Evaluation of nutritional value and safety of the green micro-algae *Chlorella vulgaris* treated with novel processing methods.

Pawel Janczyk, Carola Wolf*, Wolfgang Bernhard Souffrant
Research Institute for the Biology of Farm Animals, Research Unit of Nutritional Physiology, Wilhelm Stahl Allee 2, 18196 Dummerstorf, Germany
** Office for Veterinary and Foodstuff Examination of the Mecklenburg-Vorpommern (LVL), Thierfelderstr. 18*
18059 Rostock, Germany

Abstract

Three green micro-algae C. vulgaris (C1) preparations were investigated – singly spray dried; electroporated and spray dried, and ultrasonized and spray dried. Nitrogen-balance study was accomplished. Ultrasonized C. vulgaris was investigated for its safety in prolonged (33 days) feeding study. Apparent crude protein digestibility for spray dried C. vulgaris was 46.9 ± 12.7% (mean ± SD), for electroporated C. vulgaris 44.3 ± 7.5%, for ultrasonic treated algae 56.7 ±13.7%. PER was 1.4 ± 0.3; 1.0 ± 0.5; 2.1 ± 0.3, respectively. N-balance was 41.86 ± 32.8 mg; 31.3 ± 17.3 mg; and 66.7 ± 30.1 mg, respectively. The differences between nutritional parameters for ultrasonized and electroporated C. vulgaris were statistically significant (p<0.05 in HSD-Tukey test). There were no negative effects of algal feeding on blood biochemistry and hematology. Histology of gut, livers and kidneys revealed no changes in organ structure. The digestibility of C. vulgaris was enhanced by ultrasonic treatment and reduced by electroporating, thus the ultrasonication may be a helpful technological process in practical processing of the green micro-algae in food industry. Furthermore, feeding green micro-algae has shown no adverse effects and so there is no hazard combined with feeding the animals with the micro-algae.

Keywords *Chlorella vulgaris, nutritional value, digestibility, uric acid, allantoin, rats, safety*

Introduction

The nutritive value of outdoor or indoor cultured *C. vulgaris* is of interest to the food industry, especially in countries, where the weather conditions do not allow massive culture of higher plants. Nevertheless, first results of such studies were equivocal, depending upon the technological process used to treat the algal mass. Autoclaving, cooking or different drying processes not only helped to destroy the algal cell wall, but also led to denaturation of amino acids and/or active substances of chlorella cells (*Bock et al., 1967; Komaki et al., 1998; Lin, 1969; Lubitz, 1963; Saleh et*

al., 1985). The main problem to deal with, when micro algae are being fed, is the robust cell wall, which restricts the access of the gut enzymes to the cell components. In order to facilitate the host enzymes' access to the algal cell components, three different technological processes were applied in this study, i.e. single spray drying, and electroporation and ultrasonication, each followed by spray drying. The influence of these processes on the digestibility of the *C. vulgaris* was investigated in a study undertaken in rats.

Another potential problem connected to feeding green algae is that they contain large amounts of nucleic acids. Nucleic acids are degraded through a series of reaction which end in the formation of uric acid (in humans) and allantoin (in most mammals). Eventually uric acid overproduction can lead to gout or renal disease (*Komaki et al., 1998; Lubitz, 1963*). In our study we also investigated, if this really is a problem and if there are any negative side effects of feeding large amount of the micro-algae during prolonged period of time.

Materials and methods

Chlorella vulgaris

The unicellular green algae, *C. vulgaris* (C1) were obtained from the Institute for Cereal Processing Ltd. (IGV), Nuthetal, Germany, where it had been cultivated in a closed photobioreactor PBR 4000 using sunlight (*Pulz, 2000*). For feeding studies on animals, three different types of algae have been used – singly spray dried (SD-A), electroporated (ES-DA) and ultrasonic treated (US-DA) with following spray-drying.

Animals and experimental protocols

Two experiments on male Wistar rats obtained from Charles River Laboratories, Germany, were accomplished. In order to determine the nutritional value of protein of each differently processed micro-algae, 24 rats were housed individually at room temperature of 21.5 ± 1 °C, with light regime 12/12 hrs in metabolic cages (Tecniplast, Italy), that allowed separate and quantitative collection of uneaten food, urine and feces. Rats were divided into four groups, 6 animals each and received diets as summarized in Table 1. The group fed with casein was an internal control group. Two trials, each consisting of an adaptation (7 days) and balance period (7 days) were carried out. The mean body weight was 135 – 136 g and 140-147 g at the beginning of the first and second trial's adaptation period, respectively. Each group of rats was fed the respective diet in both adaptation and balance period according to their body weight (10 g per 100 g BW) and the diets contained 150 mg N in 10 g DM. Feed intake was recorded and urine and feces were collected every day during the balance period.

Table 1. Feed components

Group	CAS		S-DA		ES-DA		US-DA	
	1 trial	2 trial	1 trial	2 trial	1 trial	2 trial	1 trial	2 trial
N-free mixture	41.7 %	50.0%	41.67%	50.0%	41. 7 %	50%	41. 7 %	50.0%
Corn starch	48.5%	40.2%	37.33%	29.0%	36.9%	28.6%	37.1%	28.8%
Casein + 3% methionin	9.8%		9.8%		-	-	-	
Untreated *C. vulgaris*	-		21.0%		21.0%	-	-	
Electroporated *C. vulgaris*	-		-		21.4%	21.4%	-	
Ultrasonic treated *C. vulgaris*	-		-		-	21.2%	21.2%	

Hydrochloric acid was added to urine and feces samples for storing until analyzes were done. Samples were weighed and the N-content, as well as dry matter were analyzed. DM content in pure algae powder, feed, uneaten feed and fecal samples was accomplished using the Weender standard procedure. N-content in all samples was analyzed according to Dumas and was used for evaluation of N balance. Amino acids were determined using automated amino acid ion chromatography. Nucleic acids were isolated from 500-mg algae samples according to the procedure described by *Schoenhusen et al. (1988; 2004*). The preliminary purification for removal of chlorophyll was accomplished by 10 times extraction with ethanol (95%) and a mixture of ethanol and HCl (10%). N-balance was used for calculation of apparent crude protein (cPD) and amino acids digestibility (cAAD), net protein utilization (NPU) and protein efficiency ratio (PER). Endogenous and metabolic nitrogen were calculated using factors obtained in previous nitrogen-free experiments on rats, which had been done in the Institute. Using these data true crude protein digestibility (tPD) as well as biological value (BV) of crude protein was then calculated.

In order to evaluate the safety of the micro-algae feeding, 16 rats (2 groups of 8 animals), weighing app. 70 g were housed as described above for consecutive 33 days. The control group (CAS) was fed diet containing casein as the sole protein source. In the experimental group (US-DA), the sole protein source was ultrasonicated and spray-dried *C. vulgaris,* the food was prepared as described above. Water was given in nipple bottles *ad libitum*. Rats were weighed on day 0, 12, 19, 26, and 33. Feed and water intake as well as urine production were recorded daily throughout the whole experiment. Urine was also collected daily for further analyses.

Blood samples were taken via tail bleeding on day 0, 12, 19 and 26. Collected serum was stored frozen at-25°C till analyzed. On day 33 all rats were sacrificed. Anesthetized rats were exsanguinated by cardiac puncture. An aliquot of full blood and serum were collected. Organs (liver, heart, lungs, kidneys, testicles, stomach, small and large intestines and spleen) were cut out and weighed. The length of stomach (*curvatura major*), of the whole small intestine, caecum, and colon

were recorded. Kidneys, livers, and intestines were shock-frozen in liquid nitrogen and stored in -80°C until analyzed.

Allantoin concentration in urine and deproteinized serum was measured according to *Borchers (1977*). For uric acid determination in urine and serum enzymatic Trinder method (*Barthelmai et al., 1962; Trinder, 1969*) was applied. Serum biochemistry and blood hematology was done at the Office for Veterinary and Foodstuff Examination of the Mecklenburg-Vorpommern (LVL), Rostock, Germany, using standard procedures.

The frozen liver and kidney samples were sliced on cryotome, obtained slices were stained classically with Mayer's haematoxylin and eosin and observed under light microscope.

Statistical analysis

Statistics was done using Statistica Software version 6.0. ANOVA followed by Tukey HSD-Test determined statistical significance of the data by a p-value <0.05.

Results and discussion

The composition of *C. vulgaris* is shown in Table 1. The strain of *C. vulgaris* used in our experiments contained amino acids in amounts comparable to the amount determined by Saleh et al. (1985), who used *C. vulgaris* (no strain named) from outdoor culture, but much lower than that observed by *Komaki et al. (1998*) in *C. vulgaris*: K5 (no cultivation method mentioned). The differences in cultivation methods are the main source of differences in protein and minerals contents of one strain of algae (*Priestley, 1975; Spoehr, 1948*).

Table 2. Composition of spray dried and technologically modified Chlorella vulgaris

Component	SD-A	ES-DA	US-DA	Component	SD-A	ES-DA	US-DA
DM (%)	95.77	95.74	96.67	Tyrosine (TYR)	2.69	2.95	2.74
N (g/100g DM)	7.14	6.99	7.07	Phenylalanine (PHE)*	4.62	4.57	4.63
cP (g/100g DM)	44.65	43.72	44.16	Histidine (HIS)*	1.61	1.52	1.67
AA (g/16 g N) (total)	81.29	80.92	83.47	Lysine (LYS)*	5.01	4.72	5.01
Aspartate (ASP)	8.02	8.01	8.21	Arginine (ARG)*	5.88	7.42	5.89
Threonine (THR)*	3.80	4.11	4.15	Proline (PRO)	4.16	4.14	4.36
Serine (SER)	2.89	3.26	3.22	Cystine (CYS)	1.53	1.55	1.36
Glutamate (GLU)	9.63	8.49	9.63	Methionine (MET)*	2.06	1.84	2.11
Glycine (GLY)	5.04	4.94	5.19	Tryptophan (TRP)*	0.99	0.83	1.08
Alanine (ALA)	6.75	6.68	7.60	RNA (mg/g DM)	8.17	8.27	9.53
Valine (VAL)*	5.29	5.01	5.28	DNA (mg/g DM)	4.99	4.92	5.55
Isoleucine (ILE)*	3.78	3.61	3.76	RNA + DNA (g/100g DM)	1.32	1.32	1.51
Leucine (LEU)*	7.54	7.27	7.57				

* - essential amino acids in monogastric animals; SD-A – spray dried, ES-DA – electroporated, US-DA - ultrasonized algae

The nucleic acid content of the green algae used in our study was about 1.3-1.5 % of air DM. *Saleh et al. (1985)* found a nucleic acids content of 4.06 % DM, other authors reported a nucleic acids content of 5 – 6 % of DM. The ratio RNA/DNA in our study was 1.6 – 1.7 (RNA equaled to app. 63 % of nucleic acids), others reported the percentage of RNA to be of 88 - 95%, with the rest consisting of DNA (*Narasimha et al., 1982*). This difference was most probably due to method used in our laboratory for purification of the algae.

Animals were in good health throughout the whole experimental period in both experiments.

Normal growth was observed with the internal control group fed casein. Of the test treatments, the best growth was obtained in the group fed ultrasonic treated *C. vulgaris*, the least growth in the group fed electroporated algae (Table 3). The aPD of the spray dried algae was 46.9 ± 12.7 % (mean ± SD) which was similar to the electroporated *C. vulgaris* with 44.3 ± 7.5%. The aPD of the ultrasonic treated algae 56.7 ± 13.7% was higher than both the other treatments but only significantly higher than the electroporated algae. Nitrogen was retained in amounts of 41.9 ± 32.8 mg, 31.3 ± 17.3 mg and 66.7 ± 30.1 mg, in the rats fed untreated, electroporated and ultrasonized algae, respectively. The PER was 1.4%, 1.0%, 2.1%, respectively. BV for spray dried, electroporated and ultrasonic processed *C. vulgaris* was 93.0 ± 9.8%, 93.6 ± 10.0% and 100.7 ± 5.0%, respectively. The tPD was 53.4 ± 12.8%, 50.9 ± 7.5% and 63.3 ± 13.8%, respectively. The digestibility of amino acids, as shown in detail in Table 4, was the highest for ultrasonic treated algae and the lowest for electroporated *C. vulgaris*.

Physical or chemical treatment of algal cells is necessary for destroying the cell wall, but not every process will increase the nutritional value of green algae. *Komaki et al. (1998)* investigated the influence of high-pressure homogenization (HPH) on *C. vulgaris*: K-5 and found, that the digestibility of algal protein decreased because of the high pressure homogenization. Similarly in our experiments electroporation leads to less digestibility of algal protein. However, we also found, that ultrasonication enhances the nutritional value of the green algae, which is clearly confirmed by all the measured nutritional parameters.

Table 3. Weight gain and parameters of nutritional value of protein of C. vulgaris (n=12/group, mean ± SD)

Group	dWG (g)	aPD (%)	tPD (%)	NPU (%)	BV (%)	PPV (%)	PER
CAS	4.9 ± 0.9 ^{a}	85.2 ± 1.1 ^{a}	92.1 ± 1.1 ^{a}	98.4 ± 2.4 ^{a}	106.8 ± 2.6 ^{a}	64.8 ± 2.3 ^{a}	3.2 ± 0.6 ^{a}
S-DA	1.8 ± 0.5 ^{b}	54.8 ± 5.4 ^{b}	61.3 ± 5.5 ^{b}	58.2 ± 7.4 ^{bc}	94.7 ± 6.5 ^{b}	27.1 ± 6.8 ^{bc}	1.4 ± 0.3 ^{bc}
ES-DA	1.2 ± 0.7 ^{bc}	44.9 ± 2.8 ^{bc}	51.6 ± 2.7 ^{bc}	48.4 ± 5.3 ^{bc}	94.0 ± 9.5 ^{b}	16.7 ± 5.4 ^{bc}	1.0 ± 0.5 ^{bc}
US-DA	2.7 ± 0.5 ^{bd}	59.8 ± 4.4 ^{bd}	66.5 ± 4.4 ^{bd}	67.5 ± 6.0 ^{bd}	101.4 ± 3.0 ^{a}	35.7 ± 6.2 ^{bd}	2.1 ± 0.3 ^{bd}

$^{a-d}$ – data in one column marked with different letters differ significantly ($p<0.05$)

The results show that the cell content from ultrasonic treated *C. vulgaris* is easier accessible for digestive enzymes, which would suggest that the technology of ultrasonic treatment destructs algae walls better than electroporation. The cell rupture is also more intensive than in the spray-dried only (untreated) green algae. It may also be that electroporation not only destructs the cell wall, but also the molecules inside the cells, hence reducing the nutritional value of the algae. These observations have a great practical value for the use of the green algae as a food component and indicate the effects of the three technological processes on *C. vulgaris* preparations.

The daily water intake was similar in both groups for the first three weeks. Starting from day 20 – 21, rats from the group fed algae reached a maximal water intake of 15 ml/rat/day, in contrast to rats from casein group, which continued to drink more and were consuming app. 25 ml water/rat/day by the end of experiment (Fig. 1). This pattern was also observed for feed intake (Fig. 2). These findings correlated with animals weight gain (Fig. 3). Urine production (Fig. 4) during the whole experiment reflected the water intake and so the curve pattern looks similar to the one for water intake. During the first 3 days of the experiment, when rats adapted to the feed, uric acid and allantoin excretion in urine was similar in both groups.

Table 4. Apparent digestibility of amino acids (aAAD) (%, n = 12/group, mean ± SD)

Group / aAAD	CAS	S-DA	ES-DA	US-DA
ASP	82.45 ± 2.81 ^{a}	52.42 ± 12.30 ^{b}	45.80 ± 7.03 ^{bc}	61.52 ± 10.31 ^{bd}
THR*	84.93 ± 2.82 ^{a}	44.77 ± 14.06 ^{bc}	42.83 ± 6.71 ^{bc}	57.98 ± 11.23 ^{bd}
SER	74.65 ± 4.26 ^{a}	39.24 ± 18.13 ^{bc}	40.91 ± 8.30 ^{bc}	56.11 ± 13.68 ^{bd}
GLU	86.06 ± 2.16 ^{a}	55.42 ± 11.14 ^{bc}	44.13 ± 7.06 ^{bd}	62.36 ± 9.71 ^{bc}
GLY	73.18 ± 5.13 ^{a}	52.60 ± 11.25 ^{b}	44.98 ± 6.74 ^{bc}	62.03 ± 10.17 ^{bd}
ALA	75.31 ± 4.61 ^{a}	55.12 ± 10.17 ^{bc}	49.11 ± 6.66 ^{bc}	66.70 ± 8.42 ^{bd}
VAL*	87.08 ± 2.27 ^{a}	50.68 ± 10.79 ^{b}	42.02 ± 7.35 ^{bc}	59.25 ± 10.79 ^{bd}
ILE*	82.99 ± 2.81 ^{a}	44.80 ± 12.91 ^{b}	36.83 ± 8.34 ^{bc}	53.64 ± 12.47 ^{bd}
LEU*	91.88 ± 1.55 ^{a}	53.49 ± 10.80 ^{bc}	45.98 ± 6.20 ^{bc}	62.99 ± 10.12 ^{bd}
TYR	92.79 ± 2.98 ^{a}	35.34 ± 37.85 ^{b}	47.86 ± 10.66 ^{b}	55.23 ± 14.53 ^{b}
PHE*	92.22 ± 1.56 ^{a}	47.56 ± 11.65 ^{bc}	40.29 ± 7.23 ^{bc}	57.83 ± 10.81 ^{bd}
HIS*	93.75 ± 1.20 ^{a}	51.54 ± 14.20 ^{bc}	44.62 ± 8.70 ^{bc}	62.62 ± 11.53 ^{bd}
LYS*	91.06 ± 1.53 ^{a}	53.61 ± 10.51 ^{bc}	46.66 ± 7.01 ^{bc}	62.75 ± 9.31 ^{bd}
ARG*	89.98 ± 2.12 ^{a}	71.99 ± 7.10 ^{b}	74.20 ± 3.34 ^{b}	77.38 ± 5.96 ^{b}
PRO	94.93 ± 0.82 ^{a}	58.92 ± 10.30 ^{bc}	51.11 ± 6.02 ^{bc}	68.28 ± 8.50 ^{bd}
CYS	31.69 ± 16.41 ^{a}	45.68 ± 10.57	37.17 ± 6.57	47.96 ± 16.11 ^{b}
MET*	95.38 ± 0.83 ^{a}	62.10 ± 8.01 ^{bc}	55.23 ± 7.40 ^{bc}	71.61 ± 7.90 ^{bd}
TRP*	91.48 ± 2.23 ^{a}	77.63 ± 15.14	66.56 ± 28.26 ^{b}	82.24 ± 10.32
Total AA	87.72 ± 2.14 ^{a}	53.43 ± 11.70 ^{bc}	48.12 ± 6.33 ^{bc}	63.00 ± 9.90 ^{bd}

* - essential amino acids for monogastric animals

$^{a-d}$ – data in line marked with different letters differ significantly ($p<0.05$)

The elimination of these products (Figure 5 and 6) increased then in the group fed micro-algae and was significantly higher than in casein group for next two weeks. In the last 10 – 12 days of the experiment uric acid and allantoin elimination was more or less equal in both groups. The amount of eliminated uric acid held in between 3 and 13 µmol/day, allantoin between 280 and 500 µmol/day.

All measured values were comparable with findings of other researchers that applied the same analytical methods of allantoin determination (*Koguchi et al., 2002; 2003).*

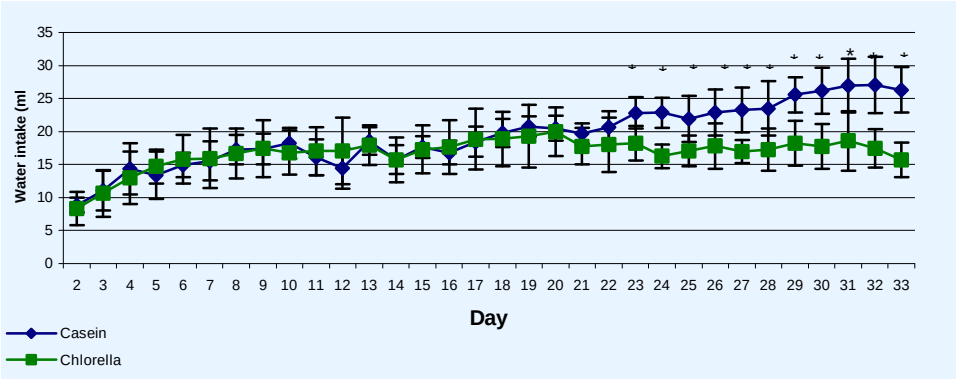

Figure 1. Daily water consumption during feeding rats with *C. vulgaris* and casein (n=8, mean ± SD). *-significant difference, p<0.05

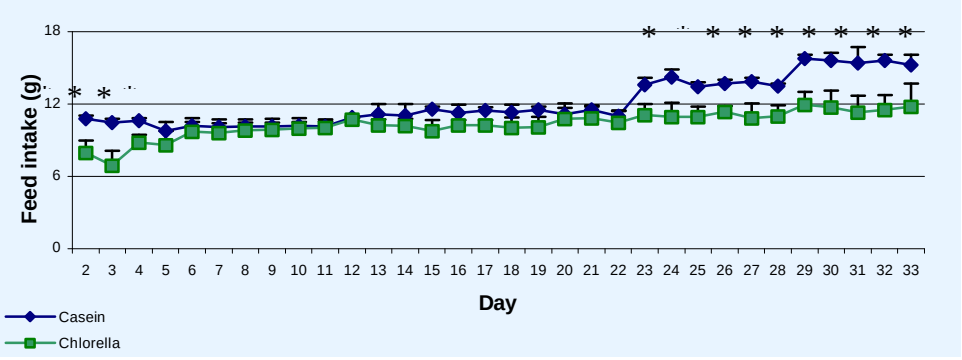

Figure 2. Daily feed intake during feeding rats with *C. vulgaris* and casein (n=8, mean ± SD). * - significant difference, p<0.05

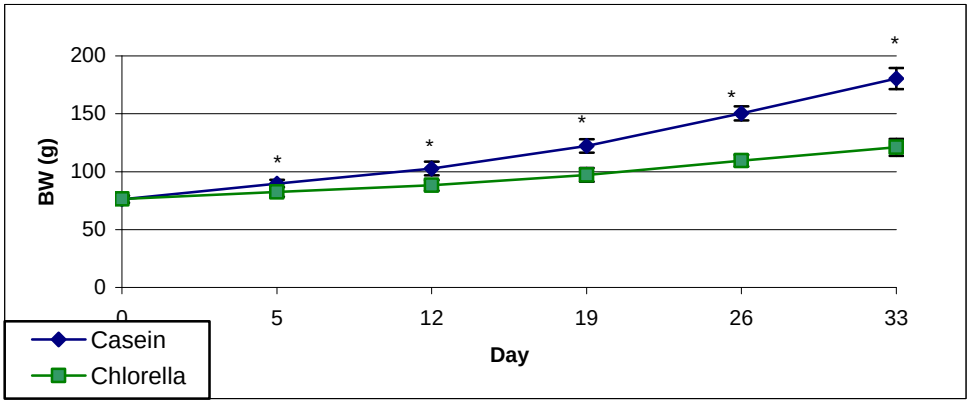

Figure 3. Body weight of rats feed with *C. vulgaris* and casein (n=8, mean ± SD). * significant difference, p<0.05

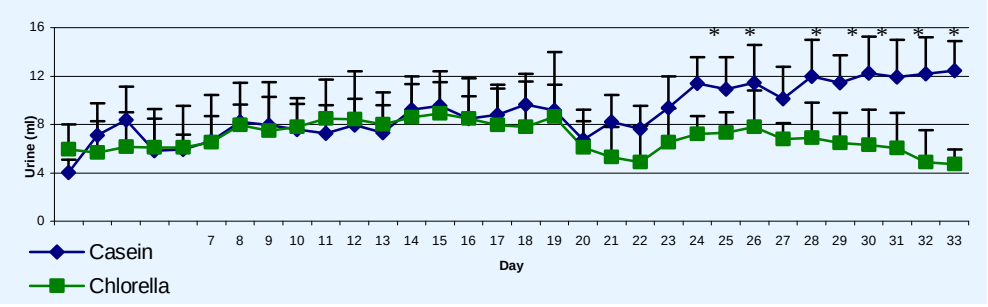

Figure 4. Daily urine production after casein vs. micro-algae feeding. * significant difference, p<0.05

Feeding large amount of nucleic acids may stimulate the enzymatic system at the very beginning, and after a while there might be a stop signal and the purines are not further metabolized in excess, or – on the other hand, microorganisms of the gastrointestinal tract may totally degrade the nucleic acids yet in the gut, before they are even absorbed (*Greife, 1986). As Koguchi et al. (2002; 2003*) showed dietary fiber influences RNA-derivatives absorption, where the electric charge in different pH or viscosity play crucial role. One can not exclude, that at least partially, the dietary fiber present in algal cells (first of all in the cell walls) may decrease amount of nucleic acids available for gut enzymes and further absorption, thus reducing uric acid and allantoin production and final excretion in urine.

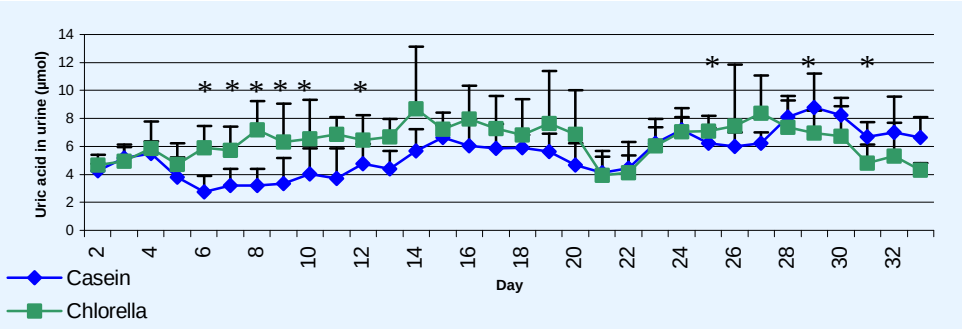

Figure 5. Daily excretion of uric acid in rats' urine during feeding with casein or ultrasonicated and spray-dried *C. vulgaris* (n=8/group; mean ± SD), * - significant difference between groups, p<0.05

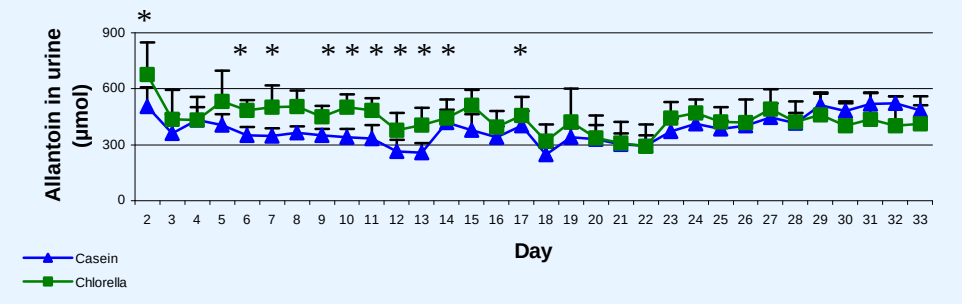

Figure 6. Daily excretion of allantoin in rats' urine during feeding with casein or ultrasonicated and spray-dried *C. vulgaris* (n=8/group; mean ± SD), * - significant difference between groups, p<0.05

The range of serum concentrations of uric acid measured in both groups was between 60 and 130 µmol/L (Fig. 7). The serum allantoin concentration remained stable in the range between 2 and 4 mmol/L (Fig. 8). The values of serum uric acid and allantoin concentration obtained in our study were comparable to findings of *Koguchi et al. (2002; 2003).*

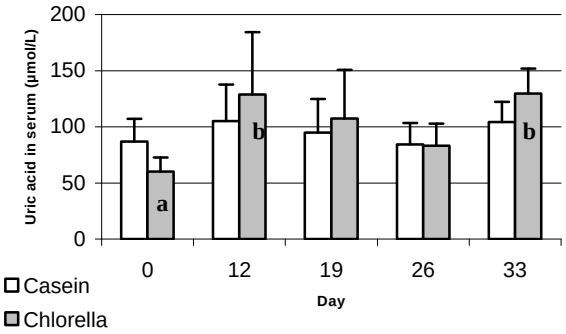

Figure 7. Serum uric acid concentration in rats fed casein or ultrasonicated and spray-dried *C. vulgaris* (n=8/group, mean ± SD), a, b – values shown in bars with different letter differ significantly, p<0.05

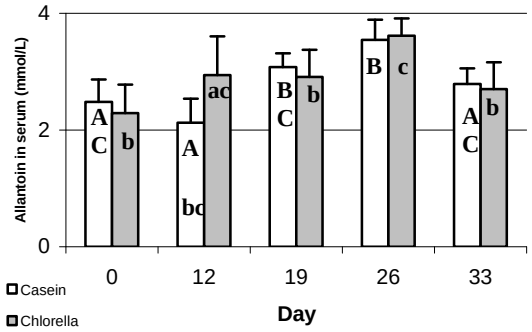

Figure 8. Serum allantoin concentration in rats fed casein or ultrasonicated and spray-dried *C. vulgaris* (n=8/group, mean + SD), A-B, a-c – values represented by bars marked with different letter differ significantly, p<0.05

Biochemical parameters were determined in serum collected from blood taken on day 12, 19, 26 and 33 of the experiment. Activities of alanine (ALAT) and aspartate aminotransferases (ASAT) were stable during the whole experiment and did not differ between groups. The alkaline phosphatase (ALP) activity in the group fed *C. vulgaris* was significantly lower than in casein group (Table 5). Serum total protein concentration did not differ significantly between groups and remained stable during the whole experiment. Urea and creatinine concentrations in rats' serum did not differ between groups. It should be noted that the activities of enzymes and concentrations of total protein and metabolites measured in the group fed with micro-algae did not exceed the ranges measured in the group fed casein and so they were within normal physiological ranges. The lower activity of ALP measured in serum of rats fed algal diet might have resulted from slower growth and thus

reduced activity of osteocytes that produce one of isoenzymes of ALP. Total serum protein was in both groups comparable. High activity of ASAT measured in rats' serum in both groups might have been caused by heart muscle injury during blood collection and partially due to methods used for estimation.

Table 5. Values of biochemical parameters measured in serum collected from rats fed with casein or ultrasonicated and spray-dried *C. vulgaris* in weekly intervals (mean ± SD; n=8/group)

Group	Day	ALAT (U/L)	ALP (U/L)	ASAT (U/L)	GLDH (U/L)	Total protein (g/L)	Urea (mmol/L)	Creatinine (µmol/L)
CAS	12	31 ± 4	632 ± 126 ^{ac}	166 ± 35 ^{a}	21 ± 4	52 ± 3 ^{a}	1.4 ± 0.6	42 ± 3 ^{a}
	19	30 ± 4	562 ± 85 ^{A}	159 ± 34 ^{a}	24 ± 5	54 ± 3 ^{a}	1.5 ± 0.9	47 ± 5 ^{ac}
	26	31 ± 2	559 ± 49 ^{c}	148 ± 29	24 ± 6	55 ± 2	1.2 ± 0.3	84 ± 16 ^{b}
	33	32 ± 8	576 ± 202	113 ± 17 ^{b}	16 ± 3	63 ± 12 ^{b}	1.3 ± 0.5	64 ± 15 ^{bc}
US-DA	12	32 ± 9	405 ± 60 ^{bc}	124 ± 33	24 ± 5 ^{a}	49 ± 2	2.3 ± 1.3	46 ± 10 ^{A}
	19	32 ± 7	353 ± 50 ^{B}	116 ± 20	25 ± 3 ^{a}	55 ± 4	2.3 ± 0.9	52 ± 6 ^{A}
	26	35 ± 4	370 ± 56 ^{abd}	137 ± 37	24 ± 8 ^{a}	57 ± 3	2.0 ± 0.8	74 ± 19 ^{B}
	33	31 ± 5	327 ± 40	120 ± 23	12 ± 3 ^{b}	55 ± 5	2.3 ± 1.1	65 ± 17

$^{a-d}$, $^{A-B}$– values in column marked with different letters of one height show statistical significance ($p<0.05$)

For evaluation of hematological parameters blood was collected from rats' hearts at the end of experiment. In 2 cases in casein group and in 3 cases in algae group blood samples clotted and analyses were impossible. None of the obtained values pointed to any disturbance of hemopoiesis and they all remained within physiological ranges (Table 6). The only observed changes were related to blood hemoglobin level and platelet count with MCV, but there was no decrease of the erythrocytes number which was the same in both groups or clinical signs of anemia. Thrombocytes count depends partially on the sampling technique, as micro clots can be generated already in the needle, what further results in underestimation of TBC. Moreover, automatic determination of erythrocyte volume gives exact MCV but this technique was not available. This could partially explain the obtained differences.

Nevertheless, these results can at least in part be due to sub-clinic development of Fe deficiency, what would be in disagreement with literature findings. *Kapoor & Mehta (1993)* reported very good development of iron stores in liver, kidneys and hearth in rats fed other micro-algae, *Spirulina*, that were comparable when rats were fed ferrous sulfate as iron source. The micro-algae used in our experiments contained about 172 mg/100g in fresh matter, what gave a daily intake of approximately 4.0 mg Fe from micro-algae/day/rat. Rats received additively mineral and vitamin mixture containing all minerals and vitamins necessary for proper rat's growth, so there could be no dietary Fe insufficiency. Also Matsuura et

al. (1991) reported very good influence of feed supplementation with 5% *Chlorella* on recovery from dietary-induced iron deficiency anemia in rats. The differences observed in our results are therefore probably, at least in part, due to methodological and laboratory errors.

Table 6. Thrombocytes count and hematological parameters of rats fed with casein or ultrasonicated and spray-dried *C. vulgaris* (mean ± SD)*

Group	Ht (%)	RBC (106/µL)	Hb (g/100mL)	MCV (10-15L)	MCH (10-9g)	MCHC (g/100 mL)	TBC (103/µL)	WBC (103/µL)	NI (%)	NM (%)	Lymph. (%)	Mon. (%)
CAS, n=6	34.7±2.0	5.7±0.4	11.7±0.7[a]	60.8±5.9	20.4±0.9[a]	33.8±2.9	1144±115[a]	2.8±0.6	2.8±2.3	32.5±21.0	61.2±21.9	5.2±3.2
US-DA, n=5	31.6±6.2	5.7±0.4	10.7±0.5[b]	55.8±13.6	18.7±1.1[b]	34.9±7.5	1672±261[b]	3.1±0.5	0.8±0.8	22.8±6.7	73.8±7.7	2.6±2.4

Hb – hemoglobin, Ht – hematocrit, Lymph. – lymphocytes, MCH – mean corpuscular hemoglobin (Hb/RBC), MCHC – mean corpuscular hemoglobin concentration (Hb/Ht), Mon. – monocytes, NI – immature neutrophils, NM – mature neutrophils, RBC – red blood cells, TBC – thrombocytes, WBC – white blood cells,

[a-b] – values marked with different letters differ statistically, $p<0.05$

*Count of basophils and eosinophils was 0 in both groups and therefore is omitted in the table

Most of the organs were heavier in rats from the CAS group, with exception of large intestine and spleen (Table 7). Significant differences were seen for almost all organ weights. When the recorded weights were compared with $BW^{0.75}$ (Table 7) and shown as a percentage ratio, fewer differences were seen and different levels of significance appeared. The major curvature of stomach, small intestine (measured from pylorus to ileo-caecal junction), caecum and colon were longer in the group fed ultrasonicated and spray-dried micro-algae, but the difference was significant only for caecum (Table 8).

Table 7. Weights of organs taken out from rats fed casein or ultrasonicated and spray-dried *C. vulgaris* for period of 33 days (n= 8/group, mean ± SD) – absolute (g) and with respect to metabolic weight (BW0.75) shown as a percentage (%)

Organ	CAS (g)	US-DA (g)	Value of p (accuracy 0.0001)	CAS (%)	US-DA (%)	Value of p (accuracy 0.0001)
Liver	6.54 ± 0.96 [a]	4.39 ± 0.46 [b]	0.0002	13.26 ± 1.49	12.02 ± 0.84	0.0647
Stomach	1.68 ± 0.24 [a]	1.09 ± 0.09 [b]	0.0001	3.41 ± 0.4 [a]	2.98 ± 0.18 [b]	0.0203
Small intestine	5.58 ± 0.26 [a]	5.03 ± 0.50 [b]	0.0197	11.36 ± 0.74 [a]	13.79 ± 1.11 [b]	0.0002
Large intestine	1.71 ± 0.15 [a]	1.90 ± 0.18 [b]	0.0356	3.49 ± 0.35 [a]	5.23 ± 0.61 [b]	0.0000
Caecum	0.91 ± 0.28	0.98 ± 0.20	0.6002	1.87 ± 0.6 [a]	2.69 ± 0.64 [b]	0.0183
Colon	0.80 ± 0.21	0.92 ± 0.20	0.2400	1.62 ± 0.4 [a]	2.53 ± 0.52 [b]	0.0018

Spleen	0.59 ± 0.05	0.63 ± 0.12	0.4615	1.20 ± 0.08 [a]	1.72 ± 0.32 [b]	0.0024
Lungs	1.00 ± 0.10 [a]	0.71 ± 0.06 [b]	0.0000	2.04 ± 0.15	1.95 ± 0.14	0.2687
Heart	0.66 ± 0.04	0.61 ± 0.07	0.1092	1.35 ± 0.08 [a]	1.67 ± 0.15 [b]	0.0002
Kidney – left	0.87 ± 0.12 [a]	0.68 ± 0.04 [b]	0.0036	1.76 ± 0.21	1.87 ± 0.06	0.1626
- right	0.91 ± 0.13 [a]	0.71 ± 0.06 [b]	0.0025	1.83 ± 0.2	1.94 ± 0.11	0.2303
Testicle – left	1.46 ± 0.14	1.20 ± 0.32	0.0550	2.98 ± 0.27	3.28 ± 0.85	0.3620
- right	1.48 ± 0.16	1.21 ± 0.33	0.0583	3.02 ± 0.34	3.31 ± 0.9	0.4106
Urinary bladder	0.20 ± 0.07 [a]	0.11 ± 0.04 [b]	0.0099	0.39 ± 0.13	0.29 ± 0.11	0.1082
Empty carcass	144.65±7.72 [a]	89.43 ± 6.11 [b]	0.0000	293.77±5.62 [a]	244.86±6.91 [b]	0.0000

[a,b] – values marked with different letters differ significantly ($p<0.05$)

Table 8. Length of parts of gastrointestinal tract taken out from rats fed casein or ultrasonicated and spray-dried *C. vulgaris* for period of 33 days (cm, n= 8/group, mean ± SD)

Group	Stomach (major curvature)	Small intestine	Caecum	Colon
CAS	6.7 ± 1.1	92.6 ± 8.1	7.6 ± 0.9 [a]	13.3 ± 2.2
US-DA	6.9 ± 0.9	99.3 ± 3.5	8.6 ± 0.8 [b]	14.3 ± 1.6

[a,b] – values marked with different letters differ significantly ($p<0.05$)

These findings suggest the intestinal tract must have adapted to lower digestibility of the feed or there is a component present in micro-algal cells that stimulated the intestinal growth. This would be in agreement with literature findings, where extract of *C. vulgaris* stimulated growth of bone marrow or spleen cells (*Justo et al., 2001; Konishi et al., 1990; Tanaka et al., 1986*) and a proposal glycoprotein has already been isolated (Noda et al., 1996). Changes in weight and length of stomach in CAS group were due to larger feed intake in the last 10 days of experiment. Histological investigation did reveal no visible differences between groups, whereas only general situation was considered. Livers of rats fed micro-algae were smaller, what was probably due to less lipid infiltration of these organs in those animals, what was observed by *Okuda et al. (1975)* and *Sano & Tanaka (1987*). In histological evaluation of liver slices no lipid infiltration was seen in livers from rats from casein group, and was neither observed in livers from rats fed the micro-algae, so findings of these authors could have not been confirmed. The spleens were heavier in rats from algal group, what could be a result of increased iron storage or increased hematopoiesis (*Konishi et al., 1985; 1990; Tanaka et al., 1984; 1986*). No changes between groups for weights of kidneys or testicles with respect to metabolic BW were noticed. No histological changes in renal structure were observed.

Our results are somehow in opposite to findings of other authors. *Venkataraman et al. (1979*) reported the relative weights of rats' organs (with respect to 100 g BW) after 12 weeks of feeding with 24 % or 36 % of micro-algae *Scenedesmus acutus* were

lower than in group fed casein, but in this case the rats from algal groups gained more weight as the rats from casein group over the longer experimental period.

Conclusions

The crude protein digestibility and biological value of green micro-algae *C. vulgaris* was enhanced by ultrasonication and reduced by electroporating, thus the ultrasonic technique may be a valuable procedure in algal processing in food industry. Prolonged feeding the green micro-algae to rats did show no adverse effects on serum biochemistry, blood hematology or histological structure of investigated organs and so no hazard connected with feeding the micro-algae could be seen.

References

Barthelmai, W., Czok, R., 1962. Enzymatische Bestimmungen der Glukose in Blut, Liquor und Harn. Klin. Wochenschr. 40, 585-589.

Bock, H.D., Wünsche, J., 1967. Untersuchungen über die Proteinqualität von zwei Grünalgenmehlen. Jahrbuch Tierernahrung Futterung 6, 544-548.

Borchers, R., 1977. Allantoin determination. Ann. Biochem. 79, 612-613.

Greife, H. A., (1986). Studies upon the sites of bacterial breakdown of dietary purines within the gastrointestinal tract of the rat. Z. Versuchstierkd. 28, 33-40.

Justo, G.Z., Silva, M.R., Queiroz, M.L.S., 2001. Effects of the green algae *Chlorella vulgaris* on the response of the host hematopoietic system to intraperitoneal Ehrlich ascites tumor transplantation in mice. Immunopharmacol. Immunotoxicol. 23, 119-132.

Kapoor, R. & Mehta, U., 1993. Iron status and growth of rats fed different dietary iron sources. Plant Foods Hum. Nutr. 44, 29-34.

Koguchi, T., Nakajima, H., Wada, M., Yamamoto, Y., Innami, S., Maekawa, A., Tadokoro, T., 2002. Dietary fiber suppresses elevations of uric acid and allantoin in serum and urine induced by dietary RNA and increases its excretion to feces in rats. J. Nutr. Sci. Vitaminol. 48, 184-193.

Koguchi, T., Nakajima, H., Koguchi, H., Wada, M., Yamamoto, Y., Innami, S., Maekawa, A., Tadokoro, T., 2003. Suppressive effect of viscous dietary fiber on elevations of uric acid in serum and urine induced by dietary RNA in rats is associated with strength of viscosity. Int. J. Vit. Nutr. Res. 73: 369-376.

Komaki, H., Yamashita, M., Niwa, Y., Tanaka, Y., Kamiya, N., Ando, Y., and Furuse, M., 1998. The effect of processing of *Chlorella vulgaris*: K-5 on in vitro and in vivo digestibility in rats. Anim. Feed Sci. Technol. 70, 363-366.

Konishi, F., Tanaka, K., Himeno, K., Taniguchi, K., Nomoto, K., 1985. Antitumor effect induced by a hot water extract of *Chlorella vulgaris* (CE): resistance to Meth-A tumor growth mediated by CE-induced polymorphonuclear leukocytes. Cancer Immunol. Immunother. 19, 73-78.

Konishi, F., Tanaka, H., Kumamoto, S., Hasegawa, T., Okuda, M., Yano, I., Yoshikai, Y., Nomoto, K., 1990. Enhanced resistance against Escherichia coli infection by subcutaneous administration of the hot-water extract of *Chlorella vulgaris* in cyclophosphamide-treated mice. Cancer Immunol. Immunother. 32, 1-7.

Lin, Y.Ch., 1969. The supplementary effect of algae on the nutritive value of soybean milk. J.F.M.A. 68, 15-21.

Lubitz, J.A., 1963. The protein quality, digestibility, and composition of algae, chlorella 71105. J. Food Sci. 28, 229-232.

Matsuura, E., Nemoto, T., Hozumi, H., Izumi, K., Saito, Y., Ishida, H., Fukimbara, T., Kawahara, H., 1991. Effect of chlorella on rats with iron deficient anemia. Kitasato Arch. Exp. Med. 64, 193-204.

Narasimha, D.L.R., Venkataraman, G.S., Duggal, S.K., and Eggum, B.O., 1982. Nutritional quality of the blue-green algae *Spirulina platensis* Geitler. J. Sci. Food Agric. 33, 456-460.

Noda, K., Ohno, N., Tanaka, K., Kamiya, N., Okuda, M., Yadomae, T., Nomoto, K., Shoyama, Y., 1996. A water-soluble antitumor glycoprotein from *Chlorella vulgaris*. Planta Med. 62, 423-426.

Okuda, M., Hasegawa, T., Sonoda, M., Okabe, T., Tanaka, Y., 1975. The effects of Chlorella on the levels of cholesterol in serum and liver. Jpn. J. Nutr. 33, 3-8.

Pak, N., Donoso, G., Tagle, M. A., 1973. Allantoin excretion in rat. Br. J. Nutr. 30, 107-112.

Priestley, G., 1975. Algal Proteins. In: Birch, G.G., Parker, K.J., Worgan, J.T. (Eds), Food from Waste. Applied Science Publishers Ltd, London, pp. 114-138.

Pulz, O., Scheibenbogen, K., Gross, W. , 2000. Biotechnology with Cyanobacteria and Microalgae. In: Rehm H.-J., Reed G. (Eds), Biotechnology, Vol. 10, Second Edition, Weinheim, 105-136.

Saleh, A.M., Hussein, L.A., Abdalla, F.E., El-Fouly, M.M., Shaheen, A.B., 1985. The nutritional quality of drum-dried algae produced in open door mass culture. Z. Ernahrungswiss. 24, 256-263.

Sano, T. Tanaka, Y., 1987. Effect of dried, powdered *Chlorella vulgaris* on experimental atherosclerosis and alimentary hypercholesterolemia in cholesterol-fed rabbits. Artery 14, 76-84.

Schoenhusen, U., Voigt, J, Piatkowski, B., 1988. The use of ribonucleic acid as a marker for measuring microbial protein yield in the rumen. 1. Chemical determination of ribonucleic acid. Arch. Tierernahr. 38, 289-297.

Schoenhusen, U., Voigt, J., 2004. Ruminal degradability of 15N labelled ribonucleic acid in grass. Arch. Anim. Nutr. 58, 343-352.

Spoehr, H. A., Milner, H. W., 1948. The chemical composition of *Chlorella*; effect of environmental conditions. Plant Physiol., 120-149.

Tanaka, K., Konishi, F., Himeno, K., Taniguchi, K., Nomoto, K., 1984. Augmentation of antitumor resistance by a strain of unicellular green algae, *Chlorella vulgaris*. Cancer Immunol. Immunother. 17, 90-94.

Tanaka, K., Koga, T., Konishi, F., Nakamura, M., Mitsuyama, M., Himeno, K., Nomoto, K., 1986. Augmentation of host defense by a unicellular green algae, *Chlorella vulgaris*, to *Escherichia coli* infection. Infect. Immun. 53, 267-271.

Trinder, p., 1969. Determination of glucose in blood using glucose oxidase with an alternative oxygen acceptor. Ann. Clin. Biochem. 6, pp. 24.

Venkataraman, L. V., Becker, W. E., Rajasekaran, T., Mathew, K. R., 1980. Investigations on the toxicology and safety of algal diets in albino rats. Food Cosmet. Toxicol. 18, 271-275.