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ORIGINAL CONTRIBUTION

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Spirulina enhanced the skeletal muscle protein in growing rats

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Abstract *Background/Aim of the study* This study evaluates the effects of the blue green alga *spirulina* as the sole dietary source of protein on muscle protein in weaning rats. *Methods* Young (30 days) Wistar rats were fed, during 60 days, with 17% protein *spirulina* (S) and compared to rats fed 17% protein casein (C). We evaluated the muscle total protein and DNA contents and the in vitro protein synthesis and degradation rates as well the myosin protein expression. *Results* The groups presented similar body weight ($C = 427.3 \pm 8.6$; $S = 434.6 \pm 7.7$ g) and length ($C = 25.4 \pm 0.2$; $S = 25.6 \pm 0.2$ cm). Soleus muscle total protein ($C = 2.9 \pm 0.1$; $S = 2.7 \pm 0.1$ mg/100 mg) and DNA ($C = 0.084 \pm 0.005$; $S = 0.074 \pm 0.005$ mg/100 mg) contents were also similar in both groups. Protein degradation ($C = 427.5 \pm 40.6$; $S = 476.7 \pm 50.5$ pmol/mg^{-1} h^{-1}) did not differ between the groups but protein synthesis ($C = 17.5 \pm 1.0$; $S = 25.2 \pm 1.9$ pmol/mg^{-1} h^{-1}) and myosin content (western blot analyses) were higher ($P < 0.05$, t test) in *spirulina* group. *Conclusions* Although the *spirulina* proved adequate protein quality to maintain body growth, the muscle protein synthesis rates were increased by the ingestion of the experimental diet in young rats.

Key words skeletal muscle – *spirulina* – protein

Introduction

The use of algae (seaweed) in human alimentation is ancient. In the East, in particular, such source of food material have been tried extensively, in the hope of correcting the widespread protein deficiency that characterizes the nutritional status of less favored populations [22, 41]. To this day, with the continuing low level of available protein food all over the world and increasingly urgent need for low-cost food of good nutritional value, the exploration of seaweed biomasses as food sources is a matter of generalized interest.

Spirulina is a helicoidal shaped blue green alga with length of 0.2–0.3 mm [8, 19]. *Spirulina*'s special merit as a food source is that it contains 65–70% protein on dry weight basis, which is higher than any other natural food and has all eight essential amino acids to men [26]. It normally grows in naturally alkaline lakes located in arid zones. Although the alkaline water from such lakes cannot be used for irrigation, it can be used for cultivation of *spirulina* [22, 41]. Since this alga has a rapid reproduction rate, dividing three times a day, a pond devoted totally to the growth of *spirulina* can produce 125 times as much protein as the same amount area devoted to

corn, 70 times as much protein as to fish and 600 times as much as cattle [5, 16].

According to previous evaluations, *spirulina* seems to be a good alimentary protein source for human subjects. It has adequate acceptance and does not appear to exert any toxic effects, although it has slightly reduced digestibility [37, 40]. The most detailed studies on the nutritional value of *spirulina* have been conducted in rodents, mostly rats. The rodent studies covered a variety of parameters, such as body growth [7, 28, 36, 45], protein efficiency ratio (PER) and net protein utilization (NPU) [3, 31], sexual maturation [7], reproductive performance [36], hematological status [21], toxicity [6, 36, 45], among others. *Spirulina* proved adequate to maintain all these physiological makers at normal levels and did not produce adverse effects after chronic treatment. However, to the author's knowledge, there is little information about the effects of *spirulina* on tissues with high metabolic rates, such as skeletal muscle, examined at cellular and molecular levels.

The present study was designed to evaluate the effects of *spirulina* as the sole dietary source of protein in weaning rats on protein synthesis and degradation rates and myosin protein expression in skeletal muscle.

Experimental methods adopted

Animals and diets

All experiments involving animals followed the specific Brazilian resolutions of the Bioethics of Experiments with Animals (law No. 6.638 of 8 May 1979; Decree No. 24.645 of 10 July 1934, Brazilian College of Animal Experimentation). Young (30 days old at the start of the experiment) male Wistar rats, weighing 131.1 ± 22.1 g and measuring (nose-to-anus length) 20.6 ± 1.3 cm, were obtained from the animal facilities of the UNESP, Sao Paulo State University, Botucatu, SP, Brazil, and were randomly separated and maintained on an isocaloric powdered protein free diet plus *spirulina* (powdered *Spirulina maxima* sold commercially by All Chemistry do Brasil Ltda.) or a powdered protein free diet plus casein (powdered milk protein) until the adult age (90 days old). The casein diet contained 17% protein (casein), 64% carbohydrate, 7% fat, 5% fiber and was complemented with a mineral mix and a vitamin mix, in accordance to the AIN-93 [35]. *Spirulina* diet contained the same amount of protein, carbohydrate, fat and fiber as the casein diet but casein was replaced by *spirulina* as protein source. The total protein content in both diets was assessed and corrected by Kjeldahl nitrogen assay [1]. The mineral and vitamin mix composition were corrected for the mineral and vitamin contents in the *spirulina* powder. Details of the composition of both diets are described on Table 1.

Table 1 Diets composition (g/kg)

Components	Casein 17%[a]	*Spirulina* 17%
Spirulina (65% protein)[b]	–	280.0
Casein (84% protein)[c]	202.2	–
Cornstarch	397.0	386.0
Dextrinized cornstarch	130.5	130.5
Sucrose	100.0	100.0
Soybean oil	70.0	70.0
Fiber (microcellulose)	50.0	50.0
Mineral mix	35.0	35.0
Vitamin mix	10.0	10.0
L-cystine	3.0	3.0
Choline hydrochloride	2.5	2.5

[a]In accordance to the diet for growth phase, pregnancy and lactation of rodents, AIN-93G [35]
[b]Corrected values for the protein content in *spirulina* [1]
[c]Corrected values for the protein content in casein [1]

The *spirulina* powder composition was as follows. General analysis (%): protein 64.7, carbohydrates 15, lipids 6, minerals (ash) 7, fiber 8. Minerals (mg/10 g): calcium 70, iron 15, phosphorus 80, magnesium 40, zinc 0.3, manganese 0.5, sodium 90, potassium 140; minerals (mcg/10 g): selenium 10, copper 120, chromium 25. Vitamins (per 10 g): vitamin A 23,000 IU, beta carotene 14 mg, vitamin D 1,200 IU, vitamin E 1.0 mg, vitamin K 200 mcg, biotin 0.5 mcg, inositol 6.4 mg, thiamine 0.35 mg, riboflavin 0.4 mg, niacin 1.4 mg, pyridoxine 80 mcg, folate 1 mcg, cianocobalamine 20 mcg, pantothenic acid 10 mcg. Essential amino acids (mg/10 g): isoleucine 350, leucine 540, lysine 290, methionine 140, phenylalanine, 280, threonine 320, tryptophan 90, valine 400. Non essential amino acids (mg/10 g): alanine 470, arginine 430, aspartic acid 610, cystine 60, glutamic acid 910, glycine 320, histidine 100, praline 270, serine 320, tyrosine 300. Total amino acids (g/10 g): 6.2.

During the experiment, the rats had free access to food and water and were housed on a 12 h light/dark cycle at room temperature of 25°C. Food intake was monitored daily and body weight and length were measured (nose-to-anus length) once a week.

Tissue extraction

At the end of the experimental period, all animals were killed by decapitation. Blood samples were collected for serum glucose, total protein, albumin

and free fatty acid (FFA) levels measurements [32]. Samples of the liver were taken for total lipid [32], total protein [27] and DNA [18] determinations. Samples of the right gastrocnemius muscle were collected for total protein content and chymotrypsin-like and alkaline phosphatase activities assays and for myosin protein expression by western blot analysis. Left soleus muscle was also excised, weighed and analyzed for total protein and DNA contents and for myosin protein expression by western blot analysis. Longitudinal strips (70 mg) from the right soleus muscle were obtained for protein synthesis and degradation assays. The carcass was eviscerated, weighed and dried to constant weight in an oven at 100°C. It was then homogenized in a blender with benzene and extracted with several more changes of benzene. The powder was dried in the oven and weighed. Water and fat contents were calculated by difference. The dry fat-free powder was dissolved in $HClO_4$ 1 N and protein content was then measured [27].

Protein synthesis

The soleus muscle strips were preincubated for 30 min in RPMI 1640 medium (with glutamine and without red phenol and sodium bicarbonate), supplemented with bovine serum albumin fatty acid free (BSA) [1 g/l] and insulin [100 U/ml], saturated with 95%O_2/5%CO_2 gas mixture. They were then transferred into a fresh RPMI 1640 medium with the same supplementation containing 14-C phenylalanine [0.05 μCi/ml] and incubated for 2 h. At the end of the incubation, the muscle strips were homogenized in 5% trichloroacetic (TCA) and centrifuged at 2,000 rpm for 15 min at 4°C. TCA-insoluble material was washed three times with 5% TCA. The resultant pellet was solubilized in 10% sodium dodecyl sulfate (SDS) at room temperature for 30 min for determination of protein content and radioactivity incorporated to muscle proteins. Muscle protein was determined by the folinphenol method [27] and the protein-bound radioactivity was measured using scintillation counting. Protein synthesis was calculated by dividing the protein-bound radioactivity by the specific activity of the free phenylalanine in the incubation medium and expressed as nanomoles of phenylalanine incorporated per milligram of protein per 2 h.

Protein degradation

Tyrosine release from isolated muscle in presence of cyclohexamide was used as indicator of protein degradation as previously described by Fulks et al. [15]. This method makes use of the fact that the amino acid tyrosine is neither synthesized nor degraded by skeletal muscle. The soleus muscle strips were preincubated for 30 min in Krebs Ringer buffer [NaCl 1.2 mmol/L; KCl 4.8 mmol/L; $NaHCO_3$ 25 mmol/L; $CaCl_2$ 2.5 mmol/L; kH_2PO_4 1.2 mmol/L and $mgSO_4$ 1.2 mmol/L; ph 7.4], supplemented with glucose [5.5 mmol/L], BSA [1.0 g/l], insulin [5 U/ml] and cyclohexamide [5 mmol/L], saturated with 95%O_2/5%CO_2 gas mixture. They were then transferred into fresh medium of the same composition and incubated for 2 h. At the end of the incubation, samples of the incubation medium were used for the assay of tyrosine by the procedure of Waalkes e Udenfriend [46].

Western blotting analysis

The gastrocnemius muscles were homogenised in homogenising buffer (HB) (20 mmol/L Tris, 1 mM dithiothreitol, 2 mmol/L ATP and 5 mmol/L $MgCl_2$) and centrifuged at 15,000*g* for 15 min at 4°C. The resulting supernatant was analyzed for total protein content [4].

The protein samples of gastrocnemius muscle (5 μg) were resolved on 12% sodium dodecyl sulphate polyacrylamide gels, and transferred to 0.45 μm nitrocellulose membranes (Hybond-C, Amersham, UK) and blocked with 5% Marvel in PBS buffer saline, 0.1% tween, pH 7.5. Myosin antibody (Novocastra, New Castle, England) was used at 1:250 dilution and the secondary antibody, rabbit anti-mouse, was conjugated horseradish peroxidase, and was used at 1:1,500 dilution. Actin was used as the loading control, after probing the mouse actin antibody. The band expression was developed using ECL chemiluminescense (Amersham, UK). The blots were scanned by densitometry to quantify the differences using Gel Pro Analyser software (Media Cybernetics, Silver Spring, MD, USA).

Enzyme activity analysis

The enzyme *chymotrypsin-like* and alkaline phosphatase activities of the muscles were measured. Chymotrypsin-like activity was determined using the fluorogenic substrate succinyl-Leu-Leu-Val-Try-7-amino-4-methylcoumarin (Suc LLVY-AMC; 0.167 mg/ml in 100 mmol/L Tris–HCl, pH7.4; excitation 360 nm, emission 460 nm). The activity was expressed as units of fluorescence per microgram of protein, as a percentage of the control group. Alkaline phosphatase activity was measured using 37 mM *p*-nitrophenyl phosphate (PNPP) as substrate, and activity was ex-

Table 2 Body weight (g) and body length (cm) at the end of the experiment and food intake (g/100 g of body weight) during the experiment

	Casein	*Spirulina*
Body weight	427.3 ± 8.6	434.6 ± 7.7
Body length	25.4 ± 0.2	25.6 ± 0.2
Food intake	8.5 ± 0.1	7.9 ± 0.2

Results expressed as mean ± SEM of 14 rats in each group

Table 3 Carcass water (g/100 g), fat (g/100 g) and protein (g/100 g) contents at the end of the experiment

	Casein	*Spirulina*
Water	61.5 ± 1.0	59.6 ± 2.0
Fat	20.3 ± 1.3	20.8 ± 0.7
Protein	13.1 ± 0.7	12.8 ± 0.8

Results expressed as men ± SEM of 7 rats in each group

pressed in nanomoles of nitrophenol formed per microgram of muscle protein [29].

Statistical analysis

The results were expressed as mean ± SEM for the number of rats (n) indicated. When working with soleus muscle strips, n refers to the number of strips used. The data were analyzed by Student's unpaired t test and P values <0.05 were considered to indicate significant difference.

Results

After 8 weeks of diet treatment, there were no differences between casein and *spirulina* fed rats in body weight, body length, food intake (Table 2) and carcass composition (Table 3). Serum total protein, albumin, glucose and FFA levels were also similar in the two groups (Table 4). Liver (Table 5) and soleus (Table 6) muscle weight, total protein and DNA contents and protein/DNA ration did not differ between the groups. The muscle cellular activity, as analyzed by the alkaline phosphatase activity in gastrocnemius muscle, was similar in both groups (C = 11.78 ± 1.02; S = 10.49 ± 0.69 nmol μg protein^{-1}). The protein degradation rates and the *chymotrypsin-like* activity (which represents the catalytic portion of the proteasome system) in soleus and gastrocnemius muscles, respectively, were similar in both groups (Fig. 1a, b) whereas the muscle protein synthesis rates (soleus) and myosin content (gastrocnemius) (the protein expression and the densitometric analysis) were significantly higher in *spirulina* fed rats compared to casein group (Fig. 2a, b, c, respectively).

Table 4 Serum protein (g/dl), albumin (g/dl) glucose (mg/dl) and free fatty acids (FFA, in μEq/l) concentrations at the end of the experiment

	Casein	*Spirulina*
Protein	7.3 ± 0.1	7.1 ± 0.2
Albumin	3.6 ± 0.3	3.4 ± 0.1
Glucose	119.5 ± 9.3	114.8 ± 9.1
FFA	490.0 ± 42.3	440.0 ± 26.9

Results expressed as men ± SEM of 7 rats in each group

Table 5 Liver weight (g/100 g of body weight) and total protein (g/100 g), DNA (g/100 g); protein to DNA ratio and total fat (g/100 g) contents at the end of the experiment

	Casein	*Spirulina*
Weight	3.6 ± 0.1	3.5 ± 0.1
Total protein content	2.9 ± 0.3	2.6 ± 0.1
DNA content	0.125 ± 0.01	0.113 ± 0.005
Protein/DNA	24.1 ± 2.6	23.2 ± 2.1
Total fat	6.7 ± 0.5	6.6 ± 0.3

Results expressed as men ± SEM of 7 rats in each group

Discussion

The nutritional status of the animals fed casein or *spirulina* was analyzed by measuring the body weight and length and serum total protein and albumin levels, which are the traditional nutritional assessment indices for human subjects [35] and also frequently used in animal studies [12, 13, 33]. The present study showed that feeding the rats a *spirulina* protein diet from weaning until adult age leaded to a similar body weight and length gain as the control group. Equally, these findings agree with previous observations that *spirulina* as the sole protein source in the diet allows normal growth in rats [6, 7, 36, 45]. The food intake of the animals on the *spirulina* diet was slightly lower compared to the casein diet; however, this difference was not significant. A similar observation was also noted by Tranquille et al. [45] and Chen and Pan [6] in

Table 6 Soleus muscle weight (g/100 g of body weight); total protein (g/100 g) and DNA (g/100 g) contents; and protein to DNA ratio at the end of the experiment

	Casein	*Spirulina*
Weight	0.053 ± 0.003	0.054 ± 0.002
Total protein content	2.9 ± 0.1	2.7 ± 0.1
DNA content	0.084 ± 0.005	0.074 ± 0.005
Protein/DNA	34.4 ± 2.5	33.9 ± 3.9

Results expressed as men ± SEM of 7 rats in each group

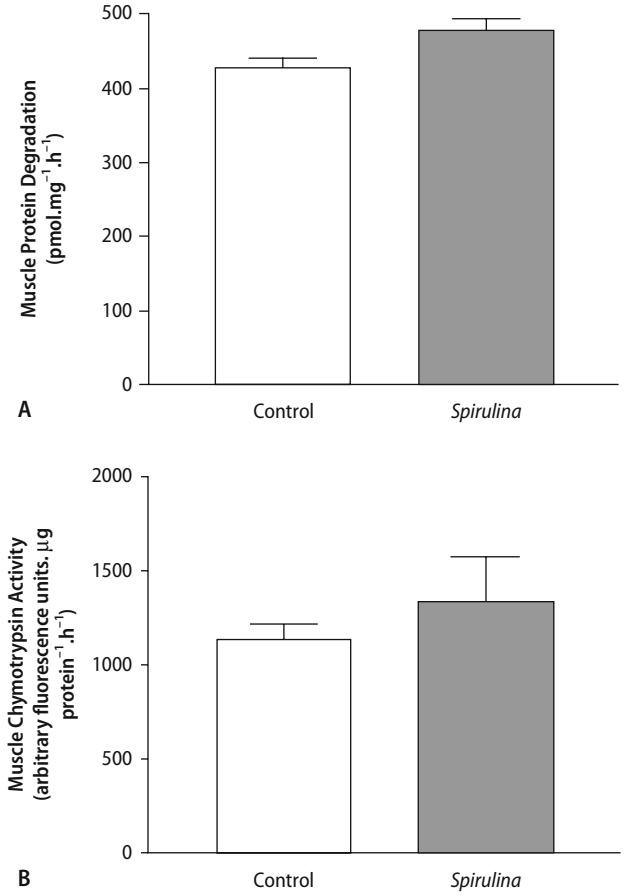

Fig. 1 Effects of *spirulina* diet on soleus muscle protein degradation (pmol/mg^{-1} h^{-1}) (**a**) and the gastrocnemius muscle *chymotrypsin-like* activity (arbitrary fluorescence units, µg protein^{-1} h^{-1}) (**b**) in weaning rats fed with casein (**c**) and *spirulina* diet (*S*), during 60 days. The results were expressed as mean ± SEM of 13 muscle strips in each group. *Significantly different ($P < 0.05$, *t* test) from casein group

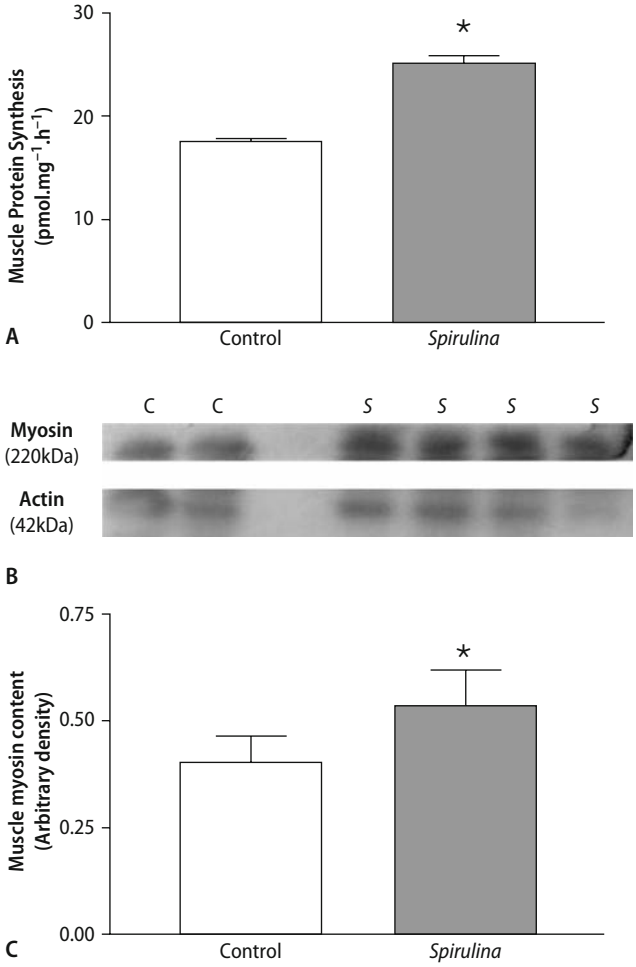

Fig. 2 Effects of *spirulina* diet on soleus muscle protein synthesis (pmol/mg^{-1} h^{-1}) (**a**) and the western blot image of the myosin and actin of the gastrocnemius muscle (**b**) and densitometry analysis (arbitrary densitometric units) (**c**) in weaning rats fed with casein (*C*) and *spirulina* diet (*S*), during 60 days. Arbitrary densitometric values of myosin expression are analyzed for eight individual western blotting. Actin was used as a loading control, and there was no difference between the groups. The results were expressed as mean ± SEM of 13 muscle samples from each group. *Significantly different ($P < 0.05$, *t* test) from casein group

their feeding studies. Serum proteins, especially serum albumin levels, are among the most extensively studied physiological markers. Several studies have demonstrated that a low serum albumin is associated with medical complications [20], including protein malnutrition [44]. No differences were observed in total protein and albumin levels between casein and *spirulina* rats. In addition, *spirulina* fed rats did not show other characteristics commonly found in infantile [44] and animal [33] protein malnutrition as hypoglycemia, fatty liver and elevated serum FFA concentrations. Together, these results suggest that feeding *spirulina* as the sole protein source in the diet did not induce signs of protein malnutrition in the rats.

According to Winick et al. [47], the total number of cells in an organ can be estimated, for practical purposes, by determining total organ DNA content and the cell size can be estimated by calculating the weight/DNA ratio or the protein/DNA ratio [47]. In rats, organ weight and protein continue to increase (hypertrophy) until about 100 days of age. By contrast, DNA (hyperplasia) reaches a maximum before this in most organs [47]. In skeletal muscle fiber, DNA reaches a maximum at about 90 days of age whereas weight and protein increment continues to approximately 140 days. Nutritional alterations during the period of hyperplasic and/or hypertrophic growth may result in altered number and/or size of cells in this tissue. According to the same authors [47], the additional measurement of total muscle mass may give further important information regarding the effects of the nutritional stress on muscle growth. Taken

together, our observations on muscle weight and protein and DNA contents indicate that *spirulina* as the sole dietary protein source did not impair neither hyperplasic nor hypertrophic growth in skeletal muscle in the young rat.

To further analyze the consequences of long term of *spirulina* protein ingestion on muscle protein we evaluated, in vitro, protein synthesis and degradation rates in the soleus muscle as well as myosin expression and chymotrypsin-like activity in the gastrocnemius muscle.

Incubated soleus muscle strips showed negative protein balance, even in presence of insulin, as demonstrated previously in incubated mouse extensor digitorium longus muscle [EDL] [43]. Despite of this fact, incubated EDL was considered a sensitive preparation to in vivo alterations of protein metabolism by nutrients [43]. It was demonstrated that the cutting the soleus muscle significantly increased protein degradation rates compared to uncut counterparts [38]. However, a single cut, one-half way through the soleus muscle, procedure adopted in the present study, did not significantly alter these rates. At least two cuts through the muscle were necessary to significantly increase the rates of protein degradation [38]. Oxygen diffusion may be improved in incubated muscles by increasing the resting tension during the incubation, which creates a thinner muscle bundle, favoring muscle anabolism. In fact, the protein balance was improved in some studies by increasing the resting tension in the muscle during incubation [2] but not in others [14]. In previous studies performed in our laboratory, where aspects of glucose metabolism in the incubated soleus muscle were evaluated, the results were not altered by the degree of tension to which the muscles were submitted during incubation [42]. On the other hand, others [38] demonstrated an increase in high-energy phosphates in the soleus muscles with the increase of the degree of tension during the incubation.

Protein degradation rates in incubated soleus muscle were similar in groups and the *chymotryspisn-like* activity, pertaining to the catalytic ubiquitin-proteasome system pathway, the main pathway for protein degradation [9], evaluated in the gastrocnemius muscle, did not show any difference between the groups. In this sense, these in vitro results suggested *spirulina* had no spoil effect on muscle proteolysis, as expected in protein-malnourished or in cachetic states. On the other hand, the protein synthesis rates in the soleus muscle were higher in *spirulina* fed rats, as well as the myosin content in the gastrocnemius muscle.

Protein synthesis and degradation assays were performed in vitro in presence of insulin, a hormone which participates in the stimulation of muscle protein synthesis, especially in young growing animals [17]. It was demonstrated that the dietary protein level affects insulin sensitivity to glucose in the rat skeletal muscle as a result of alterations in the early steps of the signal transduction pathway, including the insulin receptor (IR) levels; the lower the dietary protein content, the greater the insulin sensitivity to glucose [25]. No evaluations were made on the effects of dietary protein quality on muscle insulin sensitivity to glucose or other nutrients. Amino acids also play an important role in regulating muscle protein synthesis [11] and this modulation may be affected by dietary protein quantity or quality. It may be hypothesized that the increase in the muscle protein synthesis and myosin expression observed in the *spirulina* fed rats might be a consequence of an alteration in the signaling cascades responsible for the insulin and/or amino acid-induced stimulation of protein synthesis in skeletal muscle, since the *spirulina* diet composition has slightly higher branched-chain amino acid amount than casein diet composition. In this sense, an interesting step to be investigated is the role of mTOR pathway in mediating the observed effects.

It is known that fatty acids can affect muscle protein synthesis. In an interesting study [23], the effects of elevated circulating lipids on protein synthesis of chronically catheterized conscious rats were verified. The short-term elevation of plasma FFAs by a 5-h infusion of heparin plus Intralipid decreased muscle protein synthesis by approximately 25% under basal conditions. Therefore, another aspect that deserves further investigation is the effect of the free fatty acids contained in *spirulina* on muscle protein metabolism.

The results of the present study suggest certain caution about the nutritional value and safety of *spirulina* as protein source. The nutritional value of this protein is demonstrated by the adequate body growth and the absence of biochemical abnormalities in the tissues of the rats fed the experimental diet. However, there was one finding indicative of alteration in muscle protein: the increased protein synthesis observed in isolated soleus muscle.

An important question emerged from the results of the present study: if protein synthesis rates are higher in the *spirulina* fed group, without alteration in degradation rates, there should be expected differences in body growth and weight between the *spirulina* and the casein groups, which were not observed. Although the total carcass protein content was also similar between the two groups, in previous studies we also showed increase in protein synthesis and myosin content in response to branched-chain amino acids induced cell signaling. This point worth, to be evaluated in nearly future. Unfortunately, for the present work the nitrogen balance was not determined, which might be

of importance in evaluating the biological value of dietary *spirulina* protein. Even though, the present results showed particular effect of *spirulina* diet on protein synthesis. Further studies are now underway in our laboratory to determine and clarify the mechanisms involved in the effects of dietary *spirulina* protein on skeletal muscle protein.

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