

Flaxseed, olive and fish oil influence plasmatic lipids, lymphocyte migration and morphometry of the intestinal of Wistar rats¹

Óleo de linhaça, oliva e peixe influenciam os lipídios plasmáticos, migração de linfócitos e morfometria intestinal de ratos Wistar

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ABSTRACT

Purpose: Evaluate the effect of flaxseed, olive and fish oil on the lipid profile, preservation of villousities and lymphocyte migration in the intestinal mucosa of Wistar rats. **Methods:** Thirty Wistar male rats were divided into four groups, which received the AIN-93M diet, with changes only to their lipid source: flaxseed, olive, fish, and soy oil (control group). The serum was separated for the biochemical parameter analysis. A histological evaluation was performed in the ileal portion. **Results:** The group which was fed fish oil presented lower values when compared to the other treatments for Total Cholesterol, High-density Lipoprotein Cholesterol and Triacylglycerol ($p < 0.05$). The animals treated with fish and olive oils presented better intestinal villousities preservation. Less deposition of lymphocytes was observed in the flaxseed group ($p < 0.001$). **Conclusions:** This study demonstrated that flaxseed, olive and fish oils present different responses than soy oil for the intestinal mucosa preservation and lymphocyte proliferation in Wistar rats.

Key words: Vegetable Fats. Linseed Oil. Soybean Oil. Fish Oils. Morphology. Intestines. Lymphocytes. Rats.

RESUMO

Objetivo: Avaliar o efeito dos óleos de linhaça, oliva e peixe no perfil lipídico, preservação das vilosidades e migração de linfócitos na mucosa intestinal de ratos Wistar. **Métodos:** Trinta ratos Wistar foram divididos em quatro grupos e receberam dieta AIN-93M, modificando para cada grupo apenas a fonte lipídica: óleo de linhaça, oliva, peixe e soja (grupo controle). O soro foi separado para análise dos parâmetros bioquímicos. A análise histológica foi realizada na porção ileal. **Resultados:** O grupo que recebeu óleo de peixe apresentou menores valores de colesterol total, lipoproteína de alta densidade e triacilglicerol ($p < 0.05$). Os animais tratados com óleo de peixe e oliva apresentaram melhor preservação das vilosidades intestinais. Menor deposição de linfócitos foi observado no grupo tratado com óleo de linhaça ($p < 0.001$). **Conclusão:** Este estudo demonstrou que os óleos de linhaça, oliva e peixe apresentam diferentes respostas em relação ao óleo de soja na preservação da mucosa intestinal e proliferação de linfócitos em ratos Wistar.

Descritores: Gorduras Vegetais. Óleo de Semente do Linho. Óleo de Soja. Óleos de Peixe. Morfologia. Intestino. Linfócitos. Ratos.

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Introduction

The role diet components in the prevention or in the genesis of diseases, as well as their activity mechanisms, have been the object of studies for decades. Among the macronutrients, lipids exert great influence on the genesis of chronic diseases, such as obesity, atherosclerosis and other cardiovascular diseases¹.

According to some studies, olive, flaxseed and fish oils, among others, present several beneficial effects to the organism because they are important sources of oleic acid (omega-9), α -linolenic acid (ALA - omega-3) and eicosapentaenoic acid/docosahexaenoic acid (EPA/DHA) respectively².

The modulation of these fatty acids in the diets plays an important role in the prevention and treatment of coronary heart

diseases³, hypertension⁴, auto-immune disorders and cancer, also presenting good results for inflammatory responses⁵. The linoleic acid (LA) is metabolized to arachidonic acid (AA), the precursor of pro-inflammatory eicosanoids, such as, primarily, prostaglandin E₂ (PGE₂), thromboxane A₂ (TXA₂) and leukotriene B₄⁶, while ALA is metabolized to EPA and DHA, which are precursors of anti-inflammatory eicosanoids, such as PGE₃ and PGF₃⁷.

Inflammation is the strategy organisms use to protect themselves from a lesion in the cell, which is involved in the reparation processes, restoration of the homeostasis in the damaged sites and production of chemical mediators⁸. When cell damage occurs, the migration of lymphocytes to the damaged site generates an inflammatory process. Lymphocytes can be found in all parts of the organism, including the intestinal mucosa,

where the absorption of lipids takes place⁹. The activation of lymphocytes is usually inhibited by fatty acids, mainly those of the omega-3 series. These fatty acids, which are present in the diet, are incorporated in the membrane phospholipids in a higher proportion than the other classes. Previous studies demonstrate that the polyunsaturated fatty acids of the omega-3 series presents a beneficial effect on the preservation of the intestinal mucosa and improvement of the lipid profile². Recent investigations have shown the morphophysiological importance of the intestinal loops associated with the nutrient absorption processes¹⁰.

Another issue that still remains incongruent is speculation on the ALA activity. Whether it exerts the same benefits as the long chain omega-3 fatty acids, such as EPA and DHA, in small amounts, since their conversion into EPA and DHA is limited by the amount of omega-6 fatty acids in the diet. Hussein *et al.*¹¹ reported that the conversion of ALA into EPA varies from 0.3 to 8% in men and less than 1% of ALA is converted into DHA. It is not clear yet if ALA alone presents any direct benefits or if they are achieved through its conversion into EPA^{3,12}.

Oils have been widely studied in animal models, with diets supplemented with some kind of lipid or fatty acid in particular¹³. Trying to understand the activity of different oils, this study proposed to compare the effect of soybean oil to the effect of other oils (olive, fish and flaxseed) on the lipid profile, preservation of villusities and lymphocyte migration in the intestinal mucosa of Wistar rats.

Methods

Thirty 72-day old male rats (*Ratus norvegicus albinus*, *Mammalia*, Wistar lineage) provide by the Central Animal House at the Biological Sciences Center at the Federal University of Viçosa. All the groups received the AIN-93M diet¹⁴. The animals were separated into four homogeneous groups, with modifications only in their lipid source: soybean (n=8), olive (n=8), fish (n=7) and flaxseed (n=7). The group fed with soybean oil was considered the control group, as recommended by the AIN-93M. The rats were maintained in individual cages at a temperature of 22±1°C, in a controlled environment, with a light/dark cycle, for 12 hours, and received a diet, *ad libitum*, for 8 weeks. After this period, the animals were submitted to a fasting period of 8 hours and anesthetized with ethyl ether. Later, blood and a portion of the small intestine (ileum) were collected.

Profile of the fatty acids of the oils used

Soybean, olive, flaxseed and fish oils were extracted as recommended Folch¹⁵, and saponified and esterified as recommended Hartmann and Lago¹⁶. The identification of the fatty acid methyl esters was performed by gas chromatography using the CG-17A Shimadzu/Class model, with a fused silica column SP-2560 (biscianopropil polysiloxane), 100 m and 0.25 mm diameter and a flame ionization detector. The programming of the analysis presented an initial temperature of 140°C, being isothermic for 5 minutes, and a posterior heating of 4°C per minute up to 240°C, maintaining this temperature for 30 minutes. The temperature of the vaporizer was 250°C and the temperature of the detector was 260°C. The carrier gas used was nitrogen at 20 cm/second, at 175°C. The split of the sample in the injector was 1/50 and 1 µL of the solution was injected.

The peaks were identified by comparing the retention times with known methyl ester standards (FAME mix, Supelco®, USA) and quantified per automatic integration area.

Analysis of the plasmatic lipids

The blood samples were centrifuged at 3000rpm for 10 minutes in order to achieve the serum and were frozen at -20°C for the Total Cholesterol (TC), High-Lipoprotein Cholesterol (HDL-C) and Triacylglycerol (TAG) analyses, with the use of enzymatic “kits”.

Histological analysis

Fragments of the ileum were removed and fixed in a Carson formalin¹⁷. After dehydration in an increasing gradient of ethanol, the material was diaphanized in xylol and included on paraffin. 7 µm-thick histological sections were achieved in a rotary microtome (Olympus America Inc., CUT 4055) and colored with hematoxylin and eosin (HE).

Lymphocyte quantitative analysis

Histological sections randomly selected from three animals of each group were used in the analyses. Five laminas were achieved from each animal, each of them with 8 consecutive cuts with minimum intervals of 40 µm between them. 20 images (2048 x 1536 pixels) of three non-consecutive cuts (objective: 20x) were achieved from each slide, totaling 900 fields per experimental group. The images were obtained with a Q-Color 3 (Olympus) digital camera attached to a BX-60 (Olympus) microscope.

The counting of the number of lymphocytes present in the histological field was carried out with the use of the Image Pro-Plus® software system, version 4.5 (Media Cybernetics). The overlapping of the standard square matrix (21lines x 21 columns) in the photographed fields, and only the lymphocytes coinciding with the intersections were counted (Figure 1).

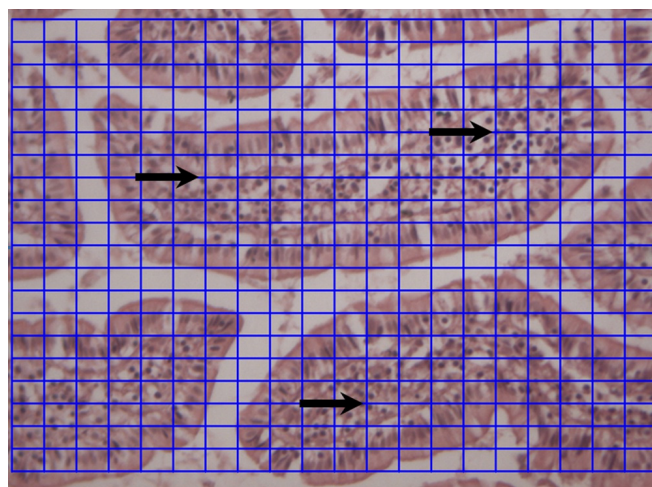


FIGURE 1 - Photograph of the histological sections of the ileal mucosa of Wistar rats. It was standardized for the counting of the lymphocytes the overlap of a standard square matrix (21lines x 21 columns) in the photographed fields, counting only the lymphocytes which coincided with the intersections, as shown in the figure. Magnification 20x. (Figure: 2048 x 1536 pixels)

Morphometry of the intestinal villusities and crypts

The images of the histological sections were captured with an objective 10x. The following measures were taken with the use of the Image Pro-Plus® software system, version 4.5 (Media Cybernetics):

Height of villous: Ten random fields were selected per animal. At least 70 villusities per experimental group were measured. Only the villusities with defined epithelium and visible conjunctives were used;

Villous width: in the villusities used in the analysis of height, three measures were taken (apical, average and basal regions) and it was considered the average value of these three measures in the same villosity was taken into consideration;

Crypt depth: the measurements of ten fields per animal were taken, where it was possible to see the basis and the apex (opening) of the crypt. At least 70 crypts were measured per experimental group.

Ethics committee

This project was approved by the Ethics Committee of the Department of Veterinary Medicine of the Federal University

of Viçosa, processed 12/2008, and the experiment was carried out according to the Ethical Principles in Animal Experimentation, adopted by the Brazilian College of Animal Experimentation (COBEA).

Statistical analysis

The variables were submitted to the normality and homocedasticity tests and, later, to the variance analysis. When necessary, a test to compare averages was performed, Tukey or Dunn's, depending on the characteristic of the variable. When the data did not meet the premises of normality, even after the proper transformations, they were submitted to the non-parametric test, with the use of the Sigma Stat® statistical software system (version 3.1), at 5% significance.

Results

The composition of the oils as well as the concentration of fatty acids is listed in Table 1, where the ω-6/ω-3 relation of the diet offered can also be observed. At the end of the experiment no difference was observed in weight gain, food consumption or food coefficient efficiency (data not shown).

TABLE 1 - Concentration of fatty acids in the oils used in the diets (per cent)

Fatty acids	Concentrations ^a			
	Soybean oil	Olive oil	Fish oil	Flaxseed oil
C14:0	ND	ND	5.86	ND
C15:0	ND	ND	0.22	ND
C16:0	7.75	11.30	10.76	6.45
C17:0	ND	ND	0.50	ND
C18:0	3.01	2.96	3.60	4.35
C20:0	ND	0.38	0.48	ND
C22:0	0.34	0.12	ND	ND
C16:1	ND	1.09	6.60	ND
C18:1 ω-9	21.36	74.01	9.98	18.00
C20:1	ND	0.25	1.08	ND
C20:2	ND	ND	3.05	ND
C18:2 ω-6	60.75	8.74	2.78	12.71
C20:4 ω-6	ND	ND	0.73	ND
C22:2 ω-6	ND	ND	0.87	ND
C18:3 ω-3	6.96	ND	0.80	58.47
C20:5 ω-3	ND	ND	24.01	ND
C22:6 ω-3	ND	ND	19.86	ND
Total ω-6	60.75	8.74	4.37	12.71
Total ω-3	6.93	0.75	44.63	58.47
ω-6/ω-3	8.77	11.65	0.10	0.22

Fatty acids concentration in soybean, olive, fish and flaxseed oils. The identification of the methyl esters of the fatty acids was performed by gas chromatography. Values are in g/100 g. *ND= undetermined value.

The group fed fish oil presented a significant difference when compared to the group fed olive oil and the control group, for the TC, HDL-C and TAG parameters. Thus, no differences

were observed among treatments related with HDL-C/TC ratio (Table 2).

TABLE 2 - Average of the values of TC, HDL-C, TG and HDL-C/TC serum of the different experimental groups (mg/dL)

		Average values							
		TC ^a		HDL-C ^b		TAG ^a		HDL-C/TC ^a	
Treatment	n	Average	p	Average	p	Average	p	Average	p
Control	8	132.31±36.10a	0.01	80.63±19.14a	-	249.58±95.67a	0.034	0.62±0.11a	-
Olive	8	138.05±37.06a	0.005	83.68±16.85a	-	267.19±55.00a	0.014	0.62±0.09a	-
Fish	7	78.83±26.11b	-	50.87±20.23b	0.003	145.83±40.02b	-	0.64±0.11a	-
Flaxseed	7	100.12±11.85ab	-	53.24±14.24ab	-	198.42±64.70ab	-	0.52±0.10a	-

Data are given as means ±SEM. ^aAverages followed by the same letter in the column do not differ by the Tukey test (P <0.05); ^bAverages followed by the same letter in the column do not differ by the Dunn's test (P <0.05). TC: Total Cholesterol; HDL-C: High-Lipoprotein Cholesterol; TAG: Triacylglycerol; HDL-C/TC: Ratio High-Lipoprotein Cholesterol/ Total Cholesterol.

The villusities of the ileum of the rats fed with the different lipid sources presented significant differences as to the height and width of the villusities and the depth of the crypts. All groups presented a greater villous height and crypt depth besides a smaller villous width than the control group. The groups treated with olive and fish oils presented higher values for the villous height

(p < 0.001) and crypt depth (p = 0.002), indicating an increase in the development of the mucosa. The width of the villusities suffered changes only in the groups treated with olive and flaxseed oils (p < 0.001). In these cases, the villusities became thinner than those in the control (Table 3).

TABLE 3 - Morphometry of the intestinal villusities and crypts of Wistar rats fed with different lipid sources

		Height of the villous ^a		Villous width ^b		Crypt depth ^a	
Treatment	n	Mean ± SEM	Median(min-max)	Mean ± SEM	Median(min-max)	Mean ± SEM	Median(min-max)
Control	3	317.52 ± 52.26	317.8 (428.5-161.9) a	86.15 ± 16.26 a	86.3(125.5-29.53)	131.55 ± 25.23	129.5(206.0-82.3) a
Olive	3	364.44 ± 63.16	380.4(445.1-170.3) b	79.25 ± 14.65 b	78.9(117.7-49.5)	146.54 ± 25.42	144.8(198.6-73.5) b
Fish	3	381.15 ± 107.51	379.8(575.4-176.9) b	85.82 ± 12.61 a	85.6(110.9-54.1)	142.49 ± 29.85	146.3(189.6-69.2) b
Flaxseed	3	322.19 ± 58.12	323.2(440.9-176.9) a	71.05 ± 12.97 c	69.9(101.8-43.8)	135.55 ± 36.36	131.2(231.6-63.0) a

Average values of villus height, villous width and crypt depth (µm). Data are given as means ±SEM and median (minimum- maximum). ^aAverages followed by the same letter in the column do not differ by the Dunn's test (P <0.05). ^bAverages followed by the same letter in the column do not differ by the Tukey test (P <0.05).

All groups exhibited less lymphocyte deposition in the intestinal mucosa (ileum) than the control group. The deposition of lymphocytes in the intestinal mucosa was statistically lower in

the animals that were treated with flaxseed oil (p=0.001). Then, lower values were observed in the groups treated with fish and olive oil, respectively (Table 4).

TABLE 4 - Average counted number of lymphocytes in the intestinal mucosa

Treatment	n	Lymphocytes	
		Average ^a	P
Control	3	16.36 ± 14.82a	0.001
Olive	3	9.38 ± 4.18b	-
Fish	3	7.81 ± 4.19bc	-
Flaxseed	3	4.70 ± 2.31c	0.001

Data are given as means ±SEM. ^aMean values with same letter in the column do not differ by the Tukey test (P <0.05).

Discussion

In the present study, the rats fed with diets containing fish oil presented a decrease in the concentration of TC, HDL-C and TAG. The olive and flaxseed oils did not differ from the control as the plasmatic lipids did. The polyunsaturated fatty acids, rich in EPA and DHA derived from sea products, presented a cardioprotective and antiatherogenic role, due to the inhibition of LDL synthesis, acceleration of LDL elimination and reductions of serum triglyceride¹⁸. It can also be observed that the lower ω -6/ ω -3 diet relates to a higher reduction of plasmatic lipids.

The reduction of the HDL fraction in the group treated with fish oil may be related to the fact that this lipoprotein is what is mainly responsible for the transport of cholesterol in rodents¹⁹. Lower levels of total cholesterol were verified in this group and, therefore, a lower demand of HDL was necessary to transport the cholesterol to the liver for metabolization.

Histology was carried out to corroborate the activity of the different lipid sources on the preservation of the structure of the intestinal mucosa in the ileum portion, which is the main location for lipid absorption. The morphometric analysis allows the measurement of these changes to occur in the mucosa as a response to the different diet components. The fish and olive group presented the highest villous height and crypt depth, while the flaxseed group presented the smallest villosity width, demonstrating that these oils exerted a positive effect on the integrity of the mucosa. These results indicate that a strong hyperplasia process occurred in these groups, aiming to guarantee the cell turnover rate in order to compensate the cell losses in the apical region of the villosities. In the small intestine, enterocytes generated from stem cells in the crypt base differentiate into absorptive cells and are finally lost from the tips of the villus, resulting in the replacement of lining cells every 2-3 days²⁰.

Campos *et al.*²¹ observed less inflammatory alterations in the intestinal wall of the rats treated with lipid emulsions rich in omega-3 fatty acids and greater protection against the development of morphological lesions. Similarly, the preservation of the morphological structure with the use of omega-3 fatty acids was also demonstrated in experimental²² and clinical studies²³. It has been discovered that long-chain fatty acids appear to be more effective intestinal stimulators and significant trophic effects on small intestinal mucosa in rats²⁴.

As to the lymphocyte counting, it was observed in this study that the group treated with flaxseed oil presented the lowest deposition of lymphocytes in the ileal portion, followed by the fish and olive groups, demonstrating that the treatments played an important role in the reduction of the migration of this inflammatory cell. Andoh *et al.*²⁵ fed rats with diets rich in omega-3 and omega-6 for 12 days after an enteritis induction. They verified significantly more severe histological changes in the animals fed with omega-6 and a greater amount of pro-inflammatory interleukins, in comparison to those fed with omega-3, suggesting a possible mechanism for the suppression of the inflammatory response.

Jeffery *et al.*²⁶ demonstrated that the supplementation of EPA and DHA inhibited the proliferation of isolated lymphocytes of the lymphatic nodes, spleen and thymus of rodents as well as their concentration in human blood, which is in accordance with the lower number of lymphocytes found in the intestinal mucosa in the groups treated with the oils which are sources of ALA and EPA/DHA present in this work.

However, the treatments with olive and fish oils provided better preservation of the ileal mucosa and the group treated with flaxseed oil reduced the migration of lymphocytes to the intestinal mucosa. These data suggest that the oils, even in small amounts in the diet, present different metabolic responses from the soybean oil.

The control group, fed with oil rich in LA, presented the greatest number of lymphocytes in the intestinal mucosa, probably due to the inflammatory response to AA, resulting from the LA metabolism²⁷. Although the modulatory role of the inflammatory response depends on the concentrations of ω -6/ ω -3 in the diet, soybean oil presented an inflammatory response superior to olive oil which presented a greater ω -6/ ω -3 relation.

Conclusion

The present study demonstrated that fish oil, flaxseed oil and olive oil exerts different effects on lipid profile, intestinal villosities preservation and mucosal lymphocyte migration of Wistar rats probably due to difference in their fatty acid composition.

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