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Original article

DIETARY SUPPLEMENTATION WITH INTEGRAL CHIA AND FLAX FLOURS AMELIORATES SYSTEMIC INFLAMMATION

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Abstract

Introduction: Chia and flax seeds are rich in alpha-linolenic acid (ALA), which is bioconverted into the active derivatives eicosapentaenoic (EPA) and docosahexaenoic (DHA) having multiple beneficial effects. However, there is limited knowledge about the anti-inflammatory effects of chia and flax integral flours diets rich in ALA.

Objective: The study aimed to evaluate the anti-inflammatory effect of dietary supplementation with integral chia and flax flours in a murine model of LPS-induced systemic inflammation.

Methods: Balb/c mice were distributed into three groups: diet A (control), diet B (supplemented with integral chia flour), and diet C (supplemented with integral flax flour). Nutritional, hematological, and biochemical determinations were performed. ALA, EPA, and DHA were assessed by GC-MS in the liver, brain, cardiac and skeletal muscles. NF-kB immunoassays were performed in kidney, liver, and peritoneal macrophages, respectively. The phagocytic capacity was determined in peritoneal macrophages and the expression of the pro- and anti-inflammatory cytokines was assessed by RT-qPCR in the kidney, liver, and spleen.

Results: Diets B and C exhibited optimal nutritional adequacy and caused increased levels of ALA, EPA, and DHA in critical tissues compared to the control. The phagocytic capacity of murine peritoneal macrophages ($p< 0.01$) and IL-10 transcription increased, whereas the expression of NF-κB, IL-1β, IL-6, and TNF-α decreased in animals fed both experimental diets.

Conclusions: This work contributes to the current knowledge of the anti-inflammatory effects of chia and flax integral flours rich in ALA and reinforces the health advantages of their consumption.

Key words: anti-inflammatory properties, diets, integral flours, linolenic acid, chia, flax

Resumen

La suplementación dietaria con harinas integrales de chia y lino tiene efectos antiinflamatorios sistémicos

Introducción: Las semillas de chía y lino son ricas en ácido alfa-linolénico (ALA), sus derivados activos eicosapentaenoico (EPA) y docosahexaenoico (DHA) ejercen probados efectos beneficiosos. Existe un conocimiento limitado sobre los efectos protectores de ambas semillas bajo la forma de harinas integrales, siendo de particular interés el efecto antiinflamatorio.

Objetivo: El objetivo de este trabajo fue evaluar el efecto antiinflamatorio de la suplementación dietaria con harinas integrales de semillas de chía y lino en un modelo murino de inflamación sistémica inducido por LPS.

Métodos: Ratones de la cepa Balb/c fueron distribuidos en tres grupos: dieta A (control), dieta B (suplemen-

tada con harina integral de chía) y dieta C (suplementada con harina integral de lino). Se efecturaron determinaciones nutricionales, hematológicas y bioquímicas. El contenido de ALA, EPA y DHA en hígado, cerebro, corazón y músculo esquelético se determinó por cromatografía GC-MS. Se realizó la inmunodetección de NF-kB en macrófagos peritoneales, riñón e hígado. Se determinó la capacidad fagocítica de macrófagos peritoneales y se evaluó la expresión de citoquinas pro y antiinflamatorias por RT-qPCR en riñón, hígado y bazo.

Resultados: Las dietas B y C mostraron una adecuación nutricional óptima y generaron niveles elevados de ALA, EPA y DHA en tejidos críticos. La capacidad fagocítica de los macrófagos peritoneales ($p< 0.01$) y la transcripción de IL-10 aumentó, mientras que la expresión de NF-κB, IL-1β, IL-6 y TNF-α disminuyó en animales de los grupos B y C.

Conclusiones: Este trabajo contribuye al conocimiento actual de los efectos antiinflamatorios de ambas harinas integrales y refuerza los beneficios de su consumo.

Palabras clave: propiedades antiinflamatorias, dietas, harinas integrales, ácido linolénico, chía, lino

KEY POINTS

- Chia and flax integral flours are a rich in ALA.
- Supplemented diets contribute to differential ω-3 PUFAs profile in tissues.
- The dietary intake of integral flours ameliorated systemic inflammation.

Omega-3 polyunsaturated fatty acids (ω-3-PUFAs), in addition to essential nutrients, have been associated with potential health benefits[1-2]. There are two types of ω-3- PUFAs, known as short-chain ω-3 PUFAs like ALA (alpha-linolenic acid, C18:3 ω-3) or long-chain ω-3 PUFAs such as EPA (eicosapentaenoic acid, C20:5 ω-3) and DHA (docosahexaenoic acid, C22:6 ω-3). Short-chain ω-3 PUFAs are present in plant oil such as flax and chia seeds, while long-chain ω-3 PUFAs are usually found in marine products[1-2]. Although short-chain ω-3 PUFAs are more common and less expensive, the potential health benefits of ω-3 PUFAs have been related only to long-chain ω-3 PUFAs[1]. Compelling data from epidemiological and interventional studies have demonstrated an inverse correlation between long-chain ω-3 PUFAs and the risk of suffering from some chronic inflammatory diseases[1].

In the Western diet, the most consumed ω-3 PUFA is ALA. It is considered an essential fatty acid because it can not be synthesized by humans[1]. It bioconverts in the liver to EPA and DHA and to a lesser extent in the brain to DHA, a process that involves several steps orchestrated by multiple elongases, desaturases, and β-oxidases[2-5]. The efficient conversion of short to long-chain ω-3 PUFAs (EPA and DHA) in the liver can increase the levels of these fatty acids in critical tissues such as the brain, cardiac, and skeletal muscles, where they exert their protective effects and provide health benefits[1, 6, 7].

Omega-3 fatty acids are well-known dietary supplements that optimize physiological functions and alleviate pathological conditions, including inflammation[5]. Moreover, long-chain ω-3 fatty acids (EPA and DHA) have been shown to promote an anti-inflammatory eicosanoid profile and NF-kB gene expression, thereby reducing pro-inflammatory cytokines[8-11]. In this study, a murine model of systemic inflammation induced by lipopolysaccharide (LPS) was implemented[12-14].

Chia and flax seeds have been studied in various research models for their potential to alleviate systemic inflammation in human diseases[4, 5, 15, 16]. Previous dietary studies often utilized whole seeds, defatted flours, or seed oils[4, 15, 17]. However, after an extensive literature review, we did not find any studies reporting the dietary supplementation of integral chia and flax flours and their effects on EPA and DHA levels in crucial tissues, as well as their potential role as anti-inflammatory agents.

This work aimed to evaluate the anti-inflammatory effect of dietary supplementation with integral chia and flax flours rich in ALA in the amelioration of LPS-induced systemic inflammation in a murine model.

The hypothesis is that the dietary intake of integral chia and flax flours would contribute to attenuating inflammation through various mechanisms evaluated by multiparametric assays.

Material and methods

Animals

Male Balb/c mice (25-30 g, n: 30, age: 3 weeks) were obtained from the animal house of the Facultad de Medicina, Universidad Nacional del Nordeste (UNNE), Argentina. All animals were housed under standard laboratory conditions with a room temperature of 25.0 ± 2.0 °C, relative humidity of 55–65%, and a 12 h light/dark cycle. They had *ad libitum* access to food and water. *In vivo* experiments were conducted in accordance to the Institutional Animal Care and Use Committee (IACUC) of the Facultad de Medicina, UNNE (Resolution #0002-IACUC/17), followed the ARRIVE guidelines (Animal Research: Reporting of *in vivo* Experiments) and the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978).

Experimental procedures

The experimental setup included three groups with 10 mice per group: (I) control group on diet A (balanced commercial murine chow, Cooperación S.A.®, Buenos Aires, Argentina), (II) diet B (supplemented with integral chia flour), and (III) diet C (supplemented with integral flax flour). Experimental diets B and C were formulated to provide the recommended ω-3 contribution for murine nutrition[18]. The mice were provided with food *ad libitum* for 70 days (10 weeks).

Samples sizes were based on prospective power analysis (80%), using the magnitude of group differences and the standard errors within each group observed in our preliminary studies.

Initially, the macronutrients centesimal composition of the integral flours and the experimental diets were determined (Table 1).

The recommended intake of ω-3 fatty acids (FA) for mice is about 1.3 g ω-3 FA/kg diet[19]. The experimental diets B and C were supplemented by doubling the recommended amounts of ω-3 FA (2.6 g /kg diet). The nutritional composition of diets B and C is presented in Table 2.

The FA profiles of the integral flours (Table 3) and the diets (Table 4) were determined using *gas chromatography and mass spectrometry* (GC/MS). The FA methyl esters (FAMEs) were identified by comparing their retention times to those of commercial standards and those provided by the International CYTED Net (208RT0343) GC/MS.

The body weight and diet consumption of mice were measured weekly using an Ohaus portable balance (TA 3001) with a precision of 0.1g.

At the end of the experimental period, five animals from each group were injected with LPS (serotype 0127: B8, Sigma, St. Louis, MO, USA) at a dose of 1.3 mg/kg i.p[20] to induce systemic inflammatory condition. After 24 h, the mice were anesthetized with pentobarbital (60 mg/kg i.p) to obtain blood samples by cardiac puncture. Euthanasia was performed by cervical dislocation. These mice were used to obtain different tissue samples for further assays.

The remaining five unstimulated mice were injected with the same volume of sterile saline solution and used to obtain peritoneal macrophages for primary cultures stimulated with LPS (section 2.5).

Hematologic parameters and biochemical determinations

Biochemical determinations were carried out using a spectrophotometer (Metrolab 1600 Plus) and Wiener Lab reagents. The measurements included total proteins, albumin, triglycerides, total cholesterol, and glucose mea-

Table 1 | Macronutrients centesimal composition of the integral flours and the experimental diets

Nutrients	Integral chia flour	Integral flax flour	DIET A	DIET B	DIET C
Humidity (%)	6.63 ± 1.20	6.91 ± 1.60	12.20 ± 2.14	9.66 ± 2.12	10.78 ± 1.79
Ashes (%)	4.93 ± 0,80	3.57 ± 2,10	7.00 ± 1.96	4.19 ± 1.77	3.87 ± 1.87
Total fibers (%)	45.01 ± 2.10	53.15 ± 1.90	61.36 ± 1.64	70.80 ± 1.88	69.80 ± 1.92
Carbohydrates (%)	16.00 ± 1.70	7.43 ± 1.60	10.12 ± 1.45	4.60 ± 1.54	4.00 ± 1.96
Lipids (%)	8.48 ± 1.80	17.13 ± 1.70	4.05 ± 1.89	5.60 ± 1.74	5.20 ± 1.69
Proteins (%)	18.95 ± 2.00	11.81 ± 1.75	25.66 ± 1.88	19.00 ± 1.81	21.00 ± 1.79
Energetic value (kcal/100g)	332.16 ± 42.32	414.01 ± 33.89	250.11 ± 21.01	406.40 ± 14.11	410.00 ± 17.89

Values are the mean ± SD from three samples. Humidity (air stove), ash (AOAC 14.006), total fat (Butt method), crude fiber (AOAC 7.065), total proteins (AOAC 2057, Kjeldahl), and carbohydrates (indirect method).

Table 2 | Nutritional composition of diets B and C

Ingredients	Content g/100 g DIET B	DIET C
Integral chia flour	5	-
Integral flax flour	-	2.70
River fish flour	8	8
Corn starch	22	24.40
Skim milk powder	17.80	17
Sunflower oil	0.90	0,90
Bovine fat	1.80	1.60
Cowpea flour (variety red)	40	40
Egg white powder	4.50	5.50

Diets were designed according to the experimental mice's nutritional requirements following the recommendations of the National Research Council for laboratory mice feeding[18]. Native natural ingredients from the Argentine Northeast (NEA) and others of commercial origin were used

surements in plasma samples. Hematological parameters were evaluated using a Mendray BC 2800 VeT adjusted to murine standard values.

Phagocytic capacity assay of peritoneal murine macrophages (Mø)

A non-invasive assay using colloidal carbon particles was performed to evaluate the phagocytic capacity through the morphological changes of cultured adherent cells[21]. The peritoneal macrophages of mice previously stimulated *in vivo* with LPS were used.

Briefly, the outer layer of the peritoneum was carefully cut and the peritoneal cavity was gently exposed. The peritoneal lavage was performed with 5 ml of sterile phosphate buffer saline pH 7.2 (PBS). Cell pellets were washed and Mø were placed over sterile degreased glasses in 35 mm culture dishes. Then, 30 µl of Pelikan

Table 3 | Fatty acids profile of integral chia and flax flours

Fatty acids	Integral chia flour	Integral flax flour
C 14:0	nd	0.03 ± 0.01
C 16:0	5.32 ± 0.08	5.57 ± 0.32
C 16:1 9c	0.04 ± 0.01	0.08 0.01
C 17:0 ANTEISO	0.09 ± 0.02	nd
C 17:0 ISO	nd	0.03 ± 0.01
C 17:0	0.02 ± 0.01	0.05 ± 0.01
C 17:1 9c	0.02 ± 0.01	nd
C 18:0	2.03 ± 0.26	4.78 ± 0.08
C 18:1 9c	4.54 ± 0.01	18.19 ± 0.78
C 18:1 11c	0.63 ± 0.06	0.77 ± 0.08
C 18:1 12c	0.07 ±0.01	nd
C 18:2 9c12c (Linoleic)	19.17 ± 0.91	13.24 0.02
C 18:3 6c9c12c (γ-linolenic)	0.15 ± 0.01	nd
C 18:3 9c12c15c (γ- linolenic)	67.59 ± 0.71	56.76 ± 0.23
C 20:0	0.15 ± 0.01	0.12 ± 0.01
C 20:2	0.03 ± 0.01	nd
C 20:3 (γ-6)	0.06 ± 0.01	nd
C 20:3 (γ-3)	0.02 ± 0.01	nd
C 22:0	nd	0.15 ± 0.01
C 24:0	0.05 ± 0.01	0.09 ± 0.01
C 22:4	0.02 ± 0.01	nd
NI	nd	0.15 0.01
Total	100 ± 0.04	100 ± 0.03

NI: unidentified; Nd: not detected
Values are expressed as the mean (% of total FAMEs) from integral chia flour and integral flax flour. Data are expressed as mean ± SD from three independent experiences. It can be seen that both integral flours contain ALA (C 18:3 9c12c15c: α- linolenic fatty acid, ω-3), the amount of which is higher than any other type of fatty acid

Table 4 | Fatty acids profile of diets

Fatty acids	DIET A		DIET B DIET C
C 10:0	0.04 ± 0.05	0.03 ± 0.02	0.03 ± 0.02
C 12:0	0.03 ± 0.02	0.04 ± 0.01	0.04 ± 0.02
C 14:0	1.17 ± 0.07	1.35 ± 0.23	1.44 ± 0.09
C 14:1 9c	nd	0.12 ± 0.02	nd
C 15 ISO	nd	nd	0.04
C 15:0 ANTEISO	0.20 ± 0.06	0.08 ± 0.02	0.06 ± 0.04
C 15:0	0.04 ± 0.06	0.15 ± 0.01	nd
C 16:0	0.22 ± 0.02	16.14 ± 0.01	8.20 ± 0.04
C 16:1 9c	30.55 ± 0.06	1.38± 0.04	1.62 ± 0.02
C 17:0 ISO	3.03 ±0.04	0.18 ± 0.03	0.21± 0.20
C 17:0 ANTEISO	0.36 ± 0.05	0.17 ± 0.01	0.14 ± 0.04
C 17:0	0.15 ± 0.01	0.35 ± 0.01	0.37 ± 0.01
C 17:1 9c	0.25 ± 0.01	0.12 ± 0.04	0.10± 0.01
C 18:0	21.48 ± 0.03	10.78 ± 0.87	11.53 ± 0.07
C 18:1 9t	0.21 ± 0.03	0.12 ± 0.01	0.15 ± 0.01
C 18:1 10t	0.35 ± 0.14	0.18 ± 0.01	0.23 ± 0.02
C 18:1 11t	0.89 ± 0.16	0.57 ± 0.01	0.51 ± 0.06
C 18:1 9c	19.31 ± 0.09	29.53 ± 0.34	35.96 ± 0.08
C 18:1 11c	0.98 ± 0.07	1.02 ± 0.01	1.12 ± 0.08
C 18:2 9c12c (Linoleic)	11.68 ± 0.01	18.95 ± 0.76	18.73 ± 0.78
C 18:3 6c9c12c (γ-linolenic)	0.17 ± 0.02	0.29 ± 0.07	0.02 ± 0.12
C 18:3 9c12c15c (α-linolenic)	8.11 ± 0.04	17.21 ± 0.99	18.09 ± 0.05
C 20:0	0.03 ± 0.01	0.05 ± 0.02	0.20 ± 0.04
C 20:1 5c	nd	0.02 ± 0.03	nd
C 20:1 8c	nd	0.24 ± 0.01	nd
C 20:2	0.08 ± 0.02	0.07 ± 0.01	nd
C 20:3 ω-6	nd	0.09 ± 0.01	0.12 ± 0.01
C 20:3 ω-3	nd	0.02 ± 0.01	0.03 ± 0.01
C 20:4	0.11 ± 0.02	0.12 ± 0.03	0.38 ± 0.14
C 22:0	0.52 ± 0.02	0.41 ± 0.08	0.47 ± 0.03
C 24:0	0.04 ± 0.02	0.22 ± 0.05	0.21| ± 0.04
Total	100 ± 0.14	100 ± 0.05	100 ± 0.17

Nd: not detected
Values are expressed as the mean (% of total FAMEs) from diets, diet A, diet B, diet C by GC-MS spectrometry. Data are expressed as mean ± SD from three independent experiences

#17 Schwarz Black colloidal carbon particles (ref. 70306 4340503), were added to each dish. The medium was removed after 60 min of incubation at 37°C. Adherent cells were washed twice with 1 ml of phosphate-buffered saline (PBS) for removing the excedent of medium and colloidal carbon particles.

The cells were fixed with a 0.4% paraformaldehyde solution. A semi-quantitative evaluation was obtained by the measurement of the % intracellular colloidal carbon area, determined by the ImageJ 1.53s software (Wayne Rasband and contributors. National Institutes of Health. USA). Images were taken using an Optika Italy-B-290 microscope with a built-in camera. Five random fields per sample were processed (X400 original magnification).

Obtention of murine peritoneal macrophages (Mø) and setting of primary cell cultures

Peritoneal macrophages without *in vivo* challenge of LPS were collected as described in 2.4. Cells were seeded in 24-well plates with Dulbecco's modified Eagle's medi-

um (DMEM) containing 10% fetal bovine serum and 1% penicillin/ streptomycin. Cells were cultured for 2 h at 37°C in a 5% CO_2 humidified atmosphere. Mø enrichment (≥ 95%) was confirmed by nonspecific esterase staining and viability (≥ 95%) was determined by the trypan blue exclusion assay[22]. Subsequently, LPS at a concentration of 1 µg/mL (Lipopolysaccharide Escherichia coli O127:B8, γ-irradiated, Sigma, Darmstadt, Germany) was added to the cell suspension and incubated for 24 h to achieve complete activation[23].

NF-κB immunodetection

The detection of the nuclear translocation of phosphorylated NF-κB p65, serving as a biomarker of systemic inflammation, was performed using immunocytochemistry (ICC) in samples of murine primary macrophages stimulated *in vitro* with LPS. Cells were fixed with anhydrous methanol (99.8%) for 1 min. Subsequently, hydration was carried out using a series of decreasing alcohol concetrations.

Liver and kidney samples of mice fed experimental diets were assessed for NF-kB localization by immunohistochemistry (IHC). The samples were fixed with buffered formalin at room temperature. After fixation, the samples were dehydrated using increasing concentrations of alcohol and embedded in paraffin. Sections (3 µm) were deparaffinized and rehydrated. To inhibit endogenous peroxidase activity and prevent not specific labeling, the samples for ICC and IHC were incubated with 0.3% H_2O_2 in methanol for 10 min, followed by treatment with a blocking solution for 30 min.

The samples were then incubated with an NF-κB p65 antibody (A: sc-109 Santa Cruz Biotechnology CA, USA, dilution 1: 250) for 18 h at 4 °C. Immunostaining was performed using a DAKO LSAB+/HRP kit (Dako Cytomation) followed by the application of a chromogen DAB (DAKO kit) according to the manufacturer´s instructions. Images were captured using an Optika Italy-B-290 microscope with a built-in camera. The percentage of immunoreactive area for NF-κB p65 (µm^2) was measured in five random fields (x1000 original magnification) using the Image J software (National Institutes of Health, Bethesda, MD)[24].

Tissular Omega-3 profile

The ω-3 FA profile was analyzed by GC-MS to determine ALA, EPA, and DHA levels in cardiac muscle, liver, skeletal muscle, and brain.

The FA composition of the tissues was determined by gas chromatography using a Shimadzu (GC 2014) chromatograph equipped with a flame ionization detector. Analyses were carried out with a capillary column CP Sil 88 (100 m, 0.25 µm film thickness)[25]. The carrier gas was hydrogen with a split ratio of 1:10. The column temperature was held at 75°C for 2 min after injection, then 5°C/min to 170°C, held for 40 min, 5°C/min to 220°C and held for 40 min. The injection volume was 0.5 µL, and the column flow was 0.8 mL/min. The fatty acid methyl esters (FAMEs) were formed by transesterification with methanolic potassium hydroxide solution as an interim stage before saponification (ISO 5509:2000, Point 5 IUPAC method 2.301).

Briefly, samples from different tissues were excised after euthanasia, washed extensively with cold saline solution (0.9% NaCl), and homogenized mechanically with a polytron tissue homogenizer before total lipid extraction according to Bligh & Dyer[26]. The internal standards were added before lipid extraction. The detection limit for the main FAMEs identified ranged from 0.01% to 0.03%[25].

FAMEs were identified by comparison of their retention times relative to those of commercial standards. The values of FA content were expressed as the percentage of total FAMEs.

Real-time polymerase chain reaction analysis

Hepatic, renal, and splenic samples were preserved in RNA *later* (Sigma, St. Louis, MO, USA). Total RNA was extracted using Transzol reagent (TransGen Biotech Co., Beijing, China) according to the manufacturer's instructions. The reverse transcription of RNA was performed using M-MLV transcriptase (Promega, Madison, WI, USA) following technical guidelines. Gene expression was quantified in ECO® Real-Time PCR System (Illumina, San Diego, CA, USA) using HOT FIREPol®EvaGreen® qPCR mix (Solis Byodine, Tartu, Estonia) according to the product's datasheet. The primers were as follows (5′–3′): IL-1β-F, GAGCCCATCCTCTGTGACTC; IL-1β-R, TCCATTGAGGTGGAGAGCTT; IL-6-F, ATGAAGTTCCTCTCTGCAAGAGACT; IL-6-R, CACTAGGTTTGCCGAGTAGATCTC; TNF-α- F, ATGAGCACAGAAAGCATGATC; TNF-α- R, TACAGGCTTGTCACTCGAATT; IL-10-F, CAGCCGGGAAGACAATAACT; IL-10-R, TCATTTCCGATAAGGCTTGG; GAPDH-F, TGATGACATCAAGAAGGTGGTGAAG; GAPDH-R, TCCTTGGAGGCCATGTGGGCCAT.

The relative expression of IL-1β, IL-6, TNFα, and IL-10 was normalized to that of GAPDH and calculated using the ΔΔCt method.

Statistical analysis

Data are expressed as the mean ± standard deviation (SD). Comparisons between groups were performed using ANOVA with post hoc Bonferroni´s multiple comparisons tests. The software used was GraphPad Prism 8.0 (Graph-

Pad, San Diego, CA, USA). Differences between groups were statistically significant at $p<0.05$.

Results

Nutritional parameters

The increment in body weight was comparable among all experimental groups (Fig. 1A). Figure 1B illustrates a significant decrease ($p < 0.0001$) in food intake noticed between experimental groups fed diets B and C compared to the control (diet A). However, the feed conversion index did not show statistically significant differences. Hence, the nutritional efficiency of diets B and C were similar (data not shown).

Mice showed a healthy status without any pathological manifestation through the experimental study.

Hematological parameters and biochemical determinations

Hematological data revealed that hematocrit, hemoglobin content, total white blood cell counts, and the differential leukocyte contribution did not change significantly among the experimental groups (Table 5).

Animals fed diets B and C exhibited a significant decrease ($p < 0.05$) of plasma glucose, total cholesterol, and triglycerides levels compared to diet A (control). Even though, total protein and albumin concentrations did not show significant differences (Table 5).

Phagocytic capacity assay of murine peritoneal macrophages (Mø)

This assay was performed to evaluate the effect of ALA from experimental diets on the phagocytic capacity of peritoneal macrophages through the incorporation of colloidal carbon particles after LPS stimulation. The use of this inert model avoids the use of fresh Zymosan particles or any other type of noxa that can induce additional inflammatory triggering[27].

Figure 2 shows representative microphotographs of peritoneal Mø from experimental animals following LPS-induced systemic inflammation (1.3 mg/kg). Experimental data revealed that the Mø of groups B and C exhibited an increased percentage of the intracellular colloidal carbon area compared to the control.

NF-κB immunodetection

NF-κB is considered a key member of the orchestrated signaling pathway following LPS stimulation[25]. Once activated, free NF-κB dimers translocate to the nucleus stimulating pro-inflammatory cytokine genes transcription, as well as other target genes.

Figure 1 | **Nutritional parameters**. Lines represent 1A. Mice body weight (g), 1B. Weekly food consumption (g) of mice fed diet B and C compared to control (diet A) for 70 days (10 weeks) of food treatment. Data are expressed as the mean ± SD from three independent experiences (n = 10 mice/group). Differences among groups were analyzed by two-way ANOVA with Bonferroni's multiple comparisons tests

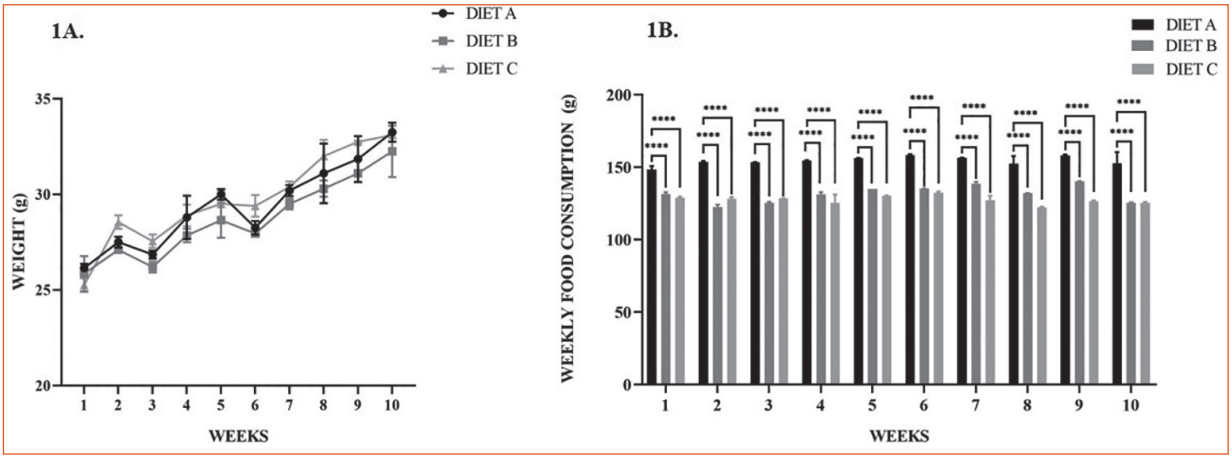


Table 5 | Hematological and biochemical parameters

Parameters	DIET A	DIET B	DIET C
Hematocrit (%)	39.83 ± 4.41	38.50 ± 5.64	37.33 ± 6.21
Hemoglobin concentration (g/dL)	11.75 ± 1.20	11.41 ± 1.55	10.58 ± 3.31
White blood cells (10^3cells/µL)	4.81 ± 1.48	4.88 ± 1.50	4.96 ± 1.46
WBC x 10^9/L	3.86 ± 1.01	3.72 ± 1.22	3.12 ± 1.68
Lym x 10^9/L	3.04 ± 0.98	4.36 ± 1.05	2.68 ± 1.13
Mon x 10^9/L	0.12 ± 0.04	0.18 ± 0.04	0.18 ± 0.08
Gran x 10^9/L	0.70 ± 0.20	1.18 ± 0.26	1.26 ± 0.50
Lym%	70.46 ± 9.27	75.86 ± 4.29	64.98 ± 4.17
Mon%	4.88 ± 2.37	2.80 ± 0.55	4.64 ± 0.37
Gran%	24.66 ± 6.95	21.34 ± 4.67	30.38 ± 4.26
RBC x 101^2/L	7.83 ± 0.42	7.30 ± 1.47	7.73 ± 1.84
HGB	116.2 ± 8.58	111.80 ± 15.48	120.80 ± 6.41
HCT	39.08 ± 2.92	35.40 ± 7.20	40.42 ± 2.37
MCV	49.94 ± 1.45	48.50 ± 0.85	47.10 ± 2.15
MCH	14.80 ± 0.40	14.64 ± 0.20	14.14 ± 0.40
MCHC	296.80 ± 5.06	303 ± 2.91	301.60 ± 6.58
RDW	12.50 ± 0.47	12.34 ± 0.52	12.46 ± 0.63
PLT x 10^9/L	986 ± 7.80	677 ± 2.60	850.2 ± 3.05
MPV fL	4.66 ± 0.47	5.16 ± 0.79	4.54 ± 0.15
PDW	16.50 ± 0.37	17.36 ± 0.89	16.60 ± 0.23
PCT %	0.42 ± 0.12	0.26 ± 0.14	0.33 ± 0.19
Glucose (mg/dL)	119 ± 5.85	92.01 ± 6.77 *	73.33 ± 5.09 *
Total cholesterol (mg/dL)	139.33 ± 8.19	89.98 ± 7.83 *	102 ± 7.79 *
Triglycerides (mg/dL)	147 ± 8.12	116.55± 7.89 *	115.16 ± 0.72 *
Total protein (g/dL)	4.81 ± 0.49	4.48 ± 0.76	4.95 ± 0.57
Albumin (g/dL)	2.55 ± 0.31	2.65 ± 0.32	2.49 ± 0.36

*Values are presented as mean ± standard desviation (SD) of three independent experiments. Significance of the differences among groups were analyzed by two-way ANOVA with Bonferroni's multiple comparisons test (*p<0.05)*

In this study, the nuclear NF-kB immunodetection significantly decreased ($p < 0.0001$) in peritoneal macrophages from animals fed-diet B and C compared to the control (Fig. 3A). Thus, our findings suggest that integral chia and flax flours may reduce NF-κB translocation from cytosol to nucleous more effectively than diet B.

Figure 3 (Fig. 3B) illustrates the immunodetection of the transcription factor NF-κB in the liver after induction of systemic inflammation. NF-κB positive area showed a significant decrease in mice fed diets B and C ($p < 0.0001$) against the control, suggesting their potential anti-inflammatory effect (Fig. 3B).

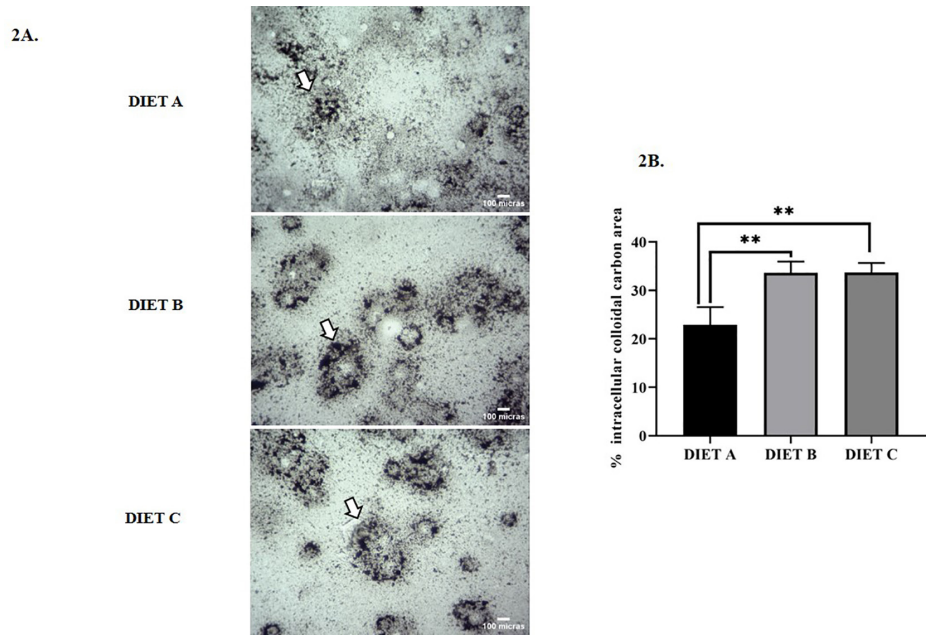
Furthermore, NF-κB immunoreactivity in renal tissue exhibited a significant decrease ($p<0.0001$) in diet C-fed mice (Fig. 3C). No statistical differences were found for diet B versus the control.

Tissular Omega-3 profile

The differential ω-3 FA profile was determined in various tissues, including cardiac (Fig. 4A), hepatic (Fig. 4B), skeletal muscle (Fig. 4C), and nervous tissue (Fig. 4D).

In terms of ALA levels, there were no significant variations observed in all tissue samples with diet C supplementation. However, con-

Figure 2 | **Phagocytic capacity assay of murine peritoneal macrophages**. A: Phagocytosis assay with colloidal carbon particles. Representative images corresponding to primary macrophages from mouse intraperitoneal lavages 24 hours after *in vivo* stimulation with LPS: control group (diet A), animals treated with a diet based on integral chia flour (diet B), and animals treated with a diet based on integral flax flour (diet C). Magnification X400. Arrows indicate colloidal carbon particles engulfed by peritoneal macrophages. The bars represent 100 µm. B: Semi-quantitative analysis of the % intracellular colloidal carbon area. The results are expressed as the mean ±SD. Image processing to evaluate the intracellular colloidal carbon area was performed using Image J software (National Institutes of Health, Bethesda, MD). Differences among groups were analyzed by one-way ANOVA with Bonferroni's multiple comparisons tests. (**p< 0.01)



sumption of diet B significantly increased ALA concentrations in the liver, cardiac muscle, and skeletal muscle, but not in the brain.

EPA levels showed a significant increase in hepatic tissue with both diets B and C, while only diet B promoted an increase in skeletal muscle.

DHA emerged as the predominant ω-3 FA in the liver, heart, and skeletal muscle of animals fed diet C compared to diet B. However, the diet B group exhibited a significant increment of DHA levels in the brain ($p < 0.0001$).

Expression of IL-1β, IL-6, TNF-α and IL-10 in renal, hepatic, and splenic tissues

To evaluate the anti-inflammatory effects of integral chia and flax flours, the expression of IL-1β, IL-6, TNF-α, and IL-10 were determined in liver, kidney and spleen by RT-qPCR.

These cytokines expression decreased in hepatic and renal tissue from mice fed-experimental diets compared to diet A, suggesting an anti-inflammatory effect of chia and flax flours rich in ALA. These findings are consistent with the reduction of NF-κB signaling pathway activation mentioned earlier. Additionally, in splenic tissue, IL-1β and IL-6 expression increased while TNF-α decreased (Fig. 5).

The expression of IL-10, an anti-inflammatory cytokine, was significantly increased ($p < 0.0001$) in renal, hepatic, and splenic tissues, further reinforcing the anti-inflammatory role of the chia and flax-supplemented diets (Fig. 5).

Figure 3 | **Immunodetection of the transcription factor NF-κB in primary macrophages, liver and kidney.** A: Immunocytochemistry of NF-kB in primary macrophages 24 h after *in vitro* induction of systemic inflammation with LPS. Representative images correspond to the control group (diet A), animals treated with a diet based on integral chia flour (diet B), and animals treated with a diet based on integral flax flour (diet C). Arrows indicate intranuclear localization of the p65 NF-κB subunit. The immunoreaction was intense in the nuclei of Mø of group A. Magnification X1000. The bars represent 30 µm. B: Immunohistochemical assay for immunodetection of the nuclear transcription factor NF-κB in liver. C: Immunohistochemical assay for immunodetection of the nuclear transcription factor NF-κB in kidney. Representative images corresponding to the liver (3b) and kidney (3c) from mice stimulated *in vivo* with LPS: control group (diet A), animals treated with a diet based on integral chia flour (diet B), and animals treated with a diet based on integral flax flour (diet C). Representative photomicrographs at X1000 magnification are shown. The bars represent 30 µm.

Semi-quantitative analysis of the % positive NF-kB area (µm²) was measured in five random fields for each group using the Image J software (National Institutes of Health, Bethesda, MD). The results are expressed as the mean ±SD. ANOVA and Bonferroni test (****p<0.0001)

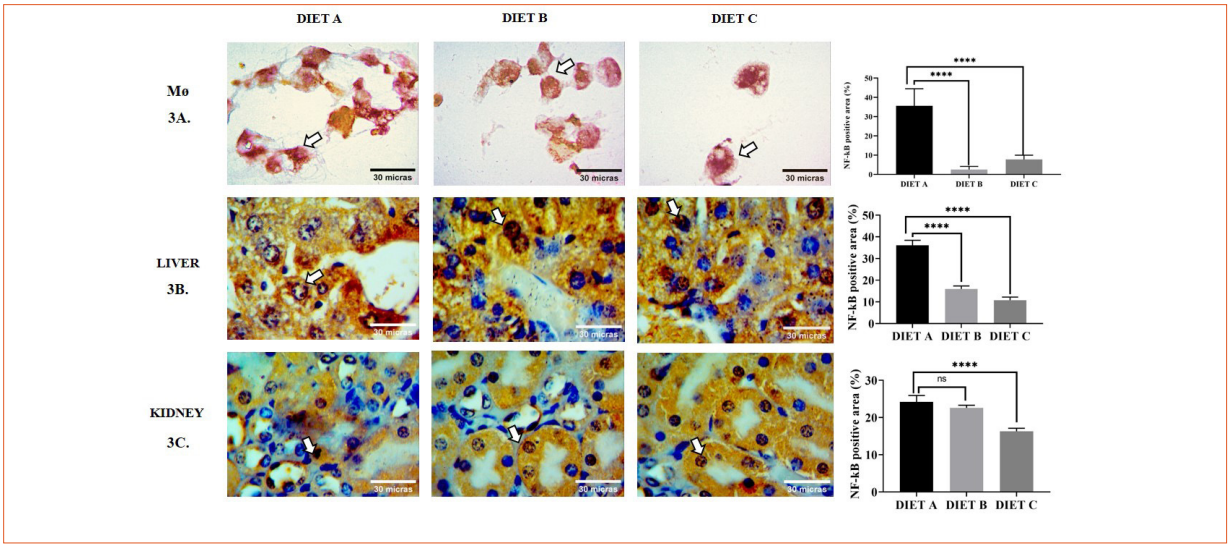


Figure 4 | **Tissular Omega-3 profile**. Bars represent methyl esters (%) of ALA (C 18:3, ω-3), EPA (C 20:5, ω-3), and DHA (C 22:6, ω-3) in cardiac muscle (4A), liver (4B), skeletal muscle (4C), and brain (4D) of mice fed with diet A, B, and C.

Data are expressed as mean ± SD from three independent experiences (n = 10 mice/group). Differences among groups were analyzed by two-way ANOVA with Bonferroni's multiple comparisons tests (*p < 0.05; ***p < 0.001; ****p < 0.0001)

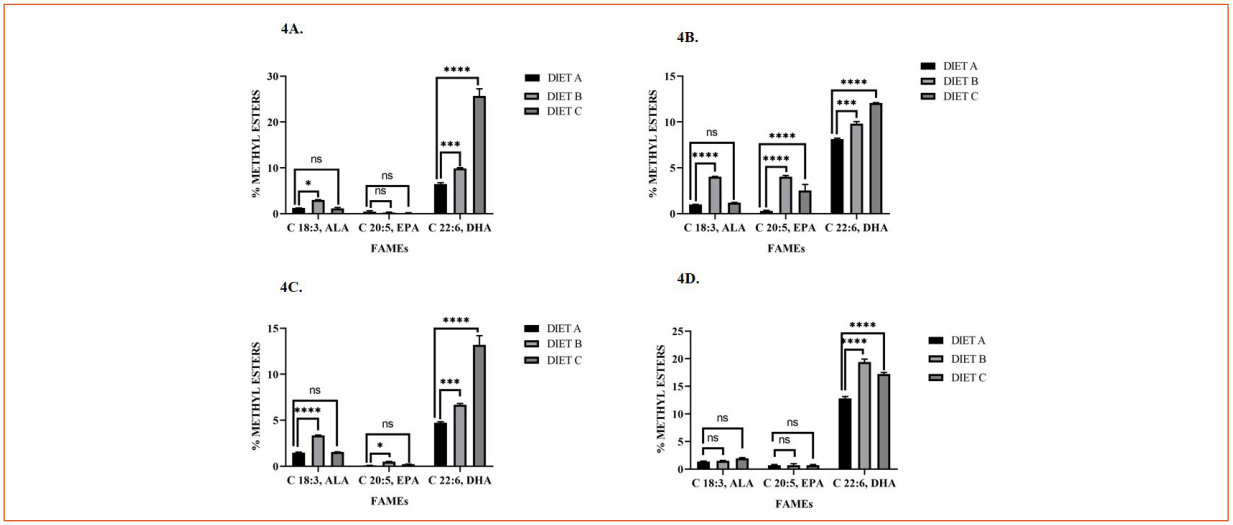
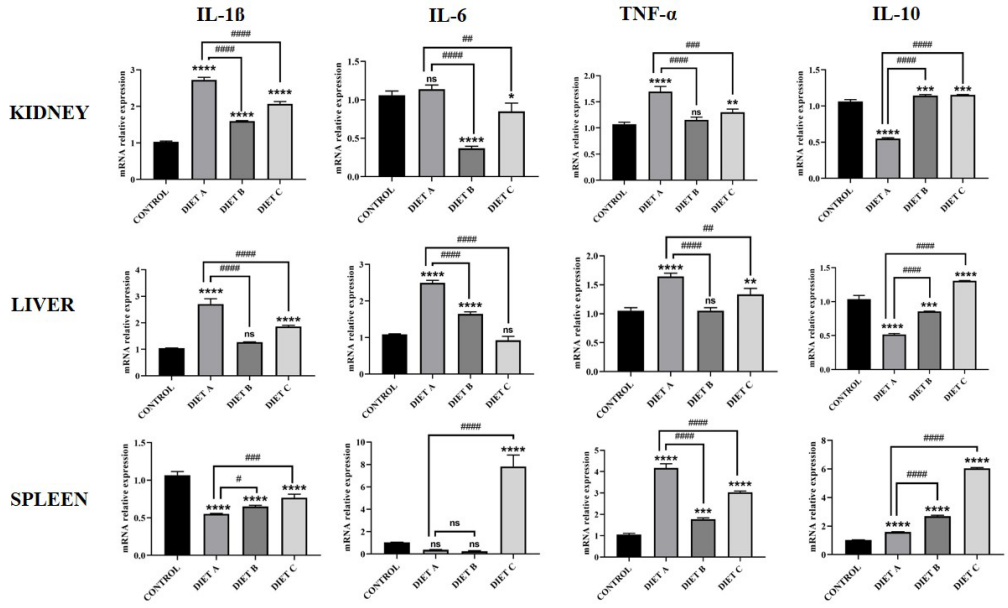


Figure 5 | **The effect of chia and flax flours in LPS-mediated expression of IL-1β, IL-6, TNF-α and IL-10 in the kidney, liver, and spleen.** Values are presented as mean ± standard deviation (SD) of three independent experiments, corresponding to the control group (diet A), animals treated with a diet based on integral chia flour (diet B), and animals treated with a diet based on integral flax flour (diet C). Differences among groups and control were analyzed by one-way ANOVA with Bonferroni's correction (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001). Differences among experimental groups (diet B and C) and diet A were analyzed by one-way ANOVA with Bonferroni's correction (#p< 0.05; ##p< 0.01; ###p< 0.001; ####p< 0.0001). RT-qPCR data were calculated using the ΔΔCt method using GAPDH as a reference gene



Discussion

This study describes the anti-inflammatory effect of dietary supplementation with integral chia and flax flours rich in ALA on systemic inflammation induced by LPS.

The mode of incorporation of these seeds represents an innovative approach to studying their properties with potential application in food matrices since other reports were performed with seed oils [28-31] or seed-defatted flours.

The experimental diets were designed according to the animal´s requirements for Balb/c mice[18-4]. The nutritional parameters of our experimental data were close to normality for the mice strain, sex, and age as described by the NRC - The Laboratory Animals[18].

On the other hand, the hematologic parameters did not change significantly and were coincident with Kelada et al[32] and according to reference values[18-33]. The biochemical parameters (glucose, total cholesterol, and triglycerides) significantly decreased in diets B and C compared to the control (diet A) in agreement with other studies[29-34].

Extensive data demonstrate that ALA is endogenously transformed into EPA and DHA, the main bioactive forms of ω-3 FA, with a higher conversion rate occurring in the liver than in the brain[6-14].

It is well known that DHA and EPA are present in varying proportions across different tissues[31, 35-38] and are strongly associated with anti-inflammatory effects[6-14].

However, there is limited knowledge regarding the ω-3 FA profiles in crucial tissues of mice fed with integral chia and flax flours rich in ALA[35].

The present study revealed variations in ALA, DHA, and EPA levels among crucial tissues through dietary supplementation.

Our results suggested that DHA was the predominant bioactive derivative of ALA in the

hearts of mice fed experimental diets, mainly through flax supplementation. Furthermore, both EPA and DHA were found in hepatic tissue. These findings agree with Watkins's work[39].

On the other hand, our study revealed that DHA was the predominant fatty acid in brain tissue, which is consistent with the findings of Valenzuela et al[30]. Their study demonstrated that ALA is converted to DHA and EPA in the brains of mice fed diets containing chia, flax, and/or salmon oils. However, to our knowledge, there are no reports available regarding the ω-3 FA profile in murine skeletal muscles from diets including integral chia and flax flours.

Our results demonstrated that the chia diet predominantly increased levels of EPA and DHA in skeletal muscles. In contrast, the flax diet only promoted the presence of DHA. These findings align with the study conducted by Apperson and Cherian[40], which showed the prevalence of DHA, AA (c20:4 ω-6), and ALA in bird's muscles after feeding them with flax seeds.

Despite the extensive evidence regarding the anti-inflammatory role of ω-3 fatty acids, the specific effect of dietary supplementation with integral chia and flax flours has not been thoroughly investigated[6-14]. Therefore, experiments were conducted to examine the potential alleviation of systemic inflammation in an LPS-induced murine model.

The experimental groups showed an enhanced phagocytic capacity of peritoneal macrophages. It has been suggested that the phagocytic plasticity of macrophages may be associated with changes in the composition and structure of the cell membrane resulting from the incorporation of ω-3 fatty acids[5-41]. Furthermore, Gutierrez et al[5] described that the dietary intake of ω-3 fatty acids (ALA, EPA, and DHA) leads to macrophage polarization from a pro-inflammatory M1 phenotype to an anti-inflammatory M2 phenotype. Although this aspect was not specifically addressed in our study, it is well-documented that M2 macrophages exhibit higher rates of phagocytosis[5, 42, 43].

In parallel, the NF-κB transcription factor plays a crucial role in regulating the immune response by controlling the transcription of genes involved in inflammation, including cytokines/growth factors, soluble cytokine receptors, adhesion molecules, and immunoregulatory molecules[31, 42, 44]. In our study, the immunodetection of p65 NF-κB in critical tissues and peritoneal macrophages decreased in the experimental groups compared to the control group after LPS challenge. It is worth noting that although the diets contained the same amount of ALA, derived from different food sources (integral chia flour vs integral flax flour), their potential for downregulating NF-κB immunoreactivity varied among the tissues. However, a recent study by Branco Ramos Nakandakari[45] investigated the protective effects of flax oil but did not find a reduction in the expression of inflammatory mediators in the murine liver. In contrast, our work revealed that integral flax flour exerted a protective effect in the context of systemic inflammation. Further studies are necessary to identify the mechanisms involved in this finding.

The anti-inflammatory effects of the experimental diets were also demonstrated by a significant decrease in the expression of IL-1β, TNF-α, and IL-6 in hepatic and renal homogenates. These results are consistent with other studies that have reported the downregulation of pro-inflammatory cytokines following dietary intake of ALA in various animal models[17, 18, 46-48]. However, it is important to note that the expression of IL-6 and IL-1β increased in spleen homogenates. In this regard, the study by Svahn et al[49] reported an increase in IL-16 levels in the spleen, while this pro-inflammatory cytokine decreased in circulation. These findings suggest that the regulation of pro-inflammatory cytokines may be more localized rather than systemic in splenic tissue. Our results reveal that the experimental diets have different capacities to downregulate the production of pro-inflammatory cytokines, highlighting the need for further research to elucidate the underlying mechanisms. The expression of IL-10 increased in the kidney, liver, and spleen following the dietary intake of integral chia and flax flours, which is consistent with the findings of Al Za'abi et al[50]. IL-10 is known to be a regulatory cytokine that inhibits inflammatory responses, thereby protecting tissues from damage.

Our results indicate that diets supplemented with integral chia and flax flours maintain the normal nutritional status of animals, improve

metabolic biochemical parameters, and contribute to a differential ω-3 PUFA profile in critical tissues. Furthermore, the present work demonstrates that these integral flours alleviate systemic inflammation induced by LPS.

Further research is needed to establish the mechanisms involved to obtaining efficient ALA bioconversion rates in different species of mammals, including humans, for make direct associations with the improvement of different pathologies or the optimization of different physiological phenomena. Additionally, it is crucial to determine the independent rols of ALA[51].

In conclusion, this study can contribute to strengthening the scientific evidence necessary for the future development of nutraceutical supplements derived from these integral seed flours. Moreover, it can assist in formulating nutritional recommendations to promote the incorporation of these natural foods into diets, given their role as rich sources of bioactive compounds.

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Conflict of interest: None to declare

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