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RESEARCH PAPER

Short-term flaxseed oil, rich in omega 3, protects mice against metabolic damage caused by high-fat diet, but not inflammation

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Abstract

It is known that long-term high-fat diet (HF) feeding drastically affects the adipose tissue, contributing to metabolic disorders. Recently, short-term HF consumption was shown to affect different neuronal signaling pathways. Thus, we aimed to evaluate the inflammatory effects of a short-term HF and whether a diet containing omega-3 fatty acid fats from flaxseed oil (FS) has protective effects. Mice were divided into three groups for 3 d, according to their diets: Control group (CT), HF, or FS for 3 d. Lipid profiles were assessed through mass spectrometry and inflammatory markers by RT–qPCR and Western blotting. After short-term HF, mice increased food intake, body weight, adiposity, and fasting glucose. Increased mRNA content of *Ccl2* and *Tnf* was demonstrated in the HF compared to CT in mesenteric adipose tissue. In the liver, TNFα protein was higher in the HF group than in CT, followed by a decreased polyunsaturated fatty acids tissue incorporation in HF. On the other hand, the consumption of FS reduced food intake and fasting glucose, as well as increased omega-3 fatty acid incorporation in MAT and the liver. However, short-term FS was insufficient to control the early inflammation triggered by HF in MAT and the liver. These data demonstrated that a 3-d HF diet is enough to damage glucose homeostasis and trigger inflammation. In contrast, short-term FS protects against increased food intake and fasting glucose but not inflammation in mice.

Keywords: Omega-3; Inflammation; Saturated fatty acids; Adipose tissue; Liver.

1. Background

Nutrients can change physiological and molecular parameters in both human and experimental models since their first consumption (acute) or after a cumulative intake (chronic) [1,2]. The consumption of diets rich in saturated fatty acids (SFAs) is an essential trigger for developing obesity and its comorbidities, such as diabetes type 2, cardiovascular diseases, and nonalcoholic fatty liver disease (NAFLD) [3]. Nevertheless, the development of the food industry increased fat consumption because it is a crucial ingredient in maintaining stability and shelf life, with flavor as an adjuvant effect [4–6].

Among fats in industrialized foods, several research groups have targeted SFAs due to their inflammatory potential. SFAs are essential for micronutrient transportation, cell membrane composition, and homeostasis; however, the overconsumption of SFAs activates Toll-like receptor 4 (TLR4), an immune system receptor, which transduces a pro-inflammatory response through NFkB [7,8]. When long-term exposure to SFAs, several tissues are impaired, such as the hypothalamus, adipose tissue, liver, and skeletal muscle [9–11]. However, other tissues have shown metabolic damage after short-term high-fat diet (HF) feeding, such as the hypothalamus and hippocampus [1,12].

Adipose tissue is well-known as essential for storage and metabolic actions. However, this tissue can also produce and recruit several chemokines, cytokines, and immune cells and secrete

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hormones such as leptin [13]. Visceral adipose tissue (VAT) is an established marker of cardiovascular diseases but it is also involved in other metabolic dysfunctions [14]. When exposed to a long-term HF, this tissue can be hypertrophied and hyperplastic, activating and attracting inflammatory macrophages, secreting proinflammatory cytokines, and impairing leptin and insulin signaling [10,15].

Specifically, mesenteric adipose tissue (MAT) exacerbates the inflammatory response due to its proximity to the gut. Once the gastrointestinal tract receives nutrients, the tight junctions change their conformation when exposed to long-term HF, facilitating access to MAT. [16,17]. Previous studies indicate that VAT may act on liver metabolism, damaging it [18,19]. In the obesity context, the VAT and MAT increase the release of cytokines and chemokines such as TNFα and MCP1 (Ccl2), which induces a pro-inflammatory stage. Furthermore, the systemic pro-inflammatory tonus is upregulated by the release of free fatty acids stored at the adipose depots. The liver is affected by a lipidic overload, due to intensified free fatty acids flow through the portal circulation. Therefore, increased subclinical inflammation and free fatty acids in the liver can induce NAFLD and insulin resistance [18,20].

On the other hand, the omega-3 fatty acid (ω3) family, composed by α-linolenic acid (ALA-C18:3), eicosapentaenoic acid (EPA-C20:5), and docosahexaenoic acid (DHA-22:6), are well characterized as anti-inflammatory [21]. Once G-protein coupled receptor 120 (GPR120) recognizes ω3, the βarrestin2 protein is attracted to the GPR120. Consequently, the TAB1 protein is attracted to βarrestin2, which disarticulates the association of TGF-beta-activated kinase 1 with MAP3K7-binding protein 1 (TAB1) and mitogen-activated protein kinase kinase kinase 7 (TAK1) [22,23]. Then, pro-inflammatory signaling is interrupted once TAK1 is responsible for c-Jun N-terminal kinases (JNK) and IkappaB kinase (IKK)-nuclear factor kappa B (NFkB) activation [22–24]. Previous studies described the benefits of ω3 supplementation through fish oil or long-term supplementation [25–28]. However, few investigations have shown the effects of ω3 in the short term [29,30].

Therefore, the present study evaluated the effects of short-term (3 d) diets rich in saturated or unsaturated fatty acids on physiological parameters and inflammatory markers in MAT and the liver. We hypothesized that the polyunsaturated ω3 diet might attenuate inflammation in mouse MAT and liver after short-term feeding.

2. Methods

2.1. Experimental animals

Four-week-old male C57BL/6J mice were acquired from the multidisciplinary center for biological investigation (CEMIB) of the University of Campinas (UNICAMP). The mice were allocated to individual cages (22±1°C) with free access to food and water. After 3 d of experimental diet consumption and 8 h of fasting (Fig. 1), mice were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (5 mg/kg). After that, the mice lost their reflections completely and were euthanized. The tissues were collected and stored at -80°C. All procedures were approved by the animal ethics committee (CEUA) of the institute of biological sciences, UNICAMP - Campinas-SP (4649-1/2017 and 5421-1/2019).

2.2. Diet composition

Mice were fed *ad libitum* with a commercial chow diet (CT group) (Nuvilab), high-fat diet (HF group), or flaxseed diet (FS group) for 3 d. The HF composition was made following a previous study [22] and was composed of 35% fat, 31% from lard, and 4% of soy oil (Table 1). The flaxseed diet was also composed of 35% fat, of which 21% was from lard and 10% was substituted for FS. The rationale for adopting this concentration of FS in substitution for the third part of lard comes from the previous finds that showed more than this (10 or 20%) could be harmful, worsening the pro-inflammatory process [22]. The composition of the commercial diet (Nuvilab) declared on the label was: crude protein 22%, crude fat 4.5%, mineral mass 10%, crude fiber 8%, calcium 1.4%, phosphorus 0.8%, and moisture 12.5% Tables 2 and 3.

Table 1
Experimental diet composition with 35% fat (312 g of lard + 40 g of soybean oil [22] or 108 g of lard + 104 g of flaxseed oil + 10 g of soybean[25]). *Q.S.P. - Starch added in Sufficient Amount To - complete 1,000 g of diet. **AIN - American Institute of Nutrition, according to Reeves, 1993.

Ingredients	High-fat diet (g)	Flaxseed diet (g)
corn starch (Q.S.P.)	115.5	115.5
casein	200	200
dextrinated starch	132	132
Sucrose	100	100
soybean oil	40	40
flaxseed oil	0	104
lard	312	208
cellulose	50	50
mineral mix	35	35
vitamin mix	10	10
l-cystine	3	3
choline	2.5	2.5
total	1,000	1,000

2.3. Food consumption and serum analysis

Mice were allocated to individual cages, and food intake was monitored daily for 3 d. After 8 h of fasting and before tissue collection, blood was collected from the caudal vein for fasting glucose and insulin measurement. The blood glucose levels were determined using a glucometer (Accu-Chek; Roche Diagnostics). Serum insulin was measured by ELISA (#ELM- Insulin, RayBiotech, Norcross, GA, USA) [12].

2.4. HOMA-IR

The homeostatic model assessment for insulin resistance (HOMA-IR) was calculated using the formula: fasting plasma glucose (mmol/L-1) × fasting plasma insulin (μUmL-1)/22.5 [31].

2.5. RT-qPCR

MAT was homogenized with TRIzol (Invitrogen) in *Tissue Lyser II* (Qiagen), and 2 μg of RNA was used for cDNA synthesis (*High Capacity cDNA Reverse Transcription*, Thermo Fisher) in *AgileCycler* (Avans). qPCR was performed with specific forward and reverse oligonucleotide primers with SYBR Green PCR Master Mix (Applied Biosystems) [12] and Taqman (GoTaq Probe qPCR Master, Promega) [25] in the *7500 Fast Real-Time PCR* (ThermoFisher). For data analysis, ΔΔCt was applied, sample amplification verification was performed, and NTC (no template control) was applied to the *melt curve*. The TaqMan primers were *Tnf* (Applied Biosystems #Mm00443258_m1), *Ccl2* (Applied Biosystems #Mm00441242_m1), and *Gapdh* (Applied Biosystems #Mm99999915_g1).

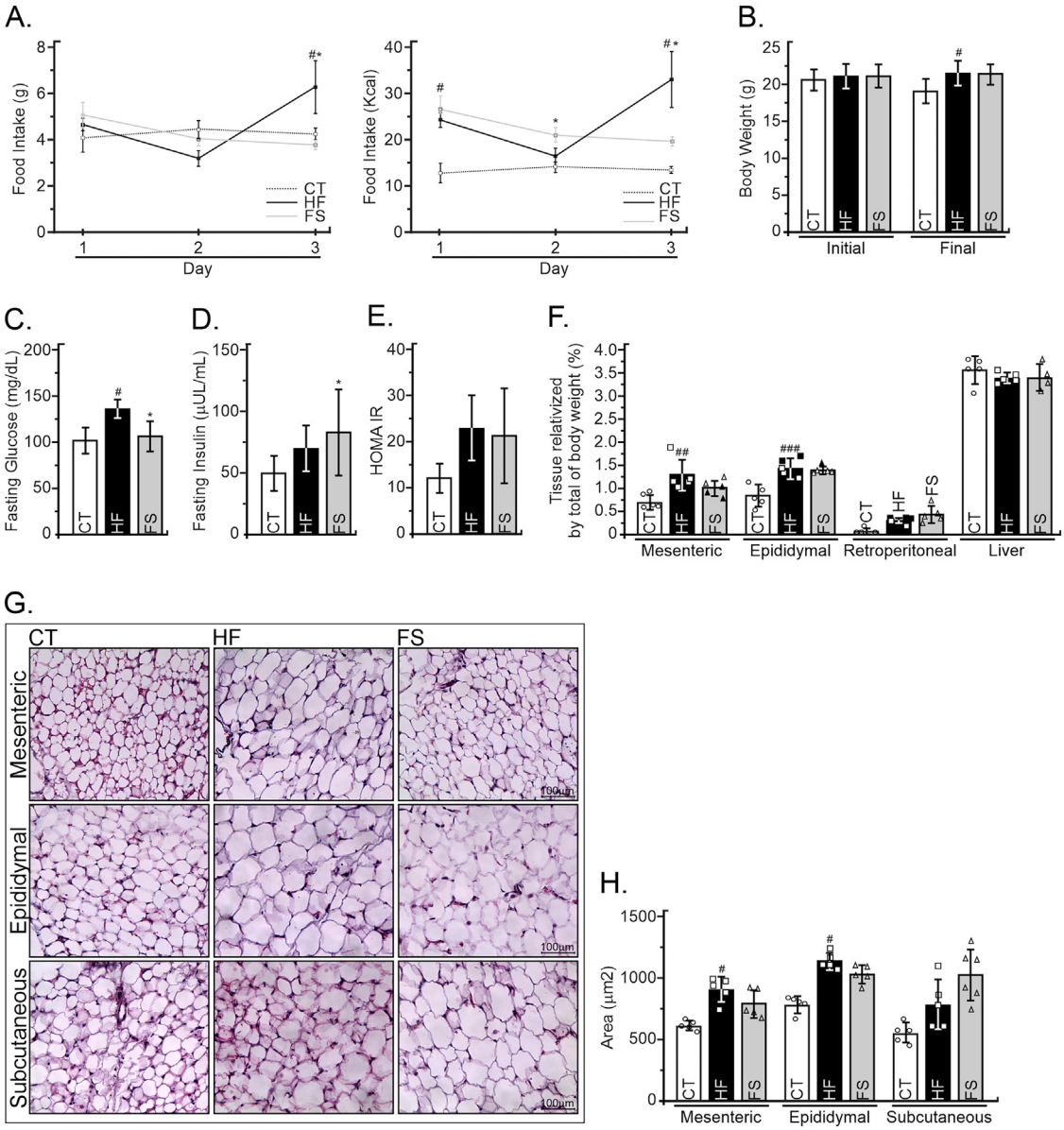

Figure 1. Physiological and morphological parameters after 3 d of exposure to experimental diets: (A) Food intake measurement (n=6 for all groups). (B) Final body weight (n=10 on CT; n=11 on HF and FS). (C) Fasting glucose (n=10 for all groups). (D) Fasting insulin (n=5 in all groups). (E). Homa IR (n=5 in all groups). (F) Mesenteric, epididymal, retroperitoneal and liver tissues (n=11 on CT; n=11 on HF and n=10 FS). (G) HE-stained histological sections of mesenteric, epididymal, and subcutaneous tissue. (H) Adipocyte area after HE staining. P<.05 #CT *vs.* HF and *HF *vs.* FS by Bonferroni's test.

2.6. Western blotting

For Western blotting analysis, the liver was collected and homogenized with extraction buffer [1% Triton-X 100, 100 mM Tris (pH 7.4), 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM PMSF and 0.1 mg aprotinin/mL] using a *Tissue Lyser II* (Qiagen) operated at maximum speed for 2 min. The liver lysates were used to determine the total protein content by the bicinchoninic acid (BCA) method (Sigma-Aldrich). After adding Laemmli buffer containing 100 mm dithiothreitol to the supernatant, the samples were heated for 10 min [32]. The samples were applied to a polyacrylamide gel for separation by SDS-PAGE and transferred to nitrocellulose membranes (0.45 mm). Ponceau staining was applied to check membrane transfer. The blocking solution (5% dry milk) was used for 1 h at room temperature and then incubated overnight at 4°C with the primary antibodies. Then, the membranes were incubated for 1 h with the specific secondary antibody. To visualize the bands, enhanced chemiluminescence was used at G-Box (Syngene) and quantified by optical densitometry using UN-SCAN-IT gel 6.1 software (SilkScientific). The antibodies utilized were TNFα (Biolegend B145868), IL1β (Biolegend B - 122), pJNK (Santa Cruz sc-135642), Tubulin (Cell 2144s), PCB (Santa Cruz sc-271493), PTP1B (Santa Cruz sc-14021), GAPDH (Santa Cruz sc-47724), F4/80 (Santa Cruz sc-25830), GPR120 (Santa Cruz sc-48203), IL18 (Santa Cruz sc-7954), and pIKBa (Cell 9246 L).

2.7. Histological analysis

MAT was formaldehyde-fixed, dehydrated with ethanol, cleared with xylene, and embedded in paraffin wax. The sections were 5 mm and obtained by microtome (Microm HM340E). The area of

Table 2
Fatty acid profile of experimental diets. The analyses were duplicate for the control diet and triplicated for the high-fat diet. Values are expressed as the mean ± standard deviation of the mean.

Fatty acids	CT	HF	FS
C10:0	ND	0.083±0.032	0.07±0.017
C12:0	ND	0.1±0.017	0.08 ±0.01
C13:0	1.44±0.099	0.467±0.163	0.387±0.195
C14:0	0.105±0.007	1.44±0.078	1.013±0.095
C14:1	ND	0.020	0.017±0.006
C15:0	0.045±0.007	0.083±0.006	0.063±0.006
C16:0	14.495±0.431	20.953±0.047	15.733±0.502
C16:1 (ω7)	0.115±0.007	2.443±0.076	1.647±0.127
C16:2	ND	ND	0.013±0.006
C17:0	0.115±0.007	0.43±0.017	0.303±0.032
C17:1	0.055±0.007	0.31±0.010	0.207 ±0.038
C18:0	3.68±0.071	11.303±0.071	9.300±0.769
C18:1 (ω9)	28.00±1.817	37.42±1.938	34.480±2.024
C18:1*t*	ND	0.105±0.021	0.090±ND
C18:2 (ω6)	46.125±0.997	21.653±0.061	19.573±0.315
C18:3 (ω6)	ND	0.0467±0.029	0.033±0.021
C19:0	ND	0.017±0.012	0.020±0.010
C19:1	ND	0.037±0.012	0.033±0.015
C18:3 (ω3)	4.69±0.226	1.853±0.075	15.783±0.363
C20:0	0.380	0.257±0.015	0.250±0.017
C20:1 (ω9)	0.290	0.757±0.032	0.563±0.055
C20:1 (ω7)	0.340	0.030	ND
C20:2 (ω7)	ND	1.005±0.092	ND
C20:2(ω7)	0.030	ND	ND
C20:3 (ω9)	ND	0.035±0.007	0.040±ND
C20:3 (ω6)	ND	0.177±0.038	ND
C20:4 (ω6)	ND	0.3±0.010	0.183±0.025
C22:0	0.37±0.028	0.087±0.012	0.133±0.015
C22:4 (ω7)	0.100	ND	ND
C22:4 (ω6)	ND	0.11±0.010	ND
ΣSat	19.19±0.552	34.773±0.176	26.983±1.378
ΣMono	28.49±1.768	39.927±0.542	36.960±1.800
ΣPoly	50.88±1.315	24.833±0.520	35.670±0.688
Σω3	4.69±0.226	1.853±0.075	15.783±0.363
Σω6	46.14±1.018	22.287±0.032	19.860±0.306
Σω6:ω3	9.84+0.258	12.038±0.484	1.258±0.010

Table 3
Primer sequences for *Ffar4* (Free fatty acid receptor 4), *Il1b* (Interleukin 1 beta), and *Gapdh* (Glyceraldehyde-3-phosphate dehydrogenase).

Gene		Sequence (5′ - 3′)
Ffar4	Forward	GTGCCGGGACTGGTCATTGTG
	Reverse	TTGTTGGGACACTCGGATCTG
Il1b	Forward	TGGACCTTCCAGGATGAGGACA
	Reverse	GTTCATCTCGGAGCCTGTAGTG
Gapdh	Forward	AACTTTGGCATTGTGGAAGG
	Reverse	ACACATTGGGGGTAGGAACA

adipocytes by hematoxylin and eosin staining was obtained by ImageJ software. For immunofluorescent analysis, sections were incubated in a blocking solution (1x PBS with 5% BSA) followed by overnight incubation with primary antibody against F4/80 (1:100) at 4°C, followed by incubation with FITC-conjugated anti-rabbit secondary antibody for 2 h at room temperature. After washing with PBS, the sections were mounted with DAPI (#H-1200 Vector Laboratories) for nucleic acid staining. The mesenteric sections were examined under a confocal microscope (Leica TCS SP5 II).

2.8. Mass spectrometry gas chromatography

For fatty acid profile analysis, experimental diets, fragments of mesenteric and liver were homogenized with 1 mL of chloroform: methanol (2:1-v:v) [33] at maximum speed for 2 min at *Tissue Lyser II* (Qiagen). The fatty acids from samples were methylated by the method proposed by Shirai, Suzuki, and Wada (2005) using 150 μL of lipid fractions. The samples were transferred to test tubes with lids, and then 1 mL of methanolic NaOH and 30 μL of BHT (Sigma-Aldrich) were added. The samples were vortexed and heated in a water bath at 100°C for 15 min. Then, 2 mL of esterification reagent was added, stirred, and heated for 5 min. Then, for all samples, 1 mL of isooctane was added. The samples were shaken, and 5 mL of saturated NaCl was added. Tube supernatant was used for analysis. Fatty acid methyl esters were analyzed using a mass spectrometer model GCMS-QP2010-Ultra (Shimadzu). The following operating conditions were examined: Stabilwax column of 30 m, with an internal diameter of 0.25 mm and a thickness of 0.25 μm (Restek, USA). High-purity helium was used as a carrier gas, with a 1 mL/min constant flow. A total of 1.0 μL of the sample was injected, with an injector split ratio of 1:15 and an inlet temperature of 80°C. The mass conditions were as follows: ionization voltage of 70 eV; ion source temperature of 200°C, with full scan mode in the mass range between 35-500, with 0.2 s/sweep speed. The initial machine temperature was 80°C for 2 min, increasing to 150°C and then 180°C for 10 min. Then, the temperature was increased to 240°C and remained there for 50 min (SHIRAI; SUZUKI; WADA, 2005).

2.9. Statistical analysis

All results were first submitted to Bartlett's test to check the symmetry. Bonferroni's test followed by one-way or two-way ANOVA was used to compare the CT *vs.* HF and HF *vs.* FS groups. When data were not normal, the Kruskal-Wallis test was performed. The statistical significance was set at $P<.05$ ($^{\#}$CT *vs.* HF and *HF *vs.* FS). All results are presented as the mean ± standard deviation using GraphPad Prism (8.0 version).

3. Results

3.1. FS protects mice against high food intake but not adipose tissue hypertrophy

Omega-3 has been widely described as a fatty acid with anti-inflammatory characteristics. In contrast, HF rich in SFAs has been extensively studied for its role in chronic inflammatory metabolic diseases such as obesity and type 2 diabetes [22,28]. We exposed the animals to the experimental diets (CT, HF, and FS) for 3 d to confirm their roles in short-term consumption. We found that the first day of HF and FS feeding did not change the food consumption in grams compared to CT feeding (Fig. 1A), but the calories from these diets were increased ($P<.05$). The consumption of both hyperlipidic diets was decreased on the second day, equalizing with the CT diet, although the HF group had a lower caloric intake than the FS group ($P<.05$). Surprisingly, on the third day, HF consumption increased again compared to CT. The FS group was similar to the CT group. Therefore, it was protected from the increment induced by the HF diet ($P<.05$), observed when measured in grams or calorie intake.

The final body weight of the HF group was higher than the CT group ($P<.05$) but not different ($P>.05$) from the FS group (Fig. 1B). Despite the body weight data, the FS fasting glucose level was lower ($P<.05$) than the HF group (Fig. 1C). Fasting insulin and Homa IR analyses explored the fasting glucose result,

which showed higher fasting insulin in the FS group. Nevertheless, there was no difference among the groups in HOMA IR (Fig. 1D and E). Moreover, the VAT (mesenteric and epididymal) of the HF group was significantly higher ($P<.01$ and $P<.001$, respectively) compared to CT (Fig. 1F). The histological analysis confirmed these data (Fig. 1G), highlighting increased adipocyte area in the mesenteric and epididymal adipose tissue in the HF compared to CT ($P<.05$). Thus, the diet containing sources of omega-3 (FS) protected mice in short-term consumption from increased food intake and fasting glucose but not from body weight and VAT accumulation.

3.2. Short-term HF consumption altered inflammatory markers in MAT

Saturated fatty acids trigger low-grade inflammation when consumed over the recommendation and constantly over long periods [34]. The VAT is closely linked to this inflammation, especially the mesentery located next to the intestine [13]. Exposure to HF for 3 d was enough to increase critical inflammatory markers, such as *Ccl2* and *Tnf,* compared to CT ($P<.05$), but no difference was noticed in FS (Fig. 2B and C). To understand whether omega-3 might have some activity associated with its receptor, GPR120, we measured the level of its gene expression (*Ffar4*) in the MAT. However, there were no differences among the groups (Fig. 2A). In addition, the consumption of HF secretes chemokines, such as *Ccl2*, which attract macrophages to the adipose tissue [13,35]. Therefore, F4/80, a macrophage marker, was measured in the mesentery through immunofluorescence, but there were no differences between the groups (Fig. 2E and F). Then, IL1β and TNFα protein levels were measured. The IL1β protein content followed the *Il1b* mRNA content, with no difference among the groups (Fig. 2D, G, and I). Although the *Tnf* mRNA levels increased in the HF compared to CT ($P<.05$), its protein content was not different among the groups (Fig. 2C, H, and J). Accordingly, 3 d of HF increased inflammatory markers, but omega-3 did not protect against it.

3.3. Fatty acid profile of the mesentery after short-term consumption of HF and FS

Following the molecular analysis of MAT, a fatty acid profile on the mesentery was performed. The consumption of HF for 3 d did not increase the summation of SFAs compared to CT (Fig 3A). Even the HF group presented higher C14:0 than CT and lower C22:0 (Fig. 3B and E). On the other hand, the FS diet decreased ($P<.05$) saturated fatty acid incorporation compared to HF (Fig. 3A), represented mainly by reduced C18:0 (Fig. 3D). In addition, monounsaturated fatty acids showed higher HF levels than CT ($P<.05$) but were not different from FS (Fig. 3F). Lastly, polyunsaturated fatty acid summation was not different ($P>.05$) among the groups (Fig. 3J). However, observing more thoroughly, it was seen that ω3 fatty acids were more incorporated in the FS group than HF ($P<.05$) but not different from CT (Fig. 3K). The higher incorporation of ω3 is explained mainly by the increased incorporation ($P<.05$) of C18:3α and C20:5 in the FS group (Fig. 3L and M).

3.4. Omega-3 does not protect liver against TNFα and PTP1B proteins induced by HF

Another tissue well known for its damage caused in obesogenic and insulin-resistant environments is the liver [36]. In addition, MAT is directly linked to the liver through portal circulation [18]. Once metabolic and mesenteric alterations were observed, hepatic analyses were performed. HF increased the protein content of PTP1B and TNFα ($P<.05$) compared to CT (Fig. 4E and H). Other proteins related to the inflammatory process were assessed (*i.e.*, IL1β, IL18, p-Iκbα, and F4/80), but there were no differences among the groups ($P>.05$). Moreover, the PCB enzyme, a pivotal enzyme in hepatic glucose production, was measured. However, no difference ($P>.05$) was observed among the groups (Fig. 4F). Finally, we assessed the protein content of GPR120, but no difference ($P>.05$) was found (Fig. 4A).

3.5. Flaxseed diet alters the liver fatty acids profile followed by high omega-3 tissue incorporation and bioconversion to longer omega-3 species

As in the MAT, the fatty acid profile of the liver was determined after molecular analysis. The consumption of experimental diets did not change ($P>.05$) the incorporation of SFAs in the hepatic tissue (Fig. 5A). On the other hand, monounsaturated fatty acids were higher ($P<.05$) in the HF group than in CT and FS (Fig. 5E), which mainly represents C18:1 (Fig. 5G). Lastly, the amount of polyunsaturated fatty acids was lower ($P<.05$) in the HF than in CT and FS (Fig. 5I). Interestingly, it showed different answers when classified into ω3 and ω6 fatty acids. The ω3 fatty acid summation was increased ($P<.05$) in the FS group compared to the HF group (Fig. 5J), which is based on the higher incorporation of C18:3α. Additionally, EPA (C20:5) and DHA (C22:6) were respectively 9.6 and 3.1 fold higher ($P<.05$) in mice fed a source of ALA (FS group) than HF (Fig. 5 K–M). The ω6 fatty acids were lower in HF than in CT ($P<.05$) but were not different from those in FS (Fig. 5N).

4. Discussion

The consumption of a HF is a crucial trigger for activating inflammatory signaling and contributes to obesity and coupled metabolic disturbances [8]. On the other hand, ω3 fatty acid has been shown to have anti-inflammatory effects in several tissues and conditions [25,26,37]. Therefore, we verified whether short-term FS diet consumption might attenuate inflammation in the MAT and liver.

Short-term HF feeding has been shown to cause disturbances in areas of the central nervous system and peripheral tissues, such as the hypothalamus, hippocampus, liver, and adipose tissue [1,12,38,39]. The effects of short-term HF feeding led to the development of hyperphagia and insulin resistance in rodent models [1,38]. Despite the impact of HF feeding being considerably studied, the short-term effects of HF with part of the lard substituted by ω3 food sources, such as FS, are poorly explored. The present study identified that short-term HF-fed mice presented higher food intake and fasting glucose levels than CT and FS mice (Fig. 1A and C). Thaler and collaborators [1] showed that a single day of HF diet increased proinflammatory genes in the hypothalamus of mice, possibly impairing its food intake. However, our results differ from Thaler's study because, in our study, mice return to high consumption of HF on the third day. In contrast, 3 d of ω3 consumption protected against food intake and fasting glucose parameters altered by the HF diet. The palatability of the same diets was previously tested [22]. The consumption of HF and FS diets was different when compared to the CT group, but identical between them. Thus, it is highly possible that some hypothalamic neuronal modulations have occurred, opening new perspectives of investigation.

Previous studies reported that short-term HF feeding could induce insulin resistance in the liver and VAT [38,39]. Consequently, the hormone keeps lipolysis abundant in VAT, releasing glycerol to the liver, and favoring gluconeogenesis [40]. The previous statement may help explain the increased fasting glucose in the HF

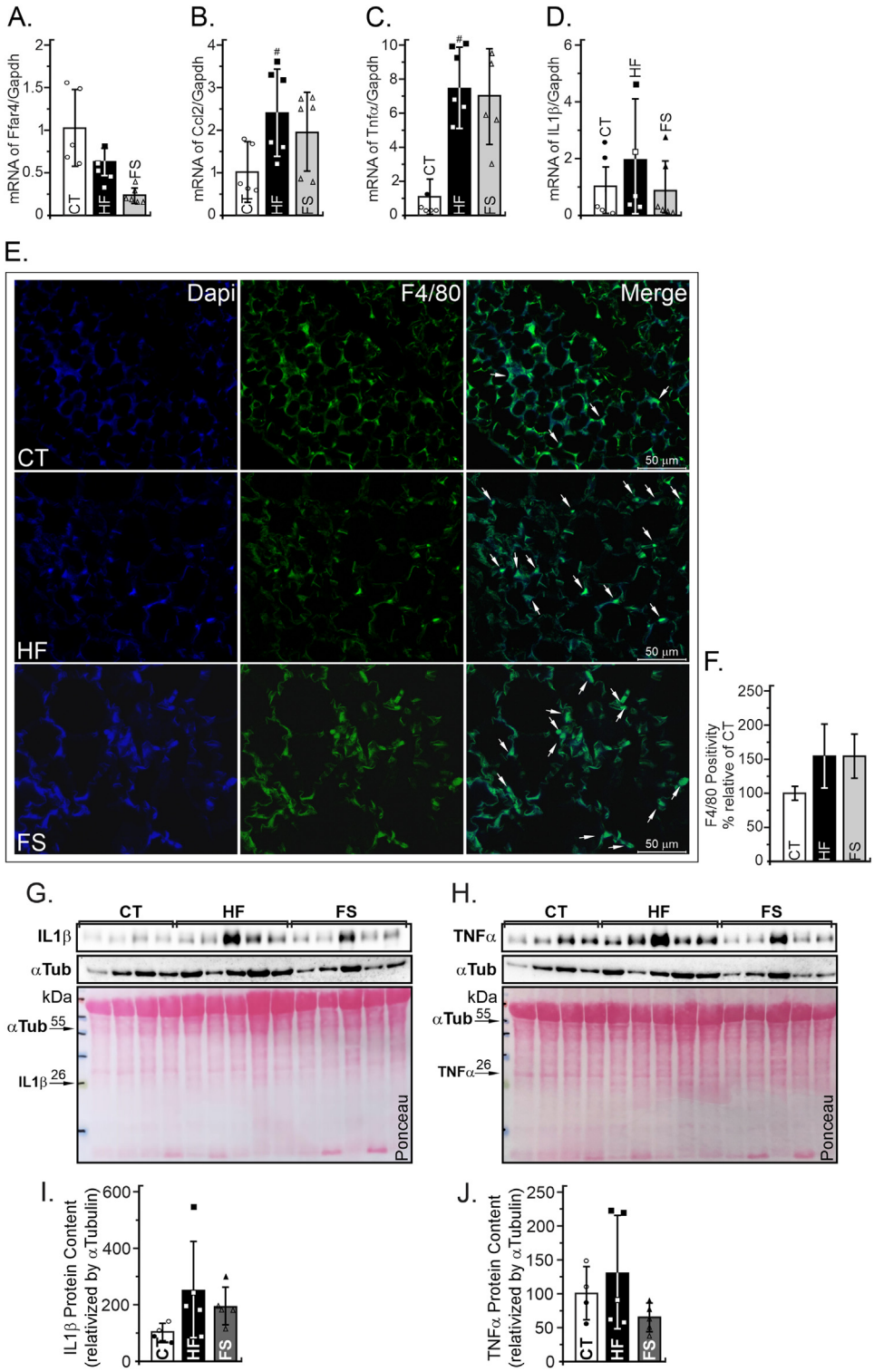

Figure 2. RT-qPCR analyses. (A) *Ffar4* mRNA levels (n=5). (B) *Ccl2* mRNA levels (n=5 on CT; n=6 on HF and FS). (C) *Tnf* mRNA levels (n=5 on CT; n=6 on HF and FS). (D) *Il1b* mRNA levels (n=5). (E) Mesenteric adipose tissue sections (6 μm) from CT and short-term HF and FS (n=3). Specific antibodies against F4/80 (Green - FitC) and DAPI (Blue) were used. The photomicrographs were acquired at 40× magnification (scale bar 50 μm), and the white represents positive labeling of DAPI and F4/80. (F) F4/80 positivity % relative to CT. (G and I) Western blotting analysis of IL1β protein. (H and J) Werten blotting analysis of TNFα protein. P<.05 $^{\#}$CT *vs*. HF and *HF *vs*. FS by Bonferroni's test.

group. FS mice showed higher fasting insulin levels, which could be a counterregulatory mechanism and contribute to reductions in glucose levels. However, since 2005 relevant investigations have been made regarding the role of the GPR120 receptor and its ability to release GLP1 [41]. Glucagon-like peptide-1 (GLP1) is an incretin produced and secreted mainly by the enteroendocrine cells (L-cells) [42], which reach the bloodstream and activates its receptor (GLP1-R) in the surface of beta-cell. The beta cell downstream signaling, mediated by GLP1 activates multiple pathways involved in insulin production and secretion. The AMPc activates PKA (Pro-

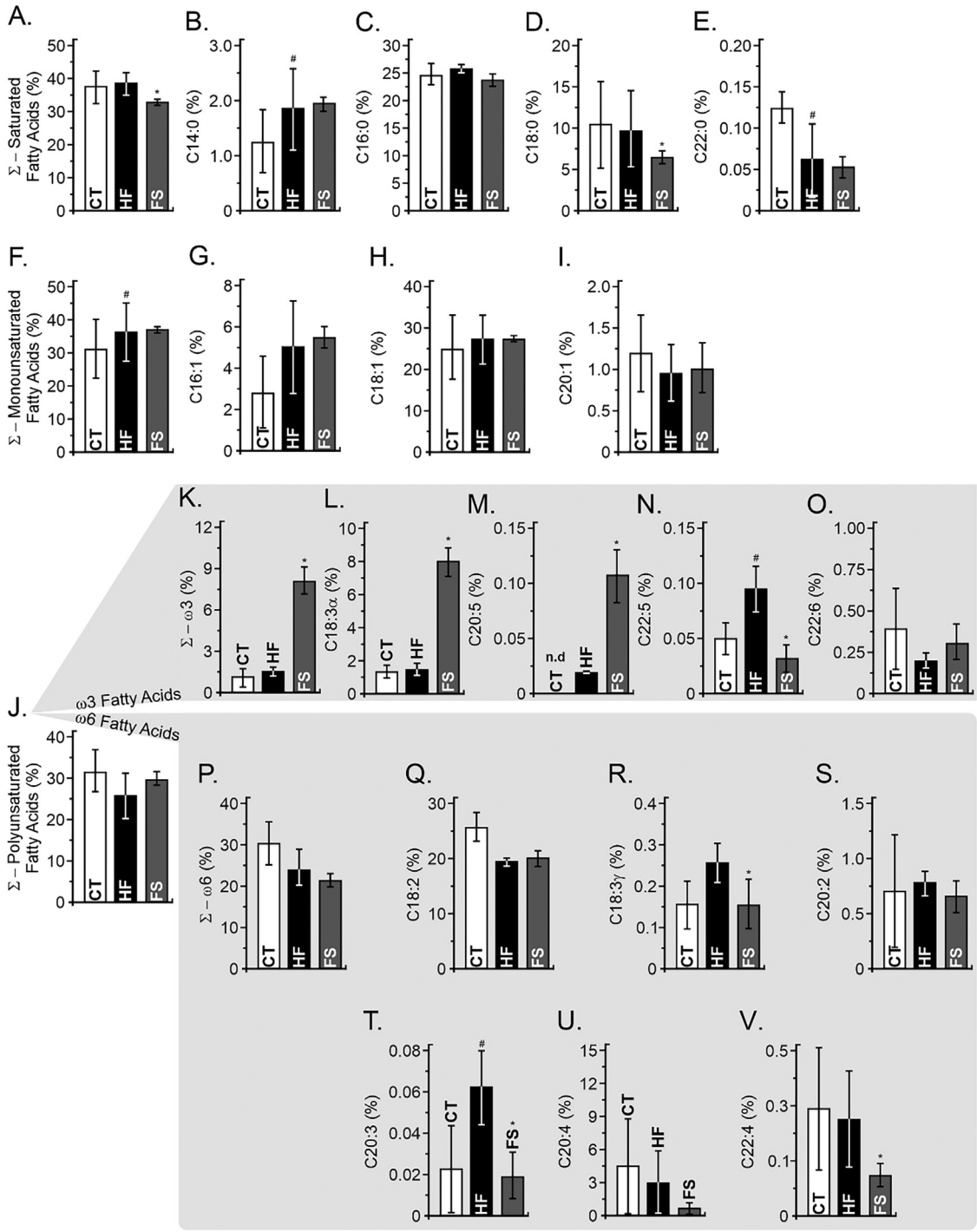

Figure 3. Fatty acid profile of the mesentery after short-term consumption of HF and FS. (A) Σ-Saturated fatty acids. (B) C14:0. (C) C16:0. (D) C18:0. (E) C22:0. (F) Σ-Monounsaturated. (G) C16:1. (H) C18:1. (I) C20:1. (J) Σ- Polyunsaturated fatty acids. (K) Σ- $\omega 3$. (L) C18:3α. (M) C20:5. (N) C22:5. (O) C22.6. (P) Σ- $\omega 6$. (Q) C18:2. (R) C18:3γ. (S) C20:2. (T) C18:3γ. (U) C20:4. (V) C22.4. $P<.05$ $^{\#}$CT *vs.* HF and *HF *vs.* FS by Bonferroni's test.

tein kinase A) and PDX1 (Pancreatic and duodenal homeobox 1), transcribing the insulin gene.

Additionally, PKA depolarized the beta cell inducing the calcium entrance and the insulin granules secretion [43]. Recently, Bianchini et al. [44] tested natural or synthetic GPR120 and GPR40 agonists. The natural compound, alpha-linolenic fatty acid, activated both receptors on the surface of enteroendocrine cells, recruiting beta-arrestin2 and Gαq/11, which mediates the GLP1 release. Also, previous works have shown that GPR120 can lead to insulin secretion through somatostatin activation [45], increasing calcium uptake and cAMP production. All of these mechanisms act independently of the inflammatory process [46].

Next, we hypothesized the changes in the incorporation of fatty lipids would prevent adipose tissue hypertrophy. Firstly, we confirmed that our short-term FS-fed model increased the $\omega 3$ fatty acid levels in the MAT and liver compared with the HF group. Second, we observed that 3 d of HF feeding was enough to expand the VAT (Fig. 1F). However, even elevated $\omega 3$ levels in the FS mice

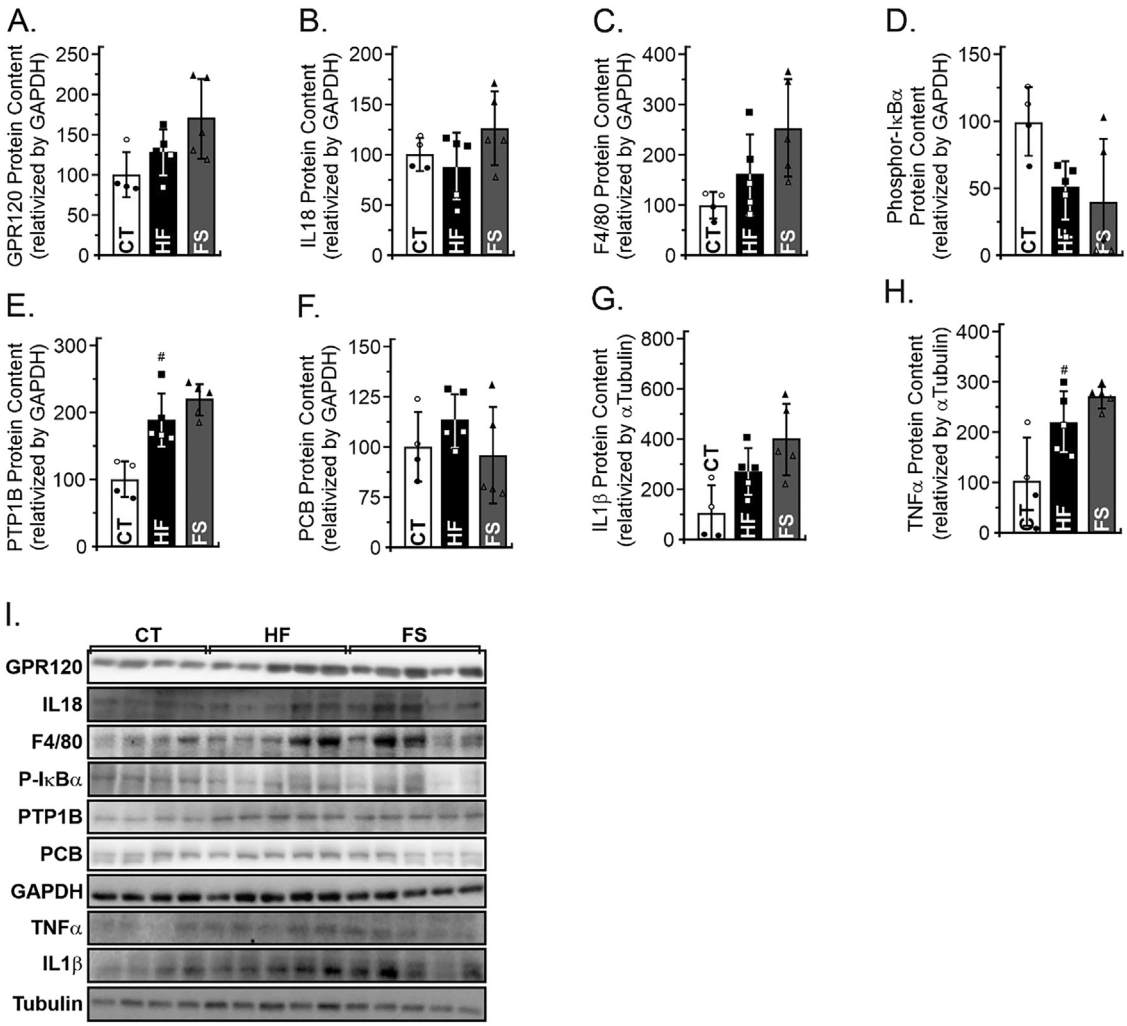

Figure 4. Short-term HF and FS molecular analysis in hepatic tissue. Protein content analysis by Western blotting (n=4 on CTL; n=5 on HFD and FS): (A) GPR120. (B) IL18. (C) F4/80. (D) p-Iκbα. (E) PTP1B. (F) PCB. (G) IL-1β. (H) TNFα. (I) Western blotting analysis (Ponceaus are in Supplementary results 1). $P<.05$ $^{\#}$CT *vs.* HF and *HF *vs.* FS by Bonferroni's test.

were insufficient to protect against body weight gain and adiposity deposition. Similar to our study, Tang et al. [47] also observed increased ω3 levels in the liver and brain of mice after short-term feeding different experimental diets containing ω3. It was also observed that ω3 reduced the total cholesterol in the liver of HF. However, no differences were found in weight gain or adiposity [47]. Based on these results, achieving some metabolic benefits by ω3 must depend on more extended ω3 consumption. It seems to be a dose- and time-dependent circumstance, due to the incremental effectiveness of ω3 tissue incorporation, supported by scientific literature [25,26].

Undisputedly, the EPA and DHA fatty acids, independently of their essentiality for mammals, are well described as exhibiting potent anti-inflammatory properties. Although the total amount of ω3 fatty acids did not prevent the HF alterations on both adipose tissue and the liver, it is relevant to highlight the EPA and DHA bioconversion from ALA fatty acid. In the adipose tissue, EPA was increased 5.75 fold, and in the liver, EPA and DHA were respectively 9.6 and 3.1 fold higher in mice fed with a source of ALA (FS group) than HF. Thus, the fatty acid bioconversion for longer fatty acids was a relevant process, which in chronic consumption could increase the bioconversion and may be more protective against inflammation.

Hypertrophy of the adipose tissue is well described due to the inflammatory environment. Increased proinflammatory cytokines and chemokines attract macrophages, characteristic of metabolic complications, such as obesity and diabetes [48]. Interestingly, Wu et al. [19] showed that after 8 weeks of HF feeding, the MAT was the first adipose tissue depot to develop inflammation compared to the other depots of VAT. Then, we evaluated the molecular parameters in MAT. The HF diet increased proinflammatory genes such as *Ccl2* and *Tnf* in MAT compared to CT, but no effects were found in FS (Fig. 2B and C). Immunofluorescence of MAT was performed to confirm the presence of infiltrating macrophages using the F4/80 marker. However, no differences among the groups were observed (Fig. 2E). Despite the absence of macrophage infiltration, the hypertrophic HF adipose tissue also presented increased mRNA levels of inflammatory cytokines and chemokines. We understand that 3 d of intervention were insufficient to change the macrophage infiltration phenotype.

Nonetheless, it is notorious for beginning a tissue "priming", which could be the first hit to the future adverse scenario. Interestingly, Lefevre et al. [49] showed a priming phenomenon occurring early in obesity and before inflammation was detected on both subcutaneous and visceral stem adipose cells from HF-diet-fed mice [49]. Different from our work, Lefevre et al. [49] point

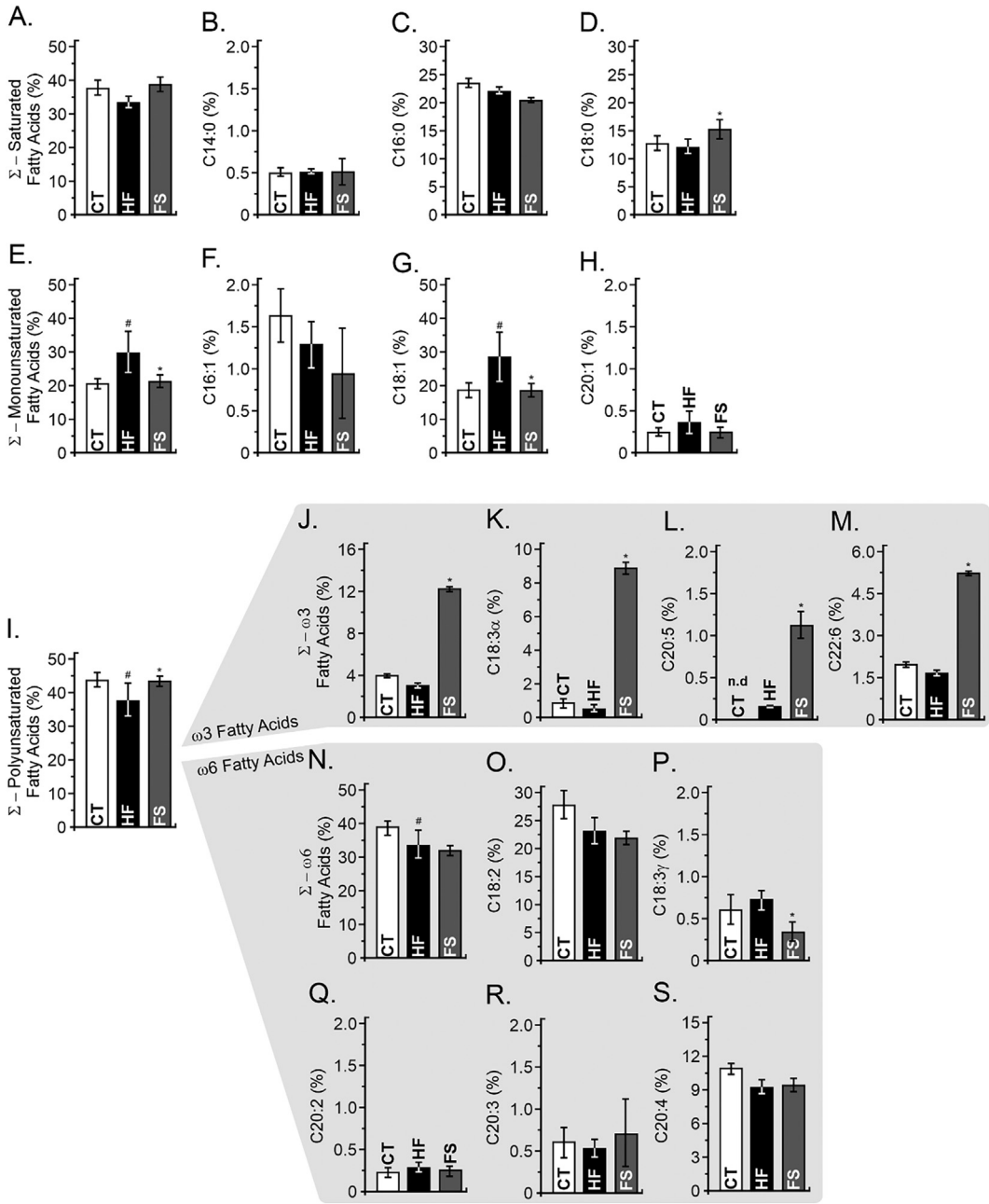

Figure 5. Fatty acid profile of hepatic tissue after short-term consumption of HF and FS. (A) Σ-Saturated fatty acids. (B) C14:0. (C) C16:0. (D) C18:0. (E) Σ- Monounsaturated. (F) C16:1. (G) C18:1. (H) C20:1. (I) Σ- Polyunsaturated fatty acids. (J) Σ- ω3. (K) C18:3α. (L) C20:5. (M) C22.6. (N) Σ- ω6. (O) C18:2. (P) C18:3γ. (Q) C20:2. (R) C20:3. (S) C20:4. $P<.05$ $^{\#}$CT *vs.* HF and *HF *vs.* FS by Bonferroni's test. N.d. means non-detected.

to IL6 as the central conductor of the priming once TNFα appears lately. Herein, TNFα protein content was not changed. Still, its gene expression was increased. Using a short-term (3-d) model in rodents, Butler et al. [50] showed the microglia priming at the hippocampus evidenced by CD11b and CX3CR1, predisposing the animals to future memory impairments [51]. Thus, it is reasonable to understand that we are evidencing a nuance with subtle but consistent characteristics and increasingly evidenced in the literature. On the other hand, FS did not attenuate the inflammatory damage. Short- or long-term high-fat feeding increases the translocation of gram-negative bacteria into MAT. Additionally, *Tnf* mRNA expression was higher after 4 weeks of HF feeding in the MAT and showed a positive correlation with the luminal and mucosal bacterial content [52]. The HF alters the permeability of the tight junctions in the intestine, allowing a more significant influx of inflammatory cytokines that can justify increased inflammation in the MAT of the HF group [53].

MAT inflammation is associated with liver damage [20]. Herein, we observed an increased protein content of TNFα and PTP1B in the liver of HF mice (Fig. 4E and H). Thus, Wu et al. [20] showed that HF feeding increased B lymphocyte cells in the MAT and promoted macrophage differentiation to inflammatory macrophages. Additionally, these immune cells can migrate to the liver and trigger hepatic inflammation . In this context, the immune system response might be the key to adipose-liver crosstalk with the portal vein. It was found that HF mice present higher levels of IL1β

in the portal blood than in the systemic blood and that IL-1β KO mice prevent NAFLD installation [54].

In addition, the mesenteric transplantation of IL1β KO mice prevented pyruvate intolerance [54]. In addition, long-term FS feeding has been observed as a crucial anti-inflammatory intervention in the liver. FS-fed mice exhibited improved hepatic insulin sensitivity and HF-induced inflammation [27,37]. Moreover, we detected increased ω3 bioavailability in the liver and a decreased ω3: ω6 ratio in the FS group compared to the HF group (Fig. 5J–M). However, the well-established anti-inflammatory effects of ω3 were not observed in the liver. A previous study showed that a high ω3:ω6 ratio increased the risk of metabolic dysfunctions [55]. Thus, increased availability of ω3 through FS could improve the ω3:ω6 ratio, which is crucial for preventing and treating metabolic diseases [56].

Insulin is a crucial regulator of adipose tissue lipolysis [40]. Previous studies reported that short-term HF feeding could induce insulin resistance in the VAT and BAT [39,57]. Once insulin cannot reduce lipolysis on VAT, more glycerol is delivered to the liver increasing gluconeogenesis. It could be partially mediated by the pyruvate carboxylase (PCB) which catalyzes the first step for gluconeogenesis, and its inhibition decreases glucose production [58]. In a short-term model of hyperlipidic diets, neither HF nor FS diet changes this behavior. Possibly, other mechanisms are involved in the control of gluconeogenesis pathways, or it could be changed in a time-dependent manner, taking more time under HF consumption to be modified.

5. Conclusion

Altogether, our results suggest that short-term HF feeding increased the inflammatory responses in the MAT and liver. On the other hand, the flaxseed diet short-term feed improved the food intake behavior, insulin and glucose homeostasis, and profoundly changed the fatty acid profiles in the tissues. However, the FS diet could not decrease the inflammation in the MAT and liver. Therefore, further studies are needed to understand the time-course response of ω3 fatty acids and their doses. This exploration reinforces the necessity to increase/improve regulation on the food label and public health programs to increase the knowledge about nutrition since excessive saturated fatty acid consumption once even short-term consumption can favor the beginning of dysmetabolic background.

Author contributions

S.C.B.R.N, R.C.G, and G.K.K were responsible for tissue extraction and physiological analysis. S.C.B.R.N, R.C.G, G.K.K, R.F.V, T.R., and V.R.M were responsible for western blotting experiments. S.C.B.R.N and F.M.S were responsible for the RT-qPCR. S.C.B.R.N and D.E.C. were responsible for the histology experiments. S.C.B.R.N, C.O.R., M.R.S, and D.E.C. were responsible for mass spectrometry gas chromatography data. S.C.B.R. N, R.C.G, G.K.K, R.F.V, V.R.M, C.O.R, M.R.S, and T.R, were responsible for the result analysis. D.E.C, J.R.P, L.P.M, A.S.R.S, and E.R.R provided laboratory support and the manuscript review. S.C.B.R.N and D.E.C conceived all experiments and wrote the paper. All authors approve this submission and are follow the journal guidelines.

Declaration of competing interests

The authors declare that there are no conflicts of interest.

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