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Food safety, hypolipidemic and hypoglycemic activities, and *in vivo* protein quality of microalga *Scenedesmus obliquus* in Wistar rats

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ABSTRACT

The present study evaluated the safety of dietary administration of the microalga *Scenedesmus obliquus* at high doses, the influence on blood biochemical profile, and *in vivo* protein quality in male Wistar rats. The microalga exhibited a high amount of fibers, carotenoids, phenolic compounds, linolenic and linoleic acid, and great antioxidant activity. The diet containing *Scenedesmus obliquus* was well tolerated, with good protein digestibility and maintenance of growth and weight of the animals. The intake of the microalga reduced triglycerides of up to 70%, atherogenic index of up to 80%, and serum glucose of up to 42% concentrations as compared with the standard diet. No alterations were observed in the analyzed organs by histology, suggesting that the microalga *Scenedesmus obliquus* can be used as potential safe food and may represent a sustainable source of food.

1. Introduction

There are over one billion people undernourished in the world, according to the Food and Agriculture Organization (FAO) (2010). Such occurrence is a consequence of diverse factors like the inefficient use of fertilizers, agricultural poor-quality land, an exponential increase in world population, and climate changes. Therefore, the obtainment of alternative food sources is a necessity to help to reduce hunger in the world (Goulão, 2016). Thus, the food security policies have focused on giving nations the ability to produce food in quantity and with the quality to meet the nutritional requirements of the population (Goulão, 2016).

The new food sources with sustainable production and nutritional value are one of the greatest challenges and interests in food research. Microalgae have been researched as a source of bio-compounds that can be used in the cosmetic, fertilizer, animal feed, food, pharmaceutical industries as well as in the biofuel production (Chen et al., 2018; El-Naggar, Samhan, Salama, Hamdy, & Ali, 2018; Kothari et al., 2017). New species of microalgae can contribute to increment the offer of value-added co-products of aquatic biomasses such as proteins, carbohydrates, and pigments, concomitant with the oil production (Kothari et al., 2017; Chen et al., 2018). Besides, microalga is an alternative source of protein, that is prone to grow in any area and region with high efficiency in the conversion of solar energy to biomass, resulting in high growth rates (10 to 50 times faster than plants) (Chen et al., 2018; Waghmare, Salve, Leblanc, & Arya, 2016). Moreover, they have superior capabilities of sequestration and conversion of CO_2 and potential for rapid growth even in areas not suitable for traditional crops like marginal sites for cultivation. Consequently, they do not compete with the soil for food production (Waghmare, Salve, Leblanc, & Arya, 2016).

Among several types of microalgae, the genus *Scenedesmus* has been recently studied. The genus *Scenedesmus* is composed of around 120 species and presents a wide morphological variation within each species. The species *Scenedesmus obliquus* is abundant in several countries, presents high tolerance at climatic variations at different values of pH, a high growth rate, and contains 40 to 56% of protein (dry mass) (Afify, El Baroty, El Baz, Abd El Baky, & Murad, 2018; Becker, 2007; Benemann, 2013; Chan et al., 2013; Rocha et al., 2019; Soares et al., 2018). This species has promising qualities to act as a new source of lutein as well as other valuable compounds (Chan et al., 2013). According to Přibyl, Cepák, Kaštánek, and Zachleder (2015), the ease of cultivation and robustness of some strains of *Scenedesmus* makes them more appropriate for sustainable large-scale production and are prone to the generation of several compounds. Afify, El Baroty, El Baz, Abd El Baky, and Murad (2018) evaluated the suitability of *Scenedesmus obliquus* to be a source of essential (Thr, Val, Met, Ile, Leu, His) and non-

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essential (Arg, Ala, Pro, Asp) amino acids. Thus, *S. obliquus* presents itself as a promising source of food, but studies of its toxicity and protein quality *in vivo* are required.

One of the concerns when proposing microalgae as a food or food ingredient for human consumption is to establish the dose safe for consumption. Short and long term feeding trials have been showed that some microalgae are safe to consume (Bigagli et al., 2017; Borowitzka, 2013; Jong-Yuh & Mei-Fen, 2005; Marles et al., 2011; Niccolai et al., 2017; Nuño et al., 2013; Sengupta, Koley, Dutta, & Bhowal, 2018; Serban et al., 2016). Some authors report the safety recommended intake of 1 to 5 g/person/day of Spirulina (*Arthrospira platensis*), with historical reports of spirulina consumption of 10 to 40 g (Marles et al., 2011; FDA, 2003; Gilroy, Kauffman, Hall, Huang, & Chu, 2000). Clinical studies described that an intake of about 10 g of spirulina per day for 6 months does not induce adverse effects (Marles et al., 2011; Yamani, Gresenguet, Kaba-Mebri, Mouala, & Rey, 2009).

The potential effect of microalgae in human nutrition is due to the biological action (Vaz, Moreira, Morais, & Costa, 2016; Raposo & De Morais, 2015; Jong-Yuh & Mei-Fen, 2005) of some species already reported in the literature. For example, *Arthrospira/Spirulina platensis, Chlorella* spp., *Isochrysis galbana* are able to act in the hypoglycemic and hypolipidemic activity, with a reduction in the glycemic and plasma concentration of total cholesterol, LDL, HDL, and triglycerides ((Bigagli et al., 2017; Jong-Yuh & Mei-Fen, 2005; Nuño et al., 2013; Sengupta, Koley, Dutta, & Bhowal, 2018; Serban et al., 2016)). *Scenedesmus obliquus* exhibits the potential to be used in food, due to the robustness of the cells, with high productivity and low costs of production. However, there are no data in the literature on the efficacy and food safety of the intake of this microalgae in high doses. Thus, some challenges must be fulfilled, among them the evaluation of the toxicity and microalgae quality *in vivo*, emphasizing metabolic and biochemistry analyses, and histological tests on kidneys and liver organs. In this context, the objective of the present work was the evaluation of *Scenedesmus obliquus* ingestion, as a source of protein in the diet of male Wistar rats, to determine: (*i*) the safety intake of the microalga in high doses (11.6 g microalga/100 g diet and 23.2 g microalga/100 g diet), (*ii*) the influence of the microalga ingestion on blood biochemical profile, (*iii*) the effect of the microalga consumption on the morphofunctional aspects in the liver and kidney, and (*iv*) the *in vivo* protein quality of the microalga.

2. Materials and methods

2.1. Production and composition of the microalgae

The microalga *Scenedesmus obliquus* was cultivated in a raceway tank (4.000 L of cultivation capacity, 0.5 g/L of stationary phase density, 12 days of cultivation), with sunlight incidence and in semi-discontinuous mode, in a medium rich in potassium chloride (173.9 mg·L^{-1}) and urea (180 mg·L^{-1}). The microalga growth curve was determined by optical density using absorbance (spectrophotometer Thermo Scientific, Multiskan GO, Germany) at 620 nm, and the biomass was separated by flocculation in the stationary growth phase. Cells were washed with distilled water and concentrated to a solids content of 10 to 15% (w/w) by using a centrifuge (Thermo Scientific, Heraeus multifuge X3R, EUA), at 3000*g* for 10 min., The biomass was then frozen at −40 °C, freeze-dried (Terroni, LS 3000, Brazil), and stored at 8 °C in sterilized containers.

2.1.1. Centesimal composition of the microalga

The dry biomass of the microalga was characterized in terms of its chemical composition for the quantification (AOAC, 2005) of moisture (925.09), ashes (923.03), lipids (920.85), protein (920.87) with a conversion factor of 4.5, and fibers (total, soluble, and insoluble). Soluble dietary fiber (SDF) and insoluble dietary fiber (IDF) were quantified according to the enzymatic–gravimetric method (985.29)(AOAC, 2005). The carbohydrate content was obtained by simple difference [Carbohydrate (%) = 100% - Protein (%) - Lipids (%) - Ash (%) - Water (%)] (AOAC, 2005).

2.1.2. Extraction, quantification and antioxidant activity of carotenoids and phenolic compounds

The extraction and quantification of pigments and phenolic compounds were carried out after the disruption of the cells of the microalga (1:10 biomass:water) by using ultrasound at 20 kHz frequency, 90% amplitude, and 5 min. Next, the biomass was frozen at −40 °C, freeze-dried, and stored in sterilized containers. The extraction and quantification of pigments, including carotenoids, were based on the methodology proposed by Wellburn (1994) for acetone extract. The extraction was started by adding pure acetone in the proportion of 30 mL for each 100 mg of microalga. The extracts were centrifuged for 10 min., at 7100*g*, after 30 min. Eqs. (1), (2), and (3) were used for quantification of chlorophyll *a*, chlorophyll *b*, and total carotenoids, following the Wellburn studies (1994) for acetone 80% extract.

$$Ca = 12.25A_{663.2} - 2.79A_{646.8} \quad (1)$$

$$Cb = 21.5A_{646.8} - 5.1A_{663.2} \quad (2)$$

$$Cc = (1000A_{470} - 1.82Ca - 85.02Cb)/198 \quad (3)$$

In wich Ca is the concentration of chlorophyll *a* (μg·mL^{-1}), Cb is the concentration of chlorophyll *b* (μg·mL^{-1}), Cc is the concentration of total carotenoids (μg·mL^{-1}).

After the extract separation, the pigments were quantified, and their concentrations (μg·mL^{-1}) were converted to % [mg of pigment·(100 mg of microalgae)$^{-1}$] using the total extract volume and the mass of the extracted biomass, from Eq. (4):

$$\%(mg\cdot(100mg^{-1})) = 100\frac{C\cdot V\cdot 10^{-3}}{m} \quad (4)$$

In which C is the concentration of chlorophyll *a*, b, or total carotenoids (μg·mL^{-1}), V is the total volume of the extract (mL), and m is the microalgae mass (mg).

The antioxidant activity of carotenoid was determined after removing the interferents, according to the methodology proposed by Howe and Tanumihardjo (2006). The acetone extract was subjected to additional extraction with hexane in the ratio of 2:3 (hexane: acetone). Deionized water was added, and the aqueous phase was separated from the organic phase. The organic phase, containing the carotenoids and interferents, such as chlorophyll and lipid, was saponified with a solution of 10% w/v KOH in ethanol (Soares, Júnior, Lopes, Derner, & Filho, 2016). After 12 h, the solution was washed with deionized water until complete separation in two phases: the greenish aqueous phase, and the yellow organic phase containing the carotenoids

The extraction and quantification of the phenolic compounds were performed according to Singleton and Rossi (1965), with modifications. Ethanol was utilized as the extraction solvent (1:10 biomass:ethanol), in which the mixture was left under stirring for 30 min. Next, the sample was centrifuged for 10 min. at 7100*g*. The total content of phenolic compounds in the extract was determined from linear regression by using an analytical curve of gallic acid (GAE), at concentrations between 0 and 1000 mg·mL^{-1} of gallic acid. The results were represented in (mg of GAE·mL^{-1} of extract and g of phenolic compounds)·(100 g^{-1} of microalga).

The method used to assess the antioxidant activity is based on the reduction of 2,2-diphenyl-1-picrylhydrazyl, DPPH, by the action of a radical species or an antioxidant, to form diphenyl-picrylhydrazine. The percent inhibition of DPPH was obtained following the method of Brand-Williams, Cuvelier, and Berset (1995) with modifications. Briefly, a solution of 60 μmol·L^{-1} DPPH in methanol was prepared. Then, aliquots of 2.9 mL of DPPH were added of 100 μL of the carotenoid extract (185.6 μg·mL^{-1}), or 100 μL of phenolic compounds extract (CF) (496.80 μg AGE·mL^{-1} extract). The solvent methanol was

used as the control system. After stirring, the mixture was left in the dark at room temperature for 30 min.

The absorbance of the samples was read after 30 min of reaction, in a spectrophotometer (Varian, Cary 50, Japan) at 517 nm, in order to obtain the percentage of inhibition, expressed by Eq. (5).

$$AA(\%) = \frac{100(Asample - Ablank)}{Acontrol} \quad (5)$$

In which, $A_{control}$ is the absorbance of the DPPH solution with methanol as the control, A_{sample} is the absorbance of the DPPH solution with the test sample, and A_{blank} is the absorbance of the sample without the DPPH solution.

2.1.3. Unsaturated fatty acid profile

The *S. obliquus* oil was extracted following the Bligh and Dyer (1959) method. The microalgal oil was converted to fatty acid methyl esters (FAMEs) to obtain the fatty acid profile of *S. obliquus*, according to Ichihara and Fukubayashi (2010). Samples of the microalga oil were diluted in chloroform:methanol solution (2:1), and the lipids were transesterified with the addition of 8 M HCl solution in methanol, followed by incubation at 100 °C for 1 h. The FAMEs were extracted using hexane, which was collected from the upper phase after centrifugation. The supernatant (solvent phase) was injected into a gas chromatograph equipped with a Flame Ionization Detector (GC-FID) (Shimadzu, GC-2010, Japan) and a capillary column of 100 m × 0.25 mm (SP-2560, Sigma-Aldrich, USA). The analysis was performed by direct injection of 1 μL of the sample. Helium gas was used as the dragging gas and maintained at a constant flow rate of 363 kPa. The FAMEs were separated using a linear heating ramp from 60 °C to 330 °C, at a heating rate of 20 °C min^{-1}, and high linear velocity for better peak resolution. Peak identification was confirmed by comparison with the standard FAME mix (SupelCo 37 FAME mix, Sigma-Aldrich, USA).

2.2. Diets

The four diets used (Table 1) were calculated based on the rodent growth formulation (AIN93G) (Reeves, Nielsen, & Fahey, 1993) adapted to provide 9.5% protein. Diets containing *Scenedesmus obliquus* were formulated, taking into account the centesimal composition of biomass and the daily amount of rodent ingestion (20 g/rat/day). The amount of added protein was calculated from the centesimal composition of casein and microalgae (Kjeldahl method 920.87; AOAC, 2005). After the formulation, the protein content of the finished diets was again quantified, in order to calculate the values of PER, NPR, and true digestibility, based on the observed protein consumption, and not only on the theoretical protein calculations. The values of 9.99%, 9.54%, and 9.02% are the corrected final protein quantities of the respective diet.

Table 1
Composition (g) of the diets used: aproteic (negative control), casein (positive control), and with microalgal biomass of Scenedesmus obliquus, the M50, and M100..

Diet (g/100 g diet)				
Ingredients	Aproteic	Casein	M50	M100
Casein (g)	–	11.46	5.73	0
Microalga (g)	–	–	11.6	23.2
Dextrinized starch (g)	13.2	13.2	13.2	13.2
Sucrose (g)	10	10	10	10
Soybean oil (g)	7	7	6.5	6
Fiber (microcristalin cellulose) (g)	5	5	3.14	1.28
Mineral mix (g)	3.5	3.5	3.5	3.5
Vitamin blend (g)	1	1	1	1
L-cysteine (g)	0.3	0.3	0.3	0.3
Choline bitartrate (g)	0.25	0.25	0.25	0.25
Corn starch (g)	59.75	48.29	44.78	41.27
Total (g)	100	100	100	100
Caloric density (cal/g)	3.95	4.39	3.82	3.77
Protein (g/100 g)	–	9.99	9.54	9.00

High amounts of *S. obliquus* were used in the formulation of the diet. Thus, the suggested daily intakes were 17.4 and 34.8 g/kg/day, which correspond to 11.6 and 23.2 g microalgae/100 g diet, or 50% and 100% of protein of the diet, respectively, considering a minimum daily intake of 8 g for weanling rats. The other ingredients of the diet (casein, maltodextrin, sucrose, and soybean oil) were adjusted to provide isocaloric diets with or without microalgae addition.

The diets containing the *Scenedesmus obliquus* were (g/100 g diet): (*a*) M50, diet with 50% of protein from the microalga and 50% of casein (11.6% of *S. obliquus)*; *(b)* M100, diet with 100% of protein from the microalga (23.2% of *S. obliquus*).

2.3. Animals and experimental design

The experiment followed the guidelines of the European Union Regulation on Care and Use of Laboratory Animals (OJ L 358; 18.12.1986) conjointly Directive 86/609/EEC of November 24, 1986, in compliance with the regulations of the Brazilian College of Animal Experimentation (COBEA) on the protection of animals utilized for experimental purposes and other scientific knowledge. The Ethics Committee on Animal Use (CEUA/UFV) of the Federal University of Viçosa, MG, Brazil, approved the project (Process No. 67/2017).

Wistar rats (*Rattus novergicus*) males, weaned (21 days old; n = 32), were used. The animals were acclimatized in plastic cages for 1 week, under controlled environmental conditions of photoperiod of 12 h light, 12 h dark, and an average temperature of 23 ± 1 °C. The animals were weighed and randomly distributed so that they were identified in individual stainless-steel cages. Thirty-two rats (n = 8 / group) were randomized into 4 groups, with both diet and water *ad libitum*. The 32 animals were schematized in 4 groups (2 control and 2 test groups). One control group received a diet without protein (negative control), whereas the other group received a diet with casein as a standard protein (positive control). The rats of the test groups were fed with diets of 50 and 100% of the protein from *S. obliquus*, as observed in Table 1. After diet consumption, the animals were inspected, visually and individually, at intervals of 15, 30, 60 min, 3 h, 6 h, 9 h, and 12 h. After that, the animals were inspected at least once a day until the end of the experiment (28 days). The observation was focused on mortality, behavioral change, and motor activity.

2.4. In vivo protein quality (FER, PER, NPR, and digestibility)

Individual dietary intake was recorded weekly during the experimental period (28 days). The animals' body weight was recorded before administration of the first diet (day 1) and once a week until the conclusion of the study (day 28).

These data were used to calculate the Feed Efficiency Ratio (FER), which is the relationship between animal weight gain (WG) and food intake (FI): FER = WG (g)/FI (g).

In order to evaluate the protein quality of the diets, the biological methods of Protein Efficiency Ratio (PER) and Net Protein Ratio (NPR) were used. The PER calculation method (Osborne, Mendel, & Ferry, 1919) measures the ratio between the weight gain (g) and the protein intake (PI): PER = WG (g) / PI (g). The NPR was determined in the 14th day of the experiment, taking the weight gain of the test group plus the weight loss of the group with aproteic diet (WLNC), regarding the protein intake of the test group (PI), following the method of Bender and Doell (1957): NPR = [WG (g) + WLNC (g)]/PI (g).

The true digestibility (TD) was evaluated between 15th and 18th days of the experiment, which normal diets were withdrawn and replaced by diet stained with 0.2% indigo carmine dye. Unmarked feces were discarded. Marked feces were collected, stored in separate containers under refrigeration (8 °C), and oven-dried with forced air

circulation (105 °C, 24 h; Toth, Model 510, Brazil). Subsequently, Afterward, the feces were put in the desiccator, weighed (Shimadzu, AUY22, Japan), and ground for the determination, in triplicate, the nitrogen content by the micro Kjeldahl method (Tecnal, TE 0363, Brazil) (920.87) (AOAC, 2005). The nitrogen content of the feces of the experimental groups was used to calculate the true digestibility, which was possible due to the implementation of a group of eight animals with aproteic diet (FAO/WHO, 1991). The calculation was made according to Eq. (6):

$$TD = \frac{100[I - (F - FK)]}{I} \quad (6)$$

In which, TD is the true digestibility; I is the amount of nitrogen ingested; F is the amount of nitrogen excreted in feces; FK is the nitrogen fecal loss of the protein-free diet group.

The relative digestibility (RD) was calculated by the ratio between TD (TD_T) of the test group and TD of the positive control (TD_C): RD = 100 (TD_T/TD_C). Thus, the result of the true digestibility of positive control is used to estimate the relative digestibility of the other groups.

The animals were anesthetized with isoflurane (Isoforine, Cristália®, Brazil) and euthanized by cardiac puncture on day 28 after 12 h fasting. The liver, spleen, and kidney were washed, weighed (Shimadzu, AUY22, Japan), and placed in formalin solution (formaldeide 10% m/v) to be histologically analyzed. Besides, blood was collected for biochemical serum analysis.

2.5. Blood biochemistry and histological analyses

The blood of the euthanized animals by cardiac puncture was collected in appropriate tubes (13 × 100 mm; BD Vacutainer®, USA) and centrifuged at 4 °C for 600 s (Fanem-204, São Paulo, Brazil) and the plasma was stored at −80 °C.

The analyzed biochemical parameters were: aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine, uric acid, glucose, total cholesterol, high-density lipoprotein cholesterol (HDL), and triglycerides. Atherogenic index (AI) was calculated by applying the formula AI = log (TGL/HDL).

Levels were measured by colorimetric methods using commercially available kits (Bioclin®, Belo Horizonte, Brazil). Analyses were conducted on a BS-200 Chemistry Analyzer (Bioclin®).

Macroscopic and histopathological examinations were conducted after the rat's euthanasia. The liver, spleen, and kidney organs were cleaned in sodium phosphate buffer solution (10 mM; Vetec, Brazil), and weighed (Shimadzu, AUY22, Japan) individually. Samples of these organs were fixed in Carson's Formalin Solution buffer solution (Carson, Martin, & Lynn, 1973) and, then, transferred to tubes (15 mL) containing an aqueous solution of ethyl alcohol (70% v/v; Vetec, Brazil) for further histopathological analysis.

The dehydration of the organs was performed in an aqueous solution of ethyl alcohol, with increasing concentration (70%, 80%, 90%, and 95%) of the alcohol, until using absolute ethyl alcohol. The organ samples remained in each alcoholic solution for 30 min. After the dehydration with absolute alcohol, the organs were included in resins (Historesin®, Leica, Germany), fixed in woodblocks to perform the microtomy. Sixteen sections of 3 μm thickness were obtained using a rotary microtome (RM 2045, Leica, Germany) and glass razors. The sections were stained with hematoxylin and eosin (Fischer, Jacobson, Rose, & Zeller, 2008) for further histological analysis. The lamina was assembled with Entellan® (Merck, Germany). The cuts were done semiserially at regular intervals of 12 μm to avoid repeating the analyses in the same cells. The preparations were analyzed with a light microscope (Olympus BX-60®, Japan) and the images captured using a photomicroscope (Olympus AX 70 TRF, Japan) in the objectives of 10 and 20x.

Table 2
Composition of microalga *Scenedesmus obliquus*

Components	g·100 g^{-1}
Moisture	10.37
Protein (factor of 4.5)	40.42
Lipids	05.57
C16: 1 (Palmitoleic)	0.29
C18: 1n9c (Oleic)	1.38
C18: 2n6t (Linolelaidic)	0.24
C18: 2n6c (linoleic)	0.28
C18: 3n3c (Linolenic)	0.95
Unidentified	0.12
Saturated	2.31
Monounsaturated	1.67
Polyunsaturated	1.47
Ashes	15.64
Carbohydrates	28.00
Total fibers	19.37
Soluble fibers	03.14
Insoluble fibers	16.23
Chlorophyll *a*	1.03 ± 0.11
Chlorophyll *b*	0.32 ± 0.09
Carotenoids	1.10 ± 0.13
Phenolic compounds	1.96 ± 0.17

2.6. Statistical analysis

Statistical analyses were performed applying the statistical software SAS (Statistical Analysis System, 1999) version 9.2, licensed by the Federal University of Viçosa. For the results referring to the biochemical data and evaluation of the protein quality, the normality of the data was verified by the Kolmogorov-Smirnov test, using a 5% significance level ($p < 0.05$). As normality was confirmed, the data were submitted to analysis of variance (ANOVA) followed by the Tukey test at 5% probability ($p < 0.05$).

3. Results and discussion

3.1. Composition of the microalga Scenedesmus obliquus

The results of the composition of microalga S. obliquus are shown in Table 2. It should be highlighted the large amount of nutrients and bioactive compounds present in this microalga species, such as proteins (40.42%), insoluble fibers (16.23%), soluble fibers (3.14%), phenolic compounds (1.96%), carotenoids (1.10%), oleic (C18: 1) 1.38%, linoleic (C18: 2) 0.95% and linolenic (C18:3) 0.28% acids. The high proportion of oleic acid is usually observed in other microalgae species.

These results are in agreement with values described in the literature for *S. obliquus*: (*a*) Afify, El Baroty, El Baz, Abd El Baky, and Murad (2018) found 40.69% of protein content; (*b*) Rocha et al. (2019) reported C18:1, C18:2 and C18:3 as main fatty acids, which are usually accumulated at high levels by several microalgae; (*c*) Wiltshire, Boersma, Möller, and Buhtz (2000) quantified about 1.1% C18:3, 0.75% C18:2, and 0.4% C18:1, in microalgal oil extracted using the Bligh & Dyer method in association with ultrasound; (*d*) Pribyl et al. (2015) observed for *Scenedesmus* sp. values of 0.68% of carotenoids in a 7-day culture in the laboratory and 2.08% in a 14-day culture with saline stress. Thus, the carotenoid concentration changed depending on the stress and the number of days of culture in the laboratory. Also, some authors point out that the major carotenoid found in this species is lutein (Chan et al., 2013; Wiltshire et al., 2000).

Extracts of carotenoid (185.6 μg·mL^{-1}) and phenolic compounds (496.80 μg AGE·mL^{-1}) showed a high percentage of inhibition of DPPH oxidation, 93.53% and 96.09%, respectively. Therefore, the higher the inhibition percentage, the higher the DPPH consumption, and the higher the antioxidant activity (Maadane et al., 2015). Under stress conditions, in some species of microalgae, the synthesis of lipids and pigments occurs in a coordinated way. Most lipids are produced in

response to stress and are accumulated in cytoplasmic oily bodies, where some carotenoids, such as β-carotene, are also deposited and may play antioxidant activity in the protection of unsaturated lipids against peroxidation (Kim, Choi, Park, & Lee, 2014).

Maadane et al. (2015) founded high antioxidant activity in microalgae *Dunalliela* sp. *Tetraselmis* sp. and *Nannochloropsis gaditana*, with DPPH inhibition capacities > 80%. These microalgae showed high total polyphenols, carotenoids, and PUFA content.

In this way, it is noticeable that the carotenoids and phenolic compounds extracted from the microalga *Scenedesmus obliquus* exhibit high antioxidant activity even when present in low concentrations. The consumption of phenolic compounds and carotenoids can help the human body to reduce oxidative damage associated with aging and diseases such as arteriosclerosis, ulcer, diabetes, and cancer (Kazui et al., 2018; Devi et al., 2009). Furthermore, the food industry is seeking the use of natural antioxidants, isolated from plants and seaweed, to replace synthetic food additives, since these non-natural additives may have harmful effects on health (Devi et al., 2009; Sengupta, Koley, Dutta, & Bhowal, 2018).

3.2. Survival, food consumption, body weights, and in vivo protein quality (FER, PER, NPR, and digestibility)

In order to collaborate with the search of new alternative and sustainable protein sources, and to support future regulatory assessments (GRAS, Novel Foods) on the use of microalga *Scenedesmus obliquus*, this study tested the ingestion of this species, in high amounts, in Wistar rats. The microalga quantity was based on amounts already used for the FDA-recognized microalga *Athrospira platensis*. Such values are between 1 and 3.5% of Spirulina in various products, such as biscuits, paste, and energy bars, and may reach 10% in medical foods or energy drinks (FDA, 2003; Marles et al., 2011; Bigagli et al., 2017). The average consumption of microalgae in g /kg/day was performed, taking into account the curves shown in Fig. 1 that results in a daily dose of 14.16 and 22.80 g/kg, respectively, for diets containing 11.6 and 23.2 g microalga/ 100 g diet. Under these experimental conditions, the human equivalent dose (HED) was determined, according to Nair and Jacob (2016). For rats with 100 (M100) and 150 (M50) g body weight, the authors suggest the use of the factors of 5.2/37 and 6/37, respectively, in the HED calculations. So, in the present work: HED (M50) = (14.16 g/kg) × (6/37) = 2.3 g/kg; HED (M100) = (22.80 g/kg) × (5.2/37) = 3.2 g/kg. For an adult with 60 kg, the HED (M50) results in a daily dose of 137 g and the HED (M100) in a daily dose of 192 g. These doses are higher than those reported for *Athrospira platensis* by (*i*) Bigagli et al. (2017): daily dose of 132 g for 60 kg human and (*ii*) Marles et al. (2011): 10 to 40 g daily dose Dih'e in Africa.

All the animals showed typical behavior and survived until the end of the experiment. Fig. 1 shows both weekly body weight and weekly food intake. Concerning to the diet ingestion, the animals from the M100 diet group (23.2% of microalga) did not present a suitable feed intake (Fig. 1), probably because of the excessive amount of microalga affected the palatability, thus the results of this group differs from both groups, the PC and M50 diet. The growth curves (Fig. 1) of the animals from the diet groups of positive control and M50 were consistent with the standard growth charts (https://www.taconic.com/rat-model/wistar-hannover-galas). Since the dietary intake of the M100 diet group was different from the control and M50, the body weight and weight gain differed significantly (Table 3).

Table 3 reveals the decrease of FER value with the increase of the microalga content in the diet. FER values showed that the ingestion of (*a*) a substantial amount of *S. obliquus* (11.6%) (M50) contributed for the animal growth and weight gain, and (*b*) high amount of *S. obliquus* (23.2%) (M100) was not efficient in the conversion of consumed feed to live weight. This behavior of the M100 diet may be associated with the higher consumption of soluble fibers compared to the control, and the high amount of phenolic compounds and other phytochemicals

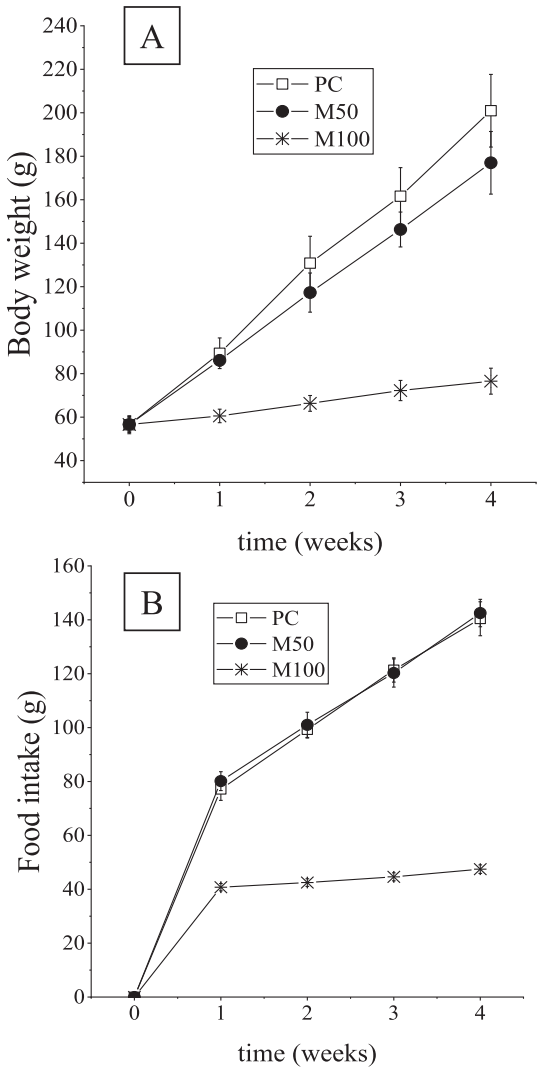

Fig. 1. Effect of diets containing 11.6% (M50) and 23.2% (M100) of microalga *S. obliquus* in Bodyweight and Food intake weekly on newly weaned rats (Wistar) as compared with the positive control (PC). (A) Body weight. (B) Food intake.

Table 3
Effect of *Scenedesmus obliquus* ingestion on total food intake, weight gain, FER, PER, NPR, feces production, feces moisture, and true and relative digestibility on newly weaned rats (Wistar) (n = 8).

Parameters	PC	M50	M100
Total food intake (28 days)(g)	438 ± 26a	443 ± 14a	174 ± 9b
Weight gain (g)	144 ± 15a	120 ± 14a	21 ± 3b
FER	0.35 ± 0.02a	0.27 ± 0.02b	0.13 ± 0.01c
PER	3.49 ± 0.16a	2.84 ± 0.20b	1.47 ± 0.23c
NPR	3.86 ± 0.10a	3.20 ± 0.15a	2.32 ± 0.13b
Feces moisture (%)	14.23 ± 1.97a	16.53 ± 1.99a	18.13 ± 1.85a
Feces production by diet consumption (%)	4.80 ± 0.97c	7.49 ± 0.97b	14.99 ± 0.97a
True digestibility (%)	93.28 ± 5.93a	83.41 ± 3.85b	78.18 ± 4.91b
Relative digestibility (%)	100.00a	89.42b	83.81b

Means followed by the same letter in the lines do not differ by the Tukey test, at the 5% level of significance. PC: positive control. M50: diet with 50% of protein from microalga and 50% of casein. M100: diet with 100% of protein from microalga.

consumed, that directly contribute to the increase of fecal volume and consequently gastric emptying (Table 3). These results corroborate with other studies of microalgae and other plant proteins (Moreira et al., 2013; Janczyk, Franke, & Souffrant, 2007).

The values of PER and NPR are necessary to determine the protein quality of a material. Results of PER for the M100 diet suggest that the *S. obliquus* protein presents quality inferior to the control protein (casein), but the NPR values point out to the effectivity of the microalga protein for animal weight maintenance (Table 3). NPR values are corrected to the endogenous losses protein. Values of PER and NPR of the M50 group indicate that the mixture of *S. obliquus* protein with another protein source was suitable to promote growth, development, and maintenance of animal tissues.

The FER and PER values found in the present research for *S. obliquus* (Table 3) are in agreement with those results reported for other species of microalgae consumed by humans. Moreira et al. (2013) evaluated the use of *Spirulina platensis* in rat feed, in proportions of 50 and 100%, and found values of, respectively, 0.25 and 0.17 for FER and 2.75 and 1.60 for PER. Janczyk et al. (2007) reported values of PER (1.4) and true digestibility (~53%) in rat experiments with M100 diets of spray-dried *Chlorella vulgaris*. True digestibility value is lower than that of the present work. The authors reported the increase of true digestibility (63%) and PER (2.1) with the M100 *Chlorella vulgaris* diets by applying ultrasound to the biomass. True digestibility remains even lower than that found for *S. obliquus*. The physical ultrasound treatment was able to damage the microalgae cell making it easy the wall rupture. Such treatment incremented the rat's growth by 1.5 times.

The results of the present work on protein quality demonstrated that the *S. obliquus* associated with a different protein source (M50 diet) showed a good conversion of the ingested food and was able to promote the growth, development, and tissue maintenance of the animals. Values of PER and NPR > 2.0 and 3.0, respectively, indicated the high-quality of the protein (Friedman, 1996). In fact, besides the good digestibility, *S. obliquus* mixed with other food sources of protein contributes to the growth and development of organs and tissues of animals, presenting itself as a potential candidate to be used in human food in different stages of growth.

3.3. Feces production and true and relative digestibility

The animals fed with diets containing *S. obliquus* presented higher production of feces as compared with the control (Table 3). This increase is directly proportional to the rise in the amount of microalga in the diet. The excretion of feces in larger quantities in rats fed by *S. obliquus* is due to the high amount of soluble fiber and phenolic compounds in the microalgae biomass. The true digestibility and relative digestibility of diets containing microalga were good (> 75%) but smaller than the control (Table 3).

The protein quality of the diet is represented by the digestibility, which can be influenced by several compounds such as inhibitors of digestive enzymes, hemagglutinins, and polyphenols, among others (García-rebollar, Cámara, Lázaro, Dapoza, & Pérez-maldonado, 2016). The presence of these compounds in the biomass of *S. obliquus* can form a complex with proteins, making it more difficult the protein digestion and subsequent absorption. These results corroborate with other studies of microalgae and other plant proteins (Moreira et al., 2013; Janczyk et al., 2007). Generally, the proteins of animal origin, such as casein, for example, have a digestibility higher than 95%, and those of vegetable origin below 80% (Friedman, 1996).

3.4. Effects of microalgae Scenedesmus obliquus on biochemical blood parameters

The intake of *S. obliquus* had no adverse effect on the liver and kidney of the animals. The damages in the hepatic and renal functions were evaluated by the level of AST, ALT, uric acid, and creatinine in the rat's blood. Such parameters for M50 and M100 diet groups showed no significant difference regarding the control (Fig. 2). Concerning the serum glucose levels, all groups showed a significant difference between them, with the values being inversely proportional to the amount of *S. obliquus* in the diet (Fig. 2A). The control diet showed the highest concentration of serum glucose (223.38 mg · dL^{-1}), and the M100 diet exhibited the lowest value (128.75 mg · dL^{-1}) (Fig. 2A). Therefore, a decrease of 42% of the serum glucose level was observed after microalgae consumption. Significant reduction in the atherogenic index (up to 80%) (Fig. 2B) with increasing consumption of microalgae was observed, demonstrating the influence of microalgae on lipid and cholesterol metabolism.

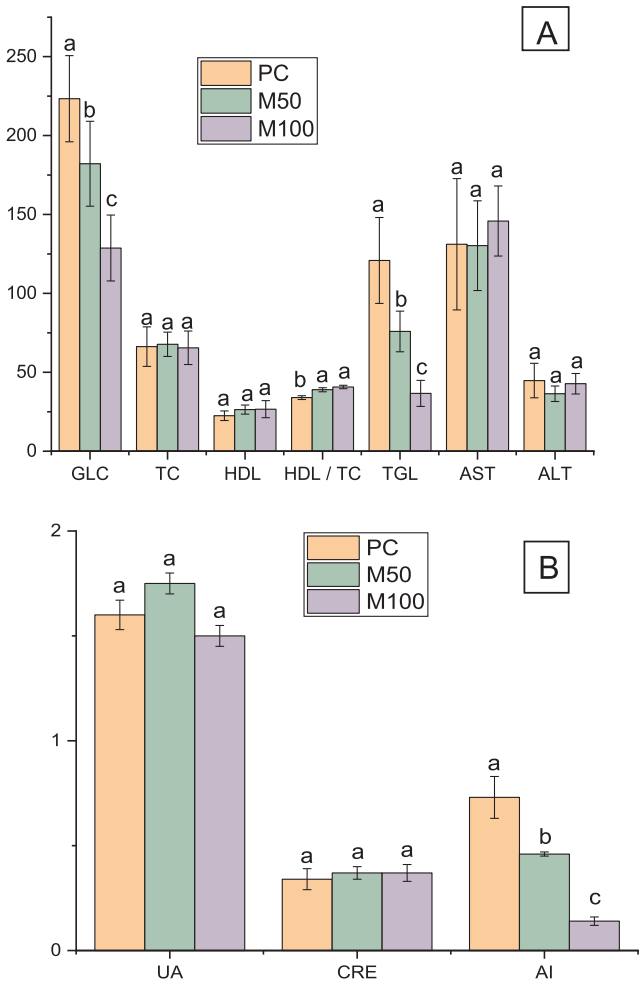

Fig. 2. Effect of ingestion of the microalga S. obliquus on the biochemical variables in Wistar rats (n = 8). (A) GLC: Glucose (mg·dL^{-1}); TC: total cholesterol (mg·dL^{-1}); HDL: HDL cholesterol (mg·dL^{-1}); HDL/Total cholesterol (%); TGL: triglycerides (mg·dL^{-1}); AST: aspartate aminotransferase (U·L^{-1}); ALT: alanine aminotransferase (U·L^{-1}). (B) UA: uric acid (mg·dL^{-1}); CRE: creatinine (mg·dL^{-1}); AI: atherogenic index. PC: positive control. M50: diet with 50% of protein from microalga and 50% of casein. M100: diet with 100% of protein from microalga. Means followed by the same letter in each parameter do not differ by the Tukey test at the 5% level of significance.

Blood triglyceride levels of the animals were reduced proportionally as the concentration of microalgae in the diet increased, and all treatments presented a significant difference ($p < 0.05$) (Fig. 2A). The M50 diet led to a lower triglyceride value (75.87 mg · dL^{-1}) compared to the control (120.88 mg · dL^{-1}) (Fig. 2A). Thus, a reduction of 70% of the triglyceride level was observed after the microalgae ingestion. The levels of total cholesterol and HDL did not present a significant difference among the microalga and control diets, but the ratio between the HDL and total cholesterol increased for the animals treated with the *S.*

obliquus diets (M50 and M100). The increase in HDL ratio concerning the total cholesterol may have been due to the presence of significant amounts of linolenic, linoleic (omega 3 and omega 6), and oleic acid in the microalga composition. The last one presented the highest proportion and could affect the lipid homeostasis.

The literature reported the high amounts of nucleic acids (4 to 6%) in the microalgal biomasses and a possible increment of the uric acid level in the body with the high intake of nucleic acid, which is due to biochemical degradation of purines (Bigagli et al., 2017; Morist, Montesinos, Cusidó, & Gòdia, 2001). Diseases such as gout attacks and urinary stones may be developed if the uric acid content increases in the human organism because of the body is unsuitable for metabolizing uric acid. Therefore, the World Health Organization recommends that the total daily consumption of nucleic acid in humans does not exceed 4 g (Gutiérrez-Salmeán, Fabila-Castillo, & Chamorro-Cevallos, 2015), which would be equivalent to approximately 100 g of *Arthrospira platensis* biomass (Bigagli et al., 2017). In the present work, the concentrations of uric acid for the diet with *S. obliquus* was normal (Fig. 2B) and resembled the quantities found in studies with other species of microalgae, such as *Arthrospira platensis*, known as Spirulina (Bigagli et al., 2017), *Isochrysis galbana,* and *Nannochloropsis oculata* (Nuño et al., 2013).

Decreases or increases in renal function parameters (uric acid and creatinine) (Fig. 2B) and liver enzymes (AST, ALT) (Fig. 2A) may indicate damage to the kidneys and liver (Sasmaz, Ozkan, Ferit, & Sasmaz, 2017). As can be seen in the consumption of microalga, *S. obliquus* did not present any significant effect on these parameters, showing no difference between them and the control. Besides, this biochemical result, in association with the liver and kidney histology analysis, confirms that *S. obliquus* did not exhibit any harmful and pathological damage in renal and hepatic functions.

The intake of the *S. obliquus* diets promoted a reduction in the blood triglycerides, an increase in the ratio between the HDL and total cholesterol, and did not influence the normal levels of the total cholesterol and HDL. Therefore, the consumption of *S. obliquus* exerts a positive effect in lipid homeostasis. The hypolipidemic activity of the microalgae *Arthrospira maxima* and *Arthrospira platensis* was reported in the literature in high-risk pro-atherogenic diets induced hypercholesterolemia, and in a well-balanced diet containing those microalgae (Bigagli et al., 2017; Colla, Muccillo-Baisch, & Costa, 2008; Kim et al., 2010; Riss et al., 2007; Sengupta, Koley, Dutta, & Bhowal, 2018; Serban et al., 2016). It should be emphasized the effect of the ingestion of *S. obliquus*, through isocaloric diets, on the reduction of triglycerides levels in healthy animals, which demonstrates the potentiality of using microalga *S. obliquus* as a nutraceutical food.

A significant reduction in serum glucose levels was verified as the concentration of microalgae in the diet increased (Fig. 2A). Ingestion of *S. obliquus* had a positive influence on glucose homeostasis, and blood glucose reduction was observed, even with isocaloric diets and healthy animals. These data corroborate the results described for other species of microalgae, such as *Chlorella vulgaris* (Ebrahimi-Mameghani, Sadeghi, Farhangi, Vaghef-Mehrabany, & Aliashrafi, 2017; Jong-Yuh & Mei-Fen, 2005); *Isochrysis galbana* (Nuño et al., 2013); *Spirulina fusiformis* (Setyaningsih, Bintang, & Madina, 2015), for which the serum glucose reduction was verified when using a balanced diet for both healthy and diabetic animals.

The high concentration of microalga in the diet (23.2%) influenced palatability, causing a decrease in food intake. Such behavior may have promoted a greater reduction in blood glucose and triglycerides. Nevertheless, this reduction effect cannot be attributed solely to food intake. The consumption of 11.6% microalga did not interfere in weight gain and food intake and significantly reduced serum glucose and triglyceride concentrations.

Improvement in the total cholesterol, triglyceride, and atherogenic index profile may be associated with the hypolipidemic and antioxidant properties of phytochemicals and peptides present in *S. obliquus*. Probably, the microalgal phytochemicals and peptides interact with cholesterol by hydrophobic interaction, promoting the reduction of the micellar solubilization of cholesterol (Hayat, Ahmad, Masud, Ahmed, & Bashir, 2014).

The positive effect of *S. obliquus* on lipid and glycemic homeostasis may be associated with its set of bioactive compounds like carotenoids, pigments, chlorophyll, phenolic compounds, polysaccharides, dietary fibers, phycobiliprotein, and unsaturated fatty acids ((Bigagli et al., 2017; J. Hussein, El-Banna, Razik, & El-Naggar, 2018; R. A. Hussein, Salama, El Naggar, & Ali, 2019; Jong-Yuh & Mei-Fen, 2005; Shalaby, 2011)). Natural microalgae products generally exhibit different biological activities such as antioxidant, hypolipidemic, anticancer, hypoglycemic, and antimicrobial ((Bigagli et al., 2017; J. Hussein, El-Banna, Razik, & El-Naggar, 2018; R. A. Hussein, Salama, El Naggar, & Ali, 2019; Jong-Yuh & Mei-Fen, 2005; Shalaby, 2011)).

Commercially grown strains of microalgae, such as *Aphanizomenon flos-aquae*, *Chlorella* sp., and *Arthrospira* sp., contain high quantities of protein, carotenoid, and phenolic compounds that have beneficial pharmacological actions (Soletto, Binaghi, Lodi, Carvalho, & Converti, 2005), such as antihyperglycemic and antihyperlipidemic activities. These biological functionalities act on the serum glucose level being useful in controlling diabetes and obesity (Hussein, Salama, El Naggar, & Ali, 2019). Hussein, Salama, El Naggar, and Ali (2019) studied the consumption of two dose levels of dried *Microcystis aerigunosa* (200 and 400 mg/kg) by diabetic rats. The authors found that the glucose level of diabetic rats was dose-dependent when compared to diabetic control rats and glimepiride-treated rats. *Spirulina platensis* also demonstrated hypoglycemic and hypolipidemic activity in diabetic rats, according to Joventino et al. (2012).

Taku et al. (2007) showed that soy isoflavones decreased serum total cholesterol and LDL significantly, but did not alter triacylglycerol and HDL cholesterol in humans. Thus, the supply of isoflavones simultaneously with soy protein would have synergistic effects or cholesterol-lowering additives.

There are numerous beneficial physiological effects attributed to linoleic acids, such as anti-atherogenic effect, fat accumulation decrease, anticancer activity, inflammation decrease, improvement of immune function, and increase of muscle mass (Miranda, Arias, Fernández-Quintela, & del Puy Portillo, 2014). Oleic acid is also considered health-friendly because diets with high amounts of monounsaturated fatty acid decreased LDL-cholesterol, plasma cholesterol, and triacylglycerol concentrations. Besides, the replacement of saturated fatty acids with *cis*-unsaturated fatty acids reduced the risk of coronary artery disease (Haug, Høstmark, & Harstad, 2007; Mensink, Zock, Kester, & Katan, 2003).

Afify, El Baroty, El Baz, Abd El Baky, and Murad (2018) analyzed the antioxidant and antiviral activities *S. obliquus* and found an inhibitory effect against the Coxsackie B virus 3. Therefore, the positive effect of microalga *S. obliquus* on the blood biochemical profile of newly weaned Wistar rats may be associated with the set of its compounds (Bigagli et al., 2017; J. Hussein, El-Banna, Razik, & El-Naggar, 2018; R. A. Hussein, Salama, El Naggar, & Ali, 2019; Jong-Yuh & Mei-Fen, 2005; Shalaby, 2011).

3.5. Histological analysis

No alterations were observed in the architecture of the liver, spleen, and kidney tissues after the ingestion of *S. obliquus*. The organ images can be observed in Figs. 3–5.

The results for liver histology did not show degeneration, hepatic inflammation, and necrosis for any of the evaluated groups. They also confirmed normal physiology with hepatocyte plaques separated by sinusoids, with well-defined nuclei of hepatocytes and observable Kupffer cells (Fig. 3).

The groups treated with microalga showed no toxic effect on renal tissues, presenting normal and healthy histological structures (Fig. 4).

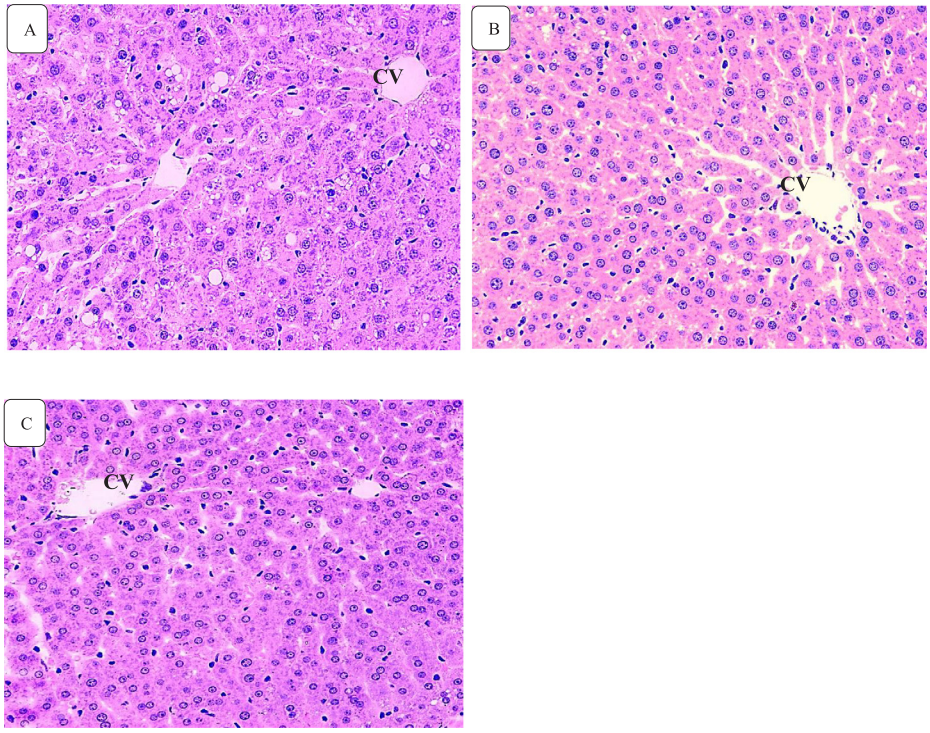

Fig. 3. Effect of microalga *S. obliquus* ingestion on hepatic tissue architecture compared to the control group. (A) Control; (B) M50; (C) M100. Central vein (CV).

Cortical structures, especially the glomerulus as a whole, reflected well defined constituent elements. The renal tubules, both proximal and distally convoluted, were observable and presented typical architecture.

The spleens of the groups treated with *S. obliquus* compared to the control also had typical structure and tissues (Fig. 5). The red pulps (RP) and white pulps (WP) were visible.

Histological analysis showed that the analyzed organs exhibit typical structure and tissues. The central vein presented a normal aspect. In general, the livers of the animals that received *S. obliquus* presented healthy appearance and normal physiology compared to the control. According to EL-Hak, Moustafa, & Mansour (2018) the architecture of the white pulps of the spleen should display normal rounded scattered follicles with a one-sided arteriole, called the central arterioles, in which the cells are arranged around the arteriole and classified into four zones: thymus-dependent zone; the germinal center; follicular

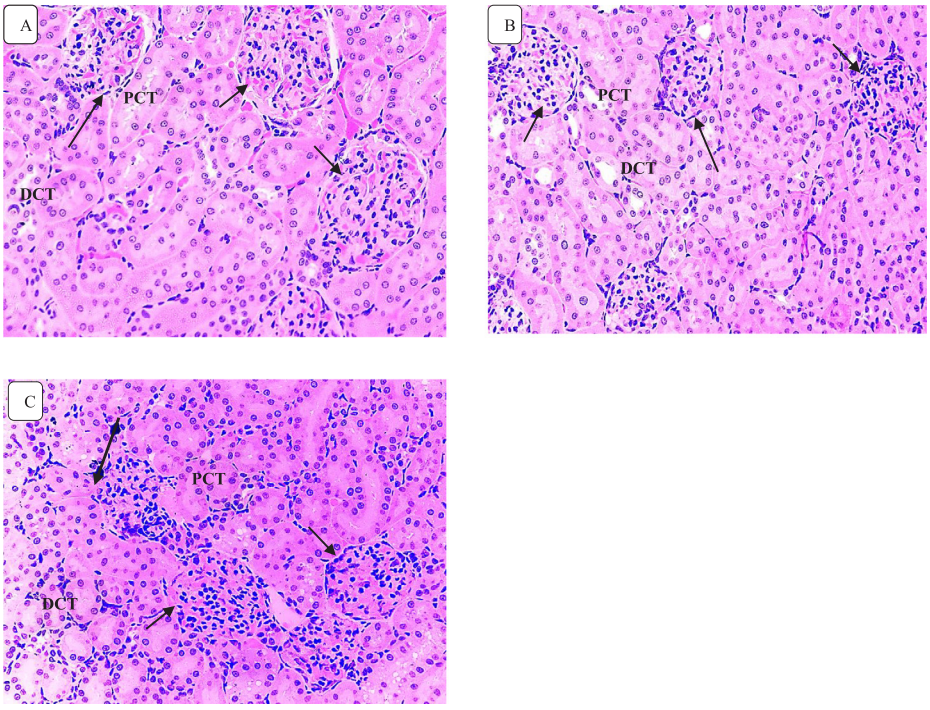

Fig. 4. Effect of ingestion of microalga *S. obliquus* on renal histological architecture compared to the control group. (A) Control; (B) M50; (C) M100. Photomicrograph of the kidney, showing normal glomerulus architecture (arrow). (PCT) proximal convoluted tubule. (DCT) distal convoluted tubule.

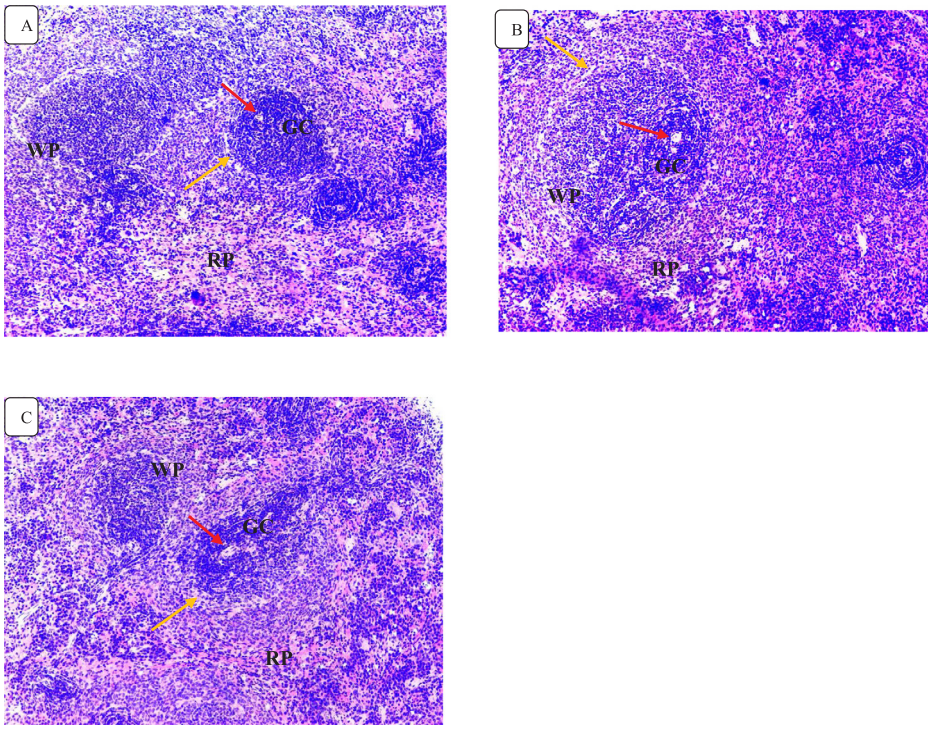

Fig. 5. Effect of microalga *S. obliquus* on the histological architecture of the spleen compared to the control group. (A) Control; (B) M50; (C) M100. Photomicrography in splenic tissue showing normal tissue architecture, white pulp (WfigureP); red pulp (RP); white pulp areas are usually represented by the germinal center (GC), arteriole (red arrow), and marginal zone (yellow arrow).

zone, and marginal zones. All these zones were observed in the organs of the animals for all groups tested.

The diets containing microalga *Scenedesmus obliquus* at concentrations of 11.6% and 23.2%, which correspond to 50% and 100% of the dietary protein, were well tolerated by the rats in a feeding period of 28 days. The formulated diet with 50% of microalga and 50% of casein promoted growth, weight gain, and tissue maintenance of the animals. Good digestibility of the microalgal protein was also observed. The intake of microalga *S. obliquus* reduces the triglycerides content (70%), atherogenic index (80%), and serum glucose (42%) concentration, even in a balanced diet. Furthermore, no alteration was observed in the analyzed organs (liver, spleen, and kidney), suggesting the use of the microalga as potential safe food. Therefore, *Scenedesmus obliquus* may represent a promising sustainable source of functional and nutraceutical foods for possible prevention and treatment of diabetes and dyslipidemias.

Ethics Statement

The authors declare that our protocol was approved by the Animal Care and Use Committee of the Federal University of Viçosa, MG, Brazil (CEUA/67/2017) ro (CEUA/001/2014). The experiments was carried out in accordance with the National Institutes of Health guide for the care and use of laboratory animals.

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