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Biological evaluation of casein and *Spirulina* in rats and encapsulation of these protein sources in liposomes

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Abstract

The work aims to study the effect of nutritional diets in rats fed casein and *Spirulina*, as well nanoencapsular via liposomes these protein sources for possible applications in food formulations . Assay was performed to evaluate the effect of these sources of protein in weight gain of the animals. We used 12 female rats, weanling (21 days), Wistar / UFPEL, in a total of 15 days, with 4 days of adaptation , initially weighing between 42.66 and 68 g . The animals were weighed, the adaptation period and the end of the experiment to determine the weight gain and feed efficiency ratio. After the biological evaluation was carried out the encapsulation process of casein and *Spirulina* by the formation of liposomes using the methodology of hydration of the lipid film , after undergoing different treatments for their preparation as sonication at 60°C for 30 min. and homogenizing (Ultra - Turrax) at 10.000 rpm for 15 min. To determine the average size and morphology of the particles we used the technique of dynamic light scattering and scanning electron microscopy, respectively. It is concluded that diets based on casein and *Spirulina* did not differ significantly in total food intake and weight gain of the animals, with coefficients of feed efficiency very close, with this, the *Spirulina* strain LEB 18 micrometer in size was efficient in growth, not interfering in the metabolism and presenting similar to the diet containing casein (C).

Introduction

Spirulina microalgae is a source of protein and fatty acids that make them important as a food supplement (Bezerra, 2006), but also has absorbable iron, and has high levels of vitamins and other minerals , phenolic compounds, phycocyanin, gamma-linolenic acid and other essential fatty acids (Belay *et al*., 1993; Von der Weid *et al*., 2000). *Spirulina* also classified as GRAS (Generally Recognized as Safe) by the FDA (Food and Drug Administration), which ensures its use as a food without risks to health, with this in Brazil , their consumption is limited to 1.6 g per day (FDA , 2003).

Due to its high nutritional value and excellent functionality, together with the low cost of production, casein appears as an ingredient in first-line enrichment and production of protein foods for specific purposes. It has been used in the production of meat products, dairy products, baked goods and chocolates (Alvim *et al*., 2002). Bovine casein can be obtained in large quantities by technological processes at low cost, both by acid precipitation (isoelectric casein) or by enzymatic coagulation (Sgarbieri, 1996). Because of the growing industry interest in the production of whey proteins, there is a very large amount of casein in the market (Alvim *et al*., 2002).

With respect to encapsulation, their main purpose is to protect a sensitive substance in the capsule or wall, physically isolating the ingredient of the environment (Assis *et al*., 2012). Applications for this technique are the increased feed since the encapsulated materials can be protected from heat, moisture or other extreme conditions, thus increasing its stability (Gibbs *et al*., 1999) industry. Nanotechnology has had a huge impact on the food sector (Huang *et al*., 1999). The main nanostructures used for encapsulation of active substances are liposomes (Lasic, 1993).

Liposomes, or phospholipid vesicles, are spherical structures composed of lipid bilayers that encapsulate part of the environment in which they find themselves. Are predominantly formed by amphiphilic insoluble molecules in water, when in aqueous environments form colloidal dispersions (Lasic, 1993). Liposomes are one of the alternatives for the encapsulation of food ingredients, presenting some advantages, being one of the oldest techniques and cheap for encapsulation (Lasic, 1993). An important feature of encapsulation in liposomes, where they can easily allow control the release of encapsulated compounds, depending on the conditions of temperature, pH and

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presence of ions to which food is subjected (Lasic, 1993).

Liposomes have been applied in the manufacture of cheeses and their use in the preparation of food emulsions. Several developments include encapsulation of food in the areas of controlled release, transport materials, methods of preparation and immobilization sweetener (Gibbs *et al*., 1999). The casein and *Spirulina* were used in this study for *in vivo* studies of their potential, and fit as core encapsulation process. The advantages of encapsulating *Spirulina* aim and casein compounds of masking undesirable flavor, delaying changes that may result in loss of flavor, color change or loss of nutritional value when the application in formulations, and also permit release directly on the body (Azeredo, 2005). The objective was to verify the weight gain and feed efficiency ratio of Wistar rats according to the two different protein sources consumed casein and *Spirulina*, as well as these protein sources were used in the encapsulation process as core.

Materials and Methods

Obtaining protein sources and wall material

The biomass of *Spirulina* provided by the Laboratory of Biochemical Engineering, FURG (LEB-18 strain), isolated pond hose, RS, Brazil (Morais and Costa, 2008) supplemented with 20% medium Zarrouk (Costa *et al*., 2004). The casein and soy lecithin were purchased in powder form in the local market of the city of Pelotas.

Preparation of biomass

The biomass of *Spirulina* LEB-18 crushed in a ball mill (Model Q298-2) and sieved in sieve shaker, reaching a particle size of 200 μm. Being packed in vacuum packaging high-density polyethylene (HDPE) with a capacity of 500 g, and stored under refrigeration at a temperature of ± 5°C until the time of analysis.

Preparation of diets

Diets were formulated according to the recommendations of Reeves *et al*. (1993) and prepared with 12% protein to fit the methodology used (Table 1).

Bioassay

The bioassay was conducted according to Reeves *et al*. (1993), with animals obtained from the Central Animal Laboratory of the Federal University of Pelotas, after approval of the project (no. 23110000978 / 2010-91) Specified by the ethics committee of the same university , all experimental procedures followed the rules of the Brazilian College of Animal Experimentation (COBEA, 1991) .

Table 1. Formulation of the casein control diet (C), and experimental diets *Spirulina* micrometer (Sm)

Ingredient	Diet ($g.Kg^{-1}$)	
	C	*Sm*
Spirulina micrometric	–	200
Casein	200	-
Soybean oil	70	70
Mineral mixture	35	35
Vitamin mix	10	10
L-cistine	3.0	3.0
Choline bitartrate	2.5	2.5
Saccharose	100	100
Wheat fiber	50	50
Corn starch	529.5	529.5

Twelve female rats, weanling (21 days) of Wistar strain / UFPel an initial weight between 42.66 and 68 g, were randomly assigned in blocks of 6 rats per diet , called control (casein-C) and *Spirulina* micrometric (Sm). The research was conducted at the Laboratory of Animal Research Center of Chemical, Pharmaceutical Sciences and Food, UFPel , and lasted 15 days and 4 days of adaptation to environmental conditions, and 11 of the experiment. The rats were individually housed in metabolic cages received daily diet and water ad libitum. The biological assay occurred in a temperature and humidity on the 22-24ºC and 65-75%, respectively, and dark / 12 h light cycle tracks. On the 15th day the rats were fasted for 12 h, were euthanized by decapitation (guillotine). The animals were weighed at the adjustment period in the beginning, the middle and end of the experiment to determine the weight gain. Data for feed intake, weight and others were recorded in spreadsheets to compare the diets fed.

Proximate composition

The proximate composition of casein and *Spirulina*, these diets containing protein and its encapsulated form sources in accordance with analytical standards, Instituto Adolfo Lutz (IAL, 2008). The conversion factor used for crude protein was 6.25. The carbohydrate content was performed by difference.

Preparation of liposomes and lyophilization of the encapsulated material

The encapsulation process followed the methodology of hydration of the lipid film, according Malheiros *et al*. (2010) and Machado *et al*. (2014), the source of lipids used for the preparation of liposomes left of phosphatidylcholine purified from soybean lecithin. First, 2 g of phosphatidylcholine

Table 2. Composition of proximal protein sources in nature, diets and liposomes (g%)

	Determinations				
	Humidity	Grays	Protein crude	Lipids	Carbohydrates
Protein Sources *in natura* (g%)					
Casein	10.51±1.64[b]	2.02±0.02 [b]	77.33±1.01[a]	0.30±0.03 [b]	9.84±0.00[b]
Spirulina	11.95 ±0.09[b]	10.88±0.08[a]	60.36 ±6.66[a]	3.05 ±0.11[a]	13.76±0.00[b]
Diets					
Casein	9.90±0.004[b]	2.89±0.04[b]	16.68±0.11[a]	7.41 ±0.11[b]	63.12±0.00[a]
Spirulina	10.40± 0.015[a]	3.67±0.32[a]	13.61±0.67[b]	7.38±0.09[b]	64.94±0.00[a]
Liposomes					
Casein	18.01±7.41[a]	22.93±2.63[a]	2.75±0.31[b]	19.31±7.55[a]	37.00±0.11[a]
Spirulina	16.12±6.63[a]	30.95 ±0.15[a]	2.08 ±0.48[b]	18.95±2.95[a]	31.90±0.00[a]

Different letters in the same column indicate significant differences by Tukey test ($p < 0.05$). Carbohydrates = 100 - (% humidity + % Ash + % Protein +% lipids). *Spirulina*(*Spirulina* micrometric)

was dissolved in 100 mL of chloroform in a round bottom flask, after its complete dispersion, the organic solvent was removed on a rotary evaporator until a lipid film was observed deposited on the walls of the flask. Traces of organic solvent were removed by storing the flask for 18h in vacuum desiccator. The resulting lipid film was dispersed by adding 20 ml of phosphate buffer pH 7.0, containing 0.2 M, 2 g of casein and *Spirulina* dissolved in each flask. The mixture in the flask was subjected to over the phase transition temperature (60ºC). After the liposomes have been subjected to different treatments sonication using ultrasonic bath (40 kHz, 700 Unique USC) at 60°C for 30 min and homogenized (Ultra -Turrax) at 10.000 rpm for 15 min. Soon after, there was the lyophilization of the material encapsulated for better conservation and use in foods, as well as for analysis in a scanning electron microscope.

Average particle size

To determine the average particle size was employed the technique of dynamic light scattering through the apparatus at a wavelength of 632.8 n, model Spectra -Physics 127 coupled to a bi -200M version 2.0 goniometer and correlator digital BI - 9000AT the Brookheaven Instruments . The liposomes were filtered through filters of 0.45 microns were used and two drops of sample dissolved in 8 ml of phosphate buffer pH 7.0, 0.2 M for the analyzes (Bruce *et al*., 2000).

Scanning electron microscopy

To determine the scanning electron microscopy, the liposomes were freeze-dried by the method of dehydration by sublimation of frozen at -80ºC for 48h product through the benchtop lyophilizer model FD 5505. Subsequently resuspended in methanol, treated in ultrasonic bath for 20 min and deposited on a silicon substrate. The substrates were attached to the sample holder of the microscope using a carbon ribbon and is then metallized with gold for 3 min and 5 mA of current, in equipment Sanyu Electron Quick Coater Model SC- 701. The images were obtained on a microscope Shimadzu SSX - 550 model, which has vacuum and coupled EDS (Li *et al*., 2010).

Statistical analysis

The results were analyzed using Statistica software , version 7.0 (2004). Analysis of variance (ANOVA), Tukey test ($p < 0.05$) for comparison of mean values of samples was performed .

Results and Discussion

The result of the chemical composition of sources of protein in nature, and liposomes diets are shown in Table 2. In Table 2, the protein sources evaluated statistically different in ash content and lipids, but the values are in agreement with the FDA (2003) for microalgae. The diets differed significantly ($p \leq 0.05$) from each other in relation to the evaluated parameters. The ash content of *Spirulina* diet was significantly higher ($p \leq 0.05$) when casein, indicating the intrinsic contribution of *Spirulina*.

According to Donato *et al*. (2010), in search of a source of protein and good quality fast playback, the alga *Spirulina platensis* is an alternative of this nutrient, compared with a protein of high biological value as casein. All parameters evaluated for the *Spirulina* and casein both in liposomes differed statistically, as the moisture content of 16.12% and 18.01% respectively, these being composed of an aqueous solution, called phosphate buffer pH 7.0. The high lipid content of the liposomes of casein and *Spirulina* refers to the composition of the lipid film, consisting of partially purified soybean

Table 3. Initial and Final Weights, weight gain, total food intake and feed efficiency ratio of rats fed a control diet, and the base of *Spirulina*

Parameters assessed	Caseín (control)	*Spirulina*
Initial weight (g)	54.00 ± 8.58[a]	51.00 ±10.33[a]
Final weight (g)	109.20 ± 8.32[a]	96.33 ±10.91[a]
Total weight gain(g)	59.66±10.54[a]	45.33±8.38[a]
Food intake (g)	108.42 ± 14.21[a]	104.15±0.56[a]
CEA*	0.509[a]	0.435[a]

Values correspond to the average estimated standard deviation (n = 6) (p <0.05); Different letters in the same row indicate statistical difference by Tukey test (α < 0.05). CEA * : Coefficient of Feed Efficiency = weight gain / food intake. *Spirulina* (*Spirulina* micrometric)

lecithin. The amount of ash in the samples found in *Spirulina* encapsulated casein is probably due to the phosphate buffer used to assist in homogenizing with both the liposome. The low protein content found for both protein sources, refers to the process of homogenisation through ultrasonic bath with stringent shock , this is due to high power and low frequency ultrasound , usually used to change the properties of a material which denatures (Santos *et al*., 2010) protein.

Table 3 presents data related to the coefficient of feed efficiency of rats *(Rattus norvegicus)* strain Wistar / UFPEL fed diets with casein and *Spirulina*. The final weight of pigs fed diets based on casein and *Spirulina* do not differ statistically. It was found that the total weight gain between the groups showed no significant difference ($p < 0.05$). According Nagaoka *et al*. (2005) comparing animals fed diets casein and *Spirulina*, found no significant difference between the experimental groups with respect to weight gain. Rogatto *et al.* (2004) obtained 0.21 CEA, in his study with a diet with 17% of the total replacement *Spirulina* protein control diet (casein) in young male Wistar rats for five weeks. The results for CEA in this study demonstrated that the protein diet with *Spirulina* was biologically as well used as the standard protein casein (recommended for the study group), and that the diet was nutritionally well balanced.

According to Table 4, the values of phosphorus and calcium did not differ statistically analyzed the diets and are close to those obtained by Marco (2008) in his study of the bioavailability of nutrients in multimixtures. Santos *et al*. (2004) found values of total protein of 5.53 g/dL for the casein diet and 4.88 g/dL to a diet supplemented with childcare multimixture, similar to the values found in this study. The levels found for Iron , did not differ significantly in this study being higher than those found by Moreira (2010) in their research on *Spirulina* as a protein source in the nutritional recovery of Wistar

Table 4. Responses of biochemical serum levels of Wistar rats

Diet	P(mg/dL)	Ca(mg/dL)	P.T.(g/dL)	Fe(µg/dL)	C.T.(mg/dL)
Casein	10.34±1.00[a]	10.02±0.33[a]	5.26±0.23[a]	140.00±51.13[a]	48.96±16.37[b]
Spirulina Micrométric	9.61±0.60[a]	9.95±0.34[a]	4.85±0.13[a]	181.50±20.60[a]	80.00±17.87[a]

P: Phosphorus; Calcium Ca; (PT): Total Protein; Fe: Iron, CT: Total Cholesterol. *Spirulina* (*Spirulina* micrometric)

rats (93 mg/dL for Casein and varied diets with *Spirulina* 79-116 mg/dL). According to the Vivarium of the Faculty of Medicine, University of São Paulo, rats *(Rattus norvegicus)* adult Wistar must submit the iron content in the range of 154 to 279 µg/dL (USP, 2010, 2010). These data are confirmed by the Saints; Madruga and Bion (2004), who found 254 µg/dL iron in rats subjected to protein recovery with casein diet for 28 days. Since Marcos (2008) elicited responses of total cholesterol of about 49.6, 64.5 to 70.2 mg/dL, and to control diet with casein obtained 70.5 mg/dL.

The Table 5 shows the results of average size and polydispersity of casein and *Spirulina* micrometer, and liposomes prepared using these sources as the core. Given the results shown in Table 5, it was observed that there was no statistical difference in the encapsulated samples compared with the control, relative to the average size. Compared to casein and pure *Spirulina*, it was found that casein and *Spirulina* had greater encapsulated particle diameter. Cafaggi *et al*. (2007) had a mean particle diameter of which varied from 180 nm to 350 nm nanoparticles made of chitosan or N-trimethyl chitosan and alginate complex is cisplatin. Have Gücer *et al*. (1993) assumed that benzalkonium chloride, due to their amphiphilic structure is incorporated into the bilayer structure, causing an increase in the diameter of the liposomes.

As Chorilli *et al.* (2007) obtained similar results to this study, observed that the presence of caffeine (CAF) increased the diameter of the liposomes, this result is possibly due to the fact that CAF, being a lipophilic active substance is inserted in the bilayer structure, causing an increase in the diameter of the liposomes. Cócera *et al.* (2003) and Lima and Castro (2006) in the analysis of liposomes composed of phosphatidylcholine and cholesterol observed a slight increase in the diameter of the vesicles formed with respect to only phosphatidylcholine (106.66 nm), due to better characteristics packaging bilayer promoted by cholesterol .

According to Jin *et al*. (2009), nanoencapsulation of lutein with hydroxypropylmethylcellulose nanocapsules with an average diameter ranging

Table 5. Average size and polydispersity of casein and *Spirulina* and the liposomes obtained

Analyses	Control*	Casein *in natura* (neat)	Liposome casein	*Spirulina* (neat)	Liposome *Spirulina*
Average size (nm)	203.07± 2.86^{d}	195.60± 4.41^{d}	263.78±3.82^{b}	215.00± 3.27^{c}	304.00± 4.81^{a}
Polydispersity index	0.194±0.05^{c}	0.429±0.06^{a}	0.444± 0.10^{a}	0.432± 0.08^{a}	0.287±0.02^{b}

* liposome without added protein source . Different letters in the same row indicate statistical difference by Tukey test ($\alpha < 0.05$). *Spirulina*: *Spirulina* micrometric

from 163 nm to 314 nm were obtained. Jhi-Joung *et al.* (2009) obtained lipid nanoparticles with different oils, which had an average diameter of the nanoparticles ranged between 180 and 200 nm. Assis *et al*. (2014), observe the average size of *Spirulina* extracts LEB-18 and Chlorella pyrenoidosa, 208.00 and 211.00 nm respectively (liposome containing methanolic extract of Chlorella and *Spirulina*) as well as 208.00 and 239.00 nm obtained for liposome containing ethanol extract of *Spirulina* and *Chlorella*. Regarding the polydispersity observed, it appears that the control sample and *Spirulina* encapsulated statistically different samples of pure casein, casein and encapsulated pure *Spirulina*. However, the results showed a low degree of polydispersity, indicating that the preparations are very homogenous with regard to size distribution . This result is in agreement with those found by Lima (2002), which states that SUV ("small unilamellar vesicles" or small unilamellar vesicles) present good homogeneity with respect to the size distribution. But many authors state that the nanoparticles size range from 1 to 100 nm (Azeredo, 2005).

For the preparation of liposomes, the choice of method of hydration of the lipid film was due to this being a simple and inexpensive process that does not require specialized equipment, and a very effective method in laboratory scale (Santos and Castanho, 2002). The following figures are shown micrographs of casein and *Spirulina* micrometer and their liposomes. By comparing the micrographs with particle size is observed that both samples had nanometer size. As Cacela and Hincha (2006) the advantage of freeze drying is an increased half-life of liposomal formulation, by its greater stability in dry state may be reconstituted with the solvent at the time of administration. According Shulkin *et al.* (1984) freeze-drying does not cause rupture of the liposomes nor significantly increases the leakage of encapsulated material. Moreover, storage in lyophilized form liposomes does not exert a

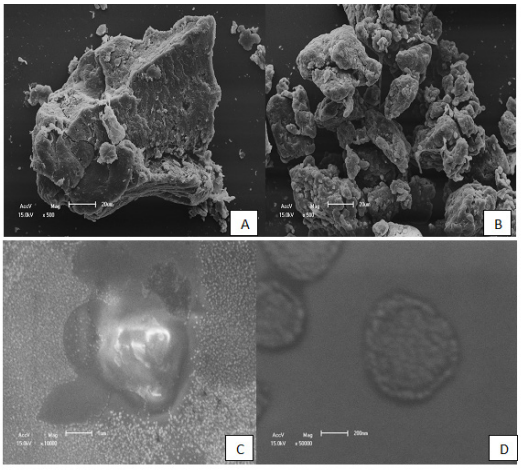

Figure 1. SEM micrographs of casein and *Spirulina* micrometer (A and B) and casein and *Spirulina* (C and D) encapsulated with soy lecithin purified using the methods of homogenization on Ultrasonic and Ultra - Turrax

significant effect on the particle size and amount of encapsulated substance (Chorilli *et al*., 2007). It can be seen in these liposomes showed that *Spirulina* cylindrical shape and apparently encapsulated. The liposomes had shown thicknesses of approximately 280 nm. Kruger (2009), in his work with chitosan nanoparticles were spherical and smooth morphology with a diameter of 20-80 nm by scanning electron microscopy. One of the advantages associated with the use of liposomes refers to preserve the nutritional quality of casein hydrolysates. Surveys reveal that encapsulation increases the stability of carotenoids, anthocyanins and betalains (Azeredo, 2005).

Conclusions

The results obtained in this work lead to the conclusion that the *Spirulina* -based diet compared to casein did not significantly affect the total food intake and weight gain of animals, promoting coefficient of feed efficiency very close to casein. It was observed that it was possible to prepare liposomes of casein and *Spirulina* on the nanometer scale using as crude lipid lecithin soy source, having this a viable opportunity in employment for encapsulating protein sources. Thus, nanotechnology can potentially be used to alter food more effectively and efficiently and can provide nutrients, proteins and antioxidants to the body.

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