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Original Research Article

A novel supplement with yeast β-glucan, prebiotic, minerals and *Silybum marianum* synergistically modulates metabolic and inflammatory pathways and improves steatosis in obese mice

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ABSTRACT

Objective: To evaluate the synergic effects of a novel oral supplement formulation, containing prebiotics, yeast β-glucans, minerals and silymarin (*Silybum marianum*), on lipid and glycidic metabolism, inflammatory and mitochondrial proteins of the liver, in control and high-fat diet-induced obese mice.
Methods: After an acclimation period, 32 male C57BL/6 mice were divided into the following groups: nonfat diet (NFD) vehicle, NFD supplemented, high-fat diet (HFD) vehicle and HFD supplemented. The vehicle and experimental formulation were administered orally by gavage once a day during the last four weeks of the diet (28 consecutive days). We then evaluated energy homeostasis, inflammation, and mitochondrial protein expression in these groups of mice.
Results: After four weeks of supplementation, study groups experienced reduced glycemia, dyslipidemia, fat, and hepatic fibrosis levels. Additionally, proliferator-activated receptor-α, AMP-activated protein kinase-1α, peroxisome proliferator-activated receptor γ co-activator -1α, and mitochondrial transcription factor A expression levels were augmented; however, levels of inhibitor of nuclear factor-κB kinase subunit α and p65 nuclear factor-κB expression, and oxidative markers were reduced. Notably, the cortisol/C-reactive protein ratio, a well-characterized marker of the hypothalamic–pituitary–adrenal axis immune interface status, was found to be modulated by the supplement.
Conclusion: We discovered that the novel supplement was able to modify different antioxidant, metabolic and inflammatory pathways, improving the energy homeostasis and inflammatory status, and consequently alleviated hepatic steatosis.

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1. Introduction

Obesity and diabetes are chronic diseases characterized by increased visceral adipose tissue and sub-acute inflammation. These conditions can lead to insulin resistance, hyperglycemia, oxidative stress [1,2], and lipotoxicity [3] as well as the development of metabolic syndrome [1–3]. Previous work has shown that these comorbidities, as well as high levels of glucocorticoids [4] can modulate the hypothalamic–pituitary–adrenal (HPA) axis and inflammatory response [5]. In some cases, these conditions and responses perturb neuroimmune endocrine system homeostasis [1,2,4,6].

Another potential outcome arising from inflammation, insulin resistance, hyperglycemia, and lipotoxicity is nonalcoholic fatty liver disease (NAFLD), a chronic liver disease that often coexists with obesity-related type 2 diabetes [7]. Previous studies have demonstrated that NAFLD compromises glycemic and lipid homeostasis [3,8–10]. The hyperglycemia [7] and the lipotoxicity [1] drive the metabolic pathways of the cells towards gluconeogenesis, glycolysis [1] and *de novo* lipogenesis [7] and the subsequent increase in free fatty acid production can cause oxidative stress [7].

Under normal physiological conditions, insulin blocks the gluconeogenesis in the liver [1]. However, it fails to do so in metabolic syndrome cases [1] and inflammatory responses, such as nuclear factor-κB (NF-κB) activation, exacerbate this disease [1,11]. Furthermore, the downregulation of adenosine monophosphate-activated protein kinase (AMPK), a master regulator of cellular energy homeostasis [12] contributes to the breakdown of glycemic and lipid homeostasis [13]. Indeed, changes in nutrition, hormone levels, and physical activity have been shown to alter the function of the mitochondria in an organ-dependent manner [13,14].

Previous studies have shown that non-pharmacological interventions can be used as strategies for treating and/or preventing inflammatory and metabolic diseases [15]. Furthermore, specific proteins and transcription factors that target the mitochondria represent potential mechanisms for these interventions.

Along these lines, natural products and derivatives thereof, often referred to as natural medicines, have been gaining attention as sources of effective therapeutics [16]. For example, polyphenols present in plants can be metabolized to form propionic acid, which is later transformed into another metabolite, butyrate. As reviewed by Juárez-Hernández et al. [17] Na-butyrate and synthetic derivatives increase prebiotic *Lactobacilli* and *Bifidobacteria* growth in the colon, consequently improving thermogenesis and basal metabolism, ultimately reducing hepatic lipid deposition, improving triacylglycerol content and insulin resistance and protecting against metabolic impairment, as well as liver inflammation and damage. Furthermore, butyrate can induce the expression of fibroblast growth factor-21, which plays a vital role in stimulating hepatic fatty acid β-oxidation [17]. In this sense, prebiotics may be a promising treatment for chronic inflammatory and metabolic diseases in humans.

The efficacy of prebiotic compounds, such as fructooligosaccharides (FOS) [18] galacto-oligosaccharides (GOS) [19] yeast β-glucans [20,21] minerals, including magnesium [22] zinc [23] and selenium [24] and plants, like *Silybum marianum* (L.) Gaerth. (Silymarin) [25] have been evaluated for the treatment of specific diseases. However, most of these studies only described the effects of the supplements being administered alone. Thus, the present study monitored the synergic effects of a novel supplement formulation containing prebiotics, yeast β-glucans, minerals and silymarin on lipid metabolism and inflammatory and mitochondrial proteins in the livers of control and high-fat diet-induced obese mice.

2. Materials and methods

2.1. Composition

The formulation of the supplement (Patent number: BR 10 2020 016156 3) developed and tested in the present study contained the following components: zinc (Zn) 0.63%; selenium (Se) 0.003%; magnesium (Mg) 4.35%; FOS 49.69%; GOS 31.05% and 1.3/1.6-(β-glycosidic bonds) yeast β-glucans (*S. cerevisiae*) 11.18% (Yes Sinergy, Campinas, São Paulo, Brazil); and *S. marianum* extract 3.11% (Ningbo Vitax Biotech Co., China). The percentages of each of the minerals were calculated based on the dietary reference values for nutrients published by the European Food Safety Authority [26]. The percentages of *S. marianum* [25] FOS [27] GOS [28] and yeast β-glucans [29] were based on previous studies and considered the body area of the animals, determined by the equation: human equivalent dose (mg/kg) = animal does (mg/kg) × 12.33 [30]. The final product was diluted in mineral water using 2% carboxymethyl cellulose as the emulsifier.

2.2. Animal model and oral supplementation

Sixty-day-old adult male C57BL/6 mice were obtained from the Central Vivarium of Mice at the University of São Paulo School of Medicine (FMUSP). All mice were maintained in an air-conditioned room at (24 ± 2)°C and subjected to a 12 h light/12 h dark cycle. The animals were subsequently divided into two groups: control and obese. The control group was fed a standard nonfat diet (NFD) that contained 3.54 kcal/g. The other group (called obese) consumed a high-fat diet (HFD) that contained 5.25 kcal/g and was composed of 30% saturated fat, mainly lard, 15.95% carbohydrate, and 20% protein [9] (Prag Soluções *Biosciences*, Jaú, São Paulo, Brazil). The mice were placed on these diets for 14 weeks and were allowed to eat the chow *ad libitum*. In week 10, the mice were further divided into the following groups: NFD vehicle (n = 7), NFD supplemented (n = 8), HFD vehicle (n = 8), and HFD supplemented (n = 9). The vehicle and experimental formulation were administered daily by gavage to ensure that all animals ingested the same amount of supplement for the last 4 weeks (28 consecutive days) of the experimental protocol. We chose this treatment period to assess the long-term effect of supplementation on control and obese animals. Animals were euthanized on 4 weeks after supplementation with excessive dose of ketamine and xylazine. All experiments were conducted in accordance with the National Institutes of Health guidelines. The protocols were reviewed and approved by the USP-FMUSP Ethics Committee (numbers: 1185/2018 and 1519/2020).

2.3. Food consumption and body index parameters

Food, water, and feeding efficiency were estimated using the following formulas [10]: food intake = initial weight of food provided – final weight of food recovered (g); water intake = initial volume of water provided – final volume of water recovered (mL); feed conversion efficiency (FCE) = body weight gain (g)/mean food intake.

These values were calculated for each cage and then divided by the number of animals in the cage (two or three animals per cage).

Additionally, the body mass index was calculated using the following formula: body mass index = body weight (g) × naso-anal length (cm)^2.

The naso-anal length was used to normalize the weekly body weight gain and final body weight [31].

Food and water intake and animal weight were recorded twice a week during the supplementation.

2.4. Body composition analysis

During the thirteenth week of the study, when animals had received vehicle or experimental supplement for three weeks, magnetic resonance imaging (MRI), with a minispec body analyzer, using Minispec software (Ettlingen, Germany), was used to quantify the body composition in terms of grams of fat/lean mass per kilogram of body weight.

2.5. Intraperitoneal glucose tolerance test and insulin curve

After week three of supplementation, the mice were weighed and fasted for 6 h. Each mouse was then intraperitoneally injected with a 10% glucose solution (1.0 g/kg body weight). Blood samples were collected from the tail at 0, 15, 30, 60, 90 and 120 min, and glycemia was measured with an Accu-Chek® Active blood glucose monitor (Roche, Mannheim, Germany) [10]; serum insulin was determined using rat/mouse insulin enzyme-linked immunosorbent assay (ELISA) kit from Milllipore/Sigma-Aldrich (catalog number EZRMI13K-St Charles, MO, USA).

2.6. Biochemistry and endocrine parameters

The levels of cholesterol, triglycerides and C-reactive protein (CRP) in the plasma were measured with a commercially available kit (Bioclin, Belo Horizonte, MG, Brazil). The low-density lipoprotein cholesterol (LDL-c) and very-low-density lipoprotein cholesterol (VLDL-c) levels were calculated according to Friedewald et al. [32].

Serum cortisol (CORT) concentrations were measured using the Cortisol Express ELISA Kit (Cayman Chemical, Ann Arbor, MI, USA), according to the recommendations of the manufacturer.

Serotonin in proximal colon was detected. Briefly, samples were weighed and placed in a two mL microtube, along with 25 μL of lysis buffer per mg of tissue. The lysis buffer was composed of 0.01 mol/L HCL, 1% L-ascorbic acid and ultrapure water, and the pH was adjusted to 8.8 with 1 mol/L NaOH. After adding 100 μL of lysis buffer, the samples were cut with fine-tipped scissors. Then another 100 μL of lysis buffer was added, along with 20 μL of the stabilizing solution (Serotonin Research ELISA kit, Labor Diagnostika Nord GmBH, Catalog number BA E-5900, Nordhorn, Germany). Next, the samples were homogenized with a microhomogenizer (MA1102, Piracicaba, SP, Brazil) for 1 min at moderate speed. Another 100 μL of lysis buffer was added, then the sample was vortexed for 1 min and centrifuged at 4000×g for 15 min at 4 °C. Finally, the supernatant was collected, and the pellet was discarded. Serotonin concentrations were measured using the ELISA kit (Nordhorn, Germany), according to the instructions of the manufacturer.

2.7. Measurement of protein carbonylation (OxyBlot)

Protein carbonylation was detected immunologically, following the separation of the proteins by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and was performed according to methods of Wardelmann et al. [33]. Briefly, after transferring the proteins from the gel to a nitrocellulose membrane, the membrane was stained with Ponceau S. The membrane was then washed with water and equilibrated in TBS/methanol 8:2 (v/v) for 5 min. Following equilibration, the membrane was washed with two mol/L HCl for 5 min and derivatized in 20 mmol/L 2,4-dinitrophenylhydrazine (DNPH) in two mol/L HCl for 10 min. Afterward, the membrane was washed twice (5 min/wash) with two mol/L HCl to remove excess DNPH. Lastly, the membrane was washed five times (5 min/wash) with TBS/methanol (1/1, v/v) and twice (5 min/wash) with TBS-T. The membrane was then blocked for 1 h in nonfat milk, washed three times with 1 × TBS-T, and incubated with an anti-DNP primary antibody (Sigma, D9656, 1:5000) overnight at 4 °C. The next day, the membrane was incubated with the respective secondary antibody conjugated with peroxidase for 1 h. The membrane was developed using chemiluminescence with luminol and hydrogen peroxide and quantified by optical density.

2.8. Thiobarbituric acid-reacting substances assay

Quantification of malondialdehyde (MDA) by thiobarbituric acid-reacting substances levels was performed in liver samples. The presence of MDA is indicative of oxidative stress, and previous work has shown that these levels can be altered in tissues following antioxidant treatment [29].

2.9. Lipid content in the liver

Lipids present in the liver were extracted and quantified, according to Folch et al. [34]. Cholesterol and triglycerides were measured using a commercially available kit (Bioclin, Belo Horizonte, MG, Brazil). Values are expressed as per gram fresh tissue weight.

2.10. Histological analysis of the liver

After 4 weeks of supplementation, mice were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg b.w.) and xylazine (5 mg/kg b.w.). The right lobe of the liver was removed, fixed in 4% formaldehyde for 24 h at room temperature and processed as described by Pessoa et al. [35]. For morphological analysis, the sections were stained with hematoxylin & eosin, and collagen content was assessed by staining the samples with Picrosirius red. Types I and III collagen fibers were evaluated under polarized light. Fibers were identified by their birefringence pattern (type I: red, orange, and yellow and type III: green). The images were captured in a microscope attached to a desktop (Leica Microsystems DMC 2900, SP, Brazil) through the LAS V4.6 program (Leica Microsystems).

2.11. Western blotting

Liver samples were lysed in RIPA buffer and centrifuged at 10,000 r/min for 15 min at 4 °C. The protein content of the supernatants was subjected to SDS–PAGE and transferred to nitrocellulose membranes using a Mini-trans blot apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Nonspecific binding to the membrane was prevented by incubating the blots in TBST-B-blocking buffer (10 mmol/L Tris, 150 mmol/L NaCl, 0.05% Tween-20, and 5% non-fat milk) for 2 h at room temperature. The nitrocellulose membranes were incubated with the specific primary antibodies overnight at 4 °C and subsequently incubated with a secondary antibody conjugated to horseradish peroxidase for two hours. The immunoblots were developed using the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Waltham, MA, USA). Immunoblots were visualized on an UVItec Alliance 4.7 System (UVITEC, Cambridge, UK) and quantified using the ImageJ software (NIH, Bethesda, MD, USA). The following primary antibodies and dilutions were used: nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB; catalog number 8242S, Cell Signaling, Danvers, MA, USA) 1:1000; nuclear respiratory factor-1 (NRF-1; catalog number 46743S, Cell Signaling, Danvers, MA, USA) 1:1000; p38 mitogen-activated protein kinase (p38 MAPK; catalog number860S, Cell Signaling, Danvers, MA, USA) 1:1000; AMPK-α1 (catalog number 2795S, Cell Signaling, Danvers, MA, USA) 1:1000; mitochondrial transcription factor A (TFAM; catalog number 19998-1AP, Proteintech, Rosemont, IL-USA) 1:1000; peroxisome proliferator-activated receptor γ co-activator -1α (PGC-1α; catalog number 66369-1, Proteintech, Rosemont, IL, USA) 1:2000; transforming growth factor-β1 (TGF-β1; catalog number B51361, Bioworld, Dublin, OH, USA) 1:1000; sirtuin 1 (SIRT1; catalog number 84695, Cell Signaling) 1:1000; superoxide dismutase [Cu-Zn] (SOD1; catalog number VPA00070, Bio-Rad, Hercules, CA, USA) 1:1000; peroxisome proliferator-activated receptor-α (PPAR-α; catalog number GTX28934, Gene Tex, Alton Pkwy Irvine, CA, USA) 1:2000; and inhibitor of nuclear factor-κB kinase subunit α (IKK-α; catalog number 26825, Cell Signaling, Danvers, MA, USA) 1:1000. Ponceau S staining was used to normalize the protein expression results.

2.12. Statistical analysis

Comparisons between groups were performed using two-way analysis of variance (ANOVA). Fisher's least significant difference test was used to make multiple comparisons when ANOVA indicated a significant difference among groups. Differences with P-value < 0.05 were considered statistically significant. All data are expressed as the mean ± standard deviation. Pearson correlation coefficients were calculated for the associations between the CRP and cortisol. All statistical analyses were conducted with the GraphPad PRISM statistical software package version 5.00 for Windows (GraphPad Software, San Diego, CA USA).

3. Results

3.1. Supplementation influenced body weight, body mass index, and lean mass in HFD-induced obese mice

The experimental protocol employed in the present study involved feeding mice with either NFD or HFD for 10 weeks and then administering either vehicle or the novel supplement for four weeks (Fig. 1A). We found that weekly weight gain (Fig. 1B), final weight (Fig. 1C), and body mass index (BMI) (Fig. 1D) of the control supplement group were not statistically different from the control vehicle group. However, the control-NFD supplement group exhibited increased food and water intake (Fig. 1 E and F), which supported the augmented FCE (Fig. 1G) and lean mass gain, as evidenced by the MRI analysis (Fig. 1H), when compared to the control vehicle group. Notably, the MRI analysis did not reveal any observable changes in fat mass (Fig. 1I) when comparing the two control groups.

On the other hand, the obese-HFD supplement group displayed less weight gain during weeks 13 and 14 than the obese vehicle group (Fig. 1B). The final weight (Fig. 1C), BMI (Fig. 1D) and FCE (Fig. 1E) of the obese-HFD supplement animals were significantly reduced, compared to the obese vehicle group. There were no detectable differences in food or water intake between these two groups (Fig. 1 E and G). Furthermore, supplementation had no influence on fat mass in either HFD group (Fig. 1I).

3.2. Novel supplement restored glucose and insulin homeostasis in HFD-induced obese mice

As expected, the HFD vehicle group was found to be glucose-intolerant (Fig. 2A) and insulin-resistant (Fig. 2D), when compared to the control and obese supplement groups. The obese vehicle HFD group had significantly greater area under the curve (AUC) values for glucose (Fig. 2B) and insulin (Fig. 2E). The obese vehicle group also displayed higher fasting glycemia (Fig. 2C) and insulin (Fig. 2F) levels than the NFD supplemented group. Notably, supplementation reversed glucose intolerance (Fig. 2A) and insulin resistance (Fig. 2D), reduced the AUC for glucose (Fig. 2B) and insulin (Fig. 2E), and decreased fasting glycemia (Fig. 2C) and insulin levels (Fig. 2F) in the obese mice. Supplement had no observable effect on the glycemic metabolism (Fig. 2A and C) or insulin levels (Fig. 2D and F) in the control-NFD supplement group.

3.3. Plasma and liver lipid levels were decreased after 4 weeks of supplementation

In the control-NFD supplement group, the circulating cholesterol, triglycerides, LDL-c and VLDL-c concentrations were similar to the control vehicle group (Fig. 3A, C, E and G). On the other hand, the HFD supplement group displayed significantly reduced plasma cholesterol (Fig. 3A) and LDL-c (Fig. 3G) levels, compared to both vehicle groups. We did not detect any significant differences in the triglyceride or VLDL-c concentrations between any of the evaluated groups (Fig. 3C and E).

As shown in Fig. 3B, D and F, supplementation modulated the lipid profile in the liver. For example, liver cholesterol concentrations were reduced in the control NFD and obese HFD supplement groups, compared to the respective vehicle groups (Fig. 3B). Additionally, triglyceride and VLDL-c concentrations were attenuated in the obese HFD supplement group when compared to the vehicle groups (Fig. 3D and F). The histological analyses further corroborated the observed reduction in liver lipid concentrations. As shown in Fig. 3H, liver sections from both supplemented groups contained less accumulated lipid droplets. Interestingly, fewer lipid droplets were present along the edge of the hepatic centrilobular vein in the obese supplement group (Fig. 3H).

3.4. Oxidative stress markers declined after four weeks of supplementation

Oxidative stress is strongly associated with an imbalance between antioxidant enzymes, mitochondrial dysfunction, and inflammation. We found that SOD protein expression was only increased in the obese supplement group when compared to the obese-HFD vehicle group (Fig. 4A). As shown in Fig. 4B, protein carbonylation was significantly reduced in the control supplement group. There was a noticeable reduction in protein carbonylation in the obese supplement group, compared to the obese vehicle; however, this result failed to reach a level of statistical significance. We also found that the obese vehicle animals had elevated MDA levels and that supplementation reduced these levels to those of the control-NFD groups (Fig. 4C).

3.5. Supplementation modulated cellular energy metabolism and mitochondrial biogenesis regulators in the liver

Next, we evaluated whether the observed supplement-induced alterations were due to changes in the expression of proteins involved in regulating cellular energy metabolism and mitochondrial biogenesis in the liver. After four weeks of supplementation, there was a significant increase in the expression of AMPK-α1 (Fig. 5A and G) in both supplemented groups (NFD and HFD), when compared to their respective controls. We also observed increased levels of PGC1-α (Fig. 5B and G), PPAR-α (Fig. 5C and G), and TFAM (Fig. 5D and G) in the obese-HFD supplement group, compared to the obese-HFD vehicle and control-NFD groups. Additionally, sirtuin 1 (SIRT1; Fig. 5E and G) and NRF-1 (Fig. 5F and G) expression levels were elevated in the control supplement group, compared to the other groups.

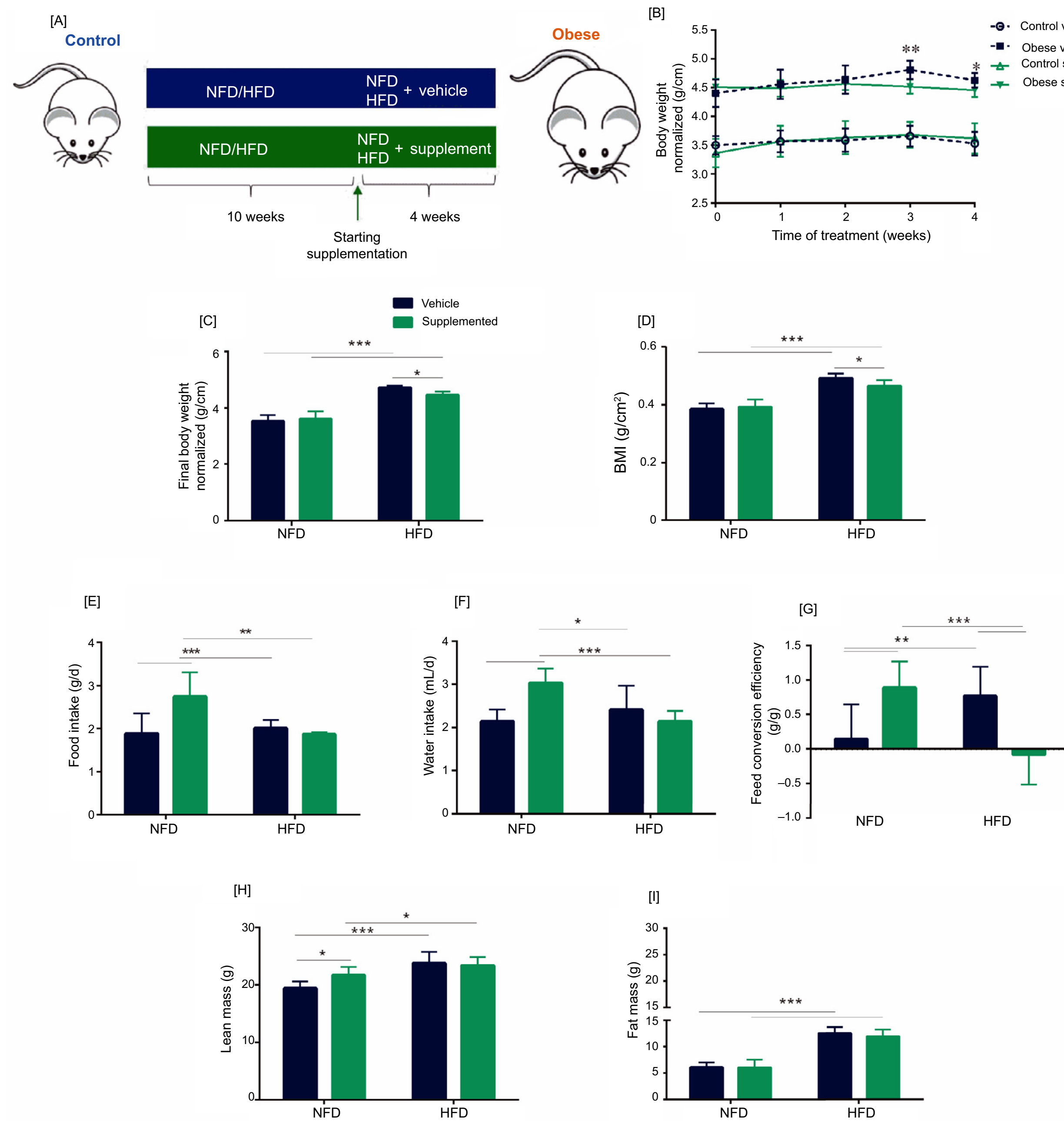

Fig. 1. Morphological and nutritional profile evaluated in nonfat diet (NFD) and high-fat diet (HFD) mice after receiving the vehicle or supplement for 4 weeks. (A) Schematic representation of the experimental design; (B) body weight during the 4-week treatment; (C) final body weight; (D) body mass index (BMI); (E) food intake; (F) water intake; (G) feed conversion efficiency; H: lean mass; I: fat mass. Results are presented as mean ± standard deviation with 7–9 animals. Statistical significance was determined using the one-way analysis of variance, followed by Fisher's least significant difference post-hoc test. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3.6. Supplementation reduced inflammatory protein expression and suppressed fibrosis in the hepatic steatosis

To assess the anti-inflammatory effect of supplementation on hepatic steatosis, we evaluated the expression of proteins involved in inflammation. The supplement reduced p38 MAPK (Fig. 6A and E), TGF-β (Fig. 6B and E), IKK-α (Fig. 6C and E) and p65 NF-κB (Fig. 6D and E) expression levels in the control-NFD and obese-HFD supplement groups. This result suggests that the novel supplement has an anti-inflammatory effect in the liver. This possibility was further corroborated by the reduction in liver fibrosis (Fig. 6F), with reduced orange-red collagen fiber type I in supplemented groups. Importantly, since the aforementioned inflammatory proteins are important activators of this status, this latter result was not entirely unexpected.

3.7. Supplementation reduced endocrine and inflammatory markers

The colon serotonin (Fig. 7A) and plasma CRP (Fig. 7B) concentrations were not significantly different between the control-NFD and obese-HFD vehicle groups. On the other hand, the control-NFD supplement group presented significantly reduced circulating cortisol concentrations compared to the control vehicle group (Fig. 7C). We did not detect a significant correlation between cortisol and CRP in the control groups (Fig. 7D). Notably, the obese-HFD supplement group exhibited significantly

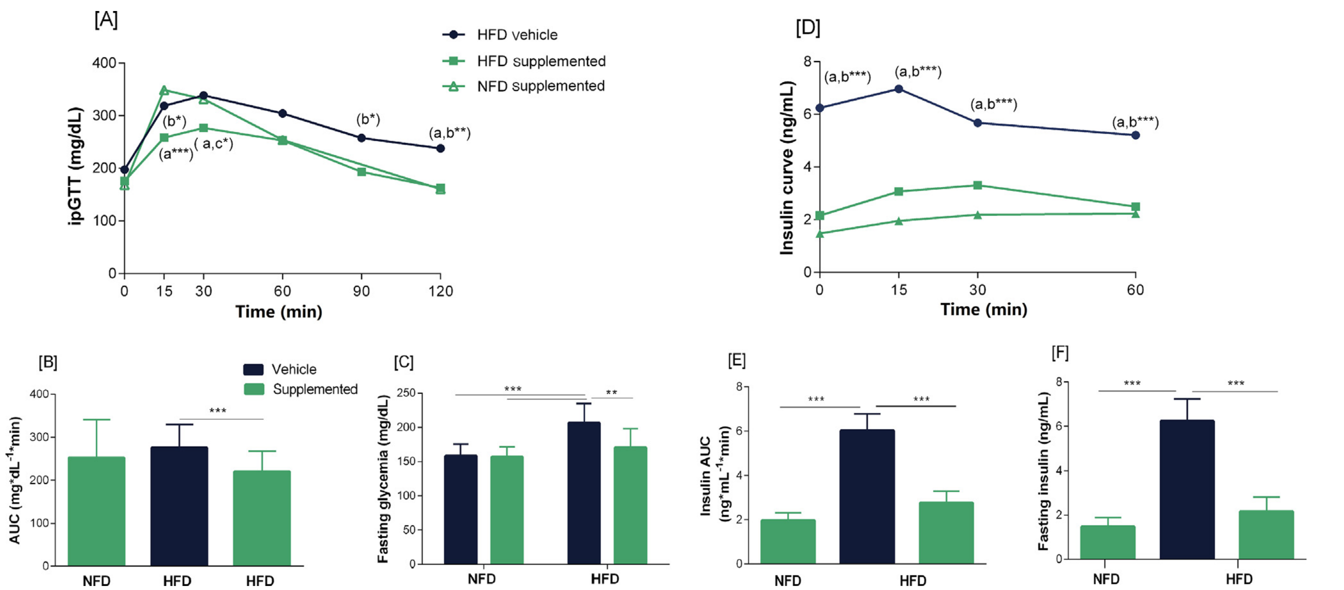

Fig. 2. Postprandial and basal glycemia in NFD and HFD mice after receiving the vehicle or novel supplement for 3 weeks. (A) Postprandial glycemia; (B) glycemia area under the curve (AUC); (C) fasting glycemia (mg/dL); (D) insulin curve; (E) insulin AUC; (F) fasting insulin (ng/mL) after 3 weeks of supplementation. Results are presented as the mean ± standard deviation. Each group contained 7–9 animals. Statistical significance was determined using the one-way analysis of variance, followed by Fisher's least significant difference post-hoc test. (a) Difference compared with NFD supplemented; (b) difference compared with HFD supplemented; (c) difference compared with HFD vehicle. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. ipGTT: intraperitoneal glucose tolerance test; NFD: nonfat diet; HFD: high-fat diet.

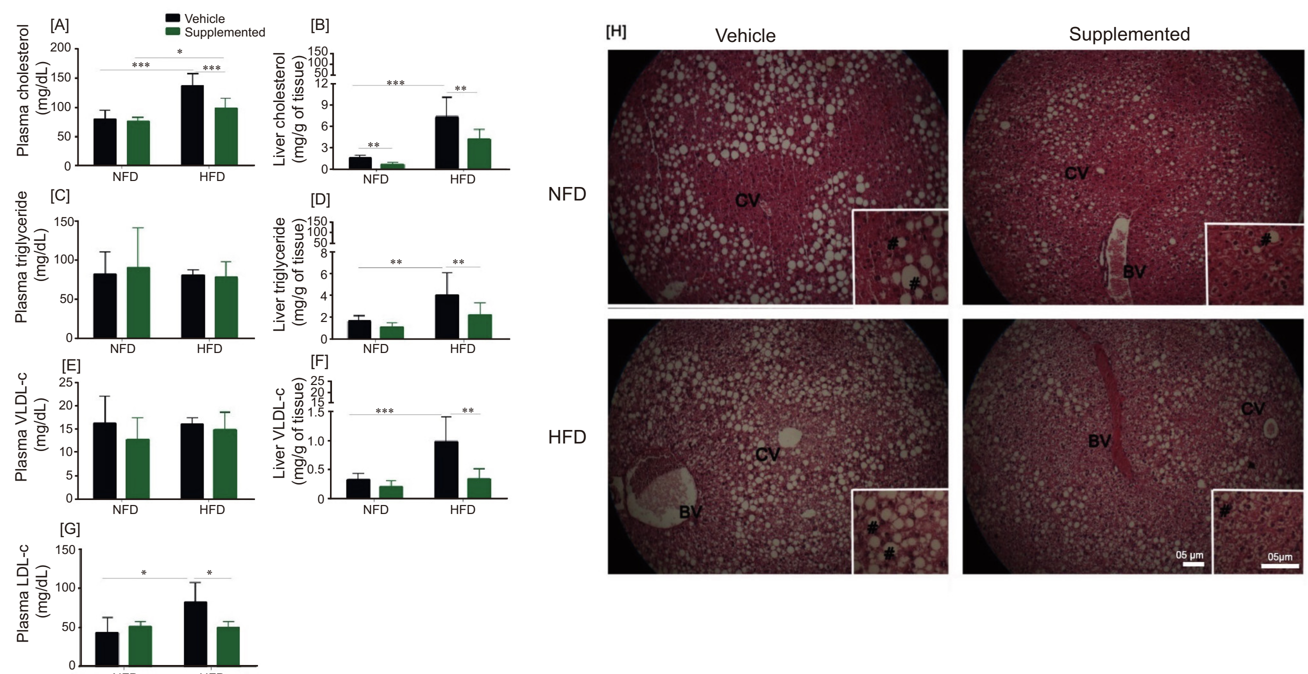

Fig. 3. Plasma and liver lipid profiles in NFD and HFD mice after daily oral administration for 4 weeks. (A and B) cholesterol; (C and D) triglycerides; (E and F) VLDL-c; (G) LDL-c. Results are presented as mean ± standard deviation. Each group contained 7–9 animals. Statistical significance was determined using the general linear model, and Fisher's least significant difference post-hoc test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. (H) The histological analysis of the liver with hematoxylin & eosin staining. Each image contains a representative histological liver section image from each group; objectives 20× and 40× (inserts). BV: blood vessel; CV: central vein; LDL-c: low-density lipoprotein cholesterol; VLDL-c: very-low-density lipoprotein cholesterol; NFD: nonfat diet; HFD: high-fat diet; (#) lipid droplets.

reduced colon serotonin (Fig. 7A), plasma cortisol (Fig. 7B), and cortisol (Fig. 7C) concentrations, compared to the obese vehicle group. In contrast to the control results, a positive and significant correlation between circulating cortisol and CRP concentrations was detected ($r = -0.913$; $P = 0.0303$; Fig. 7D). Furthermore, the obese-HFD vehicle group also exhibited a reduced CORT/CRP ratio (1.05; Fig. 7E). Importantly, the correlation between circulating cortisol and CRP concentrations was no longer detectable in the obese animals (Fig. 7D). Additionally, the CORT/CRP ratio was equivalent to the vehicle and supplement control groups (1.26 *vs.* 1.25 and 1.21, respectively).

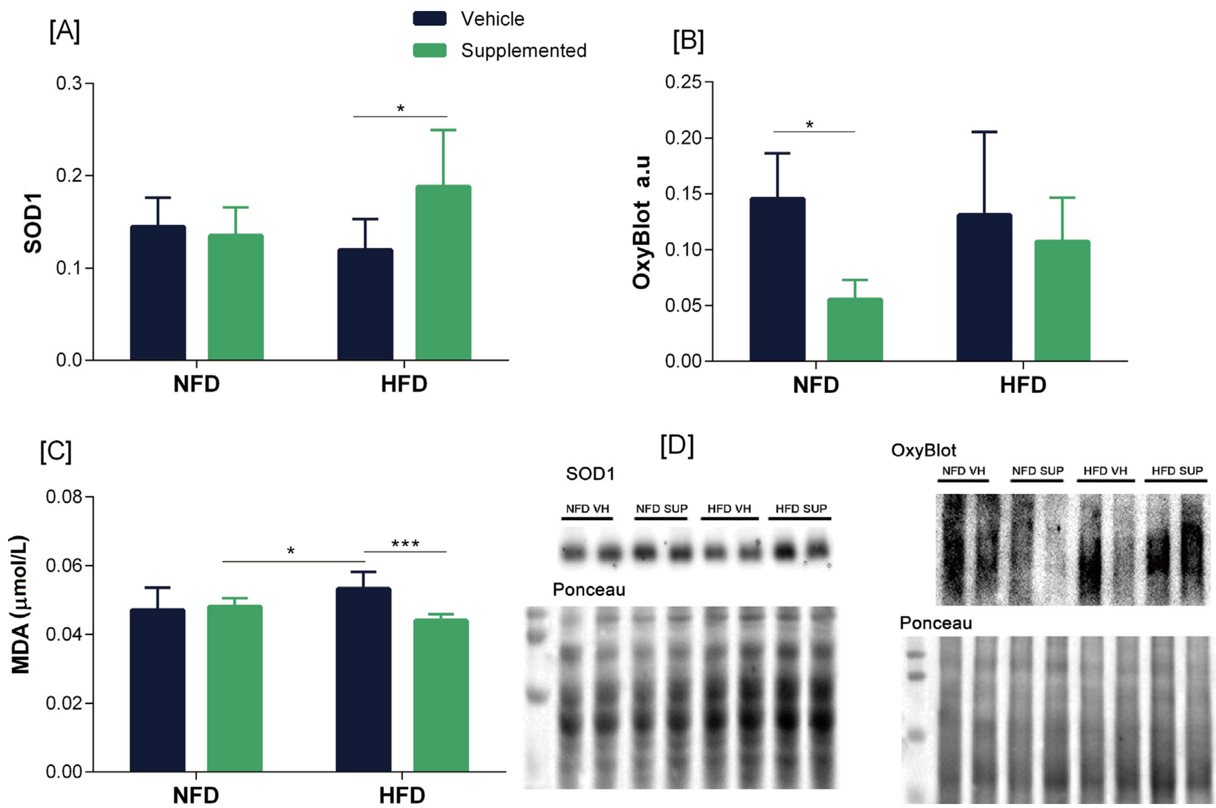

Fig. 4. Oxidative stress and antioxidant markers in NFD and HFD mice after administration for 4 weeks. (A) Superoxide dismutase [Cu-Zn] (SOD1) protein expression level assessed by immunoblot analysis. (B) Protein carbonylation assessed by OxyBlot. (C) Malondialdehyde (MDA) concentrations measured using the thiobarbituric acid-reacting substance assay. (D) Representative images of Western blot: Ponceau S staining and protein carbonylation (OxyBlot). Blots were semi-quantified, and the results are presented as mean ± standard deviation. Each group contained 7–9 animals. Statistical significance was determined using the general linear model, and Fisher's least significant difference post-hoc test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. NFD VH: control vehicle, NFD SUP: control supplemented; HFD VH: obese vehicle, HFD SUP: obese supplemented; NFD: nonfat diet; HFD: high-fat diet.

4. Discussion

Obesity and type 2 diabetes are characterized by chronic inflammation that leads to the breakdown of cellular homeostasis and disruption of the neuroimmune-endocrine axis [5,36–38]. These conditions have also been associated with increased mortality in SARS-CoV-2 infection [32]. The novel supplement contains components that can act in an integrated and synergistic way, due to their metabolism and absorption in the gut. Therefore, this supplement can effectively influence different metabolic pathways involved in cellular homeostasis. Our results show that changes in the expression of proteins involved in mitochondrial biogenesis are primarily responsible for the observed effects. Our study is the first to evaluate a formulation containing prebiotics, *S. cerevisiae* β-glucans, selenium, magnesium, zinc and silymarin (*S. marianum*) in a model of obesity and chronic inflammation.

It is known that a high intake of saturated, mainly palmitic acid and linoleic acid, and trans-fatty acids is associated with the development of NAFLD [11]. This condition shifts the metabolism of the hepatocyte away from the gluconeogenesis and glycolysis pathways [1,2] consequently contributing to insulin resistance, oxidative stress, mitochondria dysfunction [7] NF-κB activation [11] and increased free fatty acid deposits.

In individuals with obesity associated with type 2 diabetes, weight loss is a strategy that has been shown to improve inflammation and restore biochemical parameters, without pharmacological intervention [15]. The novel formulation presented here reduced weight gain, reversed HFD-induced glucose intolerance, and re-established basal insulin levels after four weeks of supplementation. Notably, the supplement also increased the FCE and lean mass in the NFD supplement group.

Reduced circulating mineral levels are closely linked with obesity and type 2 diabetes [39]. Therefore, it is likely that the observed results are a consequence of augmented mineral levels, due to the presence of yeast β-glucans and prebiotics in the gut, which increases their absorption. Additionally, these compounds also exert bifidogenic effects and increase short-chain fatty acid production, which both help to modulate carbohydrate and lipid metabolism [18,19,21]. Magnesium is essential for maintaining metabolism and initiating the glycolytic pathway [22]. Additionally, zinc improves epithelial repair and decreases NF-κB activation [23] and selenium is a potent endogenous antioxidant [24].

Finally, silymarin has been shown to exert anti-diabetic and anti-obesity effects. These properties are due to its ability to inhibit protein tyrosine phosphatase 1B, which negatively regulates the insulin and leptin signaling pathway [40]. Here, we have demonstrated that our formulation could reduce circulating and liver lipids after four weeks of supplementation. This observation is particularly relevant, since accumulated fat molecules inside the hepatocytes can alter metabolism, exacerbate inflammation, induce structural modifications, and promote mitochondrial dysfunction. These alterations can contribute to oxidative stress and are intricately linked to NAFLD [3,10].

Oxidative stress is an imbalance between the production and elimination of reactive oxygen and nitrogen species. The presence of endogenous antioxidant enzymes, such as SOD1, catalase and glutathione peroxidase, and exogenous antioxidants, including vitamins C, E, and flavonoids, protect cells against oxidative dam-

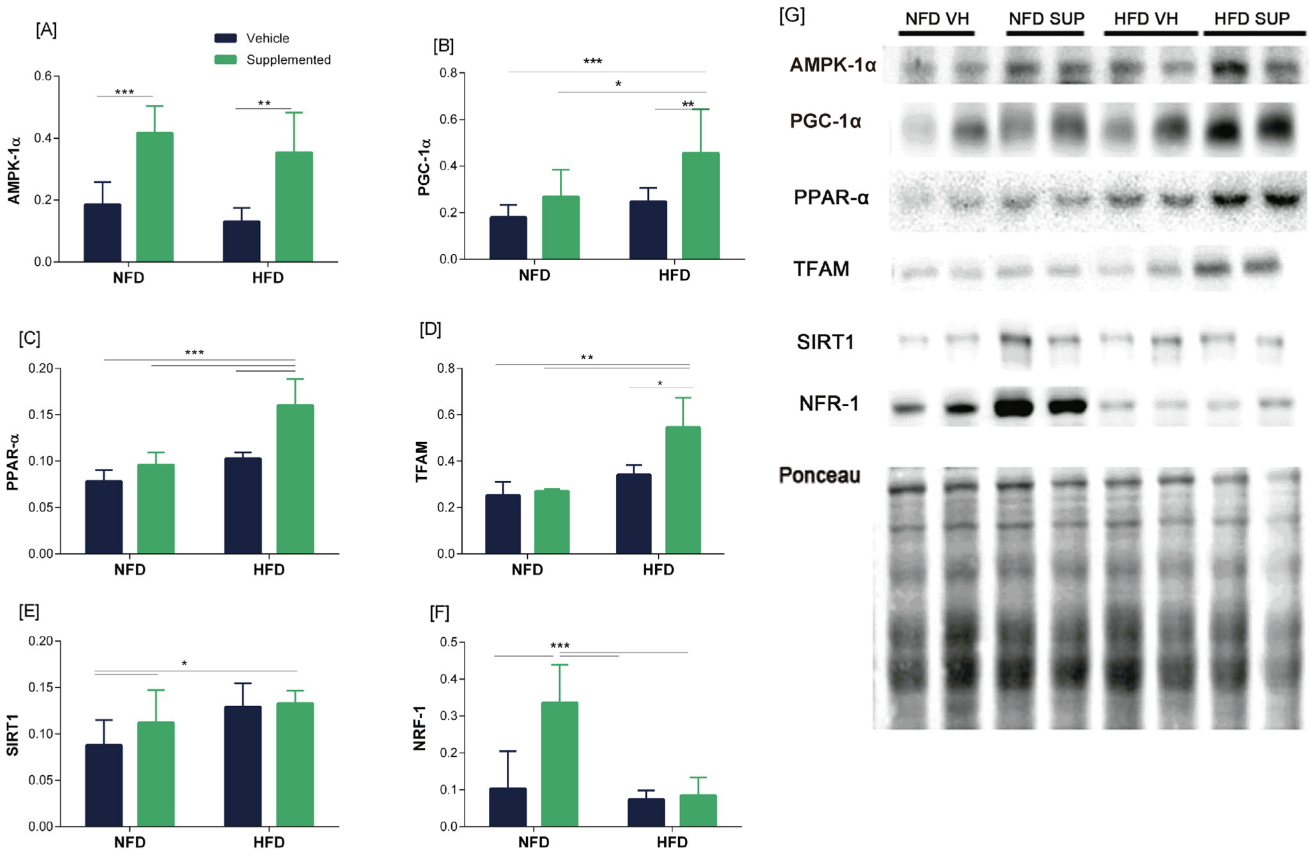

Fig. 5. Expression of proteins involved in cellular energy metabolism and mitochondrial biogenesis in NFD and HFD mice after administration for 4 weeks. The protein expression levels of AMPK-1α (A), PGC-1α (B), PPAR-α (C), TFAM (D), SIRT1 (E), and NRF-1 (F) were assessed by immunoblot analysis. (G) Representative images of Western blot and Ponceau S staining blot. Blots were semi-quantified, and the results are presented as mean ± standard deviation. Each group contained 7–9 animals. Statistical significance was determined using the general linear model, and Fisher's least significant difference post-hoc test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. NFD VH: control vehicle, NFD SUP: control supplemented; HFD VH: obese vehicle; HFD SUP: obese supplemented; NRF-1: nuclear respiratory factor-1; AMPK: AMP-activated protein kinase; SIRT1: sirtuin 1; PPAR-α: peroxisome proliferator-activated receptor-α; TFAM: mitochondrial transcription factor A; PGC-1α: peroxisome proliferator-activated receptor γ co-activator -1α; NFD: nonfat diet; HFD: high-fat diet.

age [35,41,42]. NAFLD-related lipotoxicity causes oxidative damage by increasing lipid peroxidation, which generates harmful byproducts such as MDA and carbonyl proteins; it also decreases SOD1 levels [41,42]. Previous studies have demonstrated that oral antioxidant supplementation can reduce the oxidative stress parameters [11]. Here, the novel supplement exhibited different group-dependent antioxidant effects. For example, the increased expression of PPAR-α (see below) and SOD1 and attenuated MDA concentrations were only observed in the HFD supplement group. On the other hand, supplementation only reduced OxyBlot levels in the NFD animals.

It has been demonstrated that changes in the protein expression levels of PGC-1α and PPAR-α [43,44] can contribute to a breakdown in mitochondrial homeostasis and the onset of disease [7]. PGC-1α is a transcriptional co-activator that participates in the coordinated regulation of nuclear and mitochondria-encoded genes that are involved in mitochondrial biogenesis, fatty acid oxidation, GLUT4 expression, the induction of exercise-mimetic effects [10,37] and the insulin-signaling pathway [45]. Additionally, in obesity and type 2 diabetes, PGC-1α regulates the expression of proteins involved in hepatic gluconeogenesis, lipid catabolism, and NAFLD physiopathology [46]. Interestingly, lower PGC-1α expression levels have been associated with insulin resistance in humans, which may be the result of oxidative damage elicited by the breakdown of lipidic metabolism [45].

PPAR-α is the major isoform of peroxisome proliferator-activated receptors (PPARs) in the liver. It is a sensor of genes required for lipid metabolism, activated during fasting, involved in mitochondrial fatty acid oxidation, insulin action, lipoprotein metabolism, endocrine function, and hepatoprotection [43,44]. Beyond these effects, PPAR-α produces an anti-inflammatory effect through a physical interaction with NF-κB and downregulation of inflammatory genes, such as the IL-6 receptor [47]. Previous work has shown that the livers of obese patients with non-alcoholic steatohepatitis have lower PPAR-α [48] and PGC-1α gene expression, which drastically disturbs mitochondrial structure and function [11].

Interestingly, the upregulation of PGC-1α [13,49] and PPAR-α [11,43,44] is associated with a shift towards β-oxidation in the liver, thus highlighting the ability of the mitochondria to adapt to an illness in a tissue-specific manner. In this study, we showed that PGC-1α, PPAR-α and TFAM expression levels increased in the livers of obese mice, after four weeks of supplementation. Indeed, the NRF-1-TFAM pathway contributes to mitochondrial biogenesis, lipid oxidation and mitochondrial homeostasis maintenance [50,51].

SIRT1 regulates metabolism by promoting mitochondrial biogenesis and antioxidative effects, consequently relieving mitochondrial oxidative stress via PGC-1α activation [52]. While SIRT1 pathway deficiencies have been associated with metabolic disorders such as diabetes and NAFLD [53] we did not observe any changes in the levels of this protein in the HFD groups of this study. Moreover, PGC-1α, a co-activator upstream from the SIRT1 pathway, can modulate the expression of SIRT1, NRF-1 and TFAM.

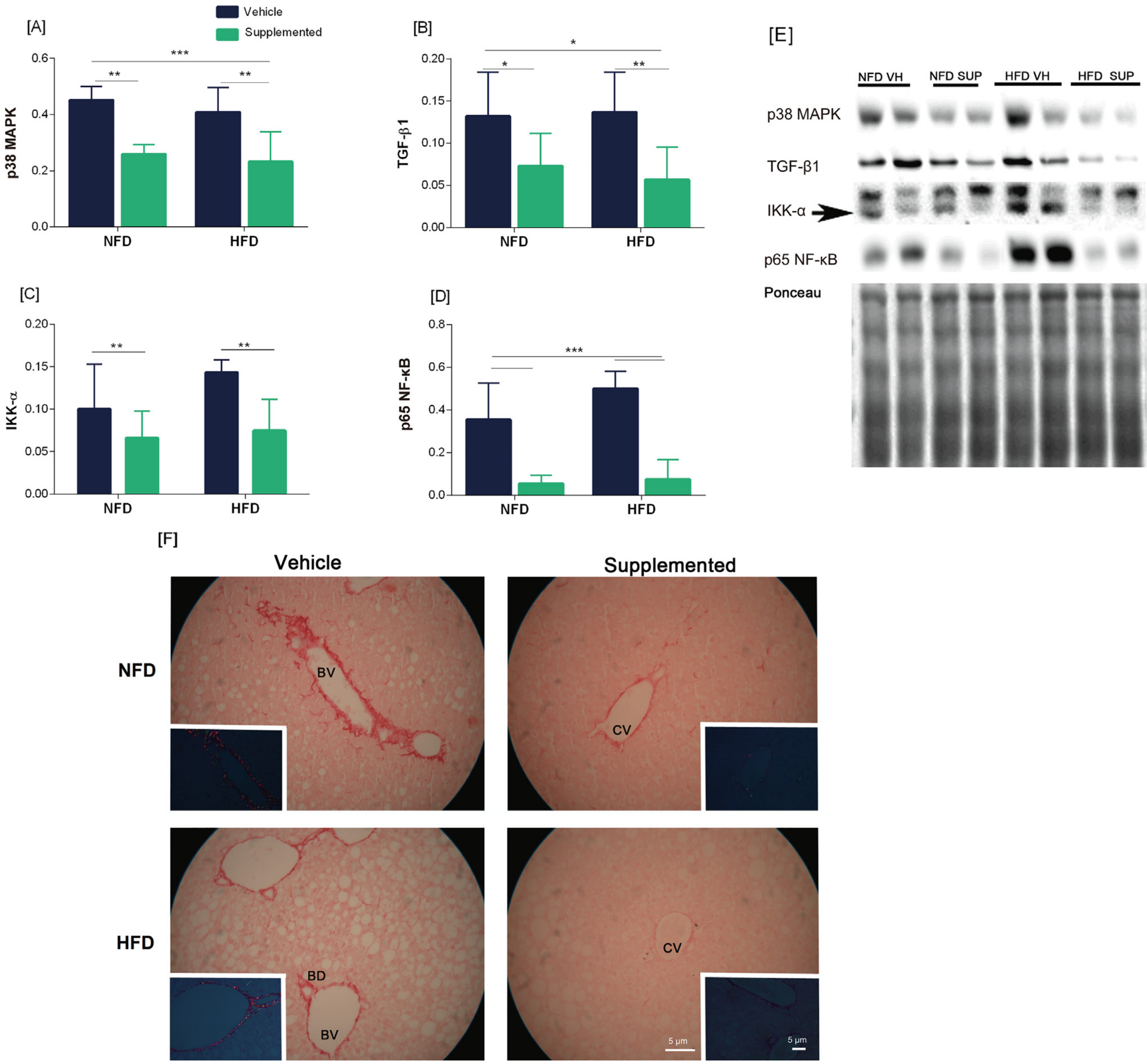

Fig. 6. Effects of supplementation on liver inflammation in NFD and HFD mice after administration of carboxymethylcellulose (vehicle) or supplement (supplemented) for 4 weeks. The protein expression levels of p38 AMPK (A), TGF-β1 (B), IKK-α (C) and p65 NF-κB (D) were assessed by immunoblot analysis. E: representative images of Western blot and Ponceau S staining blot with samples from all the evaluated groups. Blots were semi-quantified, and the results are presented as mean ± standard deviation. F: the histological analysis of the liver with picrosirius red staining (Objectives 40× and 20× inserts); Optical density of type I fibers is yellow, orange, and red values and type III fibers are green values. Each group contained 7–9 animals. Statistical significance was determined using the general linear model, and Fisher's least significant difference post-hoc test. *P < 0.05, **P < 0.01, ***P < 0.001. IKK-α: inhibitor of nuclear factor-κB kinase subunit α; p65 NF-κB: p65 nuclear factor-κ B; TGF-β1: transforming growth factor-β 1; AMPK: AMP-activated protein kinase; NFD VH: control vehicle; NFD SUP: control supplemented; HFD VH: obese vehicle; HFD Sup: obese supplemented; NFD: nonfat diet; HFD: high-fat diet; BD: bile duct; BV: blood vessel; CV: central vein.

However, it has been reported that the activation of NRF1 by PGC-1α is not essential for the up-regulation of TFAM, an observation consistent with our results [36,37,48]. Along these lines, we only observed increased SIRT1 and NRF-1 expression in the NFD supplement group and increased TFAM expression in the HFD supplement group. While the novel supplement elicited a variety of positive effects in both the control and obese groups, more studies focusing on the pathways and molecules involved in mitochondrial biogenesis in different tissues of healthy and obese individuals will need to be undertaken.

Notably, AMPK is an important enzyme regulated by the intracellular AMP/ATP ratio. This enzyme maintains the cellular energy balance, and previous studies have shown that caloric restriction and physical exercise can activate AMPK, increasing catabolic and decreasing anabolic pathways [8,12]. Thus, AMPK has become a therapeutic target for diseases such as obesity and type 2 diabetes. Interestingly, a mutation in the murine γ1 subunit leads to AMPK activation, resulting in decreased lipogenesis and liver triglycerides. Additionally, this mutation prevents fructose-induced hepatic steatosis in mice [54]. Notably, our supplement reduced the hepatic fatty acid generation (e.g., cholesterol, triglycerides and VLDL) and inflammation after four weeks, an effect probably due to increased lipid oxidation resulting from PPAR-α, AMPK- α1 a, PGC-1α and TFAM activation and IKK-α and p65 NF-κB inhibition.

It is known that NF-κB is activated by IKK-α [55] TGF-β [56] and p38 MAPK [12]. Furthermore, NF-κB activation contributes to

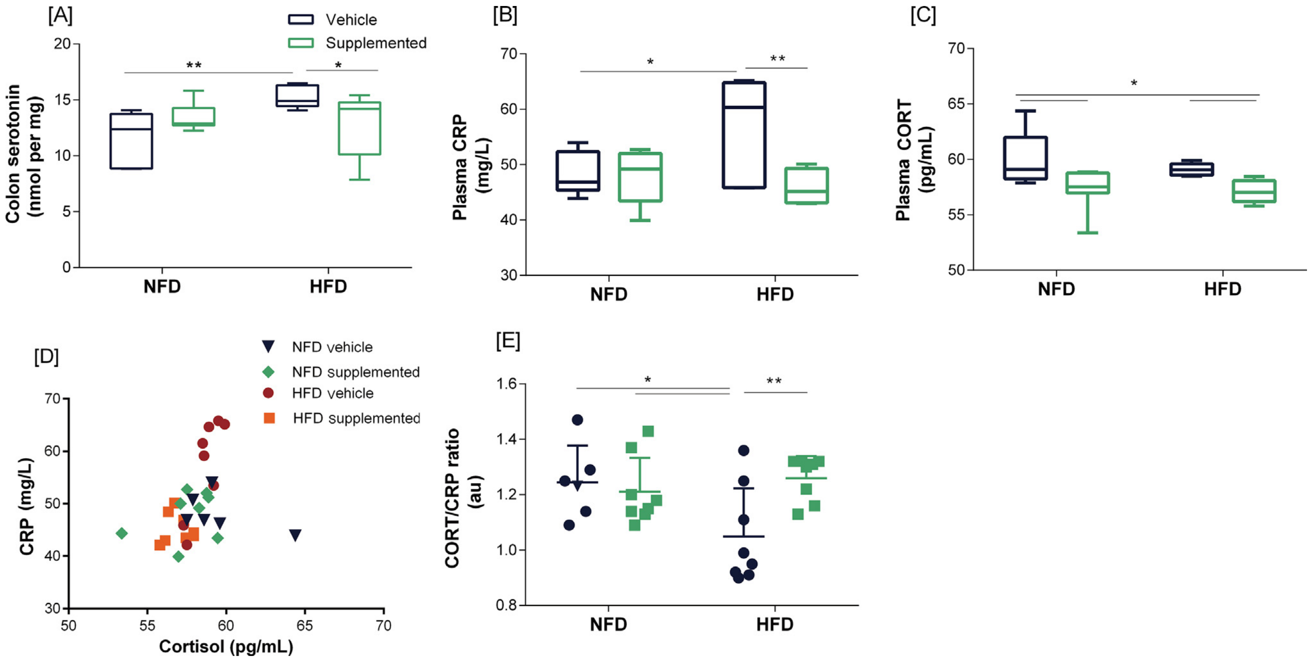

Fig. 7. Effects of supplementation on endocrine and inflammation parameters in NFD and HFD mice after 4 weeks of supplementation. (A) Colon serotonin; (B) Plasma C-reactive protein (CRP); (C) Plasma cortisol (CORT). Results are presented as box and whiskers (Min to Max). (D) CRP/cortisol correlation; (E) CORT/CRP ratio. Results are presented as scatter dot plot (mean ± standard deviation). Statistical significance was determined using the two-way analysis of variance, followed by the Fisher's least significant difference post-hoc test when necessary. Each group contained 7–9 animals. *P < 0.05, **P < 0.01, ***P < 0.001. NFD: nonfat diet; HFD: high-fat diet.

obesity-induced insulin resistance and lipid droplet accumulation, leading to inflammation and liver fibrosis in NAFLD [47,48].

Here, we showed that the supplement reduces plasma CRP, p38 MAPK and TGF-β1 expression and attenuates liver fibrosis. While most of the benefits of the formulation arise from the synergism among the compounds, silymarin has an anti-fibrogenic activity (Fig. 8). Previous studies have shown that silymarin suppresses the expression of procollagen-α1 via the downregulation of TGF-β1 and NF-κB expression, and other genes involved in the cytoskeleton organization in the liver tissue [22–25,57]. Moreover, we also observed reduced systemic inflammation, as demonstrated by the reduced plasma CRP and cortisol levels following supplementation. It has been proposed that the inflammatory status associated with obesity and type 2 diabetes contributes to neuroendocrine and neuroinflammatory dysfunction [5,37,58]. For example, patients with sleep restriction, low glucose tolerance and insulin sensitivity have an increased risk of developing obesity and diabetes. Other consequences of reduced sleep include

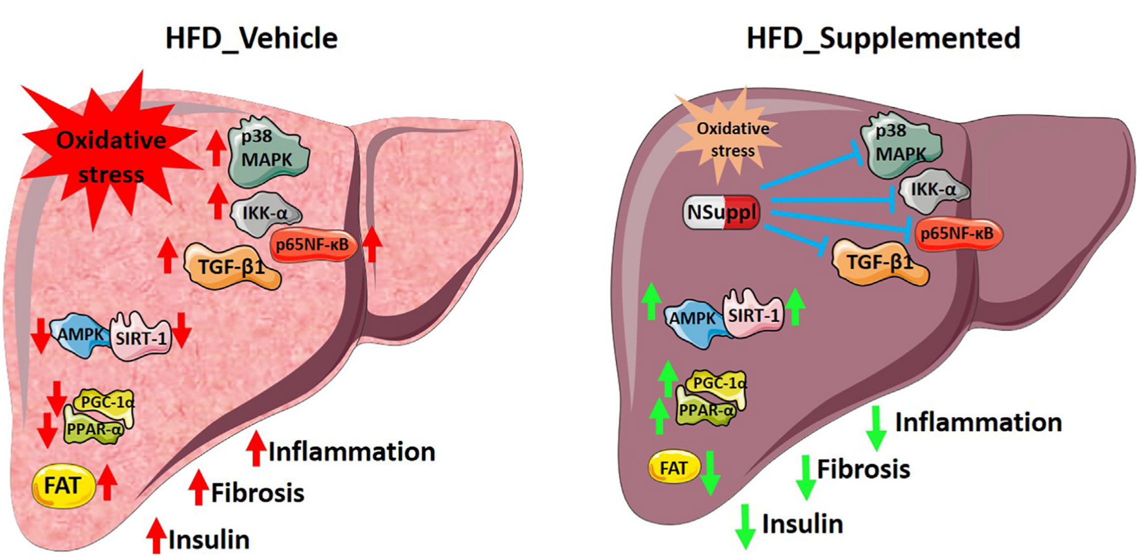

Fig. 8. Scheme illustrating the high-fat diet (HFD) and supplement-mediated effects in the liver at control animals (HFD vehicle) and treated animals (HFD supplemented). p38 MAPK: p38 mitogen-activated protein kinase; IKK-α: inhibitor of nuclear factor-κB kinase subunit α; p65 NF-κB: p65 nuclear factor-κB; TGF-β1: transforming growth factor-β 1; AMPK: AMP-activated protein kinase; SIRT1: sirtuin 1; PPAR-α: peroxisome proliferator-activated receptor-α; PGC-1α: peroxisome proliferator-activated receptor γ co-activator -1α; FAT: fatty acid; NSuppl: novel supplementation.

increased CRP levels and visceral fatty acid accumulation, which are associated with worsening metabolic syndrome and obesity [4,5,37].

In a model of HFD-induced obesity, it was found that increased plasma serotonin [59] elevated circulating CRP levels, and higher serum LDL-c and triglyceride concentrations were associated with high cortisol [60] and inflammatory cytokine levels. These alterations can contribute to HPA axis dysregulation in obese humans [5] resulting in type 2 diabetes and cardiovascular disease [60]. It is well known that CRP is produced by the liver and is a primary indicator of systemic inflammation. In this sense, it was not entirely surprising to observe a reduction in plasma CRP, since the inflammatory condition of the liver was improved after supplementation. Furthermore, supplement-induced reductions in plasma cortisol and colon serotonin concentrations appeared to modulate the endocrine system.

Previous studies investigating the HPA axis-immune interface [61] and mitochondrial biogenesis [13,14] have shown that they are naturally interconnected, and this relationship is integral to maintaining health and preventing disease. The CORT/CRP ratio is a reliable measure of homeostasis between the HPA axis and inflammation, with a low ratio indicative of inflammation, depressive symptoms, and diminished sleep quality, all of which negatively influence the human endocrine system [61]. In the present study, we found that the supplement could increase and restore the CORT/CRP ratio in HFD-induced obese animals to a level that was not different from the control groups. Thus, it is plausible that the anti-inflammatory effect of the supplement was acting at, and modulating, the HPA axis. Furthermore, we propose that the novel supplement also modulates the endocrine-neuroimmune axis, since improvements in the CORT/CRP ratio, and consequently inflammation, were observed following supplementation. Further tests will need to be conducted to accept or reject this hypothesis.

5. Conclusion

The present study demonstrated that the novel supplement promoted favorable systemic effects. In the liver, metabolic and inflammatory parameters were reduced in the NFD-control and HFD-obese supplemented mice. It is likely that modulating the expression of key proteins and pathways involved in cellular energy (AMPK-1α), mitochondrial biogenesis (PPAR-α, PGC-1α, SIRT1 and NRF-1), inflammation (p38 MAPK, IKK-α and NF-κB), and oxidative stress (SOD1, MDA and carbonyl protein) account for these benefits, after four weeks of supplementation. Furthermore, we found that the CORT/CRP ratio, a useful metric for evaluating the underlying biological perturbations associated with the HPA axis and the immune system, was altered by the integrated and synergistic effects of the supplement. Future studies investigating whether similar benefits are achieved at the hypothalamus–gut axis are necessary.

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Authors' contribution

VAN, GMM, RCM M, DAM JPO and AFMP performed experiments, data analysis, discussion of findings, and intellectual support; GCAL, JCRJ, JAF, NIV and RP contributed with technical assistance; DAM, KR, JPO and RGFC performed data analysis and discussion of findings; JCRJ, JAF, NIV and RP contributed with technical assistance; KRML contributed with intellectual support; AFMP gave conception and design of the study, discussion of findings and statistical analysis, and was adviser and supervisor in whole project and manuscript writer.

Declarations of competing interest

None to declare.

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