2. Literature overview

2.1. Taxonomy

The green micro-algae species *Chlorella vulgaris* was first described by Beijerinck in the 1890s (Oh-Hama & Miyachi, 1993). According to taxonomical grouping based on morphology and physiological properties, it belongs to genus *Chlorella*, family *Oocystaceae*, order *Chlorococcales*, class *Chlorophyceae*, division *Chlorophyta* of the kingdom *Plantae*. The taxonomical affiliation of these micro-algae has been revised several times, so that in the last revision several other green micro-algae of the genus *Chlorella,* formerly known as *C. vulgaris*, like *C. prothotecoides* or *C. kessleri* have been re-named (Anonym 3). Molecular approaches allow more objective taxonomical grouping of the micro-algae. Morphology and physiological properties do not allow complete differentiation between the micro-algal species and regrouping based on DNA-analyses is needed. According to novel taxonomical grouping based on 18S-rDNA sequencing, *Chlorella vulgaris* has been shown to belong to other class – *Trebouxiophyceae,* therefore taxonomical regrouping based on DNA sequencing is needed (Friedl, 1998).

2.2. Morphology, physiology and occurrence

Chlorella vulgaris is a spherical, eukaryotic, unicellular alga containing chlorophyll, with a cell diameter of approximately 5 – 10 μm. The cell wall contains hemicelluloses, which accounts for the stability and rigidity of the cells. It has an asexual reproductive cycle, with the production of autospores from the mature large cell, by dividing the cell into smaller units. One mature cell divides into four new ones every 16 – 20 hours. The algal cells utilize sunlight for photosynthesis. The photosynthetic rate exceeds the respiration rate of *Chlorella* cells by 10 – 100 times (Myers, 1953).

C. vulgaris as a dried powder has a dry matter of approximately 95% (Anonym 2), the crude protein content depends on the algal species, cultivation method and harvesting time, and varies from about 15 % to 88 % of dry matter (DM) with an average rate of 50 % (Fisher & Burlew, 1953; Kay, 1991; Komaki et al., 1998), and so is bigger than that of soybean meal (44%) or wheat grains (12%) (Fisher & Burlew, 1953). The final amount of harvested algal biomass depends on the cultivation procedure – the growth medium composition, lighting

regime, temperature, supply of gases and harvesting time. Maximum annual yields can be in the region of 25 tones/hectare (Spoehr & Millner, 1948; Myers, 1953; Oh-Hama & Miyachi, 1993). For instance, changing the temperature of a culture of *C. vulgaris* from 20°C to 30°, whilst keeping all other parameters the same, caused a doubling in the amount of biomass produced, (835 mg/L and 1,666 mg/L, respectively) (Miranda et al., 2001).

The lipid content of *C. vulgaris* is between 6.5 – 12.5% with very good fatty acids ratio (see Table 1), containing high amount of balanced unsaturated fatty acids (n-6 and n-3), which are essential for mammals for synthesis of important substances like prostaglandins, prostacyklines, and leukotriens, and for support of other functions, like biotransformation and transport of cholesterol. The micro-algae contain minerals in amount of 8.5 – 11.5%, carbohydrates 0.9 – 2.0% (of fresh weight). Trubachev et al. (1976) measured the components of batch or continuous *Chlorella* culture. Their results show that the dry matter of *Chlorella* contains water-soluble sugars at 2.7 ± 1.2 and 3.4 ± 1.5 % (batch and continuous culture, respectively); starch 2.4 ± 0.6 % and 4.0 ± 0.2 %; hemicelluloses 5.7 ± 1.0 % and 8.6 ± 0.8 %; crude fiber 0.8 ± 0.3 % and 0.6 ± 0.1 %; crude protein of 57.5 ± 1.2 % and 50.6 ± 0.6 %; lipids of 14.4 ± 1.6 % and 21.5 ± 2.0 %; RNA 3.9 ± 0.5 % and 3.1 ± 0.3 %; DNA 0.8 ± 0.1 % and 0.7 ± 0.06 %; ascorbic acid 240 and 120 mg/100g DM; carotene 166 and 126 mg/100 g DM; thiamin 4.3 and 3.8 mg/100g DM and riboflavin 5.8 and 4.8 mg/100 g DM.

Table 1. Fatty acids composition (% of total lipids) of *Chlorella vulgaris* cells as recorded in literature

Fatty acid	**Miranda et al., 2001**	**Yukino et al., 2002**
C16:0	3.3 ± 0.2	18.8 ± 0.9
C16:1	5.9 ± 1.2	ND
C16:2	9.2 ± 1.3	10.0 ± 0.6
C16:3	ND*	11.3 ± 0.7
C18:0	1.0 ± 0.2	ND
C18:1	2.8 ± 0.5	ND
C18:2, n-6	38.4 ± 1.8	26.5 ± 1.4
C18:3, n-3	33.0 ± 3.7	17.7 ± 1.3
n-6/n-3	1.2	1.5

*ND – not determined

Pratt & Johnson (1965, 1966, 1967) in their studies stated that *Chlorella vulgaris* contents high amounts of ascorbic acid (47 – 118 mg/100 g DM), choline (236 – 261 mg/100 g DM), panthothenic acid (1.51 – 27.79 µg/mg DM), inositol (1.55 – 2.29 mg/mg DM), thiamine (9.0 – 19.4 µg/mg DM), riboflavin (24.7 – 46.4 µg/mg DM), folic acid (6.4 – 22.0 µg/mg DM) and biotin (0.8 – 2.4 µg/mg DM), with the highest amounts harvested on days 5 – 7 of cultivation.

2.3. Nutritional value of green micro-algae protein as established in animal studies

Micro-algae have been used in human nutrition for hundreds of years. Especially in Taiwan, Japan, Chile or Mexico, the micro-algae have been used as a source of protein or fat or as a delicates (Kay, 1991; Priestley, 1975). The micro-algae used in these cases belong to many genera, mainly *Scenedesmus*, *Chlorella,* and *Spirulina,* which are different in their cell morphology, cell content and cell wall thickness. The species most often cultivated are *Scenedesmus obliquus*, *Scenedesmus acutus*, *Scenedesmus quadricauda*, *Chlorella pyrenoidosa*, *Chlorella vulgaris* and *Spirulina platensis*, but there are many others that have been grown for these purposes. Several studies have been made on the utilization of *S. obliquus* (Leveille et al, 1962; Priestley, 1975; Rydlo, 1977; Bock & Wuensche, 1967; Meffert, 1961; Witt et al, 1962; Fink & Herold, 1956, 1957; Pabst et al, 1964; Meffert & Pabst, 1963; Kraut et al., 1966; Pabst, 1974), *S. acutus* (Brune and Walz, 1978; Pabst et al., 1978) as well as *C. pyrenoidosa* (Lubitz, 1963; Merchant & Andre, 2001; Merchant et al., 2000a, b; Priestley, 1975; Lee et al., 1967), *S. quadricauda* with or without *Chlorella* spp. as a mixture (Cook, 1962; Cook et al., 1963; Hennig et al., 1970) or *Spirulina* spp. (Kapoor & Mehta, 1993a, 1993b; Yoshida & Hoshii, 1980) in animal and human nutrition.

Some studies on the utilization of *Chlorella* spp. as a source of protein exist (Saleh et al., 1985; Komaki et al., 1998; Rydlo, 1977; Bock & Wuensche, 1967; Lipstein & Hurwitz, 1980; Lipstein et al., 1980); although more interest of the researchers working with *Chlorella* is directed towards the influence of *C. vulgaris* on the immune- and other systems. This interest is caused by the possible use of these micro-algae in human medicine. However a number of studies have shown that organisms such as *Scenedesmus* spp. can be used successfully as feed additives for livestock (Venkataraman et al, 1980; Brune & Walz, 1978; Rydlo, 1977; Bock and Wuensche, 1967; Meffert, 1961; Witt et al, 1962; Witt & Schroeder, 1967; Fink & Herold, 1956, 1957; Pabst et al., 1964; Meffert & Pabst, 1963; Kraut et al., 1966; Pabst, 1974; Pabst et al., 1978).

Searching the references, one can notice that much attention has been given to considering the nutritional value and protein quality of another micro-alga, *Scenedesmus obliquus*. Fink & Herold (1956) fed growing rats for 120 days with diets containing 19.7% DM of infrared-dried *S. obliquus* (counted on basis of crude protein). Total protein source consisted in 77% of algae, 15% rye and wheat (1:1) and 8% of brewer-yeasts. Diets also contained cod-liver oil (3.1 % of DM), salts (5.5 % of DM) and starch (54.5 % of DM). The control group received defatted milk powder as protein source in amount of 21%. Rats fed algae had the same body weight gain as the control rats, but their feed intake was much higher than in the control group. It was stated that the biological value of *S. obliquus* protein was at least as good as for the skim-milk powder with no dietary liver necrosis observed. In their next publication Fink & Herold, 1957 show that increasing the algae amount to 27 % of DM, mixed with yeast (1.4 % of DM), cod-liver oil (1.8 % of DM), salts (3.8 % of DM) and starch (66.1 %), in compare to skim milk diet (24.7 % of DM) and albumen diet (10.7 % of DM) (with the same other components), caused the growth gain for algae diet to be the same as shown in the previous experiment. The next finding was that feeding for a longer time (120 days) with skimmed milk or albumen led to dietary liver necrosis, no liver pathology was seen in the algae-fed group. Also, feeding with alfalfa gave worse weight-gain results, and also led to cases of liver necrosis. The authors stated therefore, that *S. obliquus* could be a good source of protein for humans (Fink&Herold, 1957).

Cook (1962) reported that the true protein digestibility of dried green micro-algae mixture (*Scenedesmus* and *Chlorella*, ratio 10:1) as measured in nitrogen balance studies on rats to be of 65.4 ± 0.71%. The author applied the method of Mitchell (1924) with modifications of Mitchell & Carman (1926). Rats received feed containing protein at 12 % of the diet, and the micro-algae were given alone as protein source. Boiling the micro-algae for 30 minutes improved the digestibility of the algal mixture to 73.0 ± 0.79 %, autoclaving or cooking for 2 hours showed no improvement on the digestibility of this algal mixture. Net protein utilization of open-air dried micro-algae, autoclaved micro-algae, and micro-algae cooked for 30 minutes or for 2 hours was the highest for the micro-algae boiled for 2 hours (44.0 ± 1.84 %). Biological value was the highest for the algal mixture cooked for 30 minutes (56.0 ± 2.7 %). In general, the best results were achieved in this study when the *Scenedesmus-Chlorella* mixture was fed as the sole protein source to rats when first cooked for 30 minutes prior to drying. Detailed results obtained by this author are summarized in Table 2.

Narasimha et al. (1982) conducted nitrogen balance studies feeding rats diets containing *Spirulina platensis* (blue-green micro-algae) as sole protein source. The protein

amount was calculated for amount of 150 mg N/10 g DM. The micro-algae contained 58.5 % of protein in DM. The reported true protein digestibility (tPD) of *S. platensis* was 75.5 ± 1.3 %; net protein utilization (NPU) of these algae was 52.7 ± 1.7 % and biological value (BV) was 68.0 ± 1.6 %. When these algae were mixed 1:1 with barley and so fed as protein source, the true digestibility of the protein of this mixture was 81.1 ± 1.6 %, NPU was 61.2 ± 1.8 % and BV was 75.5 ± 1.8 % in compare to barley as sole protein source: 82.0 ± 1.4 %; 58.0 ± 1.7 %; 71.2 ± 1.5 %, respectively.

Table 2. Nutritional indices for protein of algal mixture consisting of *Scenedesmus* and *Chlorella* (ratio 10:1) determined by Cook (1962).

Protein source	**N intake (mg)**	**BW (g) (gain g/day)**	**TPI (g)**	**BV (%)**	**tPD (%)**	**PER**	**NPU (%)**
Dried algae	211.0	154 (1.46)	32.9	54.3 ± 3.2	65.4 ± 0.71	1.25 ± 0.04	35.5 ± 2.17
Autoclaved algae	191.0	108 (0.64)	25.2	54.5 ± 2.0	65.5 ± 0.99	0.67 ± 0.14	35.6 ± 1.58
Algae cooked 30 min.	218.3	166 (1.82)	33.2	56.0 ± 2.7	73.0 ± 0.79	1.53 ± 0.07	40.9 ± 2.26
Algae cooked 2 hours	223.0	150 (1.5)	28.9	48.7 ± 2.6	69.8 ± 0.85	1.45 ± 0.06	44.0 ± 1.84

BW – mean body weight at the end of trial; TPI – total protein intake; BV – biological value; tPD – true protein digestibility; PER – protein efficiency ratio; NPU – net protein utilization

Meffert & Pabst (1963) investigated differently processed *S. obliquus* preparations as protein sources for rats. These were: raw substance, deep frozen, boiled, lyophilized, heated and then lyophilized and roller-dried algal substance, in amounts equal to 12% of dry matter. They fed young rats, weighing app. 45-50 grams, with these algae for 10 weeks. The results show that protein efficiency ratio (PER) after physical processing such as boiling or heating followed by lyophilization is similar to the PER of roller-dried or lyophilized micro-algae. Much better growth gain per protein intake was seen after feeding algae were repeatedly frozen and defrosted. Boiling, repeated freezing and defrosting must therefore had changed

the algal cell wall so that the nitrogen from algal cells could be better utilized. Feeding with raw algae led to weight loss of about 5g at the end of the trial, 2 animals from this group died in the 9^{th} and 3 in the 10^{th} week of experiment.

Kraut et al. (1966) fed young (app. 60 g of body weight) Sprague Dawley rats with differently processed forms of *S. obliquus*: raw biomass, roller-dried, homogenized and roller-dried or homogenized. The micro-algae were fed as sole protein source in amount of 18.8% of DM for 10 weeks. Rats fed roller-dried algae grew slightly slower than rats from the control (casein) group. Feeding homogenized and roller-dried, or only homogenized algae, led to a reduced weight gain of about 50 grams/day at the end of experiment. Feeding rats with raw biomass led to weight loss. PER for the first 4 weeks was 2.7 ± 0.09; 2.1 ± 0.08; 1.83 ± 0.15 for roller-dried, homogenized and dried, homogenized micro-algae, respectively, and for raw micro-algae the PER was negative. In next 4 weeks the PER was (in respective groups) 1.65 ± 0.08; 1.43 ± 0.1; 1.56 ± 0.13 and negative; in the whole period (10 weeks) the PER was 2.05 ± 0.04; 1.66 ± 0.07; 1.65 ± 0.11 and negative, respectively. The PER for casein was in the first 4 weeks 3.03 ± 0.13, in next 4 weeks 1.80 ± 0.08, for the whole period 2.26 ± 0.05. They concluded that roller-drying increased the nutritional value of *S. obliquus* protein, whereas homogenization had no influence on the protein value.

Komaki et al. (1998) investigated the nutritional value of spray-dried *Chlorella vulgaris* (K5) and the same algae treated during spray-drying with high pressure homogenization. Rats were fed a basal diet (containing 24.5% casein in fresh matter) or experimental diets containing algae at 20% of basal diet (20% algae + 80% basal diet). The apparent digestibility of the food protein received in this study was 87.4 ± 0.39 % for feed with non-homogenized *C. vulgaris* and 88.6 ± 0.37 % for feed containing high pressure homogenized *C. vulgaris*.

Lin (1969) studied the influence of feeding soymilk enriched with *Chlorella* on female and male rats, with an initial body weight of approximately 65 grams. The exact species of algae used was not reported. Protein content was adjusted to be 20% of the whole feed by weight. Results from this experiment are shown in the Table 3. It was concluded, that *Chlorella* could improve the value of soybean milk used as a cow's milk replacement in China. These results show that adding *Chlorella* to soybean milk, independent of the concentration, stimulate weight gain in rats. Rats were fed *ad libitum* and food intake was much higher in all groups fed algae with much lower food efficiency ratio.

Pabst (1974) showed PER, BV and NPU values for *S. obliquus*, *Coelastrum proboscideum* and *Uronema* spp. [roller-dried and then heated to120ºC for 5 -10 seconds] obtained in experiments on growing rats (weighing app. 50-60 g at the beginning). The micro-algae were fed as the sole protein source (app. 18 – 22 % of feed, depending on the protein content). His results show that the highest body weight gain of rats fed micro-algae was obtained after feeding with *Scenedesmus*, then *Coelastrum* and *Uronema*, but all rats grew slower in comparison to the control, casein group. The PER was 3.21 ± 0.06; 2.48 ± 0.11; 2.47 ± 0.14 for algae, respectively (with 3.97 ± 0.14 for casein); true protein digestibility was 95.1 %; 82.8%; 77.8%; 81.8%; NPU – 88.7%; 67.3%; 53.1%; 44.9%; and BV – 93.3%; 81.3%; 68.2%; 54.9% for casein, *Scenedesmus*, *Coelastrum* and *Uronema*, respectively.

Table 3. Growth gain and nutritional indices obtained by Lin (1969) after feeding rats with soybean milk enriched in *Chlorella* or alone.

Group	**Growth gain (g/day)**		**PER**	**aPD (%)**	**BV (%)**	**Feed intake (g)**		**Feed efficiency ratio**	
	I *	II *	II	III *	III	I	II	I	II
Control (soybean milk alone)	0.8 ± 0.1	2.1 ± 0.1	1.2 ± 0.1	83 ± 8	63 ± 4	150 ± 3	140 ± 0.2	6.6 ± 1.5	4.3 ± 0.9
Soybean milk + 2% *Chlorella*	1.7 ± 0.2	2.9 ± 0.2	1.4 ± 0.1	76 ± 8	64 ± 5	253 ± 21	157 ± 6	4.4 ± 0.5	3.6 ± 0.2
Soybean milk + 4% *Chlorella*	2.1 ± 0.3	3.0 ± 0.2	1.4 ± 0.1	75 ± 11	58 ± 8	238 ± 22	159 ± 8	3.7 ± 0.7	3.5 ± 0.3
Soybean milk + 8% *Chlorella*	2.2 ± 0.2	3.1 ± 0.3	1.5 ± 0.2	73 ± 8	54 ± 3	244 ± 13	163 ± 4	3.6 ± 0.5	3.4 ± 0.5

* I - ♀ rats (fed for 30 days); II - ♂rats (fed for 15 days); III - ♀ rats (5 days trial)

Saleh et al. (1985) investigated protein value of several outdoor mass-cultured, drum-dried micro-algae: *Scenedesmus acutus, Coelastrum proboscideum* and *Chlorella vulgaris*. Young rats (app. 21 days of age) were fed feeds containing sole protein source – casein or

investigated micro-algae – at 10% of the diet. They determined biological value (BV), protein efficiency ratio (PER), net protein ratio (NPR), digestibility coefficient (DC) and net protein utilization (NPU), where NPU was calculated from equation (BV x DC)/100. The values they received are shown in Table 4.

Lubitz (1963) determined the apparent digestibility of the protein from freeze-(vacuum-) dried *Chlorella pyrenoidosa* strain 71105 in nitrogen balance study on male weanling rats (fed as 21 % by weight of the diet, equal to 10 % of the total protein in the diet, as sole protein source) to be 86%. The PER established by this author for the protein of these micro-algae was 2.19. PER recorded for *Chlorella* enriched with 0.2% of L-methionine was 2.90 and for casein was equal to 3.30.

Table 4. Nutritional parameters for *C. vulgaris* and other micro-algae obtained by Saleh et al, 1985.

Diet	**BV (%)**	**DC**	**NPU (%)**	**PER**	**NPR**
Casein	88.4 ± 1.2	94.0 ± 0.46	83.1 ± 1.4	2.7 ± 0.04	3.1 ± 0.07
Scenedesmus	76.2 ± 1.5	88.6 ± 0.84	67.5 ± 1.97	2.1 ± 0.08	2.8 ± 0.07
Chlorella	**77.9 ± 2.3**	**89.3 ± 0.64**	**69.6 ± 2.42**	**2.0 ± 0.02**	**2.6 ± 0.05**
Coelastrum	75.3 ± 2.1	89.2 ± 0.8	67.2 ± 2.42	1.9 ± 0.05	2.4 ± 0.07

Bock & Wuensche (1967) performed nitrogen balance studies on rats fed micro-algae as the sole protein source, but unfortunately the authors did not report the protein amount in the feed. They determined the apparent protein digestibility of "artificially" (no more precisely specified) dried micro-algae *Chlorella vulgaris* and *S. obliquus* to be 44.1 % and 26.1, and the true digestibility of crude protein to be 59.3 % and 32.7 %, respectively. The BV values determined for the protein from these algae, by these authors, was 52.9 ± 5.4 % and 47.0 ± 3.5 %, respectively. Treatment of the *S. obliquus* with boiling water or steam heating led to apparent crude protein digestibility of 36.7 % and 27.7 %, respectively; tPD of 52.9 % and 44.3 % and BV of 47.9 ± 2.5 % and 40.3 ± 3.0 %, respectively. Steam heating must have destroyed some algal components what resulted in worse nutritional value of the treated algae.

Yap et al. (1982) fed weaned at the 3rd day of age and artificially reared piglets on micro-algae *Spirulina maxima*, *Arthrospira platensis* and *Chlorella* sp. as substitution of 50 %

of the soybean protein (33 % of total protein in the diet). The control group received a basal diet (with ground yellow corn, soybean meal and dried skim milk as protein sources). The authors investigated daily average weight gain and blood parameters, as well as gross and microscopic changes in tissues. There were no differences between groups and no side-effects of feeding algae, and so the authors stated the micro-algae can successfully replace up to 50% of soybean meal in baby pigs diet. Authors did not give any clue about the methods used for preparing of the micro-algae used in the experiment.

In the former Soviet Union, *Chlorella* spp. (*C. vulgaris*, *C. pyrenoidosa* and others) were investigated in several kolkhozes (large collective farms). Tkachev (1966) reported on the growth performance of growing pigs (2-4 months) fed concentrate (1 kg/pig) with suspension of *Chlorella* (2 L/pig) was 21.2% better than in control group fed concentrate (1 kg/pig), whereas pigs fed the same concentrate (1 kg/pig) with a suspension of yeast (2 L/pig) gained 29.7 % more weight than pigs in the control group. Supplementation of rabbits' feed with the algae powder led to 35 % better growth performance and 25 % better feed efficiency. Broilers that received dried algae gained 20 % more weight than control ones. There is no exact composition analysis of the diets used in this reference, but it clearly shows, the *Chlorella* given as supplementation had a positive effect on growth performance in farm animals.

Hintz et al. (1966) investigated the nutritive value of micro-algae grown on sewage. These authors fed ruminants (sheep and beef steers) and monogastric animals (swine) different air-dried algal biomass, which varied with time of harvest and belonged to *Chlorella* spp., *S. obliquus* and *S. quadricauda*. Sheep sorted the feed when hay (alfalfa and oats 1:1) and algae (4 parts hay and 6 parts algae) were fed in an unpelleted form. They weighed 68 – 72 kg and received approximately 35 % of crude protein in diet. The apparent digestibility of algae was calculated by difference and was equal to 72.5 %. In similar experiment on beef steers (450 kg) the apparent protein digestibility of the algae was calculated to be 73.8 %. In another trial barrows (male pigs castrated before puberty, 40 – 46 kg) were fed algae in amount of 6 % or 10 % of the diet and the apparent digestibility of protein of these diets was 52.5 % and 55.4 %, respectively. Another trial was done on gilts (un-mated young female pigs - weighing approximately 38 kg) and they received 2.5 %, 5 % and 10 % algae in diets, which contained 15 – 16 % of crude protein. The gilts fed algae performed as well as controls with no differences between groups. Replacing part of the algae with meat or bone meal had no influence on performance.

Witt et al. (1962) compared the nutritional value of protein of the green micro-algae *S. obliquus* with soybean meal protein in an experiment on German landrace hogs (in the fattening period from 35 kg to 110 kg BW). The fresh matter of the vacuum-dried micro-algae contained 90.45 % of DM, 54.85 % of crude protein, 4.66 % of crude fat, 7.01 % of crude fiber and 14.55 % of N-free extraction substances. As protein source control group received fishmeal, and experimental groups received 75 % of algal protein with 25 % of fishmeal protein and 75 % of soybean protein with 25 % fishmeal protein and all groups were fattened with barley. The amount of fed micro-algae was then 10 % of the diet until 50 kg BW; 8 % of the diet for swine 50 – 70 kg BW; and 7 % of the diet for swine 70 – 110 kg BW. All hogs consumed the algal feed from the beginning of the experiment and the total feed intake in this group was the highest (2.71 ± 0.03 kg/day) when compared to the control (2.45 ± 0.06 kg/day) and soybean/fishmeal group (2.43 ± 0.06 kg/day). Fattening period was the shortest for the group fed micro-algae/fishmeal (93.1 days); control animals got to the slaughter weight after 95.5 days and the group fed soybean/fishmeal needed 99.9 days to get the final 110 kg BW. Daily weight gain was also the highest in the algae/fishmeal group (755 ± 15.1 kg/day, 736 ± 17.4 kg/day and 706 ± 17.6 kg/day, in respective groups). The feed efficiency ratio was therefore 3.60 ± 0.08 in the algae/fishmeal group, 3.34 ± 0.09 in control and 3.45 ± 0.07 in soybean/fishmeal group. After slaughtering of the animals no significant differences in the carcass parameters were observed between the groups. Bacon layer was the thickest in the algal group (4.65 ± 0.2 cm) compared to the control (4.42 ± 0.1 cm) and soybean/fishmeal group (4.46 ± 0.2), so the higher weight-gain in the group fed the micro-algae was due to fattening. Lumbar and abdominal muscles were the thickest in the algal group (6.7 ± 0.3 cm and 4.0 ± 0.1 cm) in comparison to control (6.3 ± 0.2 cm and 3.9 ± 0.1 cm) and soybean/fishmeal group (6.4 ±0.2 cm and 3.8 ± 0.1 cm), thus showing that the 75% of protein in diet coming from *S. obliquus* mixed with 25% of fishmeal protein was a good protein source for swine mast.

In other experiment the same micro-algae was proved for its protein quality when fed to German landrace swine as sole protein source (Witt & Schroeder, 1967). The fed amount of the micro-algae was 21.5% of the feed; the feeding trial was once again in fattening period from 30 till 110 kg BW. Control group received protein concentrate (16% fishmeal, 74% soybean meal and 10% mineral supplement) in amount of 22.5% of feed. Both groups were fattened with barley. Daily feed intake (2.7 ± 0.09 kg/day in experimental and 2.67 ± 0.05 kg/day in control group) and weight gain (0.78 ± 0.05 kg/day in experimental and 0.74 ± 0.06 in control group) was comparable in both groups. Time taken to reach the final weight was

shorter in the algal group (90 ± 6 days) in comparison to control group (95 ± 7.2 days). The carcass parameters were also comparable in both groups. Bacon thickness was 2.9 ± 0.3 cm and 3.0 ± 0.3 cm in experimental and control group, respectively. Quality of meat was also not altered through feeding hogs with micro-algae as sole protein. These results showed, the green micro-algae *S. obliquus* could be an efficient protein source for swine production.

Leveille et al. (1962) studied the protein value of different micro-algae and their mixtures, in feeding studies on rats and chicks (see Table 5 for details). Chicks fed algae grew much more slowly than a control group fed soybean with DL-methionine (0.54%), but considering only algae, a mixture of *S. obliquus* and *Chlorella ellipsoidea* gave 3 times better results than other algae. In rats the results were similar; with the difference that the control group was fed casein. Supplementation of *Spongiococcum exentricum* with DL-methionine led to increase of PER of this protein from 0.34 to 1.22, demonstrating a large deficit of this amino acid in this alga. In general, it seems that methionine is a relatively deficient amino acid in most micro-algae. The amount of glycine (essential for chicks but not for rats) was found to be low in *Chlorella pyrenoidosa*.

Table 5. Weight gain of chicks and rats and PER after different algae feeding (Leveille et al., 1962)

Protein source #	**WG (g/day) – chicks**	**PER – chicks**	**WG (g/day) – rats**	**PER – rats**
1	11.3 ± 1.2	3.04	5.3 ± 0.5	2.50 ± 0.09
2	4.1 ± 1.0	1.55	2.9 ± 0.3	1.38 ± 0.14
3	0.4 ± 0.6	0.31	1.8 ± 0.6	0.94 ± 0.19
4	0.6 ± 0.4	0.43	0.4 ± 0.3	0.34 ± 0.24
5	----	----	1.1 ± 0.2*	1.22 ± 0.18

WG – weight gain; PER – protein efficiency ratio

* The animals receiving the *S. excentricum* diet were fed the same diet, to which 0.5% of DL-methionine had been added for last 11 days.

- protein content was 18% for chicks and 15% for rats: 1 – Soybean + DL-methionine (chicks)/ casein (rats); 2 – *Scenedesmus obliquus* + *Chlorella ellipsoidea;* 3 – *Chlorella pyrenoidosa;* 4 – *Spongiococcum excentricum;* 5 – *Spongiococcum excentricum* + DL-methionine

Combs (1952) investigated the influence of vacuum-dried *Chlorella pyrenoidosa* fed at 10% supplementation (instead of soybean meal) to chicks. According to his findings, the feed efficiency ratio for such mixtures was lower than that of basal diet (2.4 to 3.1, respectively), but the growth performance of the chicks fed the algal diet was almost twice that for the control chicks (mean BW at the end of 4 wk experiment was 262 g and 135 g, respectively). Supplementation of the algal diet with 0.1% DL-methionine led to an increase in grow performance (mean BW of 298 g) but had no influence on the feed efficiency ratio. Interestingly, there was a reduction in mortality in the algal groups (100% of chicks survived the 4 weeks trial, whereas in control group only 81%). Comparing all diets to complete broiler mash,containing antibiotic as growth promoter, (mean WG after 4 weeks of 342 g) supplementation with *C. pyrenoidosa* gave quite a positive effect, but in this case extra supplementation with DL-methionine seems to be important (methionine content in the algae was 0.36 %).

Kotrbacek et al. (1994) investigated the influence of feed supplementation with *Chlorella vulgaris* biomass on chicks. The trial involved broilers from the 4th to the 56th day of life. One group received commercial mashes with 0.5% dried (not specified how) biomass of *C. vulgaris*. A second group received 0.9% dried cow's colostrums and 0.9% dried brewer's yeasts in addition to the algae (0.2%). The control group received only commercial mash. Feed was given *ad libitum* and no feed intake was measured. Weight gain was recorded on day 4 and then every 7 days starting from day 7. Furthermore, these authors determined basic hematological values and phagocytic activity of leucocytes at the age of 21, 33 and 56 days, after killing of animals. Also samples of the thyme, bursa Fabricii, spleen, ileocecal valve, Meckel's diverticulum, gonads, suprarenal glands and Harder's gland were collected and subjected to histological examination. Supplementation of chick feed with 0.5% *C. vulgaris* increased the live weight of experimental broilers only at the end of the second week of life (see Table 6). On the 21st and 33rd day of life, phagocytic activity of leucocytes increased significantly in individuals from both experimental groups in compare to controls. Development of the intestinal lymphatic tissue and Harder's gland was largely stimulated the second month of life in these groups.

Table 6. Weight (mean ± SD, g) of broilers fed feed supplemented with *Chlorella vulgaris* (Kotrbacek et al., 1994) (due to slaughtering, number of animals decreased with age).

Age of broilers (days)	**n**	**Control group**	**Group fed 0.5% micro-algae**	**Group fed 0.9% brewer-yeasts and 0.2% micro-algae**
4	90	56.8 ± 0.9	58.1 ± 0.8	58.1 ± 1.0
7	90	83.6 ± 1.7	88.1 ± 1.47	87.7 ± 1.8
14	90	190.0 ± 4.8 a	205.8 ± 4.8 b	201.3 ± 4.9
21	90	415.3 ±11.3	438.1 ± 9.8	433.9 ± 9.0
28	60	714 ± 23.4	776 ± 22.4	744 ± 20.19
33	60	1066 ± 36.7	1123 ± 33.6	1083 ± 29.8
35	30	1236 ± 56.6	1260 ± 51.5	1252 ± 43.1
42	30	1798 ± 64.5	1664 ± 61.7	1764 ± 67.7
49	30	2270 ± 86.0	2279 ± 95.1	2237 ± 79.9
56	30	2870 ± 87.6	2779 ± 118.9	2769 ± 99.4

a, b – significant difference when different letters, $p<0.05$

Koehler & Kallweit (2000) fed pregnant and lactating sows with feed supplemented with 0.8 – 1.0 % spray-dried *Chlorella vulgaris* and thereafter piglets from these sows (in period from 8th till 63rd day of life) with feed supplemented with 1 % *C. vulgaris*. The influence of algal supplementation on sows' reproductive performance will be described later, in part 2.4.3, here piglet feeding and algal influence on piglets' performance will be analyzed. The experiment schema and results are shown in Table 7. Piglets were weaned at 35th day of life. Piglets from control sows and from sows fed the micro-algae supplement were divided into two groups – control and experimental. Control piglets were fed control commercial diet; the experimental piglets received 1% of *C. vulgaris* to the diet. There were no differences between groups, so supplementation with 1% of *C. vulgaris* seemed to have no influence on piglets' development.

In experiments on rabbits (Anonym 1) pellets containing 3% of spray-dried *Chlorella vulgaris* biomass were fed to animals (91 ± 3 days of life, 2.4 – 3.05 kg BW) for 57 days.

Mean feed efficiency ratio (g daily weight gain / daily feed intake) in the group was 0.53 and was 5 times higher than in the control group (0.11). Daily weight gain was 27% higher in the *Chlorella* group than in control group. These results showed very good efficiency of the algal protein on the rabbit development.

Table 7. Feeding piglets with 1% *Chlorella vulgaris* supplementation (Koehler & Kallweit, 2000)

Parameter	**F I ***	**F II ***	**F III ***	**F IV ***
Life-born piglets	108	110	101	116
Weight/piglet at birth (kg)	1.47 ± 0.42	1.33 ± 0.26	1.53 ± 0.4	1.34 ± 0.27
Weaned piglets	87	89	84	97
Weight/piglet at the weaning (kg)	9.6 ± 2.3	9.4 ± 1.9	9.6 ± 2.3	9.3 ± 1.9
Feed intake (1^{st} d – weaning) (g/animal/day) (without algae)	25 ± 20	20 ± 16	37 ± 28	26 ± 16
Algae intake (1^{st} d – weaning) (g/animal/day)		0.2 ± 0.2		0.3 ± 0.2
dWG (birth-weaning) (g)	231 ± 60	230 ± 52	229 ± 58	226 ± 50
Piglets on 63^{rd} day of life	85	87	84	97
Weight/piglet on 63^{rd} day of life (kg)	17.2 ± 5.4	17.4 ± 3.9	17.4 ± 5.2	16.8 ± 3.9
Feed intake (36^{th} d – 63^{rd} d) (g/animal/day) (without algae)	592 ± 236	551 ± 179	562 ± 244	507 ± 138
Algae intake (36^{th} d – 63^{rd} d) (g/animal/day)		5.6 ± 1.8		5.1 ± 1.4
dWG (weaning-63^{rd} d) (g)	272 ± 133	283 ± 133	277 ± 136	268 ± 108
Feed expenditure (kg feed/kg dWG) (kg)	1.00 ± 0.24	0.99 ± 0.17	0.99 ± 0.20	0.96 ± 0.17

*Piglets from groups F I and F II came from control sows (fed no micro-algae), piglets from group F III and F IV came from sows fed 0.8 – 1.0% micro-algae. Piglets from F I and F III were fed no micro-algae, piglets from F II and F IV were fed feed with 1% micro-algae.

Table 8. Micro-algae fed to animals in different studies. For details see text.

Micro-algae	**Amount in feed**	**Animal**	**Reference**
S. obliquus	19.7% of DM 27% of DM	Rat	Fink & Herold (1956)
S. obliquus	12 % of DM	Rat	Meffert & Pabst (1963)
S. obliquus	18.8 % of DM	Rat	Kraut et al. (1966)
S. obliquus	18 – 22 % of DM	Rat	Saleh et al (1985)
S. obliquus	Sole protein source	Rat	Bock & Wuensche (1967)
S. obliquus	60 % of the diet 2.5 %, 5.0 %, 10 % of diet	Sheep, Cattle, Pig Pig	Hinz (1966)
S. obliquus	10 %, 8 %, 7 % of diet	Pig	Witt et al. (1962)
S. obliquus	21.5 % of diet	Pig	Witt & Schroeder (1967)
S. obliquus + *C. ellipsoidea*	18 % 15 %	Rat Chick	Leveille et al. (1962)
S. platensis	150 mg N/10 g DM as sole protein source	Rat	Narasimha et al. (1982)
S. platensis	61 g/kg diet	Rat	Kapoor & Mehta (1998a)
S. platensis	48 % of diet	Rat	Kapor & Mehta (1993)
Scenedesmus spp. + *Chlorella* spp. *(10:1)*	sole protein source 12 % protein in diet	Rat	Coook (1962)
S. acutus *C. proboscideum*	Sole protein source 10% protein in diet	Rat	Saleh et al. (1985)
C. proboscideum *Uronema* spp.	18 – 22 % of DM	Rat	Pabst (1974)

***Table 8**. – continued*

Micro-algae	**Amount in feed**	**Animal**	**Reference**
S. maxima *A. platensis* *Chlorella* spp.	As substitution – 50 % of soybean meal 33% protein in diet	Pig	Yap et al. (1982)
Chlorella spp.	2 %, 4 %, 8 % of soybean milk; 20 % protein in diet	Rat	Lin (1969)
C. vulgaris	20 % in diet	Rat	Komaki et al. (1998)
C. vulgaris	Sole protein source 10 % protein in diet	Rat	Saleh et al. (1985)
C. vulgaris	Sole protein source	Rat	Bock & Wuensche (1967)
C. vulgaris	10 % of soybean	Chick	Coombs (1952)
C. vulgaris	0.2 %; 0.5 % of diet	Chick	Kotrbacek et al. (1994)
C. vulgaris	1 % of diet	Pig	Koehler & Kallweit (2000)
C. vulgaris	3 % of diet	Rabbit	Anonym 1
C. pyrenoidosa	21 % of diet (10 % protein in diet)	Rat	Lubitz (1963)
C. vulgaris	5% or 10% of diet	Rat	Matsuura et al. (1991)
Chlorella spp.	2 L/animal fresh biomass	Pig	Tkachev (1966)

Table 9. Different methods used for the disruption of micro-algal cells

Method of algal treatment	**Reference**
Infrared-drying	Fink & Herold (1956)
Roller-drying	Meffert & Pabst (1963), Kraut et al. (1966)
Drum-drying	Saleh et al. (1985)
Air drying	Cook (1962), Hintz et al. (1966)
Spray-drying	Komaki et al (1998), Koehler & Kallweit (2000)
Freeze-drying (vacuum drying)	Lubitz (1963), Witt et al. (1962), Coombs (1952)
Deep freezing	Meffert & Pabst (1963)
Lyophilization (with/without preheating)	Meffert & Pabst (1963)
Autoclaving	Cook (1962)
Boiling (different time)	Cook (1962), Meffert & Pabst (1963), Bock & Wuensche (1967)
Steam-heating	Bock & Wuensche (1967)
Ultra high temperature (after roller-drying)	Pabst (1974)
Homogenization (with roller-drying or alone)	Kraut et al. (1966)
High pressure homogenization (during spray-drying)	Komaki et al. (1998)

As it can be seen from the cited literature, many studies have been performed where animals have been fed different species of micro-algae. The most commonly used micro-allga is *Chlorella vulgaris* (see Table 8). Different treatments were used in order to make the micro-algae more digestible (see Table 9). Boiling and homogenization was of some value, but there is still no really satisfactory method that results in a large increase of the crude protein digestibility and other nutritional parameters of the micro-algae. Different animal species were used for experiments, first field trials have been done – however, the results are equivocal, even though many positive effects of algal feeding on animals' growth has been observed. This is an interesting background, showing that further actions in field of algal treatment are needed to get a good product with desired effects.

2.3.1. Toxicology and safety of micro-algae in animal studies

Only a few toxicological and safety studies concerning feeding with micro-algae have been conducted. Venkataraman et al. (1979) fed the green micro-algae *Scenedesmus acutus* as sole protein source (crude protein equal to 10% and 15% of the diet) to male Wistar weanling rats for 12 weeks. The amount of the micro-algae in the diet was 24 % and 36 % by weight, respectively. The control group received casein as the sole protein source at 10 % of the crude protein of the diet (equal to 12 % of casein in the diet). Diet was fed *ad libitum* and rats were weighed weekly and food consumption was recorded daily. Feed intake in group containing 10% of micro-algal protein was comparable with control group, whereas rats fed with 15% of the micro-algal protein consumed more feed in the experimental period. Mean final body weights after 12 weeks of experiment were 189.75 ± 5.87 g, 207.13 ± 6.89 g and 252.38 ± 9.83 g in control, 10 % algal- and 15 % algal-protein group, respectively, and the differences were significant. Absolute organ weights followed the same pattern but there was a small difference in relative organ weights (g/100 g BW) between the groups (see Table 10).

Table 10. Relative organ weights (g organ/100 g body weight) of rats fed diets containing 10 % or 15 % of micro-algae or 10 % casein as sole protein source (Venkataraman et al., 1979)

Organ	**Relative organ weights of rats fed diets containing**		
	12% casein	24 % micro-algae	36% micro-algae
Liver	2.470 ± 0.167 a	2.270 ± 0.103 b	2.240 ± 0.128
Kidneys	0.526 ± 0.026 a	0.456 ± 0.027 b	0.488 ± 0.030a
Heart	0.266 ± 0.027	0.244 ± 0.017	0.260 ± 0.023
Lungs	0.650 ± 0.031 a	0.625 ± 0.027	0.600 ± 0.034 b
Brain	0.709 ± 0.040	0.658 ± 0.039	0.667 ± 0.032
Adrenal glands	0.020 ± 0.00037	0.018 ± 0.00047	0.016 ± 0.00063
Pituitary gland	0.0017 ± 0.00024	0.0017 ± 0.00028	0.0016 ± 0.00039
Thyroid gland	0.0078 ± 0.0013 a	0.0065 ± 0.0011 b	0.0065 ± 0.0011
Testes	0.947 ± 0.0050 a	0.883 ± 0.0592 b	0.737 ± 0.0253 b

a,b - values marked with different letters differed significantly (Bartlett test) ($p<0.05$)

The authors did not observed any pathological changes (no fat infiltration, no vacuolation nor distortion) in livers, hearts or kidneys from rats fed the micro-algae. There were no

hematological abnormalities in any of the groups (see Table 11). Total liver cholesterol level was significantly lower in rats fed micro-algae and mean values for 8 rats/group were as follows: 2.14, 1.75 and 1.63 mg cholesterol/g liver in control, 24 % micro-algae and 36 % micro-algae group. No deposition of cholesterol crystals was observed in any group. Serum cholesterol levels were similar in all groups. Hepatic lipid and protein levels were unchanged in the experimental groups. No harmful effect of feeding rats with green micro-algae *S. acutus* for period of 12 weeks was seen.

Table 11. Hematological data for rats fed diets containing 10% of casein, 10% or 15% of micro-algal protein as sole protein source for 12 weeks (Venkataraman et al., 1979)

Parameter	**Values for rats fed diets containing**		
	12% casein	24% micro-algae	36% micro-algae
Hemoglobin (g/100 ml whole blood)	15.4 ± 0.74	15.1 ± 0.79	14.6 ± 0.78
Red blood cells (RBC) (10^6/mm^3)	6.1 ± 0.30	6.5 ± 0.47	6.2 ± 0.25
White blood cells (WBC)			
Total (10^3/mm^3)	8.6 ± 0.48	7.4 ± 0.30	7.3 ± 0.27
Lymphocytes (% of total WBC)	75.3	74.6	73.1
Neutrophiles (% of total WBC)	18.8	19.8	20.4
Eosinophiles (% of total WBC)	2.1	2.3	2.6
Monocytes (% of total WBC)	3.8	3.3	3.1
Hematocrite (%)	50.0 ± 1.4	48.0 ± 1.5	48.5 ± 1.4

Schneegurt et al. (1995) investigated toxicological aspect of feeding cyanobacterium *Cyanothece* sp. strain ATCC 51142 to rats. Cyanobacteria, even though prokaryotic organisms are similar to eukaryotic micro-algae, they contain relative large amounts of nucleic acids, at least theoretically. In a short-term trial (2 weeks), three week old male rats were fed diet containing 5 % of the cyanobacterial biomass (equal to 13.6% of crude protein of the diet) and 15% casein. Weight gain did not differ from control group fed casein as source of protein (18% of the diet) and reached 79 grams for the whole experimental period. Uric acid plus allantoin levels in urine, liver and kidneys did not differ between groups (see Table 12). No gross pathological or histological changes were detected. Authors could find no negative influence of cyanobacterial nucleic acids on the rat.

Table 12. Uric acid plus allantoin levels in rats fed 5% cyanobacterial biomass (Schneegurt et al., 1995)

Uric acid plus allantoin	**Control group (18% casein in diet)**	**Experimental group (5% cyanobacterial biomass + 15% casein in diet)**
Urinary (mg/day)	1.27 ± 0.12	1.38 ± 0.30
Hepatic (mg/100 g tissue)	1.04 ± 0.21	1.09 ± 0.53
Renal (mg/100 g tissue)	0.61 ± 0.14	0.63 ± 0.17

2.4. Physiological effects of *Chlorella vulgaris*

Micro-algae, and especially *Chlorella vulgaris*, are of interest not only because of their high protein content and possible use in animal production as protein source, but also because they contain other active components, which make many researchers focus on possible positive effects of the microorganisms on the animal and human health.

2.4.1. Anti-tumor and immune-modulating activity

Studies on physiological properties of *Chlorella vulgaris* and its extracts have been done since the 1970s, mainly in Japan, where these algae have been used in human nutrition for several centuries. Much of the researcher interest was stimulated by the studies on aqueous extracts of multiple green algae, where active substances have been discovered and even partially characterized. Much work has been done on this aqueous extract and its influence on the immune system (animal and human).

Kojima et al. (1973) isolated a water soluble β-1, 3-glucan of molecular weight 1,250 to 1,400 Da from cell culture of *Chlorella ellipsoidea* and named it chlorellan. These authors studied the mechanism of action of this polysaccharide on phagocytic activity of the reticuloendothelial system (RES) in carbon clearance test in rats and mice. Animals received 2 mg of chlorellan / 100 g of body weight intraperitoneally or intravenously in saline. This glucan showed strong stimulating activity on peritoneal macrophages. P peritoneal injection of chlorellan gave more pronounced results than intravenous application. Also Kupfer stellate cells from animals treated with chlorellan were activated, exhibiting more intensive phagocytosis of carbon molecules. Chlorellan seems to enhance serum opsonin factor(s) (belonging to α- and β-globulins), and activates the RES-cells themselves thus elevating their phagocytic activity.

Tanaka et al. (1984) noticed an augmentation of anti-tumor resistance against fibrosarcoma (Meth-A) in female CDF1 mouse after intra tumor or into subcutaneous tissue near tumor injection of hot-water extract from *C. vulgaris* (dialyzed, lyophilized and resuspended in physiological saline, applicated in different dosages, from 20 to 500 mg/kg, over 1 – 5 days). The authors found that the mechanism of this reaction was host-dependent and required participation of T cells and macrophages, but final effector mechanism of tumor cells elimination could not be elucidated. No anti-tumor activity of *C. vulgaris* could be seen after systemic application. Konishi et al. (1985) reported the hot water extract of *C. vulgaris*

(dialyzed, lyophilized and resuspended in saline, given to female BALB/c mice in dose of 200 mg/kg, intra peritoneal – i.p.) possessed influence on polymorphonuclear cells (PMNs) in anti-tumor (anti-MethA) effect, where the mechanism of PMNs activation could have been related to acceleration of chemokinesis and superoxide generation in PMNs. This mechanism has also been identified by Tanaka et al. (1986) as the factor of augmentation of the resistance of female CDF1 mice to *Escherichia coli* (E77156:06:H1) infection, after subcutaneous injection of water extract of *C. vulgaris.* This extract contained 44.3 g of protein, 39.5 g of carbohydrates and 15.4 g of nucleic acids in 100 g of dry matter, with no detectable lipid content. The *C. vulgaris* extract was dialyzed, lyophilized and resuspended in saline and was given to animals in dose of 50 mg/kg (used as standard dose). Considering activation of PMNs by β-1-3 glucan from *Alcaligenes faecalis* (TAK) discussed in the cited article, and the presence of chlorellan described by Kojima et al. (1973), it would be concluded that chlorellan may be the active component in *C. vulgaris* cells resulting in induction of PMNs.

Morimoto et al. (1995) isolated several glyceroglycolipids from *C. vulgaris,* and tested their anti-tumor-promoting activity *in vitro*. The isolated glyceroglycolipids had no cytotoxic activity. From 7 isolated constituents, the most potent agent had anti-tumor-promoting activity was monogalactosyl diacyloglycerol containing (7Z, 10Z)-hexadecadienoic acid, at $1x10^3$ or $5x10^2$ mol ratio toward 12-O-tetradecanoylphorbol-13-acetate (TPA).

Singh et al. (1999) reported an anti-tumor activity of *C. vulgaris* (E-25) in Swiss albino mice after local skin administration (500 mg of *C. vulgaris*/kg BW/100µl acetone/day) during peri-, post-, or peri- and post-initiational stages of murine skin papillomagenesis (induced by DMBA – 7,12-dimethylbenz[a]anthracene). The postulated mechanism of papillomagenesis modulation was the influence of *C. vulgaris* on xenobiotic detoxication system. Elevation of sulfhydryl (-SH) and glutathione S-transferase (GST) levels in skin and liver was seen, what leads to reduction of cellular oxidative stress, thus possibly modulation of initiation of carcinogenesis. There were no changes in microsomal cytochrome b5 and cytochrome P-450 activities, thus no change in potentiation of neoplasia could be noted.

Tanaka et al. (1986) and Konishi et al. (1990) reported action of water extract from *C. vulgaris* on mice treated with cyclophosphamide and challenged with *E. coli*. Female CDF1 mice were first treated with cyclophosphamide (150 mg/kg, i.p.) and then were treated with *Chlorella* extract (dialyzed, lyophilized and resuspended in physiological saline, 50 mg/kg, s.c.) daily for 13 days, prior to challenge with *E. coli*. Mice survived the infection and became resistant to *E. coli*. *Chlorella* extract accelerated the recovery of PMNs in the peripheral blood; also the number of granulocyte/monocyte-progenitor cells (CFU-GM) in the spleen

increased rapidly in these mice, progressive elimination of bacteria from peritoneal cavity, spleen and liver was observed. This indicated a non-specific effectiveness of the *Chlorella vulgaris*extract in the granulocytopenic state. In studies on human beings with cytostatics-induced leukopenia it was shown, taking of 1-3 g daily for children or 5 – 6 g/day for adults resulted in increasing of leucocytes numbers and improvement of general welfare (Iarmonienko et al., 1992).

Noda et al. (1996) isolated a glycoprotein with molecular weight of 63.1 kDa purified from *Chlorella vulgaris* (CK22), containing approximately two thirds carbohydrate, mainly D-galactose, with β-1,6-D-galactopyranose backbone, and one third protein. This glycoprotein possessed anti-tumor activity in test on CDF1 mice treated with fibro sarcoma Meth A cells (originated from BALB/c mice) after intra-tumor injection of 10 mg dry weight/kg BW/injection. The amino acid sequence of the protein moiety necessary for the anti-tumor activity was also determined.

This acidic glycoprotein extracted from *Chlorella vulgaris* CK-22 mentioned above was further investigated for its purposes and the results were published by Konishi et al. (1996). CDF1 and BALB/c mice were treated with 5-fluorouracil (5-FU) (with lethal dose of 500/kg or 550 mg/kg or with sublethal dose of 250 mg/kg) and were given 50 mg/kg of the glycoprotein suspension, before or after 5-FU treatment. When the subcutaneous injection of the glycoprotein was done before treatment with 5-FU, the survival rate of mice increased. The glycoprotein prolonged survival of mice bearing Meth-A fibrosarcoma during treatement with 5-FU, without any influence on 5-FU activity. Also the myelosuppresion induced by 5-FU was restored by the *Chlorella* extract: early recovery of hematopoietic stem cells or cells responding to interleukin-3 or granulocyte/macrophage-colony-stimulatin factor was observed.

Hasegawa et al. (2002) showed that the glycoprotein extracted from *C. vulgaris* described above stimulated adherent spleen cells from C3H/HeJ mice lacking functional toll-like-receptor 4 (TLR4) to produce interleukin-12 (IL-12) p40, whereas there was significantly impaired cytokine production in adherent spleen cells from TLR2 knockout mice. The over-expression of mouse TLR2 (mTLR2) and mouse CD14 (mCD14) conferred the inducibility of nuclear factor-kappaB activation to human HEK 293 cells induced by the glycoprotein. These results suggested that TLR2 signaling was at least partly involved in the anti-tumor activity of the acidic glycoprotein extracted from *C. vulgaris*.

Yasukawa et al. (1996) have isolated several sterols from *C. vulgaris* (CK-5) and examined their anti-inflammatory and anti-tumor activity on female ICR mice. TPA was

applied to one ear of mouse (1ng/ear) to induce inflammation and examined isolates were applied onto the ear before the TPA. DMBA (50μg) and TPA (1μg) were used topically on the back of mice to induce two-stage carcinogenesis. Before each TPA-treatment 2.0 μmole of ergosterol peroxide, isolated from *C. vulgaris,* was applied topically. Two $\Delta^{5,7}$-sterols inhibited the inflammation process and ergosterol peroxide markedly inhibited the tumor-promoting effect of TPA after cancerogenesis initiation with DMBA.

Singh et al. (1998) investigated the effect of the oral administration of three doses of *Chlorella vulgaris* (E-25) – 100 mg/kg BW/day, 300 mg/kg BW/day and 500 mg/kg BW/day, administered in distilled water to Swiss albino mice during 2 weeks of gestation or lactation. The fetal and neonatal hepatic GST- and –SH levels were increased after 14 days of gavaging dams with 300 mg/kg BW/day or 500 mg/kg BW/day of *C. vulgaris*, which suggested effective perinatal passage of algal metabolites (following transplacental or translactational exposure). Decreased hepatic lipid peroxidation measured on basis of reduced production of malondialdehyde and cytochrome b5 and P-450 activity in the liver of fetuses or neonates, together with increased GST-SH levels, allowed the authors to state that the active components from *Chlorella* augment the conjugational capacity of GST and the availability of non-critical nucleophiles. The shortening of phase I of biotransformation reduces the epoxidation, thus blocking the initiation of transformation, and protecting from carcinogenesis, as well as decreasing the toxicity of xenobiotics, which need the perioxidative pathway in bioactivation.

Several groups have investigated the influence of active constituents extracted from *Chlorella vulgaris* on animals' immune response to viral and bacterial infections. Ibusuki et al. (1990) treated female ICR mice with a hot water extract from *C. vulgaris* before challenge with murine cytomegalovirus (MCMV). The lyophilized extract was resuspended in phosphate-buffered-saline (PBS). Dose of 10 mg was given i.p. twice, 3 and 1 day before the challenge with MCMV. 75 % to 100 % of mice pretreated with *C. vulgaris* extract survived the virus challenge, and the extract was effective only when given before infection. No virostatic or virocidal activity of the extract could be seen in *in-vitro* studies, when MCMV was incubated with the extract. The viral replication in target organs was inhibited when *C. vulgaris* extract was given prior to infection, also histological findings showed protection from virus-induced damage. Both facts led the authors to state that the resistance to viral infection after treatment with the algal extract must have been of host-mediated nature. Serum interferon (IFN) level – antiviral agent important in the non-specific reaction against viral infection in the early phase – in mice treated with *C. vulgaris* extract and challenged with

MCMV increased together with the activity of 2-5A synthetase (an enzyme that is induced by IFN), and probably in this way augmenting the natural killer cells (NK) activity, observed in *in-vitro* test on spleen NK against YAC-1 cells.

Dantas et al. (1999a) have demonstrated an effect of oral administration of *C. vulgaris* extract (lyophilized and resuspended in water, 50 mg/kg/day in total volume of 0.2 ml) on NK cell activity in male BALB/c mice (genetically susceptible to *Listeria monocytogenes*) infected with a sublethal dose of viable *L. monocytogenes* (i.p., 3 x 10^4 organisms/animal). The activity of spleen NK cells isolated from mice treated with the extract was tested in-vitro against YAC-1 cells, and was increased compared to the activity of NK cells taken from control mice (treated with vehicle). The infection alone also increased the NK activity. When the algal extract was given (once daily starting from 5 days before the challenge), the increase of NK activity was significantly higher than the activities of NK cells measured after the infection alone. After inoculation of mice with a lethal dose of 3 x 10^5 bacteria/animal, all control mice died. Administration of 50 mg/kg or 500 mg/kg of *Chlorella* extract prior to infection resulted in 20% and 55% survival rates, respectively, showing dose-dependent protection of the algal extract on *L. monocytogenes* infection. The hematopoietic response of granulocyte-macrophage colony-forming unit (CFU-GM) was also investigated in this study (results were published by Dantas et al., 1999b). The colony-stimulating activity (CSA) of the serum was also studied in all groups. Bone marrow cells were isolated from tibia. No effects on CFU-GM were observed in the non-infected mice receiving the extract as compared to controls. Nevertheless, the extract produced an increase in CSA levels as compared to controls. The presence of the *L. monocytogenes* infection led to a significant reduction in the numbers of CFU-GM as observed at 48 and 72 h after the infection, in spite of the significant increase in serum CSA activity. Treatment of infected animals with the *C. vulgaris* extract, lead to restoration of numbers of CFU-GM to control levels. In the treated/ infected group the increased serum CSA was significantly higher than that observed in the only infected group. These results, together with those of Dantas et al. (1999a) show that resistance to *L. monocytogenes* infection of the BALB/c mice was due, at least in part, to the increase of CFU-GM in the bone marrow of infected animals.

Hasegawa et al. (1994) administered a water extract of *C. vulgaris* at a dose of 1000 mg/kg, orally, for 10 consecutive days to female BALB/c mice. After the last dose the mice were inoculated intraperitoneally with *L. monocytogenes* (sublethal dose of 1 x 10^4 bacteria/animal). The number of bacteria in spleen and peritoneal cavity in the group pretreated with *C. vulgaris* extract was significantly lower than in control mice. When instead

of sublethal, a lethal dose (3×10^4 or 1×10^5) of bacteria was inoculated, the survival rate in the *C. vulgaris* group increased to 50% in compare to 20% in control (untreated and infected) group, indicating the protective activity of the extract against the *L. monocytogenes* infection. Flow cytometry of peritoneal exudate cells and spleen lymphocytes revealed an increase of $\gamma\delta^+$Thy1.2$^+$ cells on day 3 and 5 after infection with increase of the proportion of TCR$\alpha\beta^+$Thy1.2$^+$ cells on day 10 after infection, both in the exudates and in spleens in the group receiving *Chlorella*-extract. Augmentation of delayed type of hypersensitivity (DTH) to *L. monoctogenes* infection agreed with all other findings. These results suggested that the oral administration of *C. vulgaris* water extract effectively augmented cell-mediated immunity against *L. monocytogenes* through the enhancement in numbers of $\gamma\delta^+$T cells in the early phase of infection, and the increase of $\alpha\beta^+$T cells in the late phase of infection.

In another study, Hasegawa et al. (1995) gave a lyophilized aqueous *C. vulgaris* extract (2% w/w mixed with diet) to C57BL/6 mice with murine acquired immunodeficiency syndrome (MAIDS), induced by single i.p. infection with LP-BM5 murine leukemia virus at the age of 6 weeks. After the immunodeficient mice had been inoculated i.p. with 2×10^4 *L. monocytogenes* (4 weeks after LP-BM5 injection) they were fed the diet with micro-algae till the end of the experiment. Bacterial elimination of *L. monocytogenes* was impaired in mice with MAIDS. The DTH response to *L. monocytogenes* in MAIDS mice treated with *C. vulgaris* extract was significantly higher than that of MAIDS mice after *L. monocytogenes* infection alone. Increased numbers of CD4+CD8- and CD4-CD8+ $\alpha\beta^+$T -cells were found in the infected sites. There was no beneficial effect of the extract on protection against the *L. monocytogenes* infection in the early phase. The authors postulated the possible effectiveness of *C. vulgaris* extract in the treatment of opportunistic infection in retrovirus-induced immunodeficient (e.g. AIDS) patients.

To elucidate the mechanisms, by which the hot water extract from *C. vulgaris* augments cell-mediated immunity in the murine model of *L. monocytogenes* infection mentioned before, Hasegawa et al. (1997) examined the expression patterns of mRNA for cytokines in female C57BL/6 mice, with and without MAIDS, after infection with *L. monocytogenes*. *C. vulgaris* extract was given as 2% w/w of solid diet starting two weeks after injection of LP-BM5 (to induce the MAIDS) and lasted for four weeks. After two weeks of feeding with the extract, mice were inoculated with *L. monocytogenes*. The experiment finished two weeks later. Control mice received only the food with the extract for two weeks. The expression levels of IL-1α, IL-12, granulocyte macrophage-colony stimulating factor (GM-CSF), macrophage inflammatory protein (MIP) and tumor necrosis factor (TNF-α)

genes were significantly augmented in the peritoneal adherent cells flushed from mice from the group administered the *C. vulgaris* extract. The expression levels of IFN-γ and IL-12 mRNA were significantly higher in the spleen after *L. monocytogenes* infection, in mice treated orally with the *C. vulgaris* extract, than in normal mice, while the expression of IL-10 mRNA in the spleen was decreased by administration of the extract. In MAIDS mice, oral administration of the extract also augmented the expression of IFN-γ and IL-12 mRNA in the spleen after *L. monocytogenes* infection, while it significantly reduced the expression of IL-10 mRNA. These results suggest an activation of $\gamma\delta^{+}$T cells (shown by elevated IL-1α and IL-12) and NK cells (shown by elevated IL-12 and TNF-α) to produce IFN-γ in the early phase of *L. monocytogenes* infection. The elevation of IL-12 and TNF-α measured in the infected group fed *C. vulgaris* extract, suggests that the T cells preferentially develop into Th1 cells, which in turn produce IL-2 and IFN-γ and induce cell-mediated immunity characterized by activation of macrophages and induction of cytotoxic T lymphocytes (CTL).

Furthermore, Quieroz et al. (2002) reported a study similar to Dantas et al. (1999a), showing an increase of IFN- γ and IL-2 with no changes in IL-4 and IL-10 levels in splenocytes collected from mice infected with *L. monocytogenes* and treated with *C. vulgaris* extract. The changes in these cytokines' levels were observed 48 and 72 hours after i.p. inoculation of *L. monocytogenes* and application of the algal extract, there were no changes observed when the extract was given to non-infected mice nor in control (untreated) mice. The mode of action of *Chlorella vulgaris* extract via stimulation of IL-2 and IFN- γ production was therefore confirmed.

Justo et al. (2001) investigated the anti-tumor activity of the *Chlorella vulgaris* extract. Male BALB/c mice were inoculated i.p. with Ehrlich ascites tumor cells (6 x 10^6 viable cells/mouse) on day 0. The lyophilized extract was dissolved in distilled water and administered orally for 5 consecutive days by gavage of 0.2 ml per mouse. The investigated dosages of the extract were 50 mg/kg, 100 mg/kg and 200 mg/kg. Mice bearing a tumor, and receiving the extract, survived significantly longer than control tumor-bearing mice. Administration of the extract also protected the mice against myelosuppresion induced by the Ehrlich ascites tumor. The *C. vulgaris* extract had no influence on the number of granulocyte-macrophage progenitor cells (CFU-GM) in the bone marrow of normal mice, but it stimulated myelopoiesis in tumor-bearing mice and the number of CFU-GM was restored to control levels, with no difference between dosages of the extract. A slight reduction in spleen colony formation was noted in the tumor-bearing mice. A protective anti-tumor effect of the *C. vulgaris* extract was suggested, with a possible mechanism being stimulation of the

production, and possibly maturation of, granulocytes and macrophages. These findings were in agreement with results of Hasegawa et al. (1997). The broarder role of cytokines in myelopoiesis and hematopoesis has been discussed in the cited references.

From the existing literature one can see that *Chlorella vulgaris* possesses immunomodulating activity that could be of wide use in human or animal medicine. For these purposes hot water extract from the micro algae or purified active components, such as chlorellan, may be most useful. The correct dosages for use in humans must be established (dosages used in cited studies are summarized in Table 13) but 50 mg of lyophilized extract /kg body weight seems to be enough to assure the expected activities, at least in mouse model.

Table 13. Dosages of hot water extract from *Chlorella vulgaris*, or of extracted components of the micro-algae, used in animal studies.

Dose*	**Administration****	**Animal**	**Reference**
10 – 500 mg/kg BW in 10 ml	p.o.	Mice	Sarma et al. (1993)
20 – 500 mg/kg BW	i.t., s.c.	Mice	Tanaka et al. (1984)
50 mg/kg BW	s.c.	Mice	Tanaka et al. (1986)
50 mg/kg BW	s.c.	Mice	Tanaka et al. (1986), Konishi et al. (1990)
50 mg/kg BW in 0.2 ml	p.o.	Mice	Quieroz et al. (2003)
50 mg/kg BW, 500 mg/kg BW in 0.2 ml	p.o.	Mice	Dantas et al. (1999a)
50 mg/kg BW, 100 mg/kg BW, 200 mg/kg BW	p.o.	Mice	Justo et al. (2001)
200 mg/kg BW	i.p.	Mice	Konishi et al. (1985)
500 mg/kg BW	on-skin	Mice	Singh et al. (1999)
500 mg/kg BW	p.o.	Mice	Singh et al. (1995)
125 mg/kg BW, 500 mg/kg BW, 1000 mg/kg BW	p.o.	Rats	Tanaka et al. (1997)
1000 mg/kg BW	p.o.	Mice	Hasegawa et al. (1994)
10 mg *in toto*	i.p.	Mice	Ibusuki et al. (1990)

Table 13 *- continued*

Dose*	Administration**	Animal	Reference
C. vulgaris – 100 mg/kg BW 300 mg/kg BW, 500 mg/kg BW	p.o.	Mice	Singh et al. (1998)
C. vulgaris – 1% of diet	p.o.	Rabbits	Sano & Tanaka (1987)
C. vulgaris – 10% of diet	p.o.	Rats	Morita et al. (1999)
Chlorellan – 2 mg/100 g BW	i.p., i.v.	Rats, mice	Kojima et al. (1973)
Ergosterol peroxide – 2.0 µmol	on-skin	Mice	Yasukawa et al. (1996)
Glycolipid fraction of extract, 0.25% in diet (w/w)	p.o.	Rats	Sano et al. (1988)
Glycoprotein – 10 mg/kg BW	i.t.	Mice	Noda et al. (1996)
Glycoprotein – 50 mg/kg BW	s.c.	Mice	Konishi et al. (1996)
Lyophilized extract, 2% of diet	p.o.	Mice	Hasegawa et al. (1995, 1997, 2000)
Phospholipids fraction of extract, 0.25% in diet (w/w)	p.o.	Rats	Sano et al. (1988)

* If no component is mentioned, water extract of *C. vulgaris* is meant.

**i.p. – intra peritoneal, i.t.- intra tumor, i.v. – intravenously, p.o. – per orally, s.c. – subcutaneous

2.4.2. Other aspects of *Chlorella vulgaris* activities

Chlorella vulgaris contains several active components, not fully defined to date, that make this micro-algae interesting from the human medicine point of few. As I have already written, most of the scientific interest was towards anti-tumor activity of the micro-algae, where the glycoprotein of 63.1 kDa [isolated by Konishi et al. (1990) from the hot water extract of *C. vulgaris*] plays an important role. As this glycoprotein affects cytokine production (as described above), it is possible that other pathways, where these cytokines are also involved, could be affected.

Hasegawa et al. (2000) investigated the influence of the *C. vulgaris* extract on changes induced by physiological stress. Female C57BL/6 mice were used for the experiment. The physiological stress was induced by putting the mice in communication boxes with floor equipped with grids for electrification. Sender mice were exposed to electrical impulses and

responder mice were only exposed to emotional responses of the sender mice for 14 days. Solid food containing 2% (w/w) of lyophilized *C. vulgaris* extract was given to mice for 2 weeks before exposure to physiological stress, and during the 14 days of stress exposure. Administration of the extract prevented the usual atrophy of thymus and spleen, as well as the reduction of the granulocytes in blood and suppressed the increase of serum corticosterone level, induced by the physiological stress. The results suggested that the algal extract had prevented the apoptosis of thymocytes in stressed animals. Possible ways of decreasing the cortisol level were discussed considering the cytokines levels modulation caused by the *C. vulgaris* extract. One possibility is an effect of the extract on the hypothalamic-pituitary-adrenal axis, making it more refractory to physiological stress. The other possibility is a direct suppression of the activity of adrenal cortex to produce glucocorticoids.

Anti-ulcer activities of the water extract from *C. vulgaris* in stress-induced, cysteamine-induced peptic ulcers in rats, and in Shay's rat models, were investigated by Tanaka et al. (1997). Doses of 125 mg/kg, 500 mg/kg and 1000 mg/kg of the lyophilized extract were administered orally to rats just before stress loading. In the first two models doses higher than 500 mg/kg prevented the formation of peptic ulcer, there was no influence of ulcer formation in Shay's rat model study. This action was postulated to be due to the porous characteristic of *C. vulgaris* extract (observed in electron microscopy), which can protect the gastric mucosa, and indirectly by stimulation of T cells and macrophages, together with stimulation of IL-1 release, thus activating the "immune-brain-gut" axis (defined by Uehara et al., 1992).

Chlorell vulgaris can be used as a carrier of different substances normally not present, or present in minimal amounts, in the micro-algal cells. For this purpose, the wished constituent is added to the culture medium. Sugimoto et al. (2002) has modified *C. vulgaris* (CK22) via cultivation of the micro- algae with docosahexaenoic acid (DHA)-enriched fish oil. The effect of the oil fraction of an extract from *C. vulgaris* cultivated in this way on on radial maze performance was then investigated in studies on relatively old ICR mice. The investigated group of mice received food with 2% of the DHA-fortified oil fraction for 2 months *ad libitum*, after that time the mice were tested for learning ability related to 2 types of memory:, reference memory (the kind of information that should be retained until the next trial) and working memory (information disappearing in short time), using a partially (4 of 8) baited eight-arm radial maze. Entry into the non-baited arms and repeated entry into the visited arms were defined as reference and working memory, respectively. Administration of

the DHA-fortified *C. vulgaris* oil fraction to mice for 2 months resulted in a significant decrease in the number of working memory errors without affecting the number of reference memory errors. A significant increase in the DHA content in the brain was also observed. These results suggested that intake of the DHA-fortified *C. vulgaris* oil fraction effectively enhanced working memory of aged mice in maze performance and so its intake by Alzheimer's type patients could promise prevention and therapy for dementia in these patients.

When *C. vulgaris* CK22 enriched in DHA (final concentration was 7.6% DHA of cell DM) was fed to patients with increased serum total cholesterol (T-CHO) levels (4 g/day for two weeks, then increasing progressively to 8 and 12 g/day every 2 weeks and 20 g/day for last 3 weeks), lowering of serum level of T-CHO was observed after 6 weeks of experiment, which continued for last 3 weeks of experiment and 3 weeks beyond the modified micro-algae administration, with the same pattern for LDL-cholesterol, esterified cholesterol and phospholipids. HDL-cholesterol decreased after 9 weeks of treatment with the modified micro-algae. No side effects were observed, serum biochemical and blood parameters were not affected during the administration of *C. vulgaris*. These experiments showed DHA-fortified *C. vulgaris* could be a candidate for prophylaxis of age-related hyperlipidemia (Tanaka et al, 2002).

Tsuchida et al. (2003) conducted a placebo-controlled clinical study to investigate γ-aminobutyric acid (GABA)-rich *Chlorella vulgaris* influence on subjects with high-normal blood pressure and mild hypertension. Sixty adult participants (mean age of 48 years) with high-normal blood pressure or mild hypertension (systolic blood pressure: 145.3 ± 5.9 mmHg, diastolic blood pressure: 87.7 ± 6.5 mmHg) were randomly assigned to 4 groups (15 participants per group). Doses of 2, 4, and 6 g of GABA-rich *C. vulgaris* or 4 g of lactose (placebo) per day were ingested, throughout the eight-week treatment period, followed by a two-week withdrawal observation period. Systolic blood pressures in patients receiving 4 or 6 g *C. vulgaris*/day were significantly reduced at six and eight weeks of the micro-algae administration in comparison to the placebo group. Two weeks after discontinuation of GABA-rich *C. vulgaris* intake, the systolic blood pressure in the groups, where 4 or 6 g micro-algae were ingested, showed a rising tendency towards the level at the beginning of the experiment, while no change was observed in the placebo group. No adverse events or hematological, biochemical and urinalysis parameters were observed, throughout the whole treatment period in any of the groups. These results indicated that GABA-rich *Chlorella*

vulgaris had improved blood pressure in patients with high-normal blood pressure and mild hypertension without any adverse effects.

But not only modified *Chlorella* influences blood pressure. As Okamoto et al. (1978) showed, an alkali extract of *Chlorella* spp. (not specified), prepared by decolorization of the green micro-algae by methyl alcohol and gel fractionation, decreased blood pressure of stoke-prone, spontaneously hypertensive and spontaneously hypertensive rats after intravenous (3 mg/100 g BW), intraperitoneal (15 mg/rat) and intragastric (i.g.) (30 mg/rat) administration. The average fall of the blood pressure was of 63 mmHg 30 min. after i.v. administration, 47 mmHg 2 hours after i.p. administration and 20 mmHg 3 hours after i.g. administration. The fraction inducing these effects showed a minimum of 257 nm and maximum of 278 nm in UV absorption spectra and was positive in ninhydrin reaction and in the phenol-sulfuric acid method.

Also Iarmonienko et al. (1992) reported positive effect of *C. vulgaris* E-25 administration in hypertensive patients. After ingestion of 5 – 6 g/day of the green micro-algae the blood pressure decreased of 19 – 30 mmHg.

Micro-algae were also tested for activity in detoxification of acutely or chronically poisoned animals. Morita et al. (1999) reported that feeding male Wistar rats with feed containing 10% of *C. vulgaris* had inhibited dioxins absorption and re-absorption from gut. Dioxins were administered with contaminated rice oil (0.2 ml/rat once on day 1st in experiment 1 or 0.5 ml/rat once on day 1 in 2nd experiment). Diet containing 10% *C. vulgaris* was administered to rats from experimental group for 5 days or from day 8 to 35 (in respective experiments). In the first experiment, the fecal excretions of polychlorinated dibenzo-*p*-dioxin (PCDD) and polychlorinated dibenzofuran (PCDF) congeners in the group fed 10% *C. vulgaris* were 0.2-11.3 and 0.3-12.8 times greater ($P < 0.05$), respectively than in control group; in the second experiment the fecal excretions of respect dioxins were 0.3-3.4 and 0.5-2.5 times greater (most, $P < 0.05$), respectively, than those of the control group. These findings suggested that the administration of *C. vulgaris* may be useful in preventing gastrointestinal absorption, and for promoting the excretion of dioxin already absorbed into tissues. Therefore it could be useful in the treatment of humans exposed to dioxin. The influence of dietary fiber and chlorophyll on inhibition of dioxin absorption from gut, as well as effect of micro-algal lipids on reabsorption of dioxins in bile from the digestive tract, have been discussed as possible mechanism of action. The effect of chlorophyll prepared from *C. vulgaris* cells was then confirmed in studies on rats (Morita et al., 2001). Rats were fed the

isolated chlorophyll in 4 g diet in amount of 0.01 – 0.5%, the diet contained also 0.2 ml of dioxin mixture. The administration of chlorophyll in the diet starting from 0.01% enhanced the fecal excretion of PCDD and PCDF congeners and reduced their absorption from gut.

Quieroz et al. (2003) examined the *C. vulgaris* extract for chelating effects. C57BL/6 mice were orally administered 50 mg/kg of algal extract (lyophilized and dissolved in distilled water, 0.2 ml/mouse) during or post exposure to 1300 ppm lead. 24 hours after the last administration of *C. vulgaris* extract all mice were inoculated with a lethal dose of *L. monocytogenes*. Chelating effects of the extract were observed in the reduction of mean blood lead concentrations in the lead-exposed animals. The reduction rate in animals which received the micro-algal extract post lead-exposure was not as intensive (13.5% and 17% when *C. vulgaris* extract was given for 3 or 10 days starting 24 hours after lead exposure, respectively) as in the group where the extract was given together with exposure to lead (66.3%). Additional experiment with IFN-γ knockout C57BL/6 mice confirmed the important role of this cytokine in the protection against *L. monocytogenes* infection and immunomodulation important for lead elimination afforded by the *C. vulgaris* extract.

Rotkovska et al. (1989) investigated the influence of *Chlorella kessleri*, a green micro-algae close related to *C. vulgaris*, on bone marrow and extra-marrow hemopoiesis in adult female mice (CBA x C57B1), and survival of female Wistar rats submitted to short irradiation with 0.43 Gy/min or prolonged irradiation with 0.048 Gy/min. Mice were treated with Ivastimul *ad usum veterinarium*– freeze-dried aqueous extract of the green micro-algae, which was first dissolved in distilled water and then given i.p., i.m. or s.c. (subcutaneously) in 0.2 ml/mouse and 0.5 ml/mouse p.o., in doses of 800 mg/kg or 400 mg/kg at various times before irradiation. Rats received the product i.p., i.m., s.c. or p.o. in 0.5 ml at dose of 400 mg/kg, 24 hours before irradiation in all cases. Treatment with the *C. kessleri* extract led to an increase of stem cell numbers in the bone marrow and spleen of mice, together with an improved survival rate after irradiation. Lethal dose of gamma rays 24 hours after injection of the extract was survived by larger number of mice and rats in the treated group compared to control animals. The radio-protective effect of the *C. kessleri* extract against brief and prolonged irradiation was observed after its intraperitoneal, intramuscular and subcutaneous administration. The mechanism of this action was later reported by Vacek et al. (1990), from the same group, and activation of granulocyte macrophage-colony stimulating factor (GM-CSF) was considered to play a key role in this process.

The radio-protective activity was also later found in the *C. vulgaris*. Sarma et al. (1993) evaluated the possible role of orally fed *Chlorella vulgaris* (E-25) in modulating the gamma-ray induced chromosomal damage in whole-body irradiated (with 1 Gy delivered at dose rate of 2.6 Gy/min) male Swiss albino mice, applying a micronucleus test. Different doses of *C. vulgaris* (10 mg/kg BW to 500 mg/kg BW) were administered by gavage (in distilled water in volume of 10 ml) either chronically (once, twice or three times a day for 28 days), or as single acute dose before or after irradiation. Only doses above 400 mg/kg BW led to significant radio-protective effect in both acute and chronic pretreatments. However, in mice that received the green micro-algae in dose of 500 mg/kg, three times a day for 28 days, there was no protective effect, and a significant loss in their body weight was observed, even though the feed consumption was not altered. Interestingly, the strain of *C. vulgaris* used in this study afforded significant radioprotection even when it was administered within 0.4 hr after irradiation.

Singh et al. (1995) have also confirmed the existence of radio-protective activity after oral administration of *C. vulgaris* (E-25) to adult male Swiss albino mice, 1 hour before or immediately after exposure to sublethal gamma-rays. The magnitude of radioprotection, defined as an increase in the number of endogenous spleen colony forming units (E-CFU), was dependent on both the dose of *C. vulgaris* fed and the time of administration. The authors observed optimal E-CFU when 500 mg/kg BW of *C. vulgaris* was fed 1 hour before or immediately after irradiation. Significant recovery was observed in the number of bone marrow cells and the spleen weight. $LD_{50/30}$ for *C. vulgaris* pre- and post-treated mice were 8.66 and 9.0 Gy, respectively, compared to the control value of 7.8 Gy. The dose reduction factor (DRF) was 1.11 and 1.15 for pre-treated and post-treated mice respectively.

Okuda et al. (1975) investigated the effects of dried *Chlorella* (species not specified) on the levels of cholesterol in serum and liver of cholesterol-loaded mice and on the level of serum cholesterol of hypercholesterolemic patients. Hypercholesterolemia was induced in male mice by feeding a diet containing 2% cholesterol for 7 days. The addition of 10% dried *Chlorella* powder to the diet (containing the 2% of cholesterol) greatly depressed the hypercholesterolemia and lowered the liver trigliceride level compared with animals not receiving the micro-algae in the diet. In the human trial sixteen patients with hypercholesterolemia took part in the experiment, each of them ingested 20 tablets (5g) of dried *Chlorella* daily, for 3 months. No anti-hypercholesterolemic drugs and low fat diets were administered. At the end of experiment the serum cholesterol levels of the inpatients

were significantly lowered with the *Chlorella* ingestion. Serum cholesterol level fell in from 200 - 250 mg/dl to app. 198 - 228 mg/dl, close to normal cholesterol levels.

Sano & Tanaka (1987) reported an anti-lipidemic and anti-atherosclerotic action of spray-dried *Chlorella vulgaris* on male Japanese rabbits with diet-induced hypercholesterolemia. Rabbits were fed a diet containing 0.5% cholesterol or 0.5% cholesterol plus 1% *C. vulgaris* powder in daily amount of 100 g for 10 weeks. Administration of the green micro-algae significantly reduced serum cholesterol level, and the decline of serum total cholesterol was mainly caused by decline of β-lipoprotein cholesterol. Aortic atheromatous areas in the group fed the micro-algae was about 1/3 that of the cholesterol-group, showing a remarkable suppressive effect on atherosclerotic development. In studies on Wistar rats, Sano et al. (1988) investigated the influence of glycolipid and phospholipids fractions obtained from spray-dried *C. vulgaris*. Rats (app. 100g) were fed a diet containing 1% cholesterol, and experimental groups were fed this diet supplemented with 0.25% (w/w) of each fraction or 5% of powder (containing equivalent amounts of the fractions) in amounts of 15 g. Both fractions significantly decreased serum cholesterol and phospholipids levels, in all animals from experimental groups remarkably high secondary bile acids levels were observed in feces. These findings indicated that 1) absorption of exogenous cholesterol was suppressed by the glycolipid and phospholipid fractions isolated from *C. vulgaris*; 2) absorption of external cholic acid was inhibited and 3) conversion of cholic acid to chenodeoxycholic acid in the liver was promoted, which thereafter was excreted into the gut lumen and where it was converted into lithocholic acid. This has been considered an important mechanism of the *Chlorella vulgaris*- glycolipid and phospholipid fractions in lowering the serum cholesterol level.

Matsuura et al. (1991) fed Donryu rats, with diet-induced iron deficiency, feed containing 5% or 10% of *C. vulgaris ad libitum*. Despite the low total iron concentration (0.74 ppm and 1.39 ppm, respectively) of these *C. vulgaris* supplements, the rats recovered totally and the blood iron and hemoglobin concentrations after 30 days of feeding the algal diet were similar to the concentration of blood hemoglobin in rats from the control group fed commercial feed containing enough iron (3.25 ppm). The iron and hemoglobin concentrations in the 5%-, 10%-*Chlorella* groups and the group fed commercial diet were as follows: iron - 234.0 ± 81.8 μg/dl; 228.0 ± 79.3 μg/dl; 234.6 ± 114.0 μg/dl and hemoglobin – 15.6 ± 0.6 g/dl; 18.6 ± 0.4 g/dl; and 16.8 ± 0.3 g/dl. The authors suggested that, since the iron content of the *C. vulgaris* supplemented food was almost as low as that of the control diet, there had to exist

another mechanism of iron equilibration, leading to recovery from the iron deficiency anemia, and this mechanism remains unknown up to date.

Kapoor & Mehta (1993b) carried out a study to investigate the availability of iron from another micro-algae, the blue-green alga *Spirulina platensis*, compared to whole wheat, whole egg and standard ferrous sulphate in terms of hemoglobin formation, serum and tissue iron levels. Iron deficiency was induced in male albino Wistar rats by giving low-iron diet (9 ppm) and bleeding 1-2 ml blood at weekly intervals for a period of 21 days. The anemic rats were then given iron sources at a level of 35 ppm for 21 days (equal to 61 g of *Spirulina*/kg diet). The hemoglobin gain was significantly higher with ferrous sulphate (4.78 ± 0.43 g/dl) than with whole wheat (4.0 ± 0.91 g/dl), *Spirulina* (3.7 ± 0.69) and whole egg (3.56 ± 0.27). Feeding of ferrous sulphate, whole egg and spirulina produced significantly higher tissue iron levels than feeding of whole wheat. The authors could therefore state that bioavailability of iron from *S. platensis* and whole egg were found to be comparable to that of the standard ferrous sulphate.

2.4.3. Effect of *Chlorella vulgaris* and other micro-algae on reproduction system

Kapoor & Mehta also investigated the influence of *S. platensis* on outcome of pregnancy in rats (Kapoor & Mehta, 1993a). Rats were fed 5 different diets containing 22 % protein *ad libitum*. The protein sources were casein (30 % of diet), *S. platensis* (48 %), wheat gluten (37.4 %), *S. platensis* and wheat gluten (24 % and 18.7 %, respectively); in one diet *S. platensis* (at 48 %) was given without vitamin and mineral supplements. *S. platensis* used in these studies contained (in fresh weight) 4.28 % moisture, 45.12 % crude protein 3.02 % crude fat, 2.28 % crude fiber, 0.93 % ash, iron - 57.55 mg/100g, zinc - 10.00 mg/100g, copper – 0.75 mg/100g and β-carotene – 30.00 mg/100g. The outcome of pregnancy was assessed from litter and dam weights and litter size. Maternal weight gain (WG) was maximal when rats were fed with the *S. platensis* plus wheat gluten diet, and least with the wheat gluten diet (105.2 ± 10.46 g and 51.6 ± 4.67 g, respectively). The WG of pregnant rats fed *S. platensis* as a protein source was 96.2 ± 10.41 g. Rats receiving *S. platensis* -containing diets produced significantly higher litter size (12.6 ± 0.8 pups/litter for *S. platensis* alone, 13.0 ± 1.09 pups/litter for *S. platensis* with wheat gluten and 11.5 ± 1.02 pups/litter for *S. platensis* without vitamin and mineral mixtures) than those receiving casein and wheat gluten (9.6 ± 1.0 pups/litter and 9.8 ± 1.46 pups/litter, respectively). The weight of pups was comparable in all groups. Considering the possible modes of action of *S. platensis* on the outcome in pregnant

rats, authors mentioned a potential role of vitamin E, which plays an established role in fertility and which *S. platensis* contains in a significant quantity (19 mg/100g), but the presence of other active components in the blue-green algal cells cannot be excluded.

Ishibashi (1971) fed *Chlorella* (species not mentioned) as the protein source to female rats and investigated the influence of the green micro-algae on reproduction. No changes in vaginal opening times were observed in experimental compared to control group, the rats fed *Chlorella* were heavier at the time of vaginal opening, so there was no relationship between growth and time of the vaginal opening. In vaginal smears from the *Chlorella*-fed group more cases of continuous estrus were seen, which led the author to state the possible existence of estrogenic substance in *Chlorella* cells. The libido of rats fed *Chlorella* was slightly decreased, but after copulation a good influence on fetal growth was observed. As the text of this paper is in Japanese, I cannot cite more data contained within this study.

Pabst et al. (1978) investigated another green micro-algae, *Scenedesmus acutus*, feeding them to mice over seven generations. In the study mice of both sexes were given a diet containing 20% drum-dried micro-algae, for a period of 80 weeks. The micro-algae contained 50% protein, and were substituted for some of the protein-rich constituents of the basic diet. Authors recorded body weight, feed efficiency, reproductive capacity, life span, organ weights, hematology and blood chemistry of the test animals and compared them with those of control animals. Feeding the micro-algal diet increased body weights by 10 % over the controls, decreased litter size by 11 %, mean birth weights of pups increased by 4 % and survival of female mice by 48 %. In older females fed micro-algal diet relative liver weight increased by 15 %, absolute spleen weight increased by 19 % and absolute kidney weight increased by 13 %. There were no other significant differences between the test animals and the controls. Authors discussed possible causes of the noted changes and stated that some of these differences might have been caused by the altered feed composition rather than the algal supplement specifically. Increased feed intake could also contribute to the differences observed in the study. In Table 14 reproduction data are summarized for all generations recorded in this study.

Table 14. Reproduction data for mice of seven generations fed diet containing 20 % of green micro-algae *Scenedesmus acutus* or control diet (Pabst et al., 1978)

Gen	Age at mating (weeks)	No. of females			Mean litter size at/on			Mean BW (g) of pups at/on	
		Mated	Pregnant	With live litters	Birth	Day 4	Day 14	Birth	Day 14
Animals on control diet									
F_0	12	18	18	17	10.06	8.06	7.12	1.46	8.41
F_1	12	10	10	10	10.20	9.60	9.20	1.46	8.07
F_2	11	20	18	18	10.06	7.39	7.39	1.49	8.85
F_3	7	20	20	20	9.95	8.70	8.70	1.46	7.95
F_4	12	20	20	20	10.20	7.60	7.60	1.44	7.94
F_5	7	20	19	19	10.00	8.84	8.68	1.47	7.83
F_6	11	20	20	20	9.45	8.80	8.60	1.49	7.88
*F_5	30	20	20	17	8.23	7.59	7.58	1.45	7.85
*F_6	30	19	19	16	8.00	7.63	7.63	1.46	8.56
Animals on algal diet									
F_0	12	18	17	17	7.88	(4.24)	(3.82)	1.53	8.42
F_1	12	10	10	10	8.60	8.10	7.80	1.54	8.40
F_2	11	20	18	18	9.72	8.11	8.11	1.52	8.46
F_3	7	20	20	19	9.42	7.68	7.68	1.55	8.51
F_4	12	20	19	19	9.42	8.42	8.42	1.51	8.32
F_5	7	20	(15)**	(14)	9.79	7.93	7.50	1.51	7.96
F_6	11	20	19	19	7.74	6.37	6.21	1.56	8.81
*F_5	30	20	20	16	7.63	6.69	6.69	1.61	8.28
*F_6	30	20	18	18	7.22	6.50	6.50	1.67	10.14

* - second mating

**Values in parentheses were considered anomalous and not taken into consideration

Koehler & Kallweit (2000) reported the influence of *Chlorella vulgaris* fed to sows on their reproductive performance. Sows (that have already littered 1-5 times) were fed 25 g/sow of spray-dried *C. vulgaris* biomass as a supplement to normal feed in several periods: from 14 days before mating till 30th day pregnancy, then from 101st till 115th day of pregnancy; thereafter, in the lactation period from parturition till weaning of piglets (weaning at 35 days of life) at 50 g/sow. Sows were fed with standard feed at 2-3 kg/sow before and 4-5 kg/sow after parturition. The ratio of the micro-algae in feed was therefore equal to 0.8 – 1%. Feed intake was the same in algae- and control group (3 kg/day in the first period, 2.6 kg/day in the second period and 4.6 kg/day in the third period). The number of live-born piglets in the algae group was 11.8 ± 2.8/litter and in the controls 10.9 ± 3.1/litter. The weight of live-born litters was 16.3 ± 3.1 kg and 15.3 ± 3.1 kg, respectively. Weights of weaned piglets were 187.6 ± 27.3 kg/litter and 191.9 ± 36.5 kg/litter in respective groups. There were no significant changes observed, only tendencies in the number of live-born piglets of about 0.5 more in the algae group compared to control, and in the weight of litters about 1 kg more in algae group. No influences on feed intake or side effects after algae feeding were noted.

In a field study on laying hens the influence of 1% supplementation with *Spirulina* was investigated (Anonym 1). 100 laying hens were in the experimental, and 600 hens were in control group. Feeding with 1% of micro-algae supplementation lasted for 30 days; 14 days thereafter the hens were further observed but were fed no more micro-algae. The number of laid eggs increased from 0.38/hen in control to 0.66/hen in algae-group, in the period after algae-feeding the number was 0.3/hen and 0.48/hen, respectively. The mean egg weight increased in the algae group to 63.2 g/egg from 61.7 g/egg in control group. The general health of birds increased in algae group – counting for 100 hens only 1 hen was lost, whereas the lost in control group was 2.2 birds/100 hens. There were also more eggs from class L and XL in the algae group in compare to control, with fewer losses of eggs (25 eggs/100 hens in algae and 28 eggs/100hens in control group).

In summary the physiological properties of the green micro-algae *C. vulgaris* appears to have much potential for use in human and animal nutrition. In form of dried biomass, extracts or isolated active components, it could have a role in human medicine in cancer prevention, in chemotherapy (for its bone marrow protective activity), in the treatment of viral infections or radiotherapy/post-irradiation treatment. Furthermore, it could be applied as a preventive for aging-correlated diseases, i.e. cardiac hypertension, hyperlipidemia and Alzheimer's disease.