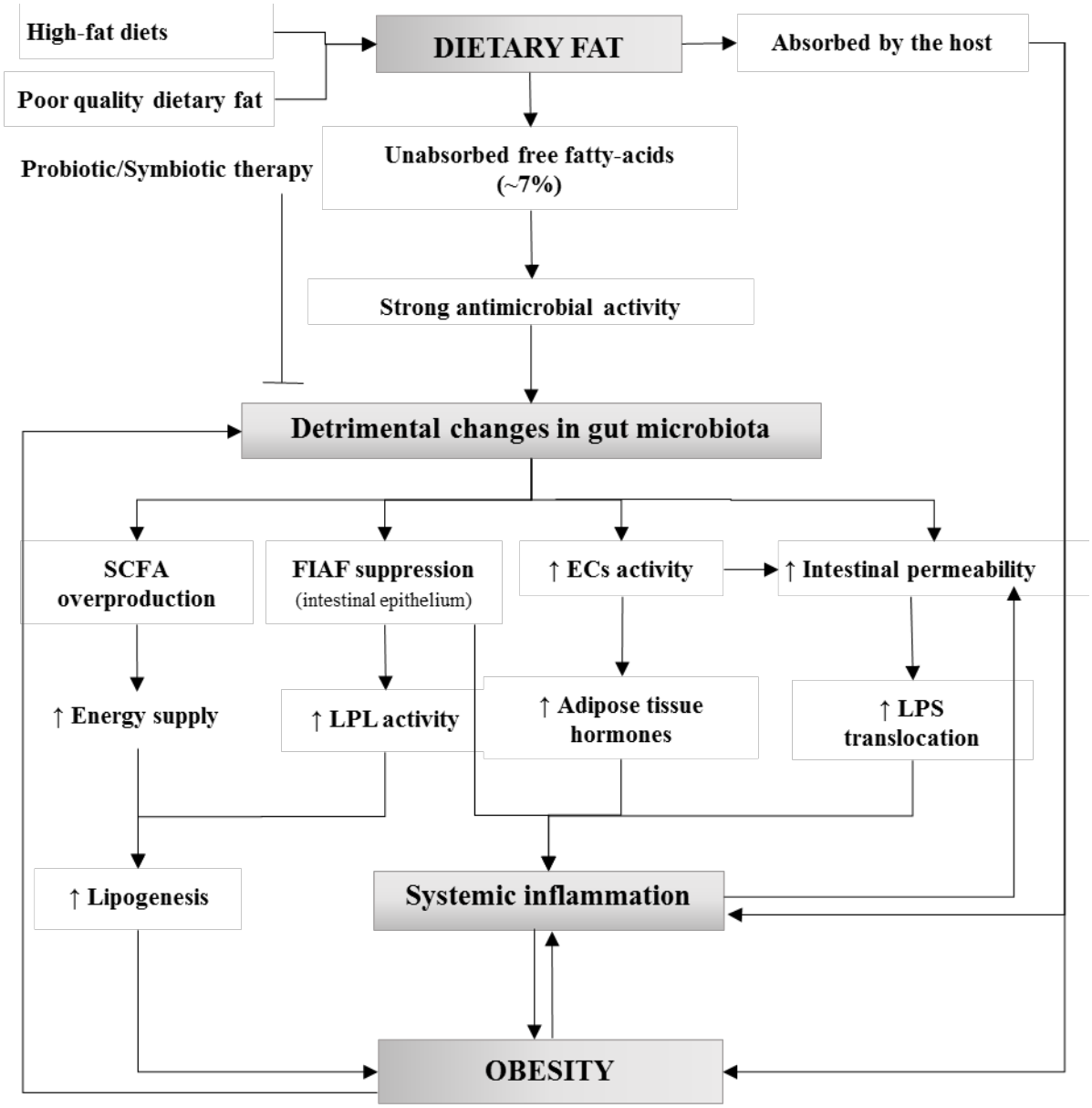

Fig. 2 Potential role of high-fat/poor quality dietary fat on gut microbiota, systemic inflammation, and obesity. SCFA: short-chain fatty acids; FIAF: fasting induced adipocyte factor; LPL: circulating lipoprotein lipase; LPS: lipopolysaccharide; ECs: endocannabinoid system.

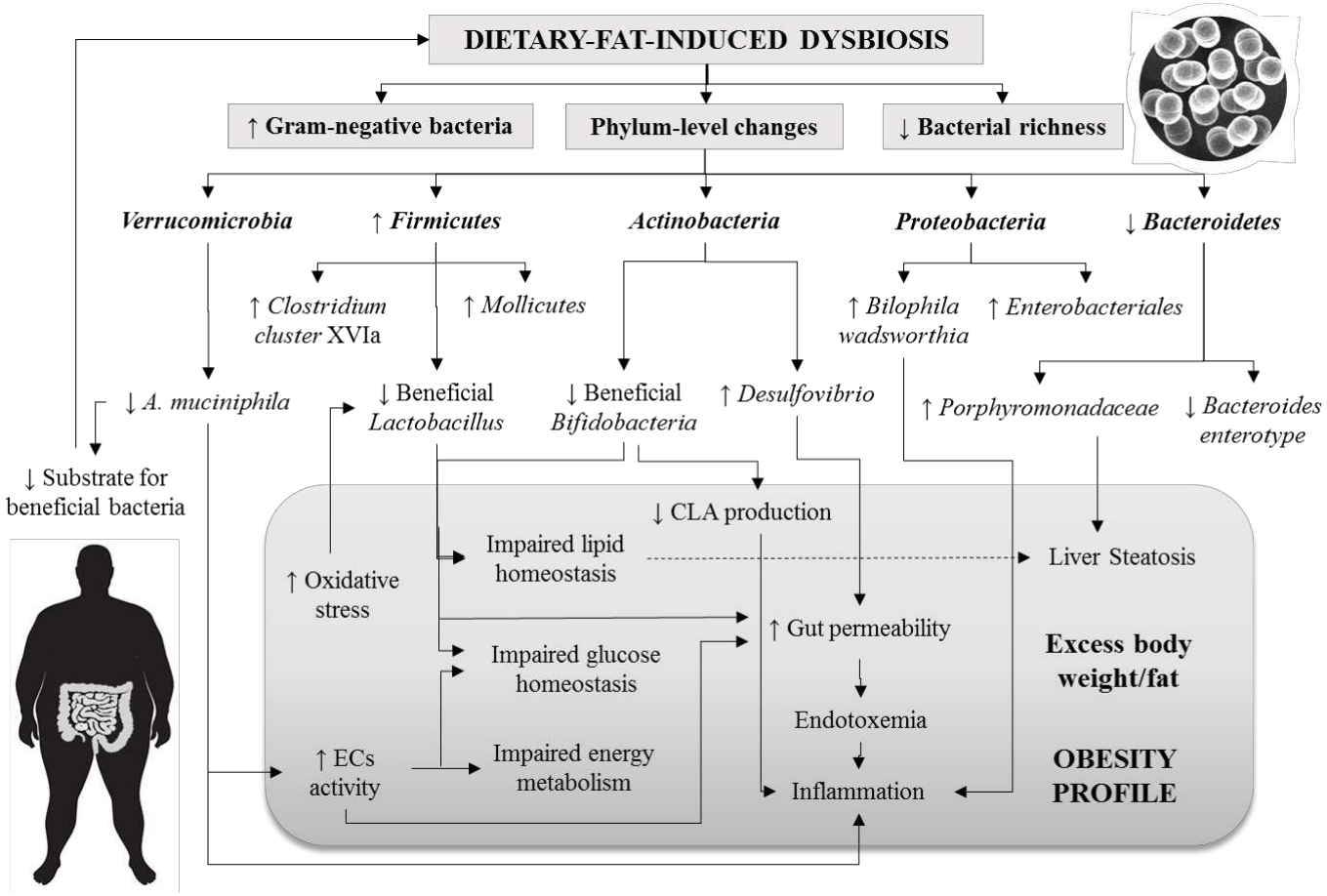

Fig. 3 Schematic model regarding dietary-fat-induced dysbiosis and metabolic disruptions related to obesity. The effects may vary according to type and amount of fat consumed. All the above-mentioned bacterial changes were related to body weight/fat gain but these relationships were suppressed in order to improve figure clarity. Arrows indicate pathways of stimulation. CLA: conjugated linoleic acid; ECs: endocannabinoid system.

4.6 ROLE OF DIETARY FATS ON OBESE DYSBIOSIS AND LOW-GRADE INFLAMMATION

Clinicians and scientific researchers have been underestimating the contribution of dietary fat on gut microbiota modulation for years, based on the argument that degradation and absorption of dietary fat mainly take place in small intestine, thus little - if any, dietary fat could reach colon in healthy individuals [54]. Small intestine harbors ~10^5 bacteria per ml, while colon harbors up to 10^{12} per ml [55]. Hence, the gut microbiota has not expected to interact substantially with dietary fat [54].

Recent findings, however, lead us to refute this argument. Gabert and colleagues [56] showed that about 7% of 13C labeled dietary fatty acids were excreted in healthy subjects stool and almost all of them (~86%) were recovered as free fatty acids. This means that fat presence in stool was not due to digestive failure, since digestive lipases were able to hydrolyze triglycerides into free fatty acids.

Free fatty acids, in turn, showed potent antimicrobial effect at very small doses [57]. It means that fat would significantly interact with gut microbiota, even if only small portion of the ingested fat reaches the colon. Furthermore, a large volume of *Lactobacillus* and other aerobics and aerotolerant bacteria which also colonize small intestine [55, 58] are closely related to obesity outcomes [58–62], and thereby likely to substantially interacted with dietary fat. Given these findings, we are convinced that dietary fat plays a relevant role in gut microbiota modulation, which could partly explain the deleterious effects of fat imbalance.

4.6.1 High-fat diets

Excessive consumption of high-energy density foods, especially those derived from fat, has an undoubtedly role on positive energy balance resulting in weight gain. However, this mechanism is insufficient to explain all metabolic dysruptions in obesity. Recognition of the relationship between high-fat diets, gut microbiome and metabolic endotoxemia is recent and can partly explain the manifestation and maintenance of a subclinical inflammatory status that favors the development of insulin resistance and associated diseases [27–29] (Table 1).

Results from animal studies revealed the supremacy of high-fat diet in promote gut microbiota disruption when compared to genetically induced obesity [27, 35]. Analyses of animal feces by 16S rRNA gene pyrosequencing showed that high-fat diet

Table 1 Summary of studies investigating the role of high-fat diets on obesity-induced dysbiosis

Study	Study population	Treatments	Main outcomes
Animal studies			
Turnbaugh et al., 2009 [10]	8 to 9-wk-old male C57BL/6J mice standardized for gut microbiota	HFD (data not shown)	- HFD increased *Erysipelotrichi* and bacilli (mainly *Enterococcus*), *Clostridium innocuum*, *Eubacterium dolichum*, and *Catenibacterium mitsuokai,* and decreased *Bacteroidetes*
Zhang et al, 2010 [16]	10 to 12-wk-old male wild type C57BL/6J and Apoa-I knockout mice	Low fat diet (5.2% of fat) or HFD (34.9%) for 25 wk.	- HFD explained 57% of the total structural variation in gut microbiota - HFD increased the *Desulfovibrionaceae* and reduced *Bifidobacterium* spp
Devkota et al., 2012 [17]	Pathogen-free C57Bl/6 mice	Low-fat diet (5% of fat) or HFD (38%) derived from milk, lard, or safflower oil during 3 wk	- Both HFD reduced the richness of the microbiota compared with low-fat diet; - Low-fat diet increased *Firmicutes* but also decreased the abundance of most of other phyla
Hildebrandt et al, 2009 [25]	14-wk-old female knockout RELMβ mice and wild-type 129Svev/C57BL/6	1 - RELMβ mice on standard chow 2 - RELMβ mice on HFD (45% of fat: lard - 87.6% and soybean oil -12.3%) 3 - Wild-type mice on standard chow 4 - Wild-type mice on HFD Experimental period: 21 wk	- HFD increased *Firmicutes* class, *Clostridiales* and *Delta-Proteobacteria* and decreased more than thirty different linages of *Bacteriodetes* on both wide-type and RELMβ mice
Turnbaugh et al., 2008 [32]	8 to 9-wk-old male C57BL/6J mice standardized for gut microbiota	Low fat, high polysaccharides diet (16% of fat) or HFD (41% as SFA and PUFA) for 8 wk.	- HFD decreased the overall bacterial diversity - HFD increased *Firmicutes*, especially *Mollicute* class, and decreased the *Bacteroidetes*
Cani et al., 2008 [59]	12-wk-old male wild-type C57bl6/J mice	Control diet or carbohydrate-free HFD (72% of fat as corn oil and lard) for 4 wk.	- HFD decreased the amount of *Lactobacillus* ssp. and *Bacteroides-Prevotella* spp. and increased *Bifidobacterium* spp - Changes in gut microbiota due to HFD consumption induced metabolic endotoxemia, increased the caecal content of LPS, and were correlated with reduced glucose intolerance, body weight gain, fat mass development, lower inflammation, oxidative stress, and macrophage infiltration in visceral adipose tissue

De La Serre et al, 2010 [60]	Male Sprague-Dawley rats exhibiting either an obesity-prone (DIO-P) or obesity-resistant (DIO-R) phenotype	Low-fat diet (10% of fat: SFA - 5.1%; MUFA - 34.7%; PUFA - 40.2%) or HFD (45%: SFA - 36.3%; MUFA - 45.3%; PUFA - 18.5%) for 12 wk.	- HFD decreased total bacterial density and the proportion of *Bacteroidales* and *Clostridiales* orders in both phenotypes - HFD increased intestinal permeability, plasma LPS, ileal inflammation associated with TLR4 activation, and decreased intestinal alkaline phosphatase, an enzyme that detoxifies LPS in DIO-P rats
Suzuki, Hara, 2010 [61]	4-wk-old Otsuka Long Evans Tokushima Fatty (OLETF) (obese strain), and Long Evans Tokushima Otsuka (LETO) (lean strain) rats	Low-fat diet (19% of fat) or HFD (53%: lard - 76.7%; soybean oil - 23.3%) for 16 wk.	- HFD increased intestinal permeability and decreased tight junction proteins (claudin-1, claudin-3, occludin and junctional adhesion molecule- 1) expression in small intestine regardless the strain
Everard et a, 2013 [65]	10-wk-old male C57BL/6 mice	Control diet or HFD (60% of fat: lard - 90.6% soybean - 9.3%) + *A. muciniphila* by oral gavage (2,108 CFU/0.2 mL) for 4 wk	- *A. muciniphila* treatment reversed HFD induced metabolic endotoxemia, adiposity, body weight, and improved body composition and reversed diet-induced fasting hyperglycemia - *A. muciniphila* administration increased the intestinal levels of endocannabinoids, the gut barrier, and gut peptide secretion
Murphy et al, 2010 [75]	7-wk-old male ob/ob mice and C57BL/6J wild-type mice	Ob/ob mice fed with low-fat diet (10% of fat) vs. wild-type mice fed either a HFD diet (45%) or a low-fat-diet (10%) for 11 and 15 wk	- HFD increased *Firmicutes* after 15 wks and decreased *Proteobacteria* after 11 and 15 wk - *Bifidobacterium* levels were lower in HFD wild-type mice when compared to lean wild-type after 11 wk
Mujico et al, 2013 [78]	8-wk-old female ICR mice	Control diet (4% of fat) or HFD (34.3%: SFA - 16.1%; MUFA – 12.7%; PUFA – 5.5%) for 19 wk	- HFD decreased the total DNA content in the feces but increased *Enterobacteriales*
Human studies			
Wu et al, 2011 [76]	Interventional study with health adults	Low-fat/high-fiber diet (13% of fat) or HFD/ low-fiber (38%) for 10 days	- HFD caused changes in microbiome composition after 24 hours of intervention and this changes were stable within 10 days of study

HFD: high-fat diet; ZO-1: zonula occludens-1; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; LPS: lipopolysaccharides; TLR4: toll-like receptor 4; wk: weeks. Fat amounts are presented as percent of total dietary energy content.

changed gut microbiota in both wild-type and RELMβ$^{-/-}$ mice [27]. These changes were characterized by increased abundances of *Firmicutes*, *Proteobacteria*, and *Actinobacteria*, followed by a reduction in abundance of *Bacteroidetes*. Since wild-type mice became obese and knockout mice remained relatively thin, authors concluded that diet effect was dominant and that high-fat diet, and not obese state, accounted for changes in microbial composition [27]. Similarly, 16S rRNA pyrosequencing of feces revealed no differences in gut microbiota composition between ob/ob leptin deficient mice and wild type mice at the beginning of experiment [35]. While low-fat diet did not change microbiota composition over the time in both wild type and genetically obese mice, *Firmicutes* ratio increased significantly from 56% to 71% when wild-type mice were fed with high-fat diet [35]. These findings suggest the supremacy of high-fat diet to impair gut microbiota by increasing *Firmicutes/Bacteroidetes* ratio compared with genetically induced obesity.

High-fat diets can increase the proportion of gram-negative bacteria, induce LPS translocation by incorporation into chylomicrons during fat absorption, and reduce intestinal mucosa integrity [20, 43] raising blood concentrations of LPS. Reduction in the expression of tight junction proteins was observed in intestinal mucosa of animals receiving high-fat diets [63–65].

Increased content of fat in diet can influence the philum *Actinobacteria*, which play an essential role on obesity maintenance [10]. These diets reduce the number of beneficial gram-positive *Bifidobacterium* species, increase plasma LPS concentrations, and induce low-grade inflammation [26]. Likewise, *Desulfovibrio* bacteria growth has been observed during high-fat diet consumption. These bacteria are gram negative, opportunistic pathogens, endotoxins producers [19, 66] and are also capable of reducing sulphate to H_2S, damaging gut barrier and promoting inflammation [18, 67].

The number of the beneficial mucin-degrading bacteria *Akkermansia muciniphila*, a member of *Verrucomicrobia* phylum that colonizes mucus layer [68], was reduced after consumption of high-fat diet [69]. *A. muciniphila* is found in about 3-5% of microbial community of healthy subjects [68, 70] and is inversely correlated with body weight in animals [69, 71] and humans [72, 73]. Close proximity of *A. muciniphila* to human intestinal epithelium has been associated with protective immune system stimulation and anti-inflammatory properties [74–76]. *A. muciniphila* could also contribute to re-establishment of a healthy mucus-associated microbiota after infection by offering oligosaccharides and SCFA from mucus and providing substrates for beneficial bacteria

growth [68, 70]. Nevertheless, causal relationship between dietary factors and *A. muciniphila* is not well established and could be influenced by energy restriction [77].

It has been emphasized that dietary fat cannot be metabolized under anaerobic conditions. Therefore, it could not serve as energy source for strict anaerobic bacteria [12]. Since most bacteria that inhabit our gastrointestinal tract are strict anaerobes (e.g. clostridia, *Bacteroides, Eubacterium, Peptostreptococcus*, and *Bifidobacterium*) [78], the use of dietary fat as an energy source for gut microbiota growt should not be a prominent mechanism for explain high-fat induced dysbiosis. Otherwise, when dietary fat content is increased, there is usually a low content of other dietary compounds such as carbohydrate and fiber [11, 27, 79, 80], and the outcome could be biased. Low carbohydrate and fiber diets could reduce energy substrates for beneficial bacteria growth such as bifidobacteria [81] and *A. muciniphila*, since administration of prebiotics was able to increase its number by ~100-fold in obese mice [71].

Despite the detrimental changes in gut microbioma due to high-fat consumption, dietary manipulations can reverse high-fat induced dysbiosis and then obesity. Whilst high-fat diet increased *Firmicutes/Bacteroidetes* ratio, marked by bloom in the class *Mollicutes* and a dramatically drop-down in overall class diversity, and promoted body weight/fat gain, reduced-fat diet diminished the bloom in *Mollicutes*, increased relative abundance of *Bacteroidetes*, and reduced fat deposition [35]. Probiotic administration is other way to manipulate high-fat induced dysbiosis and obesity, which will be further discussed.

4.6.2 Dietary fat types

Recent studies showed that different types of dietary fat (saturated fatty acid, monounsaturated fatty acid – MUFA and polyunsaturated fatty acids - PUFA), and not only the excess of fat in diet, could change gut microbiota composition and obesity profile (Table 2) [80, 82–85].

Consumption of high-SFA palm oil diet induces higher weigh gain compared to high-MUFA olive oil diet, high-PUFA safflower oil or low-SFA palm oil diet in mice [83]. This obesogenic effect was followed by a reduction in microbial diversity and an increase in *Firmicutes/Bacteroidetes* ratio. Although the above-mentioned results fit typical obesity profile [83], the study clearly indicates that overflow of SFA to distal intestine causes microbiota changes rather than obesity itself.

Habitual intake of MUFA, omega-3 PUFA and omega-6 PUFA differently affects the numbers of certain gut bacterial groups studied [85]. While MUFA and

Table 2 Summary of studies investigating the role of dietary fat types on obesity-induced dysbiosis

Study	Study population	Treatments	Main outcomes
Animal studies			
Devkota et al., 2012 [17]	Pathogen-free C57BL/6 mice	1- Low-fat diet (5% of fat) 2- HFD (38%: milk fat - 68,5%) 3- HFD (38%: lard - 50%) 4- HFD (38%: safflower oil - 87%) Experimental period: 3 wk	- PUFA (safflower oil) and SFA (milk-derived) increased *Bacteroidetes* and decreased *Firmicutes* abundances in a distinctly way of lard-based SFA diet - SFA (milk-derived) showed a significant bloom in *B. wadsworthia*, a member of the *Deltaproteobacteria*
Mujico et al., 2013 [78]	12-wk-old female pathogen-free mice	1 – Standard diet for 15 wk 2 - HFD (60.3% of fat: 91.2% from lard; 8.82% of soybean oil) for 15 wk 3 – HFD for 8 wk and HFD-supplemented with oleic acid for another 7 wk 4 – HFD for 8 wk and HFD-supplementation with a combination of n-3 fatty acids (EPA and DHA) for another 7 wk	- HFD increased body weight, which was reduced by oleic-acid supplementation. - Oleic-acid markedly increased total bacterial density and restored the proportions of bacteria that were altered due to HFA consumption - EPA and DHA supplementation increased the amounts of *Firmicutes* (especially the *Lactobacillus*) - Oleic-acid, EPA, and DHA supplementation lead to a better gut-microbiota profile, which were associated with lower body weight
de Wit et al., 2012 [79]	9-wk-old C57Bl/6J mice	Low-fat palm oil diet (10% of fat, soybean oil - 55.5% and palm oil - 44.5%) or HFD (45%) with palm oil, olive oil, or safflower oil for 8 wk	- HFD with palm oil induced the highest body weight gain and liver triglyceride content - HFD with palm oil reduced microbial richness and increased the *Firmicutes/Bacteroidetes* ratio - HFD with palm oil elevated lipid metabolism-related genes in the distal small intestine which were previously associated with the metabolic syndrome.
Patterson et al., 2014 [80]	8-wk-old wild type C57BL/6J mice	Low-fat diet (12% of fat with equal amounts of the tested fat) or HFD (45%) from palm oil, olive oil, safflower oil, or a combination of flaxseed/fish for 16 wk	- Palm oil supplementation reduced the number of *Bacteroidetes* compared to olive oil - Olive oil consumption increased *Bacteroidaceae* number compared to palm oil, flaxseed/fish oil, and high sucrose - Flaxseed/fish oil diet increased tissue concentrations of EPA, docosapentaenoic acid, and DHA, and the intestinal population of *Bifidobacterium* low-fat diet

Marques et al., 2015 [83]	Male 8 to 9-wk-old C57BL/6 mice	Standard diet supplemented with *t*10*c*12-CLA (0.5%, w/w) or with no supplementation (control) daily for 8 wk	- *t*10*c*12-CLA supplementation decreased visceral fat mass and affected lipid mass composition, but did not affect body weight - *t*10*c*12-CLA increased cecal content of acetate, propionate, and isobutyrate - *t*10*c*12-CLA reduced the *Firmicutes* to *Bacteroidetes* ratio, increased proportions of *Porphyromonadaceae* and decreased abundance of *Lachnospiraceae* and *Desulfovibrionaceae*
Human studies			
Wu et al., 2011 [76]	Cross-sectional study in healthy adults	-	- *Bacteroidetes* and *Actinobacteria* phylum were positively associated with fat, whereas *Firmicutes* and *Proteobacteria* showed the opposite association - Within each phylum, not all lower-level taxa demonstrated similar correlations with dietary components - Taxa correlated with BMI also correlated with fat and percent calories from SFA
Simões et al., 2013 [81]	Cross-sectional data was assessed in monozygotic twin pairs with distinct body weight and body fat classification were assessed for habitual dietary intake and fecal microbiota	-	- Co-twins with similar daily energetic intake had more similar numbers of *Bacteroides* spp. when compared with the ones with different energy intakes - Higher MUFA intake was associated with lower numbers of *Bifidobacterium* and slightly higher numbers of *Bacteroides* spp. - Co-twins who ingested identical levels of SFA had very similar *Bacteroides* spp. - n3-PUFA intake resulted in a significant positive association with *Lactobacillus* abundance - n6-PUFA intake was associated with decreased numbers of *Bifidobacterium*

HFD: high-fat diet; EPA: eicosapentaenoic acid; DHA: docosaexaenoic acid; SFA: saturated fatty-acids; PUFA: polyunsaturated fatty-acids; *t*10*c*12-CLA: *trans*-10, *cis*-12-conjugated linoleic acid; BMI: body mass index. Fat amounts are presented as percent of total dietary energy content.

omega-6 PUFA consumption were negatively associated with an increase in *Bifidobacterium* number, an increased ingestion of omega-3PUFA was positively associated with a higher number of bacteria from *Lactobacillus* group. Although consumption of omega-3 PUFA could be beneficial because several lactobacilli enhance the function of intestinal barrier [86], the authors found no association between BMI, microbiota composition and fatty acid intake [85]. In addition, this study [85] does not allow us to establish causal relationship between fatty acid consumption and gut microbiota composition due to its observational data.

In a metagenomic study with healthy volunteers, Bacteroides enterotype was found to be highly associated with fat consumption, in particular with MUFA and SFA [80]. Patterson and colleagues [84], studying the impact of dietary fatty acids on gut microbiota composition in mice, showed a reduction in *Bacteroidetes* at phylum level in animals fed with high-fat dietary palm oil diet compared to high-fat olive oil diet. High-fat olive oil diet, in turn, increases populations of *Bacteroidaceae* family compared to high-fat palm oil diet, high-fat flaxseed/fish oil diet and low-fat high-sucrose diet. Omega-3 rich high-fat flaxseed/fish oil diet lead to increase in *Bifidobacterium* spp. compared with low-fat high-maize starch diet. These data indicate that SFA (palm oil) consumption could lead to detrimental changes in gut microbiota but MUFA (olive oil) and omega-3 (flaxseed/fish oil) consumption could be positive to host microbial ecosystem.

Detrimental impact of SFA on gut microbiota composition and inflammation was proven in a robust study [19]. Consumption of diet high in SFA derived from milk promoted the growth of low-abundance, sulphite-reducing pathobiont, *Bilophila wadsworthia* in mice. This observation was associated with pro-inflammatory T helper type 1 (TH1) immune response. These effects were mediated by milk-derived-fat-promoted taurine conjugation of hepatic bile acids, which increases organic sulphur availability used by sulphite-reducing microorganisms like *B. wadsworthia*. Although the above-mentioned study [19] was conducted to verify the impact of SFA on intestinal inflammation, not in low-grade systemic inflammation, these data provide plausible mechanistic basis to explain why diets high in SFA diets might increase prevalence of obesity.

Gut microbiota modulation by different kinds of dietary fat could change body weight [82, 83] or visceral fat mass even in very small dose [87]. Oleic acid-derived compound supplementation reduced body weight, increased total bacterial density and restored proportions of bacteria that were increased (i.e. *Clostridium cluster* XVIa and *Enterobacteriales*) or decreased (i.e. *Bifidobacterium* spp.) due to a high-fat diet feeding

in mice [82]. In the same experiment [82], supplementation of omega-3 PUFA series (such as eicosapentaenoic - EPA and docosahexaenoic acid - DHA) significantly increased the amount of *Firmicutes* (especially *Lactobacillus* group) without reductions in body weight. This study suggests oleic and omega-3 series fatty acids potential to beneficially modulate gut microbiota, with the former benefiting weight control.

Using a very small dose of dietary *trans*-10, *cis*-12-conjugated linoleic acid (CLA) (0.5% w/w) [87], showed a significant reduction of visceral fat mass in mice wich received the supplementation as compared to the control animals. This reduction was accompanied by a beneficial decrease in *Firmicutes* to *Bacteroidetes* ratio. However, CLA supplementation increased total weight and triglicerydes concentrations in liver and promoted possibly harmful changes in gut microbiota at genus and family levels. These changes included increased numbers of *Porphyromonadaceae,* which were previously linked to non-alcoholic fatty liver disease [88]. It is important to note that increased fatty liver content could be transient and result from fast fat loss [89], and not be a consequence of detrimental changes in gut microbiota. Bifidobacteria could produce the main biologically active CLA isomers and this was associated to their ability to reduce body fat and to improve immune and inflammatory responses [78]. Physiological benefits have stimulated supplementation of CLA in safe doses in humans [90]. However, further studies are now needed to better understand the relationships between CLA consumption/production, gut microbiota, and liver diseases.

Several types of fatty acids have a potent antimicrobial activity and although their effects have been mainly explored as a way to preserve foods from pathogens, they can affect gut microbiota composition. It is important to note that antimicrobial activity of fatty acids occurs after complete enzymatic hydrolises of fat, when fatty acids are present in a freeway [17]. Thus, the modulation of fatty acids by gut microbiota could be more intense in the lower gastrointestinal tract.

Antimicrobial activity of fatty acids was well described by Desbois and Smith [17] and will not be deeply discussed here. In summary, antimicrobial activity of fatty acids is complex and depends on length of their carbon chain and presence, number, position and orientation of double bonds. Regarding the structure, the presence of hydroxyla in carboxyl group seems to be important for the antimicrobial activity of fatty acids [91]. Unsaturated fatty acids (UFA) tend to have greater activity than SFA with same length carbon chain [91, 92]. Often antimicrobial activity of PUFA increases in the same direction of the number of double bonds in their carbon chains and the naturally occurred *cis* orientation seems to have a greater antimicrobial activity than *trans* orientation [93].

Medium- and long-chain UFA (unsaturated fatty acids) tend to be more active against gram-positive than gram-negative bacterias [94]. The most potent MUFA usually have 14 or 16 carbon atoms [93] and, in SFA, 10 or 12 carbons. Antibacterial effect of SFA tends to decrease as chain length gets longer or shorter [95, 96].

Dietary fats and gut microbiota also seem to share key pathways of obesity induction. It has been proposed that some SFA (e.g. palmitic acid and lauric acid) initiate inflammatory response by acting on LPS receptor (Toll-like receptor-4 – TLR-4) in adipocytes and macrophages, which can contribute to inflammation of adipose tissue in obesity [97]. These mechanisms are also related to metabolic and immune responses related with infection by LPS [26]. Another mechanism involves the role of fatty acids in intestinal permeability through mucosal mast cells stimulation [21]. Cytokine secretion by mast cells, such as TNF-α, IL-1β, IL-4, and IL-13 may promote LPS translocation [29], thus favoring metabolic endotoxemia. Moreover, FIAF expression could also mediate inflammatory status inducted by fatty acids.

SFA, but not UFA, induces a severe proinflammatory profile in mice lacking FIAF but not in the control animals [97]. A previous study indicated a presence of protective autocrine mechanism by which high-fat diets induce FIAF expression. FIAF overexpression inhibits mesenteric lymph node macrophages uptake of proinflammatory fatty acids and consequently reduces inflammatory status [98]. Since the presence of microbiota suppresses FIAF expression in entero-endocrine cells as previously mentioned, we believe that dysbiosis could contribute to proinflammatory status by enhancing SFA uptake in mesenteric lymph node macrophages.

On the other hand, omega-3 PUFA series are recognized for their anti-inflammatory properties [22]. Although the anti-inflammatory properties of omega-3 fatty acids are well described, new mechanisms of action are still being proposed [99]. Macrophages are one of the major sources of pro-inflammatory factors and EPA and DHA could down regulate the pro-inflammatory cytokines TNFα and IL-6 production by TLR-4 ligand, indicating once again the involvement of TLR-4 pathway [100]. Thus, increased ratio of omega-3/omega-6 may favor the reduction of systemic inflammation and contribute to a reduced morbidity associated with obesity [101].

4.7 ROLE OF PROBIOTIC/SYNBIOTIC IN REVERSING HIGH-FAT DIET INDUCED DYSBIOSIS

Since high-fat diets can induce dysbiosis and obesity, it is not difficult to assume that the administration of probiotic/synbiotic could ameliorate high-fat diet induced obesity. This approach is sustained by growing body of scientific evidences from animal [59–62, 69, 102–108] and human [109, 110] studies.

Most of available studies [59–62, 102–110] included bacteria from *Lactobacillus* and/or *Bifidobacterium* group as probiotic/synbiotic and only few studies included other probiotic bacteria like *A. muciniphila* [69], *Enterococcus faecium* [108], and *Streptococcus thermophilus* [109]. Although it is too early for definitive conclusions, results from these studies so far indicated beneficial role of probiotics in preventing and even reversing body weight/fat gain [59, 62, 69, 102, 103, 105–107, 109, 110], dysbioses [59, 60, 103, 104, 107, 108], inflammation [60, 69, 104–106], gut barrier dysfunction [59, 60, 69], and metabolic disruptions [59–61, 69, 102–106, 110] due to high-fat diet consumption (Table 3).

It is important to note that the impact of probiotic/synbiotic supplementation depends of type of bacteria used to reverse high-fat diet-induced obesity [105, 107]. In non-obese healthy subjects, the use of a probiotic supplement composed by a mixture of specific bacterial strains prevents body weight and body fat gain but does not alter insulin sensibility due to high-fat diets [109]. On the other hand, the consumption of fermented milk containing *Lactobacillus casei Shirota* consumption twice a day prevents body fat gain and disruptions in glucose metabolism in a comparable population [110]. Despite their methodological differences [109, 110], *L. casei Shirota* could have a potential in reestablishing glucose metabolism after a high-fat diet consumption which needs to be further explored in clinical studies.

Influence of bacterial types on the above-mentioned relationships occurs also at a strain level [105]. While the administration of *Lactobacillus reuteri* L3 was beneficial in reduction of body weight, glucose metabolism, LPS translocation, pro-inflammatory status, and also in increase energy expenditure that were altered due to high-fat diet consumption, *L. reuteri* L10 did not show same results [105]. *L. reuteri* L3 is considered a bacterium with anti-inflammatory properties. It is also sensitive to oxidative stress generated by high-fat diets. During the consumption of a high-fat diet, the number of beneficial bacteria *L. reuteri* L3 was reduced while the number of others pro-inflammatory strains such as *L. reuteri* L8 was increased [111]. Thus, the use of *L.*

Table 3 Effects of probiotic/synbiotic on high-fat diet induced obesity

Study	Study design	Probiotic/synbiotic	Main outcomes
Animal studies			
Núñez et al., 2014 [55]	5-wk-old female mice received a conventional balanced diet or a HFD from bovine lard supplemented with milk, milk fermented by probiotic, probiotic suspension, or water over 60 d	*L. casei* CRL 431	- Milk fermented by *L. casei* decreased body weight gain due to HFD - Both *L. casei* and fermented milk reduced the increase in glucose, total cholesterol, and LDL-c serum levels due to HFD - Fermented milk improved the histology of liver and small intestine - *L. casei* increased *Bacteroides* and bifidobacteria in HFD fed animals - Both *L. casei* and fermented milk enhanced the phagocytic activity of macrophages
Raso et al., 2014 [56]	Young male rats received standard diet + placebo gavage, HFD + placebo gavage or HFD + symbiotic by gavage for 6 wk	*L. paracasei* B21060, arabinogalactan, and FOS	- Synbiotic administration down-regulated liver inflammatory markers that were elevated in HFD fed animals - Synbiotic improved glucose parameters such as fasting response, hormonal homeostasis, and glycemic control, and prevented the impairment of hepatic insulin signaling due to HFD consumption - Synbiotic also reduced cytokines synthesis in the liver and restored the HFD-dysregulated TLR 2, 4 and 9 mRNAs toward a physiological level - - Synbiotic preserved gut barrier integrity and reduced the relative amount of Gram–negative *Enterobacteriales* and *E. coli* in colonic mucosa
Song et al., 2015 [57]	7-wk-old male mice were fed with standard diet, HFD from lard source, or HFD from lard source with probiotics for 10 wk	*L. acidophilus* NS1	- *L. acidophilus* NS1 reduced the increase in total cholesterol and LDL-c due to HFD consumption - There was no significant changes in HDL-c
Karimi et al., 2015 [58]	6-wk-old male rats were fed with standard diet, HFD from beef tallow, HFD from beef tallow with probiotics, or a HFD from beef tallow with Orlistat for 15 wk	*L. casei Shirota*	- *L. casei Shirota* and Orlistat reduced the increase in body weight, body mass index, fat mass, leptin and glucose levels due to HFD consumption - HDL and adiponectin levels were higher with *L. casei Shirota* and Orlistat administration - *L. casei Shirota* was better than Orlistat in reducing body fat mass - *L. casei Shirota* and Orlistat reduced IL-6 when compared to HFD
Everard et al., 2013 [65]	10-wk-old male mice were fed with a standard diet or an HFD (60% fat) with placebo gavage, active probiotic	*A. muciniphila*	- *A. muciniphila* reversed HFD-induced metabolic disorders, including fat-mass gain, metabolic endotoxemia, adipose tissue inflammation, and insulin resistance

	gavage, or inactive probiotic gavage for 4 wk		- *A. muciniphila* increased the intestinal levels of endocannabinoids that control inflammation, the gut barrier, and gut peptide secretion - These effects were only present in active *A. muciniphila* administration
Yin et al., 2010 [98]	4-wk-old male rats were fed with standard diet or HFD with or without one of the supplemental bacteria strain for 6 wk	1 - *B.* L66-5 2 - *B.* L75-4 3 - *B.* M13-4 4 - *B.* FS31-12	- When compared with the control group, *B.* M13-4 improved body weight gains while *B.* L66-5 induced a decrease in BW - *B.* L75-4 and *B.* FS31-12 had no effect on body weight - All the probiotics reduced serum and liver triglyceride and ameliorated ectopic lipid deposition in liver - Probiotic supplementation did not show significant changes in serum insulin and glucose levels
An et al., 2011 [99]	Male rats were fed with standard diet or high-fat diet with or without probiotic for 7-wk	*B. pseudocatenulatum* SPM 1204, *B. longum* SPM 1205, and *B. longum* SPM 1207	- Probiotic reduced body and fat weights, blood serum levels (total cholesterol, HDL-c, LDL-c, triglyceride, glucose, leptin, AST, ALT, and lipase levels), and harmful enzyme activities (β-glucosidase, β-glucuronidase, and tryptophanase) - Probiotic significantly increased the supplemented bacteria fecal counts
Cano et al., 2013 [100]	6-8-wk male mice were fed a standard diet or HFD with or without probiotic for 7 wk	*B. pseudocatenulatum* CECT 7765	- *B. pseudocatenulatum* reduced serum cholesterol, triglyceride, and glucose levels and decreased insulin resistance and improved glucose tolerance in HFD-fed mice - Probiotic reduced serum levels of leptin, IL-6 and MCP-1, while increased those of IL-4 in HFD-fed mice - Probiotic reduced liver steatosis and improved the function of innate immune system - Probiotic increased bifidobacteria and reduced enterobacteria and the inflammatory properties of the gut content in HFD-fed mice
Qiao et al., 2015 [101]	9-wk-old male mices received standard diet or HFD with or without the addition of a probiotic strain (1 or 2) by gavage	1 – *L.reuteri* L3 2 – *L.reuteri* L10	- *L. reuteri* L3 (but not *L. reuteri* L10) administration reduced the increase in body weight, glucose, insulin, LPS, and pro-inflammatory cytokine levels due to HFD consumption - *L. reuteri* L3 (but not *L. reuteri* L10) also increased the energy expenditure and improved mRNA profile related to obesity genotype compared to HFD consumption
Wu et al., 2015 [102]	8-wk-old male mice received a standard diet, HFD + control gavage or HFD + probiotic by gavage for 8 wk	*L. plantarum* K21	- *L. plantarum* K21 alleviated body weight gain and epididymal fat mass accumulation, reduced plasma leptin levels, decreased cholesterol and triglyceride levels, and mitigated liver damage due to HFD - *L. plantarum* K21 downregulated the hepatic expression of PPAR-γ, improved intestinal barrier and gut microbiota composition due to HFD

Wang et al., 2015 [103]	10-wk-old male mice received standard diet or HFD with or without the addition of a probiotic strain (1, 2 or 3)	1 - *L. paracasei* CNCM I-4270 2 - *L. rhamnosus* I-3690 3 – *B. animalis* ssp. *Lactis* I-2494	- Probiotic strains attenuated weight gain and macrophage infiltration into epididymal adipose tissue and markedly improved glucose–insulin homeostasis and hepatic steatosis - Probiotic strains shifted the overall structure of the HFD-disrupted gut microbiota toward that of lean mice fed a standard diet - *L. paracasei* and *L. rhamnosus* increased cecal acetate but did not affect circulating LPS-binding protein; in contrast, *B. animalis* did not increase acetate but significantly decreased adipose and hepatic TNF-α
Prince et al., 2016 [104]	Japanese macaque juveniles exposed to a maternal control or high-fat diet were provided with probiotics for 3 months	*E. faecium, L. acidophilus, L. casei*	- Probiotics supplemented primates presented higher abundance of *Bacillus* and *Bacteriodetes* while untreated primates had a higher prevalence of *Proteobacteria* - Probiotic pretreatment did not provide protection from HFD induced dysbiosis
Human studies			
Osterberg et al., 2015 [105]	Twenty non-obese males (18-30 y), after a 2-wk of a normocaloric normofat diet, followed a high-energy HFD (rich in saturated fatty-acids from ice cream and coconut milk) with or without a probiotic for 4-wk	*S. thermophiles* DSM 24731*, L. acidophilus* DSM 24735*, L. delbrueckii* ssp. *bulgaricus* DSM 24734*, L. paracasei* DSM 24733*, L. plantarum* DSM 24730, *B. longum* 24736*, B. infantis* DSM 24737*, B. breve* DSM 24732	- Probiotic supplementation attenuated body and fat mass gain due to HFD - Probiotic did not altered insulin sensitivity and skeletal muscle pyruvate and fat oxidation
Hulston et al., 2015 [106]	Seventeen normal-weight adults consumed or not (control group) a probiotic twice a day during 4 wk treatment with a normal diet (3 wk) and a high-energy HFD (1 wk)	*L. casei Shirota*	- *L. casei Shirota* reduced body fat mass gain due to HFD consumption - *L. casei Shirota* prevented the injury on the glucose metabolism parameters like insulin sensitivity and total glucose response after HFD feeding

HFD: high-fat diet; AST: aspartate aminotransferase; ALT: alanine aminotransferase; FOS: fructooligosaccharides; LPS: lipopolysaccharide; TNF-α: tumor necrosis factor-α; IL-6: interleukin 6; MCP-1: monocyte chemotactic protein-1.

reuteri L3 could contribute to reestablishment of beneficial gut microbiota and inflammatory status. The bifidobacteria from different strains, in turn, could improve (strain *B.* M13-4), decrease (strain *B.* L66-5), or have no effect (strains *B.* L75-4 and *B.* FS31-12) on body weight gain due to high-fat diet, despite all strains improved serum and liver triglyceride [102]. The fact that bacterial strains of the same species showed different effects on inflammation and obesity, illustrates the complexity of host-bacterial cross-talk and the importance of investigating specific bacterial strains.

Certain studies deserve to be described due to the relevance of their findings [62, 69, 108]. Prince et al. [108] investigated the effect of *Enterococcus faecium*, *Lactobaccillus acidophilus*, and *L. casei* on the treatment of primates exposed to maternal high-fat diet. The authors had previously proved the influence of maternal diet on offspring out to one year of age in the same animal model [112]. While the use of probiotics provided beneficial changes in intestinal microbiome, with increased number of bacilli and *Bacteriodetes* and reduced prevalence of *Proteobacteria*, the effect was not persistent. Further, prior use with probiotics could not protect individuals from intestinal dysbiosis that is induced by a high-fat diet.

Administration of *A. muciniphila* was able to reverse high-fat diet induced metabolic disorders, metabolic endotoxemia, adipose tissue inflammation, and insulin resistance [69]. In the same study, *A. muciniphila* increased intestinal marker of endocannabinoid activities, gut barrier, and gut peptide secretion. Despite the beneficial findings with viable bacteria, heat-killed *A. muciniphila* did not show the same results [69]. Thus, probiotic cell viability is a prominent factor that deserves consideration during probiotic treatments.

Karimi and colleagues [62] compared the effects of probiotic supplementation to drug therapy on the outcomes of obesity. Both *L. casei Shirota* and Orlistat were able to reduce the increase in body weight, body mass index, fat mass, leptin, IL-6 and glucose levels due to high-fat diet consumption. Further, *L. casei Shirota* showed better results in reducing body fat mass than Orlistat. These results, in addition to offering a viable alternative to drug therapy, provide a possible and novel explanation to the mechanism of action of Orlistat.

When administered with high-fat diet, Orlistat partially inhibits hydrolysis of triglycerides, thus reducing subsequent formation of free fatty acids in the gastrointestinal tract. Until now, the weigh-reducing effect of Orlistat was attributed to reduced rate in free fatty acids absorption [113]. However, it is possible that this low amount of free fatty acids in the gastrointestinal tract also reduces the potential of high-fat diet to induce

dysbiosis by the reduction of antimicrobial fatty acids, and, thus, contribute to the results of Orlistat. Unfortunately, the study [62] did not evaluate changes in microbiota composition after probiotic and Orlistat consumption. Thus, studies, which evaluate changes in gut microbiota composition, are now urgently needed.

4.8 CONCLUSION

A growing body of scientific evidences suggests that excessive fat consumption negatively affects microbial composition and its activity, leading to obesity and systemic inflammation by mechanisms that involve the increase in SCFA conversion, intestinal permeability, LPS translocation, ECs activity, besides FIAF suppression. The role of fat consumption on gut microbiota, systemic inflammation, and obesity is complex and many questions remain to be answered by scientific community. Nevertheless, results of published studies suggest that a balanced diet in regard to fat content is critical not only for host health but also for gut microbiota. Probiotic therapy could be a complementary strategy to improve gut microbiota composition, however it does not seems to be enough to prevent or treat fat-induced dysbiosis due to its transient effects. Thus, based upon the evidences to date, high-fat diets and SFA consumption should be avoided, and MUFA and omega-3 PUFA consumption should be stimulated in order to regulate gut microbiota and inflammation, promoting body weight/fat control. We encourage scientists to conduct research, which would be able to link the antimicrobial activity of specific fatty acids to obesity-related dysbiosis.

4.9 ETHICAL STANDARDS

The manuscript does not contain clinical studies or patient data.

4.10 CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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5. ORIGINAL ARTICLE 1 – Dietary fat-induced LPS translocation is not a prominent pathway to explain acute immunological responses after consumption of coconut, extra-virgin olive, and soybean oils at usual doses

Artigo a ser submetido à revista *Lipids*

5.1 ABSTRACT

Background: Microbiome-derived lipopolysaccharide (LPS) translocation and consequent systemic low-grade inflammation could be induced by dietary fat. However, the contribution of different fat types in this phenomenon needs clarification. We acutely assessed the influence of different fat types on cytokines release and LPS translocation.
Methods: This is a randomized parallel-arm design study in which 78 excess body fat women (aged 20-41 y old, 47.23 ± 0.48% of total body fat) consumed a drink containing one of the three test oils (25 mL): coconut oil – CO ($n = 23$), extra-virgin olive oil (EVOO, $n = 31$) or soybean oil (SO, $n = 24$). On test days, women reported to the laboratory in fasting state (12 h) and blood samples were taken at baseline, 2 and 4h after starting meal consumption. Serum triglycerides and plasma LPS, IL-8, IL-1β, IL-6, IL-10, TNF-α, and IL-12p70 were assessed.
Results: LPS concentrations were not affected by time nor by group, and it was not influenced by triglyceridemia. IL-8 and triglyceridemia was higher in SO than in EVOO. CO was the only group in which there was an increase in IL-1β/IL-10 ratio after the high-fat meal consumption. LPS increase was associated with changes in total pro-inflammatory/anti-inflammatory cytokines only in CO group, and negatively associated with IL-1β/IL-10 changes in SO group.
Conclusion: Reasonable fat loads affected postprandial inflammation without changing plasma LPS concentrations. However, changes in LPS correlated with pro-inflammatory cytokines in CO, indicating the existence of a synergic mechanism between saturated medium chain fatty acids and LPS on inflammation induction.

Keywords: coconut oil; extra-virgin olive oil; soybean oil; inflammation; lipopolysaccharide; monounsaturated fatty acid; polyunsaturated fatty acids.

5.2 INTRODUCTION

Chronic low-grade inflammation and activation of the immune system are widely recognized as key pathways involved in the pathogenesis of obesity-related disorders (1). The excessive consumption of nutrients triggers metabolic signals that lead to inflammatory responses and disturbs the metabolic homeostasis in obese people (2). Thus, inflammation induced by obesity differs from classical inflammatory response, in which the immune system quickly responds to an external agent or event and can be cleared up when the stimulus is removed or neutralized. Recently, the role of the translocation of microbiome-derived lipopolysaccharide (LPS) to the bloodstream on low-grade inflammation, a phenomenon called metabolic endotoxemia, has been extensively discussed (3–5). This new idea knocks down the classical pathway of obesity-induced inflammation and triggers scientific interest on prevention of external agents capable to cause inflammation. LPS is the major component of the outer surface of Gram-negative bacteria present in the intestinal environment. It contains a wide variety of molecules that shares a common architecture: a lipid moiety, called lipid A, and a glycosidic part. Most of the LPS biological activities have been associated with the lipid moiety of the molecule, reason by which lipid A is considered to be the endotoxic component (6).

Although great attention has been given to the role of the intestinal barrier and increased intestinal permeability on the onset of endotoxemia on obesity (3), LPS incorporation into quilomicrons is a new and reasonable pathway, which could be responsible for its acute effect on systemic inflammation (4,5). Interestingly, some dietary fat types are absorbed through pathways in which the quilomicrons are not involved and they have the same fatty acids carrier as lipid A does.

Coconut oil is the best natural source of medium-chain triglycerides (MCT). It does not induce chylomicron secretion, and it contains about 43 to 53% of lauric acid (C12:0) and 16 to 21% of myristic acid (C14:0) (7). These same types of fatty acids are also present in lipid A LPS (8). Since these fatty acids can be easily oxidized, because they do not require transporters to enter the mitochondria, and studies demonstrated their potential to improve weight loss (9,10), coconut oil has been popularly consumed as an adjuvant in obesity treatment. However, the contribution of these fatty acids from dietary sources on inflammation remains unknown (11,12). On the other hand, long-chain triglycerides (LCT) induce quilomicron secretion, then could contribute to LPS

translocation, and are able to interact with LPS receptors such as Toll-like receptors 2 and 4 modulating postprandial inflammation (13).

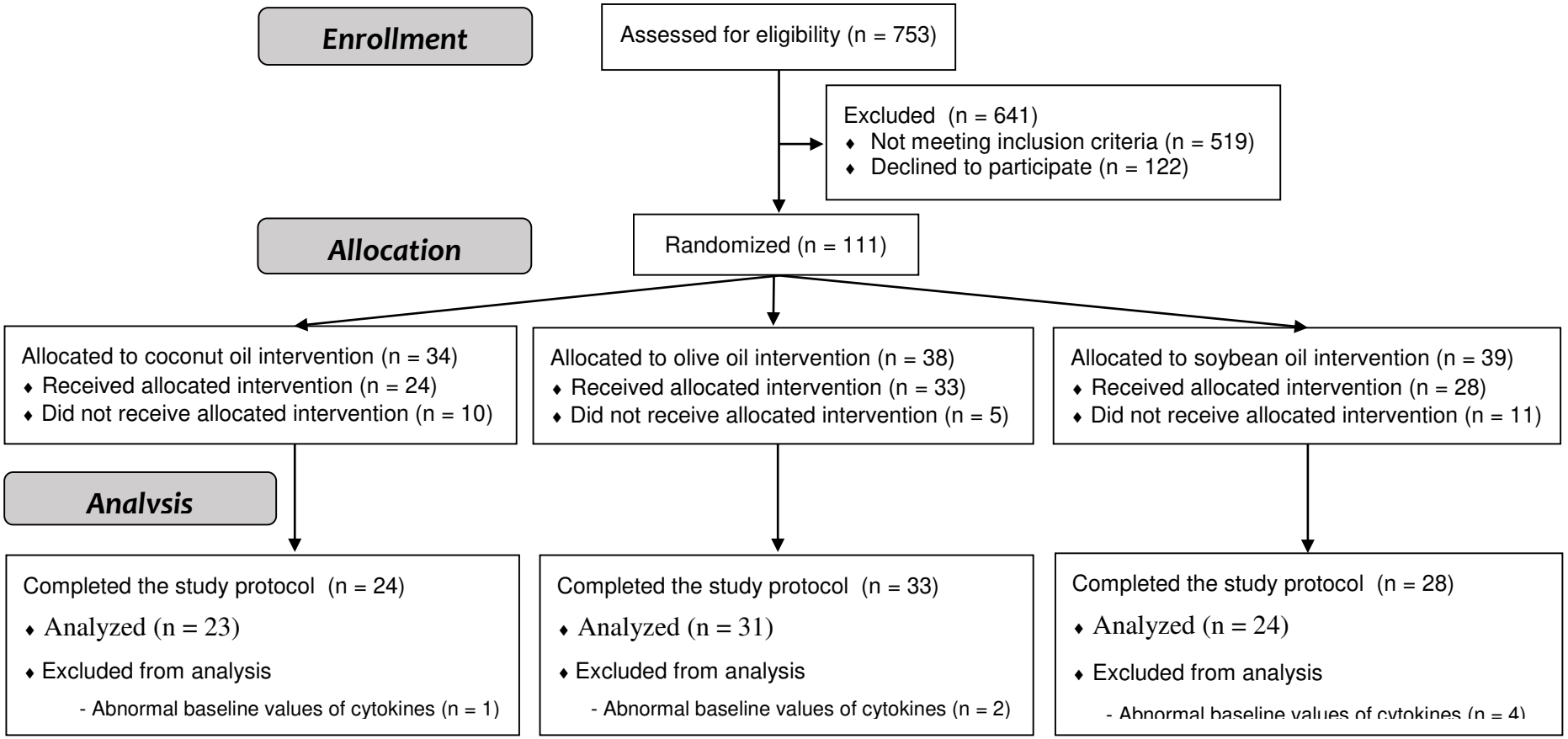
The urgent need for human studies assessing the role of different oil types on acute and long-term endotoxemia is highlighted in a very recent review (5). Therefore, we designed a study to compare the impact of oils presenting distinct fatty acid compositions (coconut oil - TMC source, extra-virgin olive oil and soybean oil - LCT sources) on postprandial markers of endotoxemia and associated metabolic inflammation. We hypothesized that 1) nonbacterial fatty acids provided by foods, mimicking a Gram negative bacterial infection, may acutely cause low-grade systemic inflammation by increasing cytokine release; 2) as incorporation into chylomicrons seems to be a key step during LPS translocation and MCT do not induce chylomicron secretion, coconut oil consumption will distinctly affect postprandial lipemia and LPS levels.

5.3 METHODS

5.3.1 Subjects

Seven hundred fifty-three women were assessed for eligibility through local advertisements and one hundred eleven apparently healthy middle-aged woman (20-41 y, BMI between 26 and 35 kg/m^2) met the inclusion criteria and were allocated to study groups (**Suppl. Fig. 1**). Potential subjects had excess body fat (> 32%), were nonsmoker, nonpregnant, and non-lactating. The exclusion criteria were the followings: alcohol consumption (>15 g of ethanol/d), elite athletes (>10 h of exercise/week), recent changes (< three months) in diet or physical activities habits, use of supplements or drugs except contraceptive ones, presence of food allergy/intolerance or aversion to tested ingredients, gastrointestinal diseases or other acute or chronic diseases besides obesity.

From the 111 initially recruited women, 26 dropped out before starting the intervention. Eighty-five eligible women were allocated in one of three experimental groups, and 78 were included in the analyses. Seven participants were excluded from the analyses due to abnormal baseline values of cytokines, characterizing asymptomatic inflammation. Power calculations indicated that 21 subjects were necessary to detect a 5% change in IL-10 and TNF-α concentrations (power = 99%, α = 5%). All recruited participants gave written consent after receiving verbal and written information about the experiment. The study protocol was approved by the Ethics Committee of Federal



Suppl. Fig. 1 CONSORT diagram showing the flow of participants through each stage of the trial. CONSORT Consolidated Standards of Reporting Trials.

University of Viçosa (protocol number: 892.467/2014), conducted in accordance with 1964 Declaration of Helsinki and registered at http://www.ensaiosclinicos.gov.br/ (identifier: RBR-7z358j).

5.3.2 Study design

This was a randomized parallel-arm design study. First served basis block randomization procedure was adopted by matching subjects in each group based on age, BMI, and body fat percentage. One week before starting the experiment, participants were instructed to not consume alcohol beverages and to maintain their usual dietary and physical activity habits. Baseline dietary intake was assessed through 24-h food records (three non-consecutive days, two week days and one weekend). A standard dinner (600 kcal, carbohydrate: 62E%, fat: 29.4E%, protein: 8.5E%) was consumed the night before the test day. On test day, women reported to the laboratory in a fasting state for anthropometric, body composition, and blood pressure assessments. Study participation was postponed if women presented any symptoms of inflammation or intestinal disorder. After the assessments, participants were assigned to one of three groups: coconut oil (CO, n = 23); EVOO (n = 31); and soybean oil (SO, n = 24). Woman consumed one of the three high-fat drinks according to the experimental group within 15 min. Antecubital blood samples were collected at baseline (T0) (right before consuming the test drink), and after 2 (T2) and 4h (T4) after consuming the test drink. No other food or beverage was consumed during the time subjects remained in the laboratory.

5.3.3 Test drinks

Coconut oil (Copra, Copra Indústria Alimentícia Ltda., Alagoas, Brazil), EVOO (Andorinha®, Sovena S.A., Algés, Portugal), and soybean oil (Corcovado, Archer Daniels Midland, Uberlândia, Brazil) were used to prepare the high-fat drinks. Test drinks consisted of a 300 mL milk-derived strawberry-flavored drink containing 25 mL of one of the previously mentioned oils. The oils tested in the study were protected from light and heat until consumption. Drinks were isocaloric and had the same nutritional composition except for the type of oil added (**Table 1**).

Table 1 Ingredients and nutritional composition of high-fat drinks (HFD) and chemical characterization of coconut oil (CO), extra-virgin olive oil (EVOO), and soybean oil (SO)

Ingredients	**CO HFD**	**EVOO HFD**	**SO HFD**
Coconut oil (mL)	25.0	0.0	0.0
EVOO (mL)	0.0	25.0	0.0
Soybean oil (mL)	0.0	0.0	25.0
Powdered milk (g)	40.0	40.0	40.0
Strawberry flavoring powder (g)	1.0	1.0	1.0
Water (mL)	280.0	280.0	280.0
Nutritional composition			
Energy content (kJ)	1,424.7	1,424.7	1,424.7
Fiber (g)	0.0	0.0	0.0
Carbohydrate (g / %E)	21.5 / 25.3	21.5 / 25.3	21.5 / 25.3
Protein (g / %E)	4.0 / 4.7	4.0 / 4.7	4.0 / 4.7
Total fat (g / %E)	26.5 / 70.0	26.5 / 70.0	26.5 / 70.0
Chemical characterization (/100 mL)	**CO**	**EVOO**	**SO**
Fatty acid profile of added oils (g)			
C8:0	5.2	0.0	0.0
C10:0	5.4	0.0	0.0
C12:0	51.6	0.0	0.0
C14:0	19.9	0.0	0.0
C14:1	0.0	0.0	0.1
C16:0	8.8	9.9	11.1
C16:1	0.0	0.7	0.1
C17:0	0.0	0.3	0.2
C18:0	3.0	2.1	3.3
C18:1	5.1	80.8	23.5
C18:2	0.7	4.9	54.3
C18:3	0.0	0.6	6.3
C20:0	0.0	0.4	0.4
C20:1n9	0.0	0.3	0.2
C20:2	0.0	0.1	0.4
Total MUFA	5.1	81.8	23.9
Total PUFA	0.7	5.6	61.0
Total SFA	94.0	12.6	15.0
Total vitamin E (mg)	16.04	31.90	189.20
Total carotenoids (mcg)	0.0	33.89	0.0
Total phenolic compounds (mg)	4.47	13.13	8.51
Total antioxidant activity (%)	2.36	68.26	9.82

Data are means. Total antioxidant activity was expressed by % inhibition of DPPH. MCFA: medium chain fatty acids; LCFA: long chain fatty acids; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; DPPH: 1,1-Diphenyl-2-picrylhydrazyl. Nutritional information was obtained from manufacturer's product information and from Brazilian Food Composition Table (39). Fatty acids profile were obtained after esterification (14) by gas chromatography. Total Vitamin E (α, β, γ and δ tocopherols and tocotrienols) (15) and total carotenoids (α-carotene, β-carotene, β-cryptoxanthin, lutein, and zeaxanthin) (17) was assessed by high-performance liquid chromatography. Total phenolic compounds (19) and total antioxidant activity to (40) were assessed by spectrophotometry.

5.3.4 Chemical characterization of oils

The fatty acids profile and quantification of antioxidant compounds of test oils were performed in triplicate. Fatty acids composition was assessed in laboratory after esterification (14) by gas chromatography (GC). Chromatographic analysis was carried out using a Shimadzu GC Solution instrument (Shimadzu Seisakusho Co., Kyoto, Japan) equipped with a flame ionization detector (FID) and a Carbowax capillary column (30 m x 0.25 mm). Briefly, 1µL of esterified sample was injected in GC with split ratio of 10. Nitrogen was supplied as the carrier gas at a flow rate of 43.2 cm/s. The initial oven temperature was 100ºC, maintained for 5 min, then increased to 220ºC at 4ºC/min. and held for 20 min. The flow rate over the column was 1.0 mL/min. The temperature of the FID and the injection port was 200ºC and 220ºC, respectively. Data handling was carried out using the software GC Solution package (Shimadzu Seisakusho Co., Kyoto, Japan).

Vitamin E content (α, β, γ and δ tocopherols and tocotrienols) was evaluated after dilution of 0.1g of each oil in 2 mL of hexane. Diluted samples were filtered (membrane porosity of 0,45 µm) and injected (30 µL for EVOO and 15 µL for soybean oil) in high-performance liquid chromatography – HPLC system (Shimadzu model SCL 10AT VP, Kyoto, Japan) (15). Carotenoid content (α-carotene, β-carotene, β-cryptoxanthin, lutein, and zeaxanthin) from oils was extracted (16) after method modifications. Briefly, ~5 g of each oil were homogenized in 60 mL of acetone during 4 min, filtered, and separated in petroleum ether. Then, 10 mL of extract were evaporated in nitrogen gas and the dry residue was resuspended in 2.0 ml of acetone. Filtered extract (0,45 µm) was injected (50 µL for olive oil and 200 µL for soybean oil) in HPLC (Shimadzu model SPD-M10 AVP, Kyoto, Japan) according to Panfili et al. (2004) (17). Total phenolic and antioxidant compounds were extracted in ethanol and mixed to Folin-Ciocalteau reagent (10%) and sodium carbonate (7.5%) before analysis on a spectrophotometer (Thermo Fisher Scientific, model Evolution^{TM} 60S, Wisconsin, USA) (18). Total phenolic compounds were analyzed according to Gutfinger (1979) (19) at absorbance of 725 nm. Antioxidant activity was evaluated by capacity of 1,1-Diphenyl-2-picrylhydrazyl (DPPH) inhibition according to Bloor (2001) at absorbance of 517 nm. The percentual of inhibition was expressed as followed: Inhibition (%) = $[(Abs_{Control} - Abs_{Extract})/Abs_{Control})] \times 100$.

5.3.4 Blood measurements

Serum (serum gel tubes) and plasma (EDTA tubes) samples were separated from whole blood by centrifugation (3,500 rpm, 4^0C, 15 min) and immediately frozen at -80^0C until analyses. Serum glucose, triglycerides (TG), total cholesterol, high-density-lipoprotein cholesterol (HDL-c), and low-density lipoprotein cholesterol (LDL-c) were quantified by an automated analyzer system (BS-200™ Chemistry Analyzer, Mindray) using available commercial colorimetric assay kits (K802, K117, K083, K071, and K088, respectively; Bioclin®, Minas Gerais, Brazil). Serum very-low-density-lipoprotein cholesterol (VLDL-c) was calculated using Friedewald et al. (1972) equations (20). Serum insulin was quantified using eletroquimioluminescence method (Elecsys-Modular E-170, Roche Diagnostics Systems). C-reactive protein (CRP) was quantified by ultrasensitive immunoturbidimetry (COBAS-Mira Plus, Roche Diagnostic Systems) using available commercial kit (K079, Bioclin®, Minas Gerais, Brazil). Insulin resistance was estimated calculating the homeostasis model assessment of insulin resistance (HOMA-IR) (21).

5.3.6 LPS measurements

Plasma samples for LPS assessments were manipulated and stored in apyrogenic recipients. LPS concentrations (T0 and T2) were determined through a chromogenic method using a Limulus Amebocyte Lysate (LAL) commercial kit (Hycult Biotech, Noord-Brabant, The Netherlands). Room temperature plasma samples were heated at 75°C for 5 min in order to neutralize endotoxin inhibitors. Aliquots of plasma (5 µL) and standards (19.8 µL for serial dilution) were diluted into microplate using 25 µL or 30 µL of pyrogen-free water, respectively. The LAL reagent (30 µl) was added to each well. After 30-min incubation, the absorbance at 405 nm was read (Multiskan Go, Thermo Scientific, USA). When the optical density of the 10 and 4 EU/mL standards differed by <10 %, the reaction was interrupted by adding 30 µL of the stop solution (acetic acid) and the absorbance was read again. Since absorbance is directly proportional to the concentration of endotoxin, a standard curve was used to calculate the LPS concentration in the samples. Four parameters logistic regression was used for fitting the standard curves. LPS concentration was expressed as endotoxin units per milliliter (EU/mL) after corrections for dilution factor (1:6).

5.3.7 Cytokine measurements

Plasma cytokines were analyzed at T0 and T4. Flow cytometry analysis was performed using a BD FACS Verse™ flow cytometer (BD Biosciences). Interleukin-8 (IL-8), interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-10 (IL-10), tumor necrosis factor-α (TNF-α), and interleukin-12p70 (IL-12p70) plasma concentrations were measured using commercial kit (Cytometric Bead Array CBA Human Inflammatory Cytokines Kit, BD Biosciences) according to the manufacturers' instructions. Data were analyzed using the FCAP Array Software v3.0 (BD Biosciences).

5.3.8 Calculations and statistical analyses

Cytokines were individually analyzed and the following ratios pro-inflammatory/anti-inflammatory were assessed: (IL-12p70 + TNF-α + IL-6 + IL-1β + IL-8 – total pro-inflammatory) / IL-10; (TNF-α + IL-6 + IL-1β) / IL-10; (IL-12p70 + IL-8) / IL-10; IL-12p70 / IL-10; TNF-α / IL-10; IL-6 / IL-10; IL-1β / IL-10; IL-8 / IL-10. Triglycerides, cytokines, cytokines ratios, and LPS numerical changes (final values – baseline values) and percentage changes [(final values – baseline values) / (baseline values)] were calculated. Total area under the curve (AUC) was calculated for triglyceridemia, from 0 to 4 hours using the trapezoid rule in GraphPad Prism 5 software (GraphPad, La Jolla, CA, USA).

Data normality was tested by Shapiro–Wilk test. Data are expressed as mean ± SEM for or median (p25-p75th percentiles values), when appropriate. Differences between tested groups were accessed by Kruskal-Wallis tests followed by post-hoc tests and Bonferroni corrections for multiple comparisons. Wilcoxon signed-rank test was used to compare data at baseline and postprandial responses, for each tested meal. Spearman's rank-correlation coefficient was used to assess the association between LPS and triglyceride AUC versus cytokine release and cytokine ratios, for each tested meal. Associations between LPS versus triglyceride AUC were also checked. Statistical analyses were conducted using SPSS 20 for Windows (SPSS, Inc., Chicago, IL, USA). The criterion for statistical significance was $\alpha \leq 0.05$ for all data analyses.

5.4 RESULTS

5.4.1 Subjects

Test meals were well tolerated by all participants (26.87 ± 0.66 y old, BMI of 30.51 ± 0.33 kg/m^2, and 47.23 ± 0.48% of total body fat). There were no significant between-group differences in baseline characteristics of participants including individual cytokines, cytokines ratios, and LPS concentrations. Baseline characteristics of the 78 study subjects according to experimental groups are shown in **Table 2**.

5.4.2 Effects of dietary oils on triglyceridemia, plasma LPS, and cytokine release

LPS concentrations were not affected by time (within-group differences) nor by dietary treatments (between-group differences). Between-group comparisons showed a greater increase in IL-8 and in triglyceridemia in SO than EVOO. However, there were no between-group differences between SO or EVOO compared with CO (**Fig. 1**). Analyses of individual cytokines also showed postprandial increases in IL-6 after the consumption of EVOO and SO ($P = 0.090$ for CO), and decreases in IL-12p70 and IL-10 in all study groups, but no significant differences between groups. SO was the only group who showed an increase in IL-8 after the high-fat meal consumption (**Suppl. Table 1; Fig. 2**).

Despite the effects in individual cytokines, results from pro-inflammatory/anti-inflammatory ratios demonstrated that CO was the only group in which there was a significantly increase in IL-1β/IL-10 ratio after the high-fat meal consumption (**Fig. 2**). All test meals promoted increase in total pro-inflammatory/anti-inflammatory, IL-6/IL-10, IL-8/IL-10, (TNF-α + IL-6 + IL-1β)/IL-10, and (IL-12p70 + IL-8)/IL-10 ratios, but there were no differences between groups (**Suppl. Table 1**).

5.4.3 Associations between triglyceridemia and plasma LPS/cytokines

There were no significant associations between triglyceridemia changes and plasma LPS changes for any experimental group. EVOO was the only group who presented significant associations between triglyceridemia and postprandial cytokines. Triglyceridemia increase was positively associated ($P < 0.05$) with IL-6 increase ($R^2 = 0.386$) and increase in the ratios IL-6/IL-10 ($R^2 = 0.386$), IL-8/IL-10 (0.405), and (TNF-α + IL-6 + IL-1β)/IL-10 ($R^2 = 0.411$).

Table 2 Baseline characteristics of study subjects according to experimental groups

	CO	**EVOO**	**SO**
Subjects (*n*)	23	31	24
Age (years)	27.78 ± 1.23	26.74 ± 1.02	26.17 ± 1.23
Physical activity (S/LA)	1 / 22	5 / 25	6 / 24
Systolic blood pressure (mmHg)	110.14 ± 2.26	113.53 ± 1.97	107.68 ± 1.95
Diastolic blood pressure (mmHg)	68.84 ± 1.86	72.63 ± 1.64	67.36 ± 1.30
Body weight (kg)	80.00 ± 2.72	80.76 ± 1.63	79.25 ± 2.20
BMI (kg/m2)	30.00 ± 0.65	30.64 ± 0.49	29.88 ± 0.62
Waist circumference (cm)	97.54 ± 1.86	97.64 ± 1.14	96.82 ± 1.57
Waist/hip circumference	0.86 ± 0.01	0.85 ± 0.01	0.85 ± 0.01
Total body fat percentage (%)	46.41 ± 1.00	47.96 ± 0.73	47.08 ± 0.78
Glucose (mmol/L)	4.84 ± 0.09	4.94 ± 0.07	4.67 ± 0.08
Triglycerides (mmol/L)	2.34 ± 0.12	2.79 ± 0.23	2.23 ± 0.23
Total cholesterol (mmol/L)	4.14 ± 0.14	4.36 ± 0.17	4.09 ± 0.17
HDL-c (mmol/L)	1.14 ± 0.04	1.27 ± 0.06	1.25 ± 0.07
LDL-c (mmol/L)	2.49 ± 0.12	2.49 ± 0.13	2.34 ± 0.14

Values are means ± SE. CO: coconut oil; EVOO: extra-virgin olive oil; SO: soybean oil; BMI: body mass index; S/LA: number of sedentary and low-active individuals ratio (41); HDL-c: high-density-lipoprotein cholesterol; LDL-c: low-density lipoprotein cholesterol. There were no significant differences between groups in baseline (One Way ANOVA, $P > 0.05$).

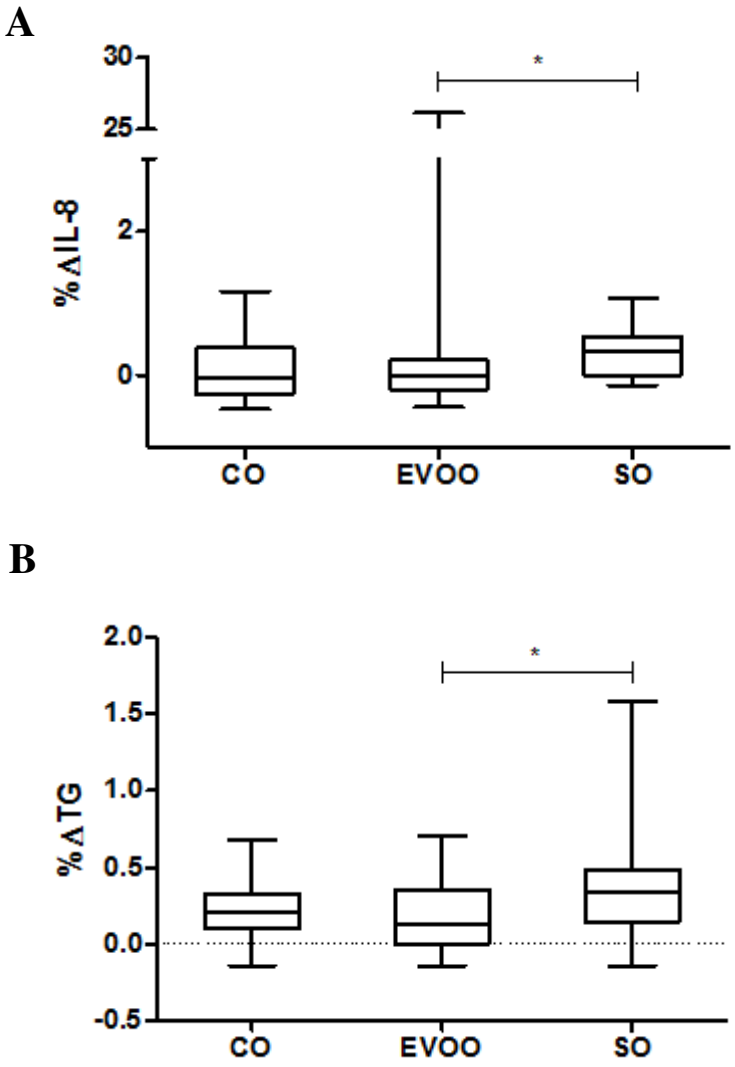


Fig. 1 Median (p25/p75) change (final values – baseline values) interleukin 8 – IL-8 values (A), and triglycerides – TG contents (B) after the consumption of drinks containing 25 mL of coconut oil (CO, $n = 23$), extra-virgin olive oil (EVOO, $n = 31$), or soybean oil (SO, $n = 24$). Data were analyzed by Kruskal-Wallis test, followed by Bonferroni corrections (*$P < 0.05$)

Suppl. Table 1 Changes in plasma lipopolysaccharides (LPS), triglycerides (TG), and cytokines according to experimental groups

	COCONUT OIL			EXTRA-VIRGIN OLIVE OIL			SOYBEAN OIL			P_{Inter}**			
	T0	**T4**	P_{Intra}*	**T0**	**T4**	P_{Intra}*	**T0**	**T4**	P_{Intra}*	**T0**	**T4**	**ΔT4**	**%ΔT4**
LPS (EU/mL)§	0,49 (0,29-0,89)	0,52 (0,32-0,86)	0,408	0,49 (0,34-0,65)	0,41 (0,33-0,81)	0,530	0,51 (0,29-0,69)	0,42 (0,38-0,77)	0,265	0,993	0,685	0,386	0,548
TG (mg/dL)	93,00 (80,00-103,00)	113,00 (90,00-123,00)	**<0,001**	97,00 (66,00-140,00)	107,00 (84,00-152,00)	**0,002**	79,50 (54,75-99,00)	101,00 (7,00-130,75)	**<0,001**	0,184	0,792	0,062	**0,046**
IL-12p70 (pg/mL)	2,68 (1,47-4,42)	1,545 (0,92-2,51)	**0,021**	2,23 (0,94-3,87)	0,92 (0,50-1,63)	**0,001**	1,60 (0,21-2,83)	1,51 (0,19-3,08)	0,401	0,222	0,123	0,068	0,145
TNF-alpha (pg/mL)	0,48 (0,00-1,18)	0,845 (0,00-1,61)	0,408	0,37 (0,00-0,67)	0,00 (0,00-0,61)	0,211	0,00 (0,00-0,24)	0,00 (0,00-1,26)	0,814	0,154	0,108	0,344	0,244
IL-10 (pg/mL)	1,3 (0,87-1,91)	0,675 (0,36-1,11)	**0,001**	1,06 (0,78-1,78)	0,72 (0,40-0,98)	**<0,001**	0,90 (0,66-1,39)	0,80 (0,58-1,13)	**0,045**	0,375	0,621	0,060	0,056
IL-6 (pg/mL)	1,835 (1,14-2,82)	2,21 (1,64-2,97)	0,090	1,87 (0,93-2,99)	2,40 (1,73-4,88)	**0,049**	1,79 (1,43-2,81)	2,92 (1,77-4,85)	**0,033**	0,981	0,429	0,246	0,151
IL-1β (pg/mL)	0,77 (0,00-2,78)	1,25 (0,00-2,40)	0,888	0,37 (0,00-1,45)	0,09 (0,00-1,36)	0,439	0,21 (0,00-1,01)	0,20 (0,00-1,63)	0,679	0,285	0,100	0,570	0,112
IL-8(pg/mL)	7,56 (6,89-11,01)	7,42 (5,32-10,23)	0,958	9,09 (6,09-10,42)	9,15 (6,95-10,14)	0,797	7,07 (5,57-8,68)	9,33 (6,85-11,76)	**0,002**	0,265	0,441	**0,022**	**0,015**
(IL-12p70+TNF-alpha+IL-6+IL-1b+IL-8)/IL-10	12,00 (8,73-16,93)	22,35 (11,80-34,94)	**0,009**	12,91 (8,78-16,11)	20,63 (14,05-31,04)	**<0,001**	10,84 (9,03-18,46)	20,53 (11,25-31,24)	**0,002**	0,984	0,854	0,790	0,663
IL-12p70/IL-10	2,35 (0,99-3,17)	2,38 (0,93-3,73)	0,205	1,79 (0,93-2,96)	1,51 (0,59-2,87)	0,871	1,24 (0,25-2,36)	1,10 (0,34-2,73)	1,000	0,167	0,097	0,508	0,440
TNF-alpha/IL-10	0,22 (0,00-0,86)	0,64 (0,00-2,28)	0,063	0,17 (0,00-0,55)	0,00 (0,00-0,88)	0,748	0,00 (0,00-0,26)	0,00 (0,00-0,81)	0,388	0,208	0,106	0,166	0,238
IL-6/IL-10	1,36 (0,89-2,07)	2,21 (1,16-4,94)	**0,023**	1,61 (1,11-2,36)	3,81 (1,96-8,21)	**<0,001**	2,09 (1,24-2,94)	3,24 (2,11-6,47)	**0,003**	0,165	0,160	0,178	0,127
IL-1b/IL-10	0,76 (0,00-1,39)	1,21 (0,00-3,22)	**0,030**	0,27 (0,00-1,10)	0,09 (0,00-1,38)	0,869	0,18 (0,00-0,84)	0,47 (0,00-1,83)	0,532	0,389	0,127	0,206	0,099
IL-8/IL-10	5,30 (4,03-10,61)	13,42 (6,47-21,32)	**0,012**	7,06 (5,10-10,89)	12,72 (7,93-21,00)	**<0,001**	7,37 (5,70-9,98)	12,33 (6,52-17,69)	**0,001**	0,615	0,835	0,703	0,530
(TNF-alpha+ IL-6 + IL-1b)/IL-10	2,80 (1,68-3,71)	4,79 (2,95-12,20)	**0,002**	2,28 (1,23-3,52)	5,68 (2,98-10,25)	**<0,001**	2,42 (1,66-4,20)	4,85 (2,73-8,93)	**0,002**	0,715	0,917	0,766	0,525
(IL-12p70 + IL-8)/IL-10	7,61 (6,02-14,98)	16,75 (8,72-27,27)	**0,030**	9,20 (6,30-12,80)	14,64 (10,25-22,07)	**<0,001**	8,85 (6,59-11,73)	14,06 (7,65-19,63)	**0,003**	0,866	0,756	0,757	0,804

Data are median (p25-p75). T0: fasting; T4: 4 h after meal; ΔT4: T4 – T0; %ΔT4: (T4 – T0)/T0; § Assessed at T0 and T2 (2h after). *Wilcoxon Signed-Ranked; **Kruskal Wallis. Bold type letters indicated significant differences ($P < 0.050$).

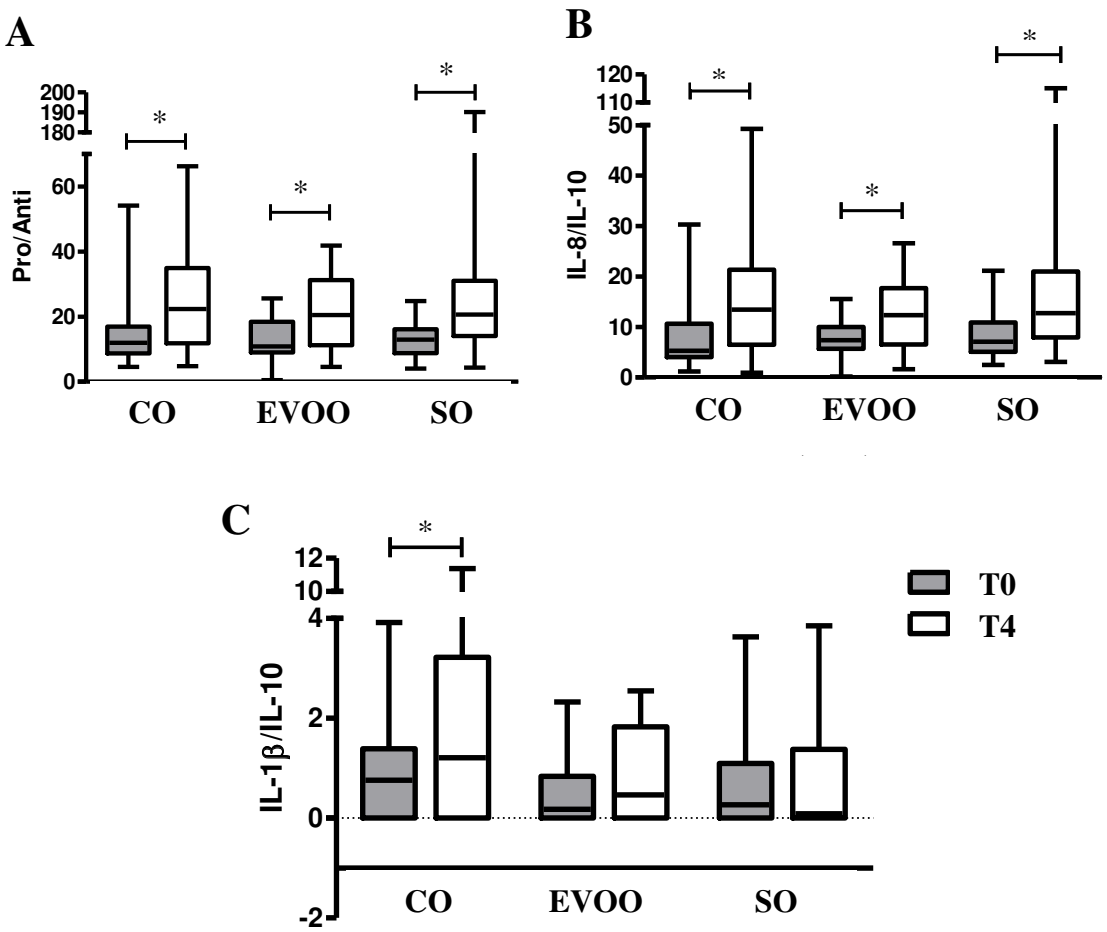


Fig. 2 Median (p25/p75) total pro-inflammatory response/anti-inflammatory [(IL-12p70 + TNF-α + IL-6 + IL-1β + IL-8)/ IL-10] (A), IL-8/IL-10 (B), and IL-1β/IL-10 (C) ratios at baseline (T0) and after 4 h (T4) of consumption of drinks containing 25 mL of coconut oil (CO, $n = 23$), extra-virgin olive oil (EVOO, $n = 31$), or soybean oil (SO, $n = 24$). Data were analyzed by Wilcoxon signed-rank test ($^{*}P < 0.050$)

5.4.5 Associations between plasma LPS and cytokines

There were no associations between dietary treatments and individual cytokines responses. However, plasma LPS concentrations increase was associated with changes in total pro-inflammatory/anti-inflammatory cytokines (R^2 = 0.489), IL-8/IL-10 (R^2 = 0.495), and (IL-12p70 + IL-8)/IL-10 (R^2 = 0.447) in CO group ($P < 0.05$). LPS concentrations increase was negatively associated with IL-1β/IL-10 changes in SO group (R^2 = -0.473, $P < 0.05$). EVOO LPS and cytokine release changes were not significantly associated with each other (**Fig. 3**).

5.5 DISCUSSION

In this study, we evaluated the role of reasonable amount of oil (25 mL), rather than excessive amount of fat loads, on postprandial plasma LPS and cytokines concentrations. We showed that the consumption of oils with distinct fatty acids profile can acutely affect cytokine release but does not affect LPS concentrations. Besides, triglyceridemia was not associated with postprandial LPS changes. Thus, our results suggest that lipid-induced LPS translocation is not a prominent pathway to explain acute immunological responses after consumption of fat at usual doses.

In our study, IL-8 increased in SO compared with EVOO, and pro-inflammatory IL-1β/IL-10 ratio increased only in CO, which is a more robust inflammatory status marker than individual the cytokine responses. Dietary fat can influence the activity and function of numerous immune system components, including antigen presentation, lymphocyte proliferation, cytokine production, granulocytes and natural killer cell activity (22,23). Scientific literature reports a suppressive soybean oil immunomodulatory effect, which is mediated by an increase in linoleic acid content of cell membranes, impairing eicosanoids synthesis (24–27). In fact, novel soybean oils containing different linoleic acid to α-linolenic acid contents affected T lymphocytes proliferative ability in different ways (28), suggesting that linoleic acid are involved in soybean oil immunomodulatory functions. However, safflower oil rich in linoleic acid increased cytokine productions such as IL-6, IL-8, and IL-10 in burned rats (29). Thus, it has been suggested that soybean oil could stimulate pro-inflammatory cytokine production and amplify stress responses in only severely stressed patients (27). The insufficient scientific evidence to support linoleic acid pro-inflammatory impact in health and obese individuals was recently described (30). Our results indicate that

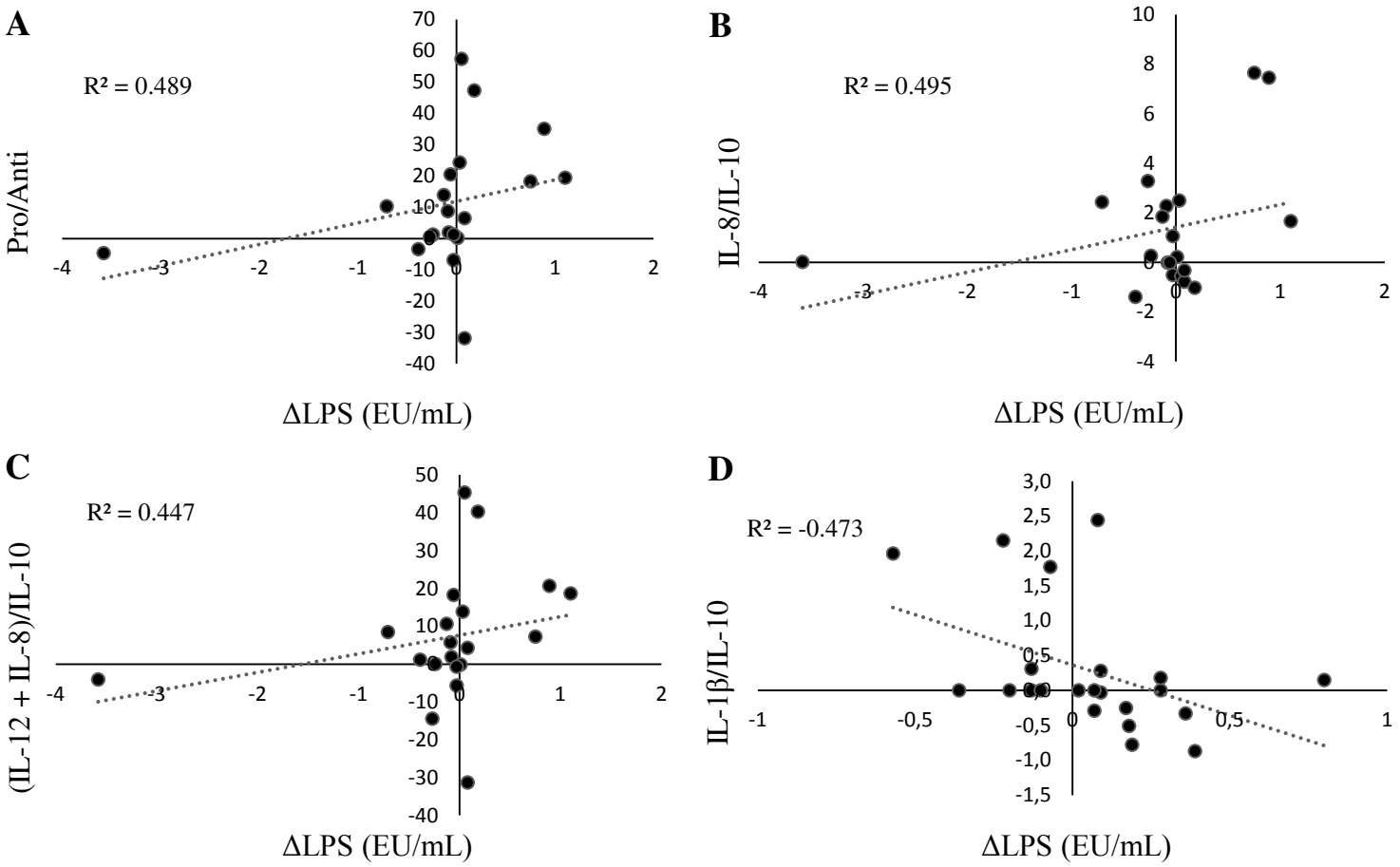


Fig. 3 Spearman correlations between changes (final values – baseline values) in lipopolysaccharides (LPS) concentrations and in cytokine releases after the consumption of drinks containing 25 mL of coconut oil (A, B, and C, $n = 23$) or soybean oil (D, $n = 24$). Pro/Anti-inflammatory markers: (IL-12p70 + TNF-α + IL-6 + IL-1β + IL-8)/ IL-10 ($P < 0.050$).

soybean oil can cause a transient pro-inflammatory status even in an obese population apparently healthy. However, we are unable to assure that this type of response would be maintained with time. Therefore, longer-term studies should be conducted to assess the effect of different types of oils, including soybean oil, on low grade subclinical inflammation.

Lauric acid is the main constituent of coconut oil and its ability to induce inflammation was showed in some (31–33) but not all (34,35) studies. A very elegant study proved that saturated fatty acid-induced activation of TLR2 or TLR4 is a fatty acid-specific effect (36), so lauric and myristic acids could stimulate cytokines release by direct interaction with receptors in immune cells. Nevertheless, the result of an in vitro study showed that only β-hydroxy lauric acid significantly stimulated IL-6 production compared to control, and that free β-hydroxy lauric and myristic acids were absent in plasma human samples (11). In our study, we used a fat load to induce changes in plasma fatty acids and impact the immune response. Interesting, changes in LPS concentration showed a clear pro-inflammatory association only in CO group. That result suggests the existence of a synergistic mechanism between saturated medium chain fatty acids and LPS on inflammation induction. The possibility of a synergic mechanism was previously documented for the long-chain saturated fatty acid palmitate, which induced secretion and TNF-α, IL-8 and IL-1β mRNA expression, and enhanced LPS-induced IL-1β secretion in ester-differentiated THP-1 cells, a model of human macrophages (37). The ability of laurate and myristate in enhancing LPS function needs further elucidation. In contrast, in our study changes in LPS concentration was negatively associated with IL-1β/IL-10 ratio in SO group. This was an unexpected result since, to the best of our knowledge; there is no reported negative association between LPS and pro-inflammatory status. LPS has long been considered as a potent inflammation stimulant. For that reason, scientists have adopted it as the main stimulation agent in immunological trials (24,37). The nature and the mechanisms involved in this relationship needs to be investigated.

In our study, triglyceridemia was associated with increased pro-inflammatory status only in EVOO group. In addition to the fact that EVOO show pro-inflammatory properties were not more prominent compared to CO and SO groups, we suggest that these results were influenced by the great sample size of this group. Between-subject triglyceridemia has large variation and could requires a large number of individuals to improve the power of such associations (38). Our study was primarily designed to assess the role of plant oil on LPS and cytokines release, and triglyceridemia association analyses were included as a way to better interpret our results.

5.6 CONCLUSION

The consumption of reasonable amounts of different plant oils distinctly affect postprandial inflammation in excess body fat women but does not affect plasma LPS concentrations. SO showed greater increase in IL-8 concentrations compared to EVOO. CO was the only group that presented postprandial IL-1β/IL-10 ratio increase. Changes in LPS concentrations were associated with pro-inflammatory profile only in CO group, suggesting the role of a synergic effect between medium-chain fatty acids and metabolic endotoxemia. The nature and the mechanisms behind the negative association between changes in LPS and IL-1β/IL-10 in SO needs to be clarified. Longer term studies are required to confirm our results.

5.7 ACKNOWLEDGMENTS

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5.8 CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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6. ARTIGO 3 - Consumption of extra-virgin olive oil improves body composition and blood pressure in excess body fat women: a randomized, double-blind, placebo-controlled clinical trial

Artigo a ser submetido à revista BJN

6.1 ABSTRACT

Background: Despite the fact that extra-virgin olive oil (EVOO) is widely used in obese individuals for treat cardiovascular diseases, the role of EVOO on weight/fat reduction remains unclear. We investigated the effects of the consumption of energy-restricted diet containing EVOO on body composition and metabolic disruptions related to obesity.
Methods: This is a randomized, double-blinded, placebo-controlled clinical trial in which 41 excess body fat adult women (mean ± SD: 27.0 ± 0.9y old, 46.8 ± 0.6% of total body fat) daily received high-fat breakfasts contained 25mL of soybean oil (control group, n = 20) or EVOO (EVOO group, n = 21) during nine consecutive weeks. Breakfasts were associated to energy-restricted normofat diets (-2090kJ, ~32%E from fat). Anthropometric, dual-energy X-ray absorptiometry were assessed, and fasting blood was collected on the first and last day of the experiment.
Results: Fat loss was ~80% higher on EVOO compared to the control group (mean ± SE: -2.41 ± 0.33kg vs. -1.33 ± 0.42, P = 0.037). EVOO also reduced diastolic blood pressure when compared to control (-5.05 ± 1.60mmHg vs. +0.25 ± 1.16mmHg, P = 0.011). Within-group differences (P < 0.050) were observed for HDL-c (-2.89 ± 1.17mmol/L) and IL-10 (+0.88 ± 0.08pg/mL) in control group, and for serum creatinine (+0.04 ± 0.01µmol/L) and alkaline phosphatase (-3.26 ± 1.78IU/L) in the EVOO group. There was also a trend for IL-1β EVOO reduction (-0.28 ± 0.14pg/mL, P = 0.060).
Conclusion: EVOO consumption reduced body fat and improved blood pressure. Our results indicate that EVOO should be included into energy-restricted programs for obesity treatment.

Keywords: extra-virgin olive oil; soybean oil; body fat; blood pressure; adiposity; monounsaturated fatty acid; polyunsaturated fatty acids.

6.2 INTRODUCTION

Obesity results from complex interactions between genetic and lifestyle factors. High fat diets consumption has been considered one of the main factors predisposing fat gain (1–3). However, the role of dietary fat on obesity pathogenesis remains unclear. In the last decade, old certainties regarding dietary fat have been questioned, and some have been abandoned. Recently, traditional recommendations of replacing animal fat by plant fats have been under increased scrutiny due to opposite scientific evidences suggesting that polyunsaturated fatty acid could increase cardiovascular and death risks more than saturated fat (4,5).

Extra virgin olive oil (EVOO) is a high-quality oil rich in monounsaturated oleic acid (55 to 85% of fatty acid content), which contains more than 230 chemical constituents with antioxidant activity such as vitamin E, carotenoids, and phenolic compounds (6). Due to the well-established beneficial effects of that oil over CVD risk (7–10) and the strong association between CVD and excess body fat, the consumption of energy-restricted diet containing EVOO has been adopted in weight loss programs. However, the benefits of EVOO over CVD have been inadvertently extrapolated for weight/fat loss promotion without adequate scientific evidence (11,12)

The current hypothesis that EVOO could also contribute to weight/fat loss is mostly based on observational evidence demonstrating that the consumption of Mediterranean diet rich in olive oil was significantly less likely to favor obesity (13–15). Results from these observational studies are wisely difficult to interpret because habitual use of olive oil in salads and vegetable based dishes within the Mediterranean diet is also associated with the consumption of other functional low density foods (12,16). Furthermore, randomized clinical trials about this topic are scarce, and presented inconclusive and controversial results (12,17). In some clinical trials, the great discrepancy in the dietary intervention applied to the control and test groups may have favored the reduction in body weight/fat in response to olive oil consumption (18,19). On the other hand, other clinical trials reported no influence of olive oil on body weight/fat (20) or even an increase in abdominal obesity (21) when it was incorporated into Mediterranean-diet. When consumed associated with an energy-restricted non-Mediterranean diet, olive oil reduced less body weight than medium-chain triacylglycerol – MCT (22).

Despite the fact that the incorporation of good oil source into energy restricted diets can improve palatability and favor compliance of the traditional energy restricted

low-fat diet (23), there is no clear evidence supporting the effect of EVOO to improve body weight/fat loss. Therefore, we investigated the effect of the consumption of EVOO into an energy restricted non-Mediterranean diet on body weight/fat. Additionally, we assessed the role of EVOO on systemic inflammation, cardiovascular, hepatic, and renal functions, which can be impaired due to lipotoxicity.

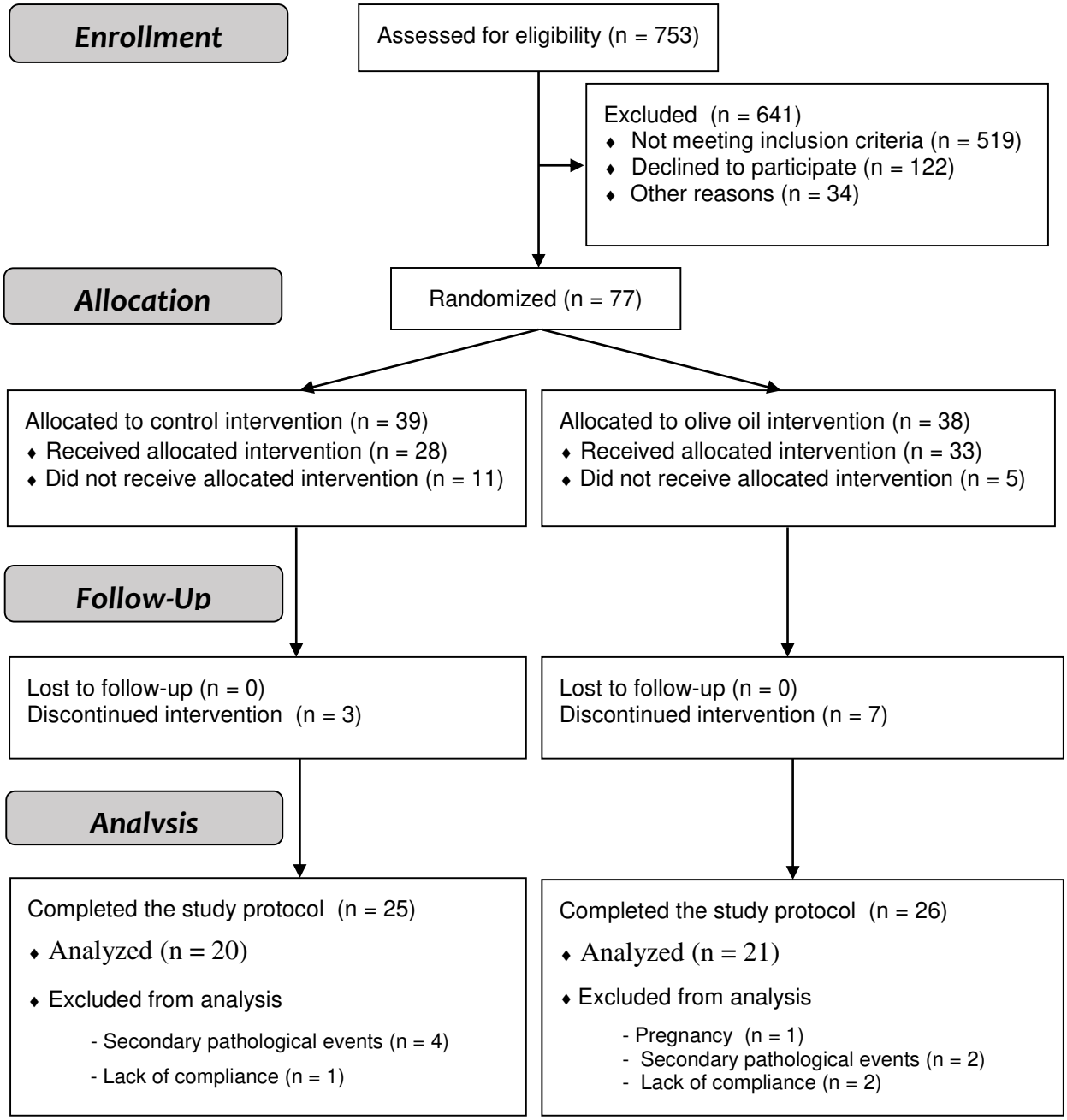
6.3 METHODS

6.3.1 Subjects

Seven hundred fifty-three woman were assessed for eligibility through local advertisements and seventy-seven apparently healthy middle-aged woman (19-41 y, BMI between 26 and 35 kg/m^2) met the inclusion criteria and were allocated to study groups (**Suppl. Fig. 1**). Potential subjects had excess body fat (> 32%), habitually used soybean oil as cooking oil; were nonsmoker, nonpregnant, and non-lactating. The exclusion criteria were the followings: alcohol consumption (>15 g of ethanol/d), elite athletes (>10 h of exercise/week), habitual consumption of olive oil (more than 8 mL/d), recent changes (< three months) in diet or physical activities habits, use of supplements or drugs except contraceptive ones; presence of food allergy/intolerance or aversion to tested ingredients, gastrointestinal diseases or other acute or chronic diseases besides obesity.

From the 77 initially recruited women, 16 dropped out before starting the intervention. Sixty-one eligible women were included the study, 51 completed the adopted protocol, and 41 were included in the analyses. The reasons by which eight women were excluded from the analyses were the following: pregnancy (n = 1), secondary pathological events (n = 6), and lack of compliance (n = 3). Sample size was calculated (24) considering 10% difference in body weight, and a statistical power of 90%.

All recruited participants gave written consent after receiving verbal and written information about the experiment. The study protocol was approved by the Ethics Committee of Federal University of Viçosa (protocol number: 892.467/2014), conducted in accordance with 1964 Declaration of Helsinki and registered at http://www.ensaiosclinicos.gov.br/ (identifier: RBR-7z358j).



Suppl. Fig. 1 CONSORT diagram showing the flow of participants through each stage of the trial. CONSORT Consolidated Standards of Reporting Trials.

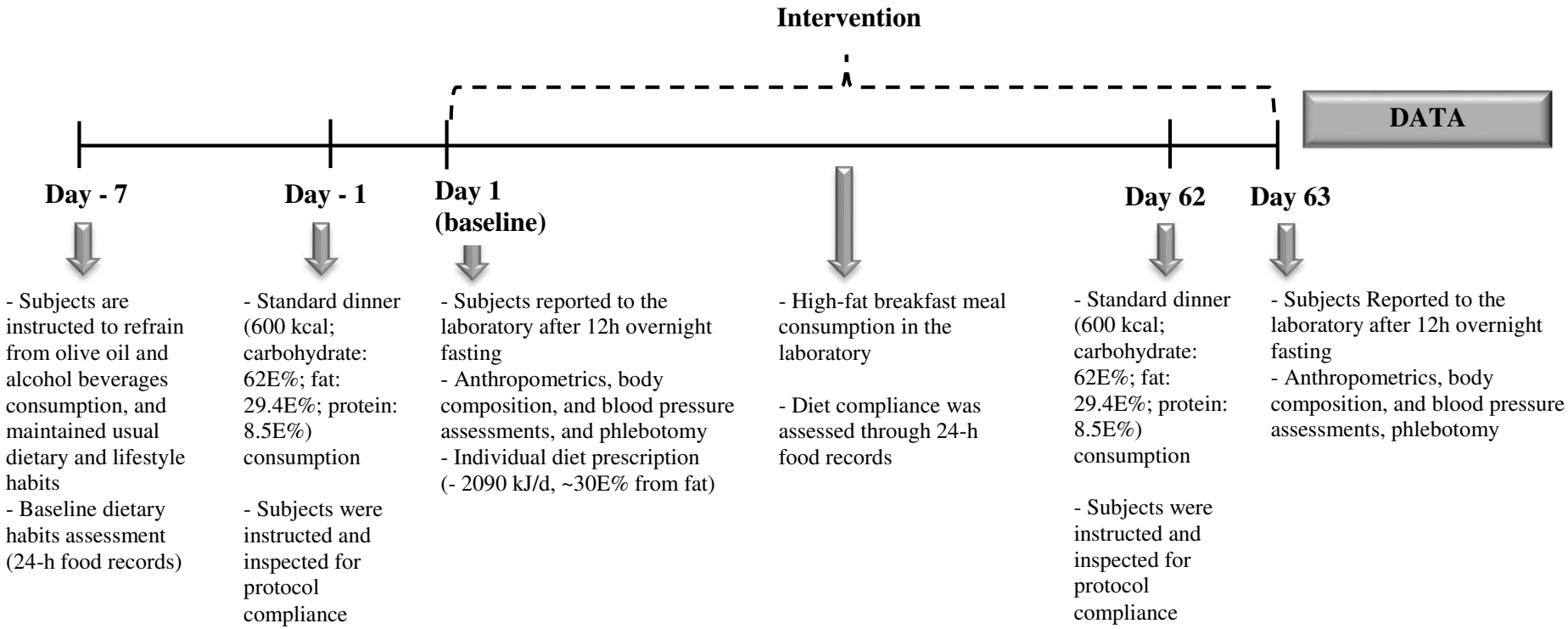
6.3.2 Experimental design

This was a nine consecutive weeks (± 5 days) of duration double-blinded, randomized, parallel, placebo-controlled clinical trial, in which subjects were randomly assigned to control (soybean oil) or interventional (EVOO) groups. The tolerance of ± 5 days to end the experiment was required to prevent impairment on anthropometric/body composition parameters assessments due to hormonal changes. First served basis block randomization procedure was adopted by matching subjects in each group based on age, BMI, and body fat percentage. The allocation on the control or interventional groups was concealed from the investigators. High-fat drinks were served into colored cups to avoid visual identification of the type of drink tested. There was no description or dietary information about the breakfasts on those cups. Therefore, neither subjects nor investigators were aware of the treatment assignments.

One week before beginning the trial, selected women refrained from eating olive oil, were instructed to not consume alcohol beverages and to maintain their usual dietary and physical activity habits. A standard dinner (600 kcal, carbohydrate: 62E%, fat: 29.4E%, protein: 8.5E%) was consumed the night before the test day. Women reported to laboratory in a fasting state for anthropometric, body composition, and blood pressure assessments at baseline and on the last day of the experiment. Study participation was postponed if women presented any symptoms of inflammation or intestinal disorder. After the assessments, subjects underwent blood collection and consumed a high-fat breakfast containing 25 mL of soybean oil or EVOO for breakfast. The amount of oil (25 mL) added to the drinks was based on the range of olive oil usually consumed by Mediterranean population (25 to 50 mL/d) (25) without exceeding the fat consumption recommendations (26). During the other study days, high-fat breakfasts were daily provided in the laboratory as part of an energy restricted non-Mediterranean diet and woman were released to follow the prescribed diet in free-living conditions. Habitual food intake, physical activity level, and prescribed diet compliance were also assessed (**Suppl. Fig. 2**).

6.3.3 Breakfasts

Olive oil (Andorinha®, Sovena S.A., Algés, Portugal) and soybean oil (Corcovado, Archer Daniels Midland, Uberlândia, Brazil) were used to prepare the high-fat drinks (300 mL of a milk-derived flavored drink containing 25 mL of the



Suppl. Fig. 2 Schematic representation of study protocol (control group: *n* = 20; EVOO group: *n* = 21). FFQ: Food frequency questionnaire, EVOO: extra-virgin olive oil.

previously mentioned oils). Both oils were protected from light and heat until their consumption. During all the experimental period, the high-fat drinks were prepared with low-fat powered milk and flavored with fruits, chocolate powder, or instant coffee. Low-fat cookies were also offered in order to avoid monotony. Breakfasts from both groups had identical nutritional composition, except regarding the type of added oil.

EVOO and soybean oil fatty acids profile and quantification of antioxidant compounds were performed in triplicate. Fatty acids composition of EVOO was assessed in laboratory after esterification (27) by gas chromatography (GC). Chromatographic analysis was carried out using a Shimadzu GC Solution instrument (Shimadzu Seisakusho Co., Kyoto, Japan) equipped with a flame ionization detector (FID) and a Carbowax capillary column (30 m x 0.25 mm). Briefly, 1µL of esterified sample was injected in GC with split ratio of 10. Nitrogen was supplied as the carrier gas at a flow rate of 43.2 cm/s. The initial oven temperature was 100ºC, maintained for 5 min, then increased to 220ºC at 4ºC/min. and held for 20 min. The flow rate over the column was 1.0 mL/min. The temperature of the FID and the injection port was 200ºC and 220ºC, respectively. Data handling was carried out using the software GC Solution package (Shimadzu Seisakusho Co., Kyoto, Japan) (**Suppl. Table 1**).

6.3.3 Dietary assessments

Energy restricted nutritionally balanced diets were individually prescribed by a single dietitian. The type of foods prescribed and the macronutrient distribution were maintained during the intervention to reduce the influence of prescribed diets beyond fats on results. No other high MUFA food besides the 25 mL of EVOO for the EVOO group was prescribed, and a food substitution list was used to subsidize food choices. Saturated fat energy requirements were estimated according to total energy expenditure for overweight/obese women (26). Then, caloric restriction (-2090 kJ/d) was applied. Physical activity levels (28) were used to obtained physical activity coefficients (1.00 for sedentary or 1.16 for low-active individuals) (26). Three non-consecutive days (two week days and one weekend day) 24-h food records were applied to assess food intake on the week before baseline, and during the experimental period. Macro- and micronutrient intakes were analyzed by a single dietitian using DietPro software (version 5.2i, Agromídia, Viçosa, Brazil), and were based on reliable composition tables (29–31).

Suppl. Table 1 Nutritional composition of high-fat breakfasts, and chemical characterization of the oil types used to prepare these breakfasts

Breakfasts meals (mean ± SD of six menus)	**CONTROL BREAKFAST**	**EVOO BREAKFAST**
Energy content (kJ)	1663.6 ± 33.9	1663.6 ± 33.9
Fiber (g)	1.8 ± 2.4	1.8 ± 2.4
Carbohydrate (g)	32.9 ± 1.7	32.9 ± 1.7
Protein (g)	4.1 ± 0.4	4.1 ± 0.4
Total fat (g)	27.7 ± 0.1	27.7 ± 0.1
Sodium (mg)	100.3 ± 25.1	100.3 ± 25.1
Chemical characterization	**SOYBEAN OIL**	**EVOO**
Fatty acid profile of added oils (%)		
C14:1	0.1	0.0
C16:0	11.1	9.9
C16:1	0.1	0.7
C17:0	0.2	0.3
C18:0	3.3	2.1
C18:1	23.5	80.8
C18:2	54.3	4.9
C18:3	6.3	0.6
C20:0	0.4	0.4
C20:1n9	0.2	0.3
C20:2	0.4	0.1
Total MUFA	23.9	81.8
Total PUFA	61.0	5.6
Total SFA	15.0	12.6

MCFA: medium chain fatty acids; LCFA: long chain fatty acids; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; DPPH: 1,1-Diphenyl-2-picrylhydrazyl. Nutritional information was obtained from manufacturer's product information and from Brazilian Food Composition Table (29). Fatty acids profile was obtained after esterification (27) by gas chromatography.

6.3.5 Anthropometric, body composition, and blood pressure measurements

Anthropometric measurements were assessed by a single investigator. Body weight were measured on a digital platform scale with a resolution of 0.5 kg (Toledo®, Model 2096PP/2, São Paulo, Brazil), while subjects were barefoot and wearing lightweight clothing. Height was measured to the nearest 0.1 cm using a wall-mounted stadiometer (Wiso, Chapecó, SC, Brazil). BMI was calculated by dividing body (kg) by height (m) squared. Waist, hip, neck, and thigh circumferences, as well as sagittal abdominal diameter were measured in triplicate as described by Vasques et al. (32) by a single examiner. The average of the two nearest of the three collected measurements was recorded. Waist circumference and sagittal abdominal diameter were measured in four

distinct regions: the narrowest waist; umbilical level; immediately above the iliac crests; and the midpoint between the last rib and iliac crest. Waist/hip, waist/height, and waist/thigh ratios, as well as conicity index (CI) were calculated following formula: CI = [waist circumference (m)] / [0.109 √(body weight (kg)/ height (m))] (Tailor et al., 2000). Blood pressure was measured by an automatic Omron HEM-7200 device (Omron Inc., Dalian, China) in both arms, according to Mancia et al. (33).

Dual energy X-ray absorptiometry scan (DXA) (model Prodigy Advance, GE Healthcare Inc., Waukesha, WI) was performed to assess changes in body composition according to manufacturer's instructions. Values of lean mass, total body fat, and fat distribution (truncal, gynoid, and android regions) were obtained.

6.3.6 Metabolic biomarkers

Antecubital blood samples were collected in the fasting state (12 h). Serum (serum gel tubes) and plasma (EDTA tubes) samples were separated from whole blood by centrifugation (3,500 rpm, 4^0C, 15 min) and immediately frozen at -80^0C until analyses. Serum glucose, triglycerides (TG), total cholesterol, high-density-lipoprotein cholesterol (HDL-c), low-density lipoprotein cholesterol (LDL-c), uric acid, urea, creatinine, alkaline phosphatase (ALP), γ-glutamyltransferase (Gamma GT), aspartate amino transferase (AST), and alanine amino transferase (ALT) were quantified by an automated analyzer system (BS-200™ Chemistry Analyzer, Mindray) using available commercial colorimetric assay kits (K802, K117, K083, K071, K088, K139, K056, K067, K021, K080, K048, and K049, respectively; Bioclin®, Minas Gerais, Brazil). The serum very-low-density-lipoprotein cholesterol (VLDL-c) was calculated using Friedewald et al. equations (34). Serum insulin was quantified using eletroquimioluminescence method (Elecsys-Modular E-170, Roche Diagnostics Systems). Ultrasensitive C-reactive protein (US-CRP) was quantified by immunoturbidimetry (COBAS-Mira Plus, Roche Diagnostic Systems) using available commercial kit (K079, Bioclin®, Minas Gerais, Brazil). The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated to estimate insulin resistance according to the equation proposed by Matthews et al. (35). Atherogenic Index (TG/HDL-c ratio) were also calculated (36).

Flow cytometry analysis was performed using a BD FACS Verse™ flow cytometer (BD Biosciences). Interleukin-8 (IL-8), interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-10 (IL-10), tumor necrosis factor-α (TNF-α), and interleukin-12p70 (IL-12p70) plasma concentrations were measured using commercial kit (Cytometric Bead Array CBA Human Inflammatory Cytokines Kit, BD Biosciences) according to the

manufacturers' instructions. Data were analyzed using the FCAP Array Software v3.0 (BD Biosciences).

6.3.7 Statistical analysis

Data were typed by two independent investigators to ensure data reliability. Group data were coded before the data analyses for blindness. Statistical analyses were carried out on SPSS 17 for Windows (SPSS, Inc., Chicago, IL, USA). Data are expressed as mean ± standard deviation (SD) for descriptive variables or mean ± standard error (SE) and median (interquartile range) for overall data. Individual outlier values were excluded before analyses. Data normality and homoscedasticity were assessed by Shapiro-Wilk and Levene tests, respectively. Differences in baseline values were assessed by chi-squared test (categorical variables), Student's t-test or Mann-Whitney's test (numerical variables). Paired Student's t-test or Wilcoxon signed-rank test were used to assess within group differences. Differences between dietary intervention were assessed over absolute delta values (9 weeks - baseline) by Student's t-test or Mann-Whitney's test. Pearson's or Spearman's correlation coefficients were used to assess the relation between fat reduction and metabolic biomarkers. A 5% level of significance was adopted.

6.4 RESULTS

6.4.1 Subjects

Forty-one women concluded the study protocol and were included in the analyses. Participants were 27.00 ± 0.85 y old, presented 46.81 ± 0.58% of total body fat, and 30.17 ± 0.43 kg/m^2 of BMI (overweight: n = 23 or 56.10%; obese: n = 18 or 43.90%). There were no significant between-group differences in baseline food intake and in all anthropometric, body composition, blood pressure, and metabolic variables assessed in this study, except for diastolic blood pressure and TNF-α which EVOO presented higher values (**Table 1**). None of the participants had systolic blood pressure higher than 139 mmHg and only one EVOO group participant had diastolic blood pressure ranging from 90 to 99 (first state of hypertension). Despite the fact that none of the participants showed symptoms of acute inflammation during the test days, five of them presented a clear inflammatory cytokines profile and were excluded from final analysis. Eight participants from both groups presented TNF-α concentration below the

detection limits of the assay kit. Six participants from the control group and five from the EVOO group had no detectable concentrations for IL-1β. That did not occur for the other cytokines.

Table 1 Baseline characteristics of study subjects according to experimental groups

	CONTROL	EXTRA-VIRGIN OLIVE OIL
Subjects (*n*)	20	21
Age (years)	27.20 ± 6.08	26.81 ± 4.96
Physical activity (S/LA)	6/14	3/18
Systolic blood pressure (mmHg)	108.45 ± 2.10	114.76 ± 2.43
Diastolic blood pressure (mmHg)	67.45 ± 1.48 [a]	74.48 ± 1.88 [b]
Body weight (kg)	77.56 ± 2.01	77.55 (13.15)
BMI (kg/m2)	29.71 ± 0.63	30.46 ± 0.61
Waist circumference (cm)	97.67 ± 1.55	98.89 ± 1.62
SAD (cm)	19.57 ± 0.45	19.65 ± 0.41
Total body fat (kg)	37.02 ± 1.38	34.42 (11.17)
Total body fat percentage (%)	46.61 ± 0.72	47.00 ± 0.92
Total lean mass (kg)	38.44 (4.58)	39.01 ± 0.86
Energy intake (kJ/d)	7744.87 (2777.73)	8341.40 ± 434.68
Fiber intake (g/d)	19.16 ± 1.53	21.03 ± 1.52
Carbohydrate intake (g/d)	229.04 (66.68)	260.68 ± 16.13
Protein intake (g/d)	78.23 ± 5.33	81.84 ± 3.51
Total cholesterol intake (mg/d)	222.05 ± 16.97	250.77 ± 20.81
Total fat intake (g/d)	61.82 (25.98)	67.75 ± 5.04
C18:1	11.74 ± 0.99	13.82 ± 1.15
C18:2	7.48 (3.64)	9.67 (5.22)
C18:3	0.71 (0.48)	0.87 (0.47)
Total MUFA	16.43 (9.11)	20.23 ± 1.46
Total PUFA	10.55 (5.18)	12.84 (11.20)
Total SFA	20.14 (12.33)	21.12 ± 1.50

Values are means ± SE or median (interquartile range). Waist circumference values were measured at umbilical level. BMI: body mass index; S/LA: number of sedentary and low-active individual ratios (28); SAD: Sagittal abdominal diameter; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; SFA: saturated fatty acids. Different letters in the same line indicates significant differences (Student's t-test, $P < 0.05$).

6.4.2 Dietary assessments

There were no between-group differences in the prescribed diets for energy, macronutrients, fiber, and sodium contents (data not shown). As expected, food intake analyses during experiment showed reduction in energy and macronutrients intake values in both groups compared to baseline due to energy restriction. However, despite the great

reduction in total body fat in EVOO group, dietary intake during the experiment only differed between control versus EVOO groups for C18:1, C18:2, C18:3, total monounsaturated fatty acids, and total polyunsaturated fatty acids ($P < 0.001$), reflecting the differences in the fatty acid profile of the supplemented oils. Group total dietary fat consumption was equivalent to 35.61 ± 2.13% on control and 34.42 ± 1.32% on EVOO ($P = 0.393$ for between group comparisons). MUFA consumption corresponded to 9.34 ± 0.63% of daily caloric intake in control group and to 16.70 ± 0.65 in EVOO, while PUFA consumption was equivalent to 13.06 ± 1.12 in control group and to 5.00 ± 0.34 in EVOO ($P < 0.001$ for both group comparisons) (**Fig. 1**).

6.4.3 Anthropometric, body composition, and blood pressure measurements

Body weight, BMI, and most of the evaluated anthropometric parameters except waist/thigh ratio ($P = 0.068$ for control and $P = 0.053$ for EVOO) reduced with time in response to energy restriction in control and EVOO groups. In addition, both groups showed weight reductions on total fat and specific fat mass sites (truncal, gynoid, and android regions), but not on lean mass. However, while body fat percentage was not affected in the control group (-0.73 ± 0.40%, $P = 0.064$), EVOO presented a significant reduction (-1.49 ± 0.31%, $P < 0.001$; $P = 0.121$ for between-group differences). Total body lean mass percentage was not affected in the control group (0.68 ± 0.46%, $P = 0.161$), but there was an increase in EVOO (1.45 ± 0.36, $P = 0.001$; $P = 0.195$ for between groups differences). Furthermore, EVOO presented great reduction on total body fat than control (-2.41 ± 0.33 kg vs. -1.33 ± 0.42, $P = 0.037$). Fat loss was ~80% higher on EVOO compared to control group. EVOO also reduced diastolic blood pressure (-5.05 ± 1.60 mmHg, $P = 0.005$), while control had no influence on that parameter (+ 0.25 ± 1.16 mmHg, $P = 0.832$; $P = 0.011$ for between-group differences) (**Fig. 2**).

6.4.4 Metabolic biomarkers

Serum glucose reduced in both groups after the intervention without a significant difference between groups ($P = 0.811$). Despite no between-group changes in metabolic biomarkers, HDL-c reduced (- 0.07 ± 0.03 mmol/L, $P = 0.042$) and IL-10 increased (+ 0.25 ± 0.07 pg/mL, $P = 0.025$) only in the control group. On the other hand, EVOO was the only group in which creatinine increased (+ 0.04 ± 0.01 µmol/L, $P = 0.011$) and

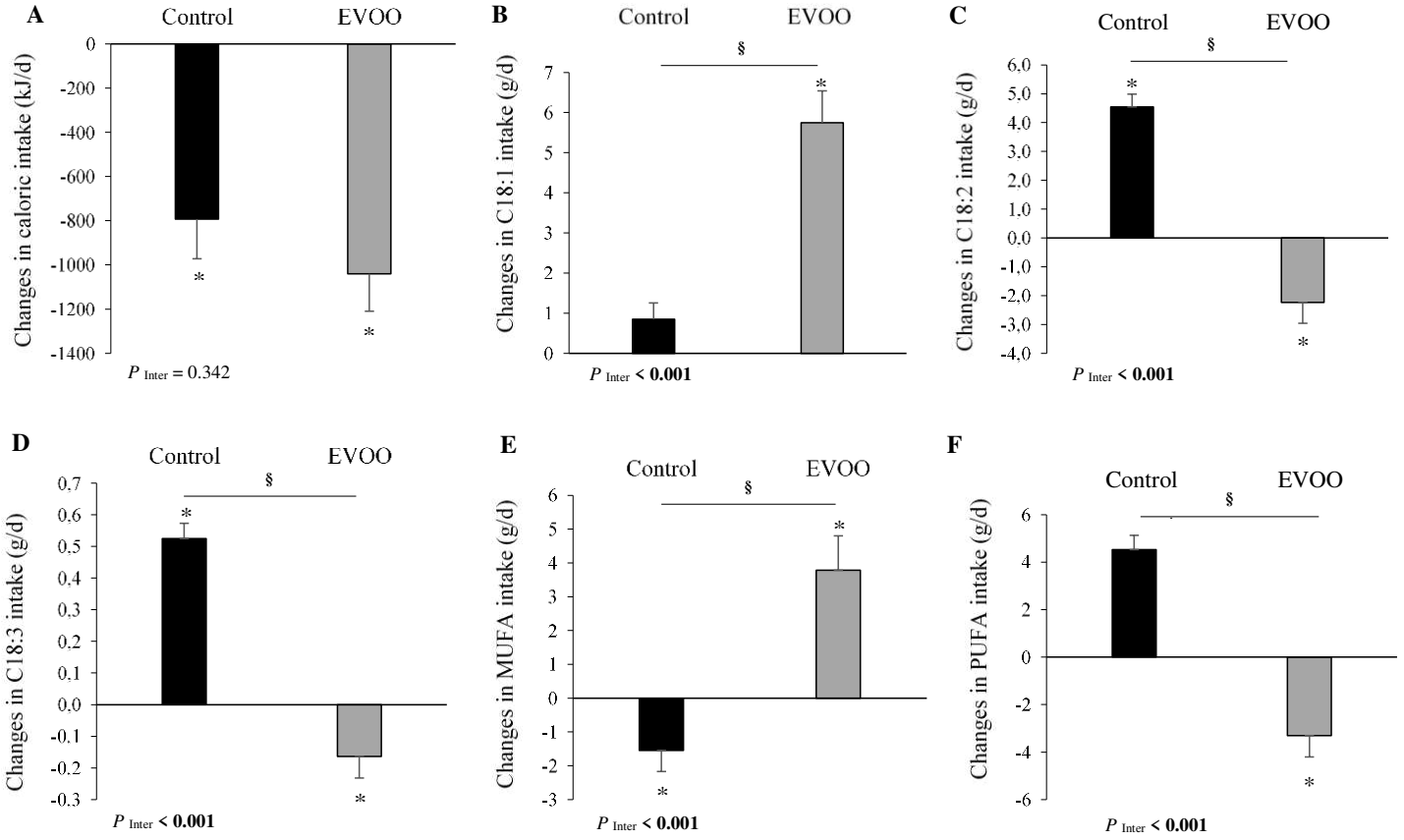


Fig. 1 Mean ± SE energy (A), C18:1 (B), C18:2 (C), C18:3 (D), monounsaturated fatty acids – MUFA (E), and polyunsaturated fatty acids - PUFA (F) consumption changes (9 week values – baseline values). Energy restricted nutritionally balanced diets (-2090 kJ/d) containing 25 mL of soybean oil (control group, $n = 20$) or extra-virgin olive oil – EVOO (EVOO group, $n = 21$) were prescribed. *Within-group significant differences (paired Student's t-test, $P < 0.05$). P_{Inter} values indicates between-groups differences (Student's t-test or Mann-Whitney's test, § $P < 0.050$).

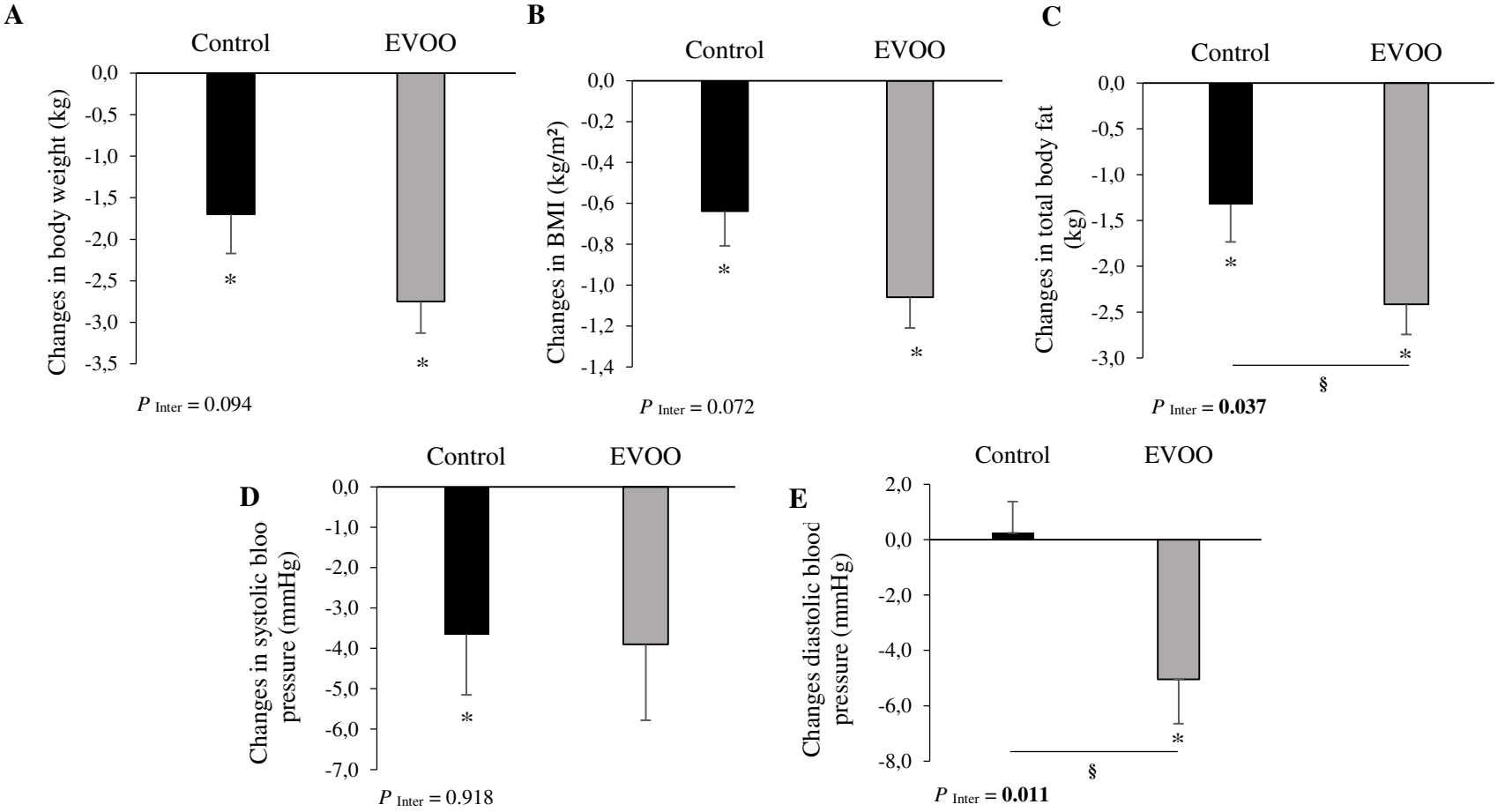


Fig. 2 Mean ± SE body weight (A), Body Mass Index – BMI (B), total body fat (C), systolic blood pressure (D), and diastolic blood pressure (E) changes (9 week values – baseline values). Energy restricted nutritionally balanced diets (-2090 kJ/d) containing 25 mL of soybean oil (control group, $n = 20$) or extra-virgin olive oil – EVOO (EVOO group, $n = 21$) were prescribed. *Within-group significant differences (paired Student's t-test or Wilcoxon signed-rank test, $P < 0.05$). P_{Inter} values indicated between-groups differences (Student's t-test or Mann-Whitney's test, §$P < 0.050$).

alkaline phosphatase reduced (- 3.26 ± 1.78 IU/L, $P = 0.042$) (**Table 2**). There was a trend to a significant reduction in IL-8 in EVOO (- 0.11 ± 0.09, $P = 0.060$) (**Fig. 3**). Correlations analyses indicated positive association between changes in total body fat and changes in alkaline phosphatase ($R^2 = 0.488$, $P = 0.005$) and negative associations between changes in total body fat and changes in serum creatinine ($R^2 = - 0.360$, $P = 0.021$).

Table 2 Metabolic biomarkers assessed before (baseline) and after dietary intervention (9 weeks) according to experimental groups

	CONTROL ($n = 20$)		**EXTRA-VIRGIN OLIVE OIL** ($n = 21$)	
Metabolic biomarkers	**Baseline**	**9 weeks**	**Baseline**	**9 weeks**
Glucose (mmol/L)	4.76 ± 0.09	4.63 ± 0.08	4.86 (0.50)	4.68 ± 0.04
Triglycerides (mmol/L)	0.98 ± 0.09	0.87 ± 0.07	1.27 ± 0.13	1.09 ± 0.09
Total cholesterol (mmol/L)	4.26 ± 0.19	4.05 ± 0.163	4.45 ± 0.20	4.25 ± 0.15
HDL-c (mmol/L)	1.19 ± 0.06 [a]	1.09 (0.32) [b]	1.31 ± 0.07	1.26 ± 0.05
LDL-c (mmol/L)	2.42 ± 0.15	2.36 ± 0.14	2.52 ± 0.15	2.42 ± 0.12
Triglycerides/ HDL-c	0.90 ± 0.12	1.03 ± 0.07	0.79 (0.55)	0.91 ± 0.08
Uric acid (µmol/L)	206.40 ± 7.73	218.30 ± 10.71	208.77 ± 8.92	203.42 ± 8.92
Creatinine (µmol/L)	51.27 ± 0.88	52.16 ± 0.88	50.39 ± 1.77 [a]	53.92 ± 1.77 [b]
AP (IU/L)	61.05 ± 3.47	57.65 ± 4.16	63.70 ± 4.89 [a]	59.50 ± 4.85 [b]
Gamma GT (IU/L)	21.94 ± 0.60	21.11 ± 0.87	19.05 ± 1.43	19.79 ± 1.10
AST (IU/L)	34.00 ± 1.56	31.67 ± 1.70	30.00 (14.00)	28.06 ± 1.59
ALT (IU/L)	16.00 (7.25)	15.00 (8.00)	17.70 ± 1.84	16.11 ± 1.49

Values are means ± SE or median (interquartile range). HDL-c: high-density-lipoprotein cholesterol; LDL-c: low-density lipoprotein cholesterol; ALP: alkaline phosphatase; AP: alkaline phosphatase; Gamma GT: γ-glutamyltransferase; AST: aspartate amino transferase; ALT: alanine amino transferase; Different letters in the same line indicates significant differences (paired Student's t-test or Wilcoxon signed-rank test, $P < 0.05$). Between-group delta values (9 week - baseline) are not significantly different.

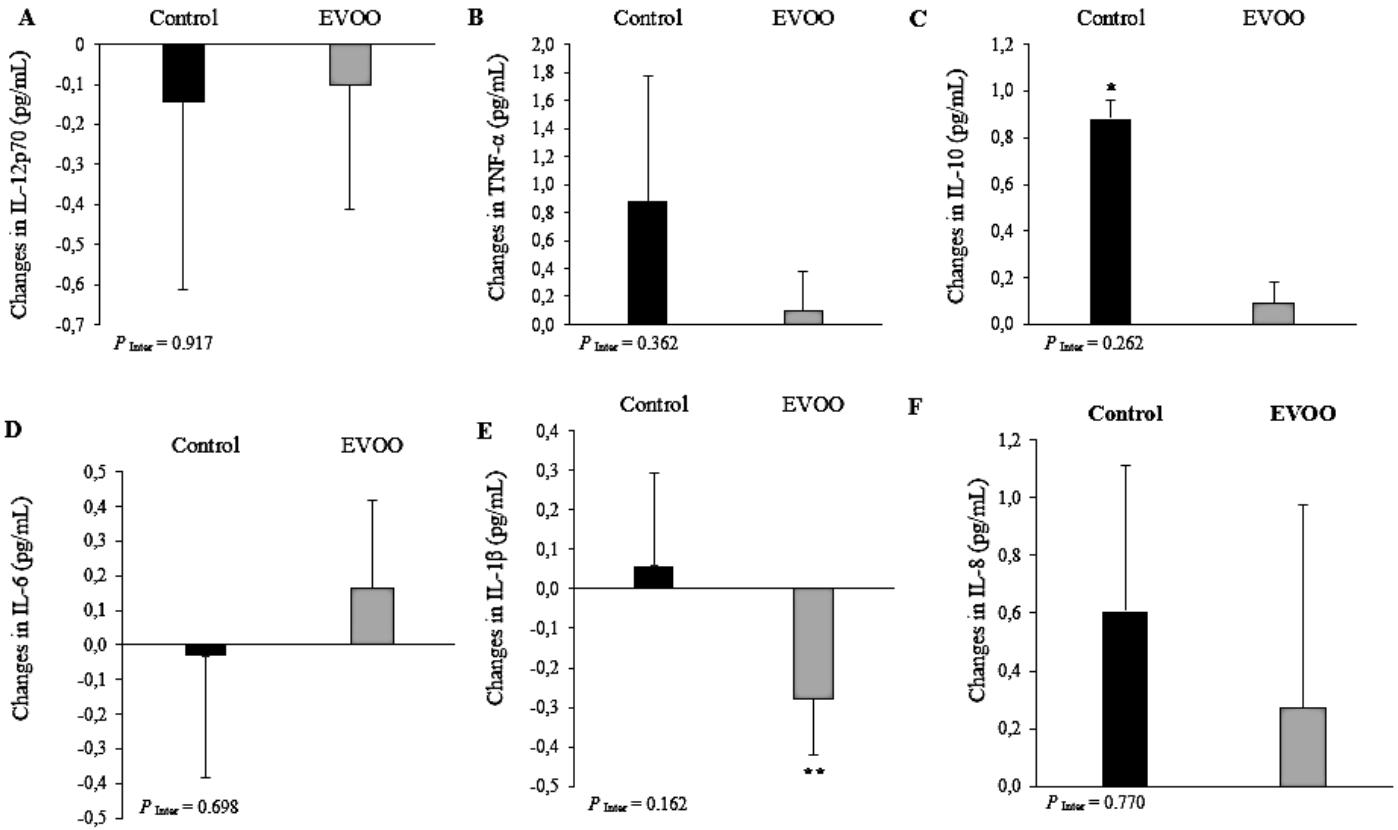


Fig. 3 Mean ± SE interleukin-12p70 - IL-12p70 (A), tumor necrosis factor-α - TNF-α (B), interleukin-10 - IL-10 (C), interleukin-6 – IL-6 (D), interleukin-1β - IL-1β (E), and interleukin-8 – IL-8 (F) concentrations changes (9 week values – baseline values). Energy restricted nutritionally balanced diets (-2090 kJ/d) containing 25 mL of soybean oil (control group, $n = 17$) or extra-virgin olive oil – EVOO (EVOO group, $n = 19$) were prescribed. *Within-group significant differences (paired Student's t-test, $P < 0.05$). **Trend to significant differences (paired Student's t-test, $P = 0.060$). P_{Inter} values indicates between-groups differences (Student's t-test or Mann-Whitney's test).

6.5 DISCUSSION

This study was design to assess the effects of EVOO incorporated into an energy-restricted non-Mediterranean diet program on body weight, body composition and metabolic biomarkers in excess body fat woman. The main findings of the present study is that the consumption of EVOO increases total fat loss and reduces diastolic blood pressure compared to the control soybean oil group. To the best of our knowledge, this paper provides the first clinical evidence that EVOO consumption increases body fat loss due to energy restricted program even when not incorporated into a Mediterranean diet. Analysis of food consumption during the experiment demonstrated that our high-fat breakfasts significantly changed daily consumption of dietary fatty acids. EVOO group increased body fat loss, which was independent of an increase in caloric restriction. Furthermore, our results show that while IL-10 increased only in the control group, HDL-c concentrations reduced in that same group. On the other hand, serum creatinine increased, alkaline phosphatase reduced, and there was a trend for IL-1β reduction in the EVOO group along the nine experimental weeks.

It has been widely suggested that consumption Mediterranean diet rich in olive oil can prevent type 2 diabetes mellitus (37,38) metabolic syndrome (37), and obesity (17,37). However, randomized clinical trials in which the effect of olive oil on body weight/fat was investigated are scarce and presented conflicting results (20–22,39). In a recent study (46) involving 7447 asymptomatic high-CVD risk individuals, daily consumption of 50 mL of EVOO for 4.8 years associated with an unrestricted-calorie, high-vegetable Mediterranean diet reduced body weight and promoted less central adiposity gain compared with the consumption of a low fat diet. In our study, the consumption of energy-restricted normo fat diet containing 25 mL of EVOO a day reduced total body fat compared to 25 mL/day of soybean oil. Additionally, to the aforementioned study, our findings support the prescription of EVOO not only for preventing weight gain, but also for promoting body weight/fat loss.

The current hypothesis that EVOO could improve body composition was mainly based in the effect of oleic acid (C18:1) on stearoyl-CoA desaturase 1 (SCD1) (13). This enzyme catalyses a key step in the endogenous biosynthesis of MUFA from saturated fatty acids. The preferential substrates for its action are palmitic acid and stearic acid, which are converted by SCD1 into palmitoleic acid and oleic acid, respectively (40). The influence of increased SCD1 activity on obesity is supported by mice with natural or SCD1-direct mutations. SCD1-deficient mice consume 25% more food but accumulate

less fat and are considerably thinner than normal mice (41,42). In addition, SCD1-deficient animals consume more oxygen and have higher rates of β-oxidation in liver and fat tissue (53). The lack of SCD1 also beneficially modulates the expression and activity of some genes related to adiposity (54). According to this hypothesis, SCD1 activity is regulated by the amount of substrate and final product. Thus, while consumption of the saturated fatty acids palmitic acid and stearic acid acts as substrate, stimulating SCD1 action and favoring obesity, oleic acid down regulates SCD1 activity, favoring weight loss (13). The effect of EVOO consumption on SCD1 expression and activity must be investigated in metagenomic studies.

The role of EVOO in reducing blood pressure is supported by a growing body of scientific evidence (45–48). Despite the fact that minor components characteristic of olive oil could contribute to the cardioprotective activity of EVOO, such as a-tocopherol, polyphenols, and other phenolic compounds, Terés et al. (48) demonstrated that its high oleic acid content is responsible for the antihypertensive effects of olive oil consumption. This effect is likely to be attributed to the incorporation of oleic acid into cell membranes, which regulates membrane lipid structure in such a way as to control G protein-mediated signaling, causing a reduction in blood pressure (48). In our study, EVOO significantly reduced (~ 5 mmHg) diastolic blood pressure compared to the control (soybean oil). Soybean oil could be considered a good control for assessing blood pressure due its little effect on that parameter (48). Therefore, our results suggested that EVOO could contribute to control hypertension.

There is still no consensus about the role of EVOO on dyslipidemia. While some studies reported beneficial increase in HDL-c (48,49) and reduction in LDL-c (51), others showed no significant changes in lipid profile (47,52–55). In our study, EVOO presented cholesterol-neutral effect, since HDL-c reduced in the control group at the end of the experiment. Our results corroborated with those reported by (56), in which there was a decrease in HDL-c concentrations after the consumption of ~50 g of soybean oil and maintenance of HDL-c in response to the consumption of similar amount of olive oil. The authors attributed the reduction to soybean oil linoleic acid high content and the maintenance to the competition between olive oil chylomicron remnants and HDL for hepatic lipase (56). Thus, olive oil could prevent HDL-c postprandial decrease, and maybe contribute for a more favorable lipid profile.

We observed a significant, but no clinically relevant, increase in serum creatinine in the EVOO group. This was an unexpected result, since creatinine was assessed as a biomarker of renal function and we expected that EVOO could protected kidneys from

obese lipotoxicity (57). However, we believe that the increase in serum creatinine was a reflect of lean mass preservation during the study, since creatinine is a lean mass content marker and EVOO was the only group in which lean mass percentage increased at the end of the experiment. On the other hand, there was a reduction in alkaline phosphatase in EVOO. Despite the fact that alkaline phosphatase is not specific from liver, data from animal studies provide some evidences that polyphenols from olive oil could improve liver function by reducing lipid peroxidation in this tissue (58,59). Thus, the slight reduction in that enzyme may reflect and improvement in liver function. This result deserves to be confirmed in individuals with non-alcoholic fatty liver disease.

In our study, there was a significant increase in IL-10 in the control group. Soybean oil was provided to the control group to match fat consumption between groups, but was responsible for an increased consumption of α-linolenic acid (C18:3) in that group. Increased consumption of α-linolenic acid can down-regulate inflammatory pathways and reduce plasma levels of IL-10 (60). In turn, EVOO showed a trend for IL-1β reduction. A very similar effect of olive oil was observed in another study conducted by Kremer et al. (61). In that study, the effect of fish oil versus olive oil (placebo) on active human rheumatoid arthritis was investigated. Olive oil consumption led to unexpected beneficial effects on the improvement of clinical aspects of the disease. These benefits were associated with decreased macrophage IL-1 production, although not to the same extent as the fish oil group (61). As IL-1β has potent and vast pro-inflammatory effect over a number of cells including macrophages, monocytes, and dendritic cells (62), the role of EVOO on IL-1β deserves to be further explored.

Our study has several strengths, including the rigorous subjects' eligibility criteria, the use of DXA for body composition assessments, use of double blind protocol, double digitation of data, controlled breakfasts consumption, and evaluation of diet compliance. Our study also has limitations. Despite the fact that we selected woman with very high body fat content (~ 48% at baseline), they were also young and it is possible that we were not able to detect the influence of dietary treatment in some metabolic biomarkers (e.g. some cytokines which were not detected). Furthermore, women are more prone to present changes in anthropometric parameters and body compositions due to menstrual cycle. Despite our efforts to reduce the influence of water retention, we cannot assure that our results were not affected by participant hormonal fluctuations. Finally, the interference of EVOO higher diastolic blood pressure at baseline in our results cannot be totally neglected.

6.6 CONCLUSION

Daily consumption of 25 mL of extra-virgin oil (EVOO) associated with an energy-restricted Western-diet increased body fat loss and reduced blood pressure. The beneficial effects of EVOO were independent of an increase in caloric restriction, indicating a positive direct role of this oil on adiposity. EVOO also increased serum creatinine, reduced hepatic alkaline phosphatase, and tended to reduce IL-1β concentrations. The intriguing impact of EVOO on SCD1 expression and activity must be better explored in metagenomic studies.

6.7 ACKNOWLEDGMENTS

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6.8 CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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7. CONLUSION

- Dietary fats have great potential to induce changes in gut microbiota and can directly or indirectly influence obesity induced by dysbiosis;
- Regardless of the lack of human studies, consumption of high-fat diets rich in saturated fat can impair the balance between microbiota/host and trigger mechanisms that induce of obesity, which are mediated by LPS. In contrast, monounsaturated or polyunsaturated fatty acids of omega-3 series may contribute to the establishment of a beneficial microbiota capable to maintain a healthy body weight. Probiotic and synbiotic are effective nutritional strategies for reversing dysbiosis. However, once these strategies are adopted healthy dietary lipid profile should also be consumed due to the transient their benefits;
- Consumption of reasonable doses of different vegetable oils affects the inflammatory pattern in distinct ways without, however, interfering on LPS plasma concentration. Soybean and coconut oils have the potential to stimulate, in a transient manner, systemic inflammation. The effects of these oils, as well as the interactions between specific fatty acids and LPS-induced inflammation, need to be investigated in long-term studies;
- Regular consumption of extra-virgin olive oil contributed to the elimination of body fat and to improve blood pressure. The mechanisms involved in these outcomes need to be elucidated, especially the effect of the consumption of that fat source on SCD1 gene epigenetic changes;
- Further analyses of lipid profile, markers of oxidative stress, dosages of protein-binding LPS, vitamin E, carotenoids, and of phenolic compounds in plasma could be interesting to better interpret our results;
- We encourage the conductance of further studies to assess the role of dietary fats on gut microbiota composition, fecal SCFA, secondary bile acids contents, and intestinal permeability.