4. Results

4.1. Experiment 1 – Evaluation of efficacy of novel technological processing of micro-algae in nitrogen balance study on rats

In order to evaluate the protein value of differently processed *C. vulgaris*, an N-balance study on rats was accomplished. The experiment proceeded without any complications, what the results obtained in the internal control (casein) group confirmed fully. All results recorded from the experiment are summarized in tables 25 to 27, to give a better and clearer overview. The data obtained in the casein group are shown together with the data recorded in other groups, but they are not meant to be compared with algal groups. They show the potential of rats' growth, i.e. what is the potential for feed intake in rats of this age and strain. Statistical analysis was performed for all groups, including the casein group, but in discussion only the data for groups fed with *C. vulgaris* will be considered, as the aim of this experiment was to see whether alternative, or additional, processing (electroporation and ultrasonication) could improve the nutritional value of algal protein compared to simply spray-drying micro-algae.

The intake of nitrogen was similar in all groups, although rats from the group fed electroporated micro-algae as sole protein took about 10 mg N/day less than rats from other groups (Table 24). These rats excreted the most nitrogen in their feces. The lowest N-excretion in feces was recorded in the group fed ultrasonicated *C. vulgaris*, and this difference was significant compared to the group fed electroporated micro-algae. Similarly, the amount of nitrogen excreted with urine was lowest in the group fed ultrasonicated micro-algae. Thus, rats fed electroporated micro-algae have retained the smallest amount of nitrogen and the N-balance was the biggest in the group fed ultrasonicated micro-algae.

Looking at the recorded data for the daily weight gain (dWG), one notices that, the feed containing protein from ultrasonicated *C. vulgaris* led to the best gain of weight (app. 2.7 g/d) compared to feeds containing protein from algae proceeded in other ways (1.2 – 1.8 g/d) (Table 25). The apparent crude protein digestibility (aPD) was 54.79 ± 5.35 %; 44.94 ± 2.82 %; 59.81 ± 4.44 % in the group S-DA, ES-DA and US-DA, respectively. Considering the whole N-balance, a productive protein value (PPV) was determined. The values of this parameter were: 27.09 ± 6.77 %; 16.71 ± 5.43 %; 35.65 ± 6.23 % in groups S-DA, ES-DA and US-DA, respectively.

Table 24. Daily nitrogen intake and its elimination in experiment 1 (mg/day; n=12/group, mean ± SD).

Group	N – intake	N in feces	N in urine	N-balance
CAS	211.36 ± 11.94 ^{a}	31.38 ± 2.73 ^{a}	42.99 ± 4.45 ^{a}	136.99 ± 10.48 ^{a}
S-DA	205.60 ± 20.74	92.47 ± 10.48 ^{bc}	56.55 ± 5.89 ^{b}	56.58 ± 17.93 ^{bc}
ES-DA	194.70 ± 8.91 ^{b}	107.30 ± 8.81 ^{c}	54.86 ± 9.49 ^{b}	32.53 ± 10.86 ^{c}
US-DA	204.14 ± 14.68	82.14 ± 11.16 ^{b}	49.35 ± 6.47	72.65 ± 12.21 ^{b}

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

In previous N-balance experiments on rats accomplished in the FBN, regression formulas for evaluation of endogenous and metabolic nitrogen were generated (see part 3.1.5 f. and g.). Using these parameters, net protein utilization (NPU), true protein digestibility (tPD) and biological value (BV) of protein sources were calculated. The exact data are shown in Table 25. The highest values were for the group fed ultrasonicated green micro-algae, the lowest for the group fed electroporated *C. vulgaris*. The difference was significant for all parameters excluding the biological value.

Table 25. Weight gain and parameters of nutritional value of protein of *C. vulgaris* (n=12/group, mean ± SD)

Group	dWG (g)	aPD (%)	tPD (%)	NPU (%)	BV (%)	PPV (%)	PER
CAS	4.28 ± 0.89 ^{a}	85.15 ± 1.09 ^{a}	92.14 ± 1.09 ^{a}	98.42 ± 2.44 ^{a}	106.82 ± 2.58 ^{a}	64.77 ± 2.25 ^{a}	3.23 ± 0.59 ^{a}
S-DA	1.82 ± 0.51 ^{c}	54.79 ± 5.35 ^{bc}	61.29 ± 5.53 ^{bc}	58.17 ± 7.42 ^{c}	94.74 ± 6.50 ^{b}	27.09 ± 6.77 ^{c}	1.40 ± 0.28 ^{c}
ES-DA	1.20 ± 0.67 ^{c}	44.94 ± 2.82 ^{c}	51.55 ± 2.66 ^{c}	48.41 ± 5.25 ^{c}	93.96 ± 9.51 ^{b}	16.71 ± 5.43 ^{c}	0.98 ± 0.52 ^{c}
US-DA	2.67 ± 0.54 ^{b}	59.81 ± 4.44 ^{b}	66.48 ± 4.43 ^{b}	67.49 ± 5.97 ^{b}	101.38 ± 2.95	35.65 ± 6.23 ^{b}	2.08 ± 0.30 ^{b}

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

Protein efficiency ratio (PER) was 2 times higher in the US-DA group (2.08 ± 0.30) compared to group ES-DA (0.98 ± 0.52) and 1.5 times higher compared to group S-DA (1.40 ± 0.28).

Table 26 shows the values for apparent digestibility of amino acids (aAAD) in casein (internal control) and investigated groups. As mentioned at the beginning of this chapter, data from casein group were compared statistically with data from other groups, but they will not be further taken under consideration.

Table 26. Apparent digestibility of amino acids (aAAD) (%, n = 12/group, mean ± SD)

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

* - essential amino acids for monogastric animals

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

Cysteine was better digested in group fed ultrasonicated *C. vulgaris*. As in case of crude protein, apparent digestibility of all amino acids was higher in group US-DA, compared to S-DA or ES-DA. The difference was significant almost in all cases (see Table 26).

4.2. Experiment 2 – Evaluating of feeding spray-dried micro-algae to rats on blood biochemical parameters

In this experiment 11 adult rats were fed commercial rat food enriched with 20% (w/w) of spray-dried algal powder in order to investigate any changes in uric acid concentrations. Rats weighed approximately 200 g at the beginning of the experiment.

Uric acid concentrations in plasma were measured at weekly intervals and are shown in Table 27. The mean values after 2 weeks of feeding this diet fell from 201 ± 100 µmol/L to 131 ± 32 µmol/L but increased again to 278 ± 162 µmol/L after 4 weeks. It is noteworthy to mention, there were very high deviations between animals (concentrations of uric acid for each animal in this study are summarized in Table II in the Appendix).

The results of other biochemical analyses of plasma collected from rats fed green algae are shown in Table 27. The aspartate aminotranferase (ASAT) activity decreased after algae feeding, whereas alanine aminotransferase (ALAT) activity remained unchanged. Alkaline phosphatase (ALP) increased after one week of feeding with *C. vulgaris* and then decreased. Urea and creatinine concentrations remained at almost the same levels during the whole experiment.

Table 27. Biochemical parameters in plasma from rats fed green algae (n=11, mean ± SD).

Parameter	**Day 0**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
ALAT (U/L)	35 ± 8 ^{ab}	30 ± 12 ^{ab}	23 ± 4 ^{a}	28 ± 6 ^{a}	44 ± 24 ^{b}
ASAT (U/L)	104 ± 8 ^{a}	70 ± 17 ^{b}	77 ± 26 ^{bc}	87 ± 23 ^{ab}	96 ± 22 ^{ac}
ALP (U/L)	198 ± 71 ^{b}	410 ± 186 ^{a}	244 ± 47 ^{b}	199 ± 59 ^{b}	122 ± 58 ^{b}
Urea (mmol/L)	9 ± 2	8 ± 1	8 ± 2	7 ± 1	8 ± 2
Creatinine (µmol/L)	65 ± 19	63 ± 21	63 ± 12	78 ± 17	74 ± 15
Uric acid (µmol/L)	201 ± 100	189 ± 127	131 ± 32 ^{a}	186 ± 87	278 ± 162 ^{b}

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

Animals were in good health throughout the whole experimental period. In conclusion, the health status of rats was not altered, all serum enzymes activities, together with serum metabolites levels measured, were in their normal physiological ranges during the experiment.

4.3. Experiment 3 – Nitrogen balance study and feeding trial undertaken to measure prolonged effect of micro-algae on rats

This experiment was undertaken in order to investigate the influence of prolonged feeding of rats with micro-algae, as the sole source of protein, on animals' growth and metabolism.

4.3.1. Feed and water intake, weight gain, urine production and protein efficiency ratio

Changes in water consumption can reflect primary changes at pituitary level or changes in renal function. As the micro-algae contain high level of nucleic acids, which can, at least in theory, affect renal function via uric acid hyper production or renal calculi formation, we recorded the daily water intake in the experiment.

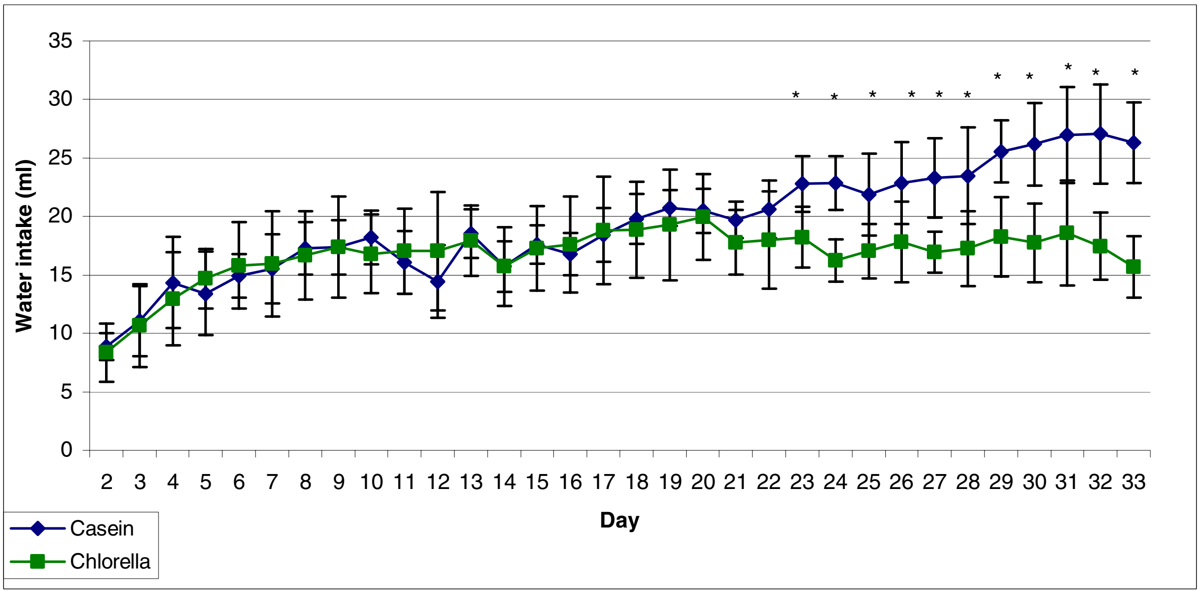
The daily water intake was similar in both groups for the first three weeks. Starting from day 20 – 21, rats from the group fed algae reached a maximal water intake of 15 ml/rat/day, in contrast to rats from casein group, which continued to drink more and were consuming app. 25 ml water/rat/day by the end of experiment (see Fig. 9).

This pattern was also observed for feed intake. Rats from the algal group ate less feed at the beginning of the study, in the adaptation period of 4 first days, and then the feed intake was quite equal for the next 7 days. From the beginning of the third week rats fed the *C. vulgaris* started to eat less than rats fed casein, but the amount of eaten feed slowly, but gradually, increased from 10 to 12 g feed/rat/day (Fig. 10). Rats fed casein ate about 15-16 g feed/rat/day at the end of experiment.

Rats fed ultrasonicated *C. vulgaris* gained weight more slowly than rats fed casein (Fig.11). Both groups started with the same mean body weight of app. 75 g. Rats from the algae group finished with weights of 121 ± 7 g (mean ± SD) and rats fed casein finished with the significantly higher mean body weight of 180 ± 9 g.

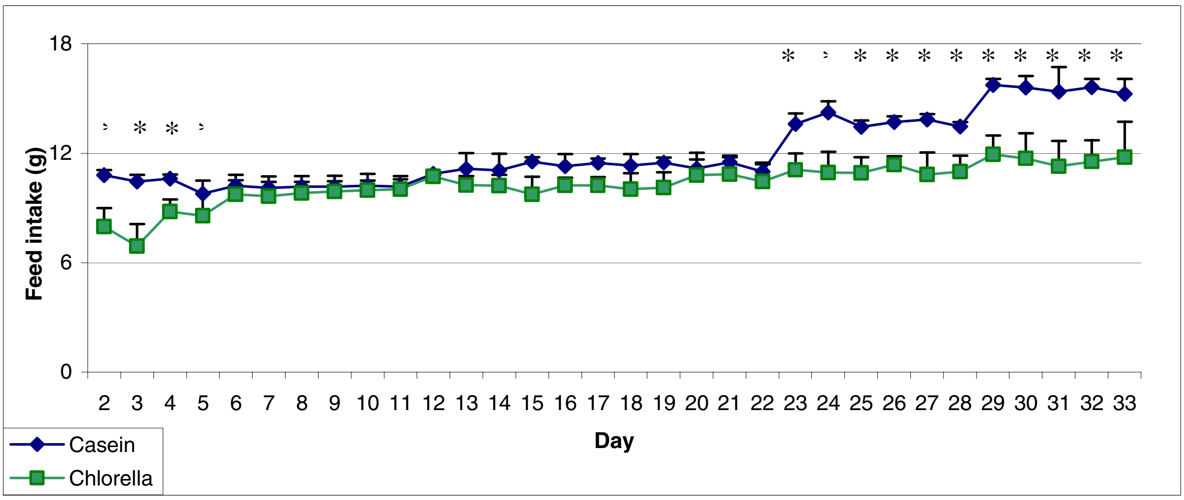
Urine production (Fig. 12) during the whole experiment reflected the water intake and so the curve pattern looks similar to the one for water intake.

Figure 9. Daily water consumption during feeding rats with *C. vulgaris* and casein (n=8, mean ± SD).



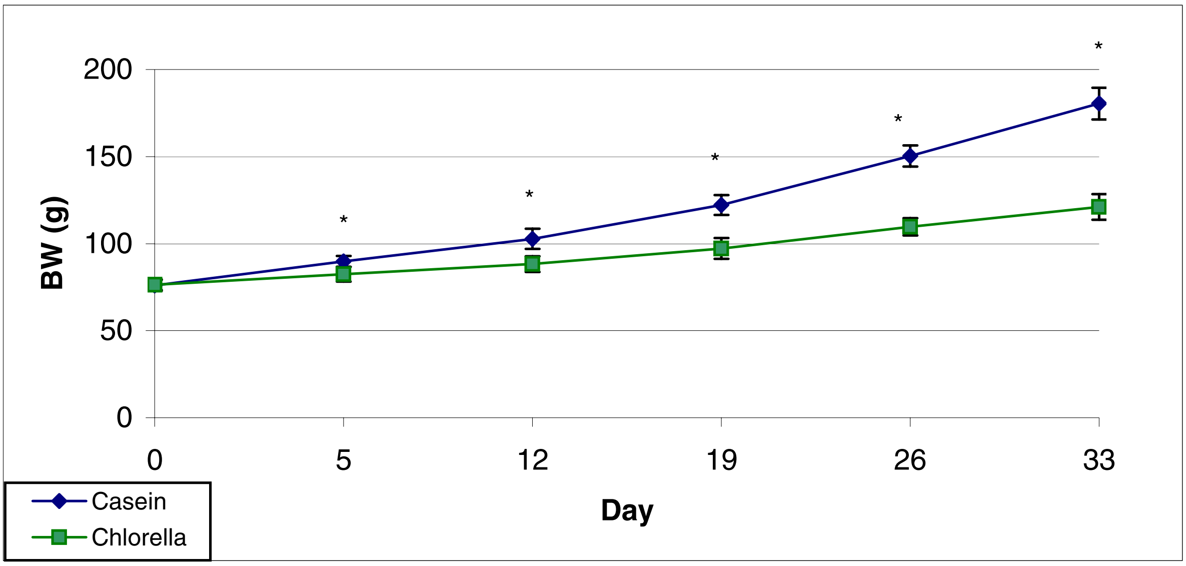
* - significant difference, p<0.05

Figure 10. Daily feed intake during feeding rats with *C. vulgaris* and casein (n=8, mean ± SD).



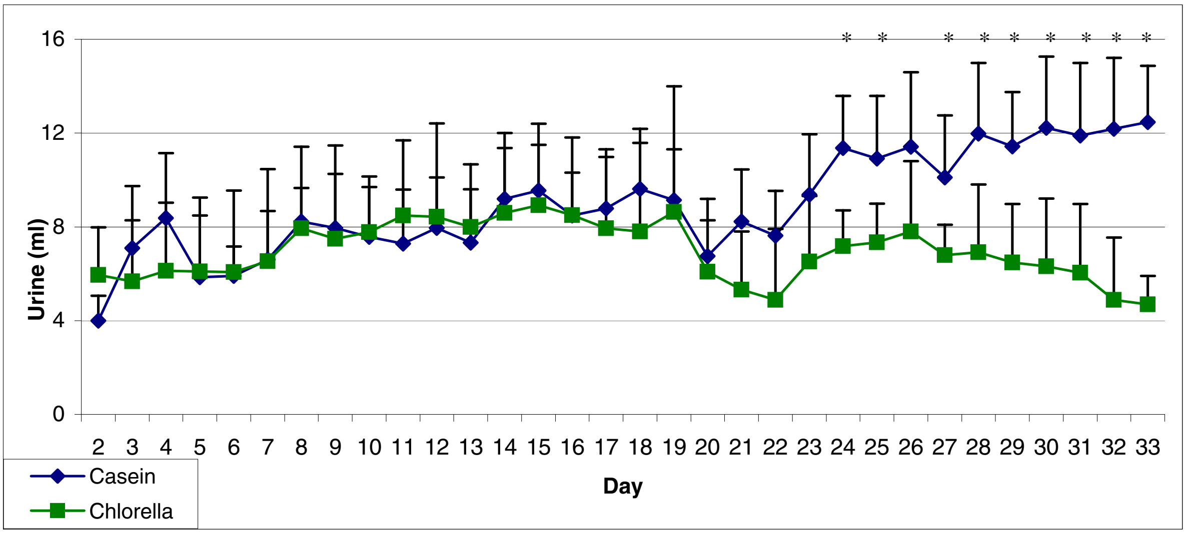
* - significant difference, p<0.05

Figure 11. Body weight of rats feed with *C. vulgaris* and casein (n=8, mean ± SD).



* significant difference, $p<0.05$

Figure 12. Daily urine production after casein vs. micro-algae feeding.



* significant difference, $p<0.05$

4.3.2. N-balance and nutritional parameters

The N-balance trial was performed for a second time, with the difference that rats taken for this experiment were younger than in the first experiment and weighed 76 g on average (76 ± 3 g). The collection of leftover food, urine and feces was done between day 5 and 12 of the whole experiment. This time only ultrasonicated and spray-dried *C. vulgaris* was investigated, in order to verify the previous results. The results obtained in both experiments are compared.

Nitrogen intake and excretion in feces and urine, together with N-balance is shown in Table 28. Rats fed the micro-algae retained less nitrogen than rats fed casein.

Table 28. Daily nitrogen intake and nitrogen elimination in Experiment 3 (mg/day, n=8/group, mean ± SD).

Group	N-intake	N in feces	N in urine	N-balance
CAS	140.61 ± 8.12	15.16 ± 1.46 ^{a}	30.80 ± 2.68	94.65 ± 10.15 ^{a}
US-DA	145.00 ± 7.41	58.97 ± 5.58 ^{b}	33.76 ± 3.42	52.27 ± 3.92 ^{b}

^{a,b} – values in column marked with letters differ significantly ($p<0.05$)

The daily weight gain and calculated parameters of nutritional value of protein are summarized in Table 29. In Tables 30 a) and b) data obtained in Experiment 1 and 3 (named in the table as trial 1 and trial 2) are compared. The parameters of protein nutritional value agree in both age groups, showing that there is no influence of age on the calculations. Daily weight gain is unsurprisingly higher when rats taken for experiment are older (starting weight of app. 135 - 145 g). The value of PER differs also depending on the age (thus depending on the weight gain), as shown in Table 31, where values of PER calculated in the Experiment 3 are shown, week to week. The value of this parameter increased in each group, starting from 2.12 ± 0.41 in casein group and 0.93 ± 0.29 in algal group in the first week to 3.38 ± 0.37 and 1.59 ± 0.37, respectively, in the 4th week, at the end of experiment. However, the maximal value of PER was determined in week 3 (3.74 ± 0.3 in casein group and 1.82 ± 0.39 in algae group), this was the period of most intensive growth of the rats.

The values of nutritional parameters for the algae group were significantly lower than for the casein group.

Table 29. Weight gain and parameters of nutritional value of protein of ultrasonicated *C. vulgaris* and casein (n= 8/group, mean ± SD)

Group	dWG (g)	aPD (%)	tPD (%)	NPU (%)	BV (%)	PPV (%)	PER
CAS	1.86 ± 0.43 [a]	89.21 ± 0.97 [a]	95.23 ± 0.99 [a]	98.95 ± 3.05 [a]	103.91 ± 2.70 [a]	67.14 ± 3.63 [a]	2.10 ± 0.39 [a]
US-DA	0.83 ± 0.27 [b]	59.39 ± 2.44 [b]	64.68 ± 2.42 [b]	64.14 ± 3.42 [b]	99.17 ± 3.57 [b]	36.10 ± 2.97 [b]	0.91 ± 0.28 [b]

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

Table 30 a). Comparison of weight gain and parameters of nutritional value of protein of ultrasonicated *C. vulgaris* and casein obtained in two independent trials (mean ± SD).*

Group / trial	dWG (g)	aPD (%)	tPD (%)	NPU (%)	BV (%)	PPV (%)	PER
CAS / 1 n=12	4.28 ± 0.89 [a]	85.15 ± 1.09 [a]	92.14 ± 1.09 [a]	98.42 ± 2.44 [a]	106.82 ± 2.58 [a]	64.77 ± 2.25 [a]	3.23 ± 0.59 [a]
CAS / 2 n=8	1.86 ± 0.43 [b]	89.21 ± 0.97 [c]	95.23 ± 0.99 [a]	98.95 ± 3.05 [a]	103.91 ± 2.70 [ac]	67.14 ± 3.63 [a]	2.10 ± 0.39 [b]
US-DA / 1 n=12	2.67 ± 0.54 [c]	59.81 ± 4.44 [b]	66.48 ± 4.43 [b]	67.49 ± 5.97 [b]	101.38 ± 2.95 [bc]	35.65 ± 6.23 [b]	2.08 ± 0.30 [b]
US-DA / 2 n=8	0.83 ± 0.27 [d]	59.39 ± 2.44 [b]	64.68 ± 2.42 [b]	64.14 ± 3.42 [b]	99.17 ± 3.57 [b]	36.10 ± 2.97 [b]	0.91 ± 0.28 [c]

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

*No statistical differences for tPD, NPU, BV and PPV between trials were obtained. aPD differs significantly between trial within CAS group, but there is no difference within algal group. No statistical comparison for dWG and PER within groups was done, because these parameters depend heavily on the animals' age.

Table 30 b). Comparison of apparent amino acids digestibility of casein and ultrasonicated and spray-dried *C. vulgaris* obtained in two independent trials (%, mean ± SD)

Group/trial aAAD	CAS / 1 n=12	CAS / 2 n=8	US-DA / 1 n=12	US-DA / 2 n=8
ASP	82.45 ± 2.81 [a]	85.77 ± 1.54 [a]	61.52 ± 10.31 [b]	58.83 ± 3.32 [b]
THR*	84.93 ± 2.82 [a]	87.34 ± 1.55 [a]	57.98 ± 11.23 [b]	55.25 ± 3.46 [b]
SER	74.65 ± 4.26 [a]	83.74 ± 1.89 [a]	56.11 ± 13.68 [b]	53.98 ± 3.33 [b]
GLU	86.06 ± 2.16 [a]	90.40 ± 0.89 [a]	62.36 ± 9.71 [b]	61.37 ± 2.95 [b]
GLY	73.18 ± 5.13 [a]	77.68 ± 2.05 [a]	62.03 ± 10.17 [b]	58.49 ± 3.03 [b]
ALA	75.31 ± 4.61 [a]	78.74 ± 2.03 [a]	66.70 ± 8.42 [b]	64.17 ± 2.46 [b]
VAL*	87.08 ± 2.27 [a]	90.36 ± 1.08 [a]	59.25 ± 10.79 [b]	59.96 ± 2.73 [b]
ILE*	82.99 ± 2.81 [a]	87.46 ± 1.60 [a]	53.64 ± 12.47 [b]	55.90 ± 3.73 [b]
LEU*	91.88 ± 1.55 [a]	93.37 ± 0.62 [a]	62.99 ± 10.12 [b]	60.63 ± 2.63 [b]
TYR	92.79 ± 2.98 [a]	91.52 ± 1.09 [a]	55.23 ± 14.53 [b]	30.02 ± 8.59 [c]
PHE*	92.22 ± 1.56 [a]	93.71 ± 0.62 [a]	57.83 ± 10.81 [b]	57.53 ± 2.99 [b]
HIS*	93.75 ± 1.20 [a]	94.62 ± 0.52 [a]	62.62 ± 11.53 [b]	61.10 ± 3.13 [b]
LYS*	91.06 ± 1.53 [a]	91.97 ± 0.76 [a]	62.75 ± 9.31 [b]	62.46 ± 3.31 [b]
ARG*	89.98 ± 2.12 [a]	90.27 ± 1.05 [a]	77.38 ± 5.96 [b]	72.89 ± 2.16 [c]
PRO	94.93 ± 0.82 [a]	95.59 ± 0.48 [a]	68.28 ± 8.50 [b]	64.90 ± 2.31 [b]
CYS	31.69 ± 16.41 [a]	70.32 ± 3.88 [b]	47.96 ± 16.11 [c]	56.20 ± 3.18 [bc]
MET*	95.38 ± 0.83 [a]	98.26 ± 0.56 [a]	71.61 ± 7.90 [b]	88.37 ± 1.38 [c]
TRP*	91.48 ± 2.23 [a]	97.94 ± 0.95 [a]	82.24 ± 10.32 [b]	80.89 ± 2.57 [b]
Total AA	87.72 ± 2.14 [a]	90.73 ± 0.88 [a]	63.00 ± 9.90 [b]	62.25 ± 2.77 [b]

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

No statistical differences between trials were noted except for TYR, ARG and MET in algal group and CYS in casein group.

* - essential amino acids in monogastric animals

Table 31. Protein efficiency ratio of casein and ultrasonicated and spray-dried *C. vulgaris* obtained in Experiment 3 (mean ± SD)

Group	**Week 1**	**Week 2**	**Week 3**	**Week4**
CAS	2.12 ± 0.41 $^{a, A}$	2.9 ± 0.22 $^{a, B}$	3.74 ± 0.3 $^{a, C}$	3.38 ± 0.37 $^{a, D}$
US-DA	0.93 ± 0.29 $^{b, A}$	1.42 ± 0.29 $^{b, B}$	1.82 ± 0.39 $^{b, C}$	1.59 ± 0.37 $^{b, BC}$

$^{a-b}$ – data in column marked with different letter differ significantly, $p<0.05$

$^{A-D}$ – data in line marked with different letter differ significantly, $p< 0.05$

4.3.3. Internal organs

The absolute weights of organs collected after 33 days of feeding rats on casein or ultrasonicated and spray-dried *C. vulgaris* as sole protein source are shown in Table 32. Most of the organs were heavier in rats from the group fed casein, with exception of large intestine and spleen, which were heavier in the algal group. Significant differences were seen for almost all organ weights, except for caecum, colon, spleen, heart and testicles. The empty carcass (without organs) was about 60 % heavier in the casein group as compared to the algal group, this difference was also significant (145 vs. 89 g).

Although for statistical significance a threshold of 0.05 was taken, in most cases the p value was < 0.01 (Table 32).

Table 32. Weights of organs taken out from rats fed casein or ultrasonicated and spray-dried *C. vulgaris* for period of 33 days (g, n= 8/group, mean ± SD).

Organ	**CAS group**	**US-DA group**	**Value of p (accuracy 0.0001)**
Liver	6.54 ± 0.96 [a]	4.39 ± 0.46 [b]	0.0002
Stomach	1.68 ± 0.24 [a]	1.09 ± 0.09 [b]	0.0001
Small intestine	5.58 ± 0.26 [a]	5.03 ± 0.50 [b]	0.0197
Large intestine	1.71 ± 0.15 [a]	1.90 ± 0.18 [b]	0.0356
Caecum	0.91 ± 0.28	0.98 ± 0.20	0.6002
Colon	0.80 ± 0.21	0.92 ± 0.20	0.2400
Spleen	0.59 ± 0.05	0.63 ± 0.12	0.4615
Lungs	1.00 ± 0.10 [a]	0.71 ± 0.06 [b]	0.0000
Heart	0.66 ± 0.04	0.61 ± 0.07	0.1092
Kidney – left	0.87 ± 0.12 [a]	0.68 ± 0.04 [b]	0.0036
- right	0.91 ± 0.13 [a]	0.71 ± 0.06 [b]	0.0025
Testicle – left	1.46 ± 0.14	1.20 ± 0.32	0.0550
- right	1.48 ± 0.16	1.21 ± 0.33	0.0583
Urinary bladder	0.20 ± 0.07 [a]	0.11 ± 0.04 [b]	0.0099
Empty carcass	144.65 ± 7.72 [a]	89.43 ± 6.11 [b]	0.0000

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

A better comparison of organs is made, when their masses are considered with respect to metabolic weight ($BW^{0.75}$). When the recorded weights were compared with $BW^{0.75}$ (Table 33) and shown as a percentage ratio, fewer differences were seen and different levels of significance appeared. The weights of stomach, small and large intestines (also caecum and colon separately), spleen and heart differed significantly between groups. Small and large intestines as well as spleens and hearts were heavier (with respect to metabolic body weight) in the algal group, whereas stomachs and livers taken out from rats from this group weighed less than the ones taken from rats fed on casein. Empty carcasses of rats fed micro-algae, were also smaller than the carcasses of rats fed casein, when compared on the basis of metabolic body weight.

Table 33. Weights of organs taken from rats fed casein or ultrasonicated and spray-dried *C. vulgaris* for period of 33 days (n= 8/group, mean ± SD) with respect to metabolic weight ($BW^{0.75}$) shown as a percentage (%)

Organ	**CAS group**	**US-DA group**	**Value of p (accuracy 0.0001)**
Liver	13.26 ± 1.49	12.02 ± 0.84	0.0647
Stomach	3.41 ± 0.4 [a]	2.98 ± 0.18 [b]	0.0203
Small intestine	11.36 ± 0.74 [a]	13.79 ± 1.11 [b]	0.0002
Large intestine	3.49 ± 0.35 [a]	5.23 ± 0.61 [b]	0.0000
Cecum	1.87 ± 0.6 [a]	2.69 ± 0.64 [b]	0.0183
Colon	1.62 ± 0.4 [a]	2.53 ± 0.52 [b]	0.0018
Spleen	1.20 ± 0.08 [a]	1.72 ± 0.32 [b]	0.0024
Lungs	2.04 ± 0.15	1.95 ± 0.14	0.2687
Heart	1.35 ± 0.08 [a]	1.67 ± 0.15 [b]	0.0002
Kidney – left	1.76 ± 0.21	1.87 ± 0.06	0.1626
- right	1.83 ± 0.2	1.94 ± 0.11	0.2303
Testicle – left	2.98 ± 0.27	3.28 ± 0.85	0.3620
- right	3.02 ± 0.34	3.31 ± 0.9	0.4106
Urinary bladder	0.39 ± 0.13	0.29 ± 0.11	0.1082
Empty carcass	293.77 ± 5.62 [a]	244.86 ± 6.91 [b]	0.0000

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

The length of major curvature of stomach and of the whole gut was also measured. The major curvature of stomach, small intestine (measured from pylorus to ileo-caecal junction), caecum and colon were longer in the group fed ultrasonicated and spray-dried micro-algae, but the difference was significant only for caecum (Table 34).

Table 34. Length of parts of gastrointestinal tract taken out from rats fed casein or ultrasonicated and spray-dried *C. vulgaris* for period of 33 days (cm, n= 8/group, mean ± SD)

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

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

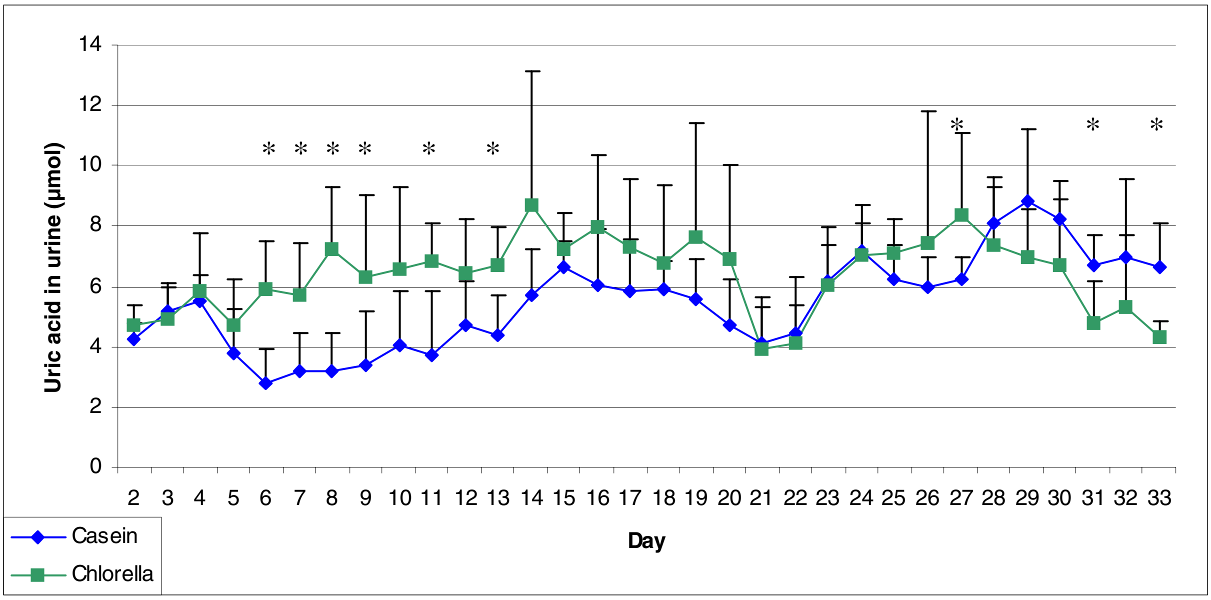
4.3.4. Allantoin and uric acid

Nucleic acids present in feed are catabolized to purine and pyrimidine bases by intestinal phosphorylases. After absorption, pyrimidines are catabolised by the liver to carbon dioxide and ammonia that are easily excreted, whereas purines are metabolized to uric acid and allantoin, which are then excreted with urine. Allantoin is the final product of purine metabolism in most animals and is formed from uric acid by uricase. Humans have lost uricase in the evolution process so, unlike other animals, uric acid is the final product of purine metabolism in human beings. Changes of serum levels of these metabolites, as well as changes in daily excretion with urine, are of clinical and diagnostic importance.

During the first 3 days of the experiment, when rats adapted to the feed, uric acid and allantoin excretion in urine was similar in both groups. The elimination of these products increased then in the group fed micro-algae and was significantly higher than in casein group for next two weeks. In the last 10 – 12 days of the experiment uric acid and allantoin elimination was more or less equal in both groups. The amount of eliminated uric acid held in between 3 and 13 µmol/day, allantoin between 280 and 700 µmol/day. Detailed excretion of both metabolites is shown in Figure 13 and 14.

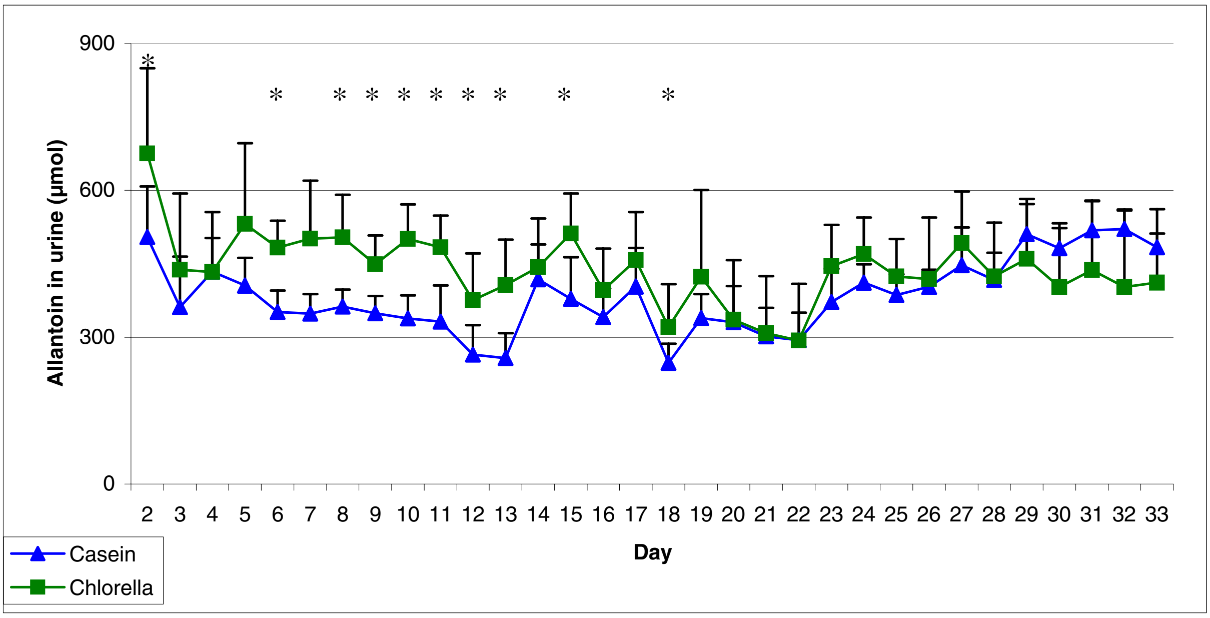
The shape of the curves of daily urinary excretion of allantoin is not exactly the same as the shape of the curves for uric acid excretion. For example, starting from day 5, the uric acid excretion rises and allantoin excretion slightly declines, but from day 13 – 14 the tendencies in both curves are more similar.

Figure 13. Daily excretion of uric acid in rats' urine during feeding with casein or ultrasonicated and spray-dried *C. vulgaris* (n=8/group; mean ± SD)



* - significant difference between groups, $p<0.05$

Figure 14. Daily excretion of allantoin in rats' urine during feeding with casein or ultrasonicated and spray-dried *C. vulgaris* (n=8/group; mean ± SD)

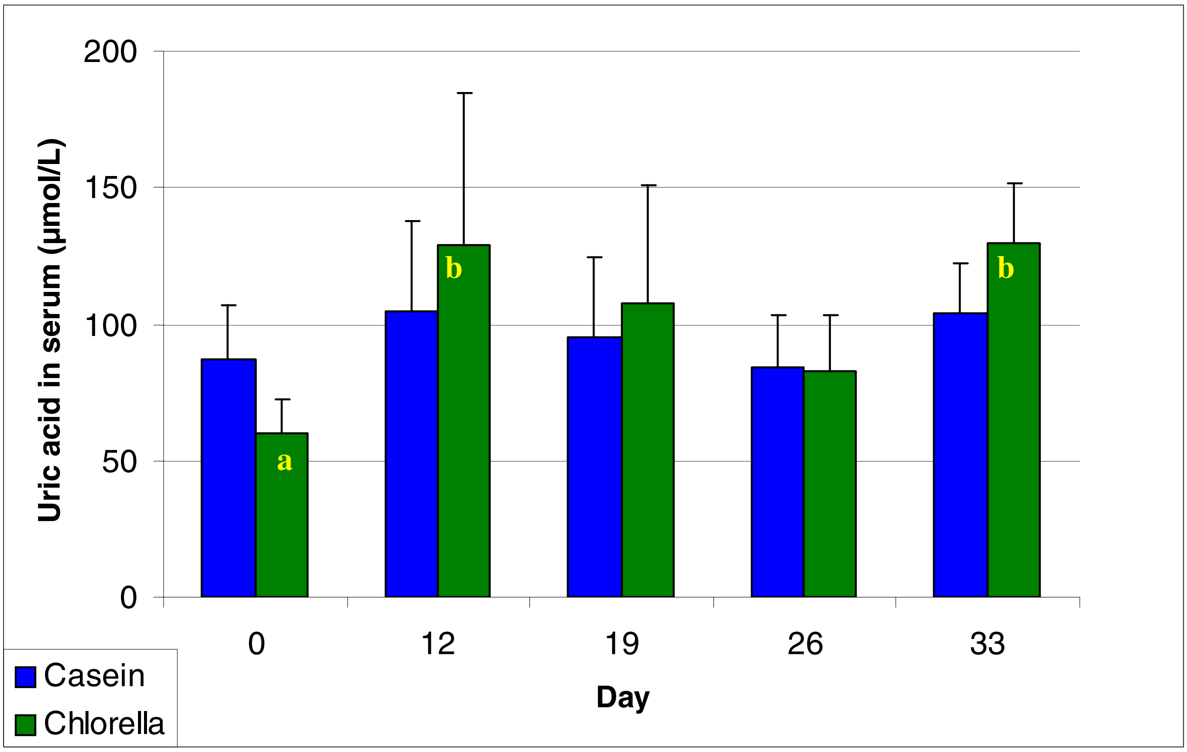


* - significant difference between groups, $p<0.05$

The range of serum concentrations of uric acid measured in both groups was between 60 and 130 µmol/L (Fig. 15). There was a significant difference between the concentrations recorded in algal group on day 12 and 33 compared to day 0. But there were no differences in the serum concentrations of uric acid between groups. Generally, no big fluctuations of uric acid concentrations in serum were noted during the whole experimental period.

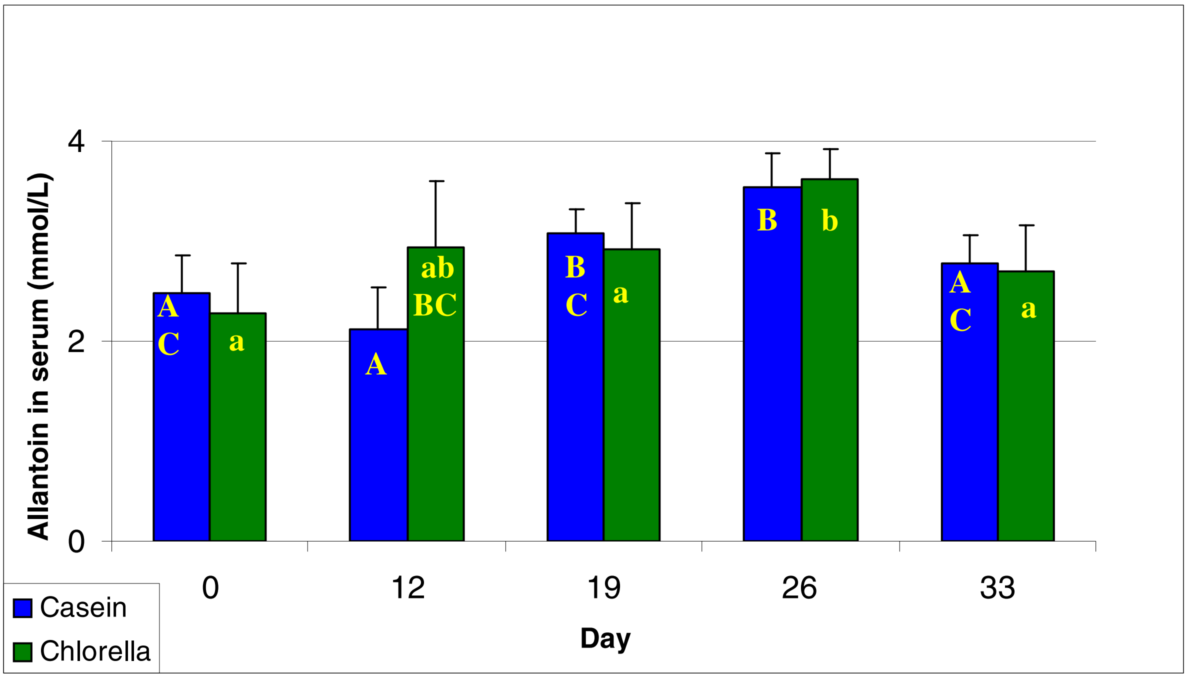
The serum allantoin concentration remained in the range between 2 and 4 mmol/L (Fig. 16). Statistics did show couple of significant differences between the concentrations measured on following days within groups, but only in one case (day 12) a significant difference was seen between groups. Similarly, as in case of uric acid, the allantoin concentrations did not vary during the experiment.

Figure 15. Serum uric acid concentration in rats fed casein or ultrasonicated and spray-dried *C. vulgaris* (n=8/group, mean ± SD)



a, b – values shown in bars with different letter differ significantly, p<0.05

Figure 16. Serum allantoin concentration in rats fed casein or ultrasonicated and spray-dried *C. vulgaris* (n=8/group, mean + SD)



A-C, a-b – bars marked with different letter of one height differ significantly, p<0.05

4.3.5. Biochemical blood parameters

Biochemical parameters were determined in serum collected from blood taken on day 12, 19, 26 and 33 of the experiment. The amount of serum from day 0 was not enough for all analyses and only uric acid and allantoin concentrations were determined in these samples (for serum uric acid and allantoin concentrations see section 4.3.4.).

Activities of alanine (ALAT) and aspartate aminotransferases (ASAT) were stable during the whole experiment and did not differ between groups. The alkaline phosphatase (ALP) activity in the group fed *C. vulgaris* was significantly lower than in casein group. Serum total protein concentration did not differ significantly between groups and remained stable during the whole experiment. Urea concentration in rats' serum was also quite stable during the whole experimental trial and even though its concentration was slightly lower in the casein group, there was no significant difference between groups. A slight increase of creatinine concentration in serum collected on day 26 and 33 was seen, but nevertheless there was no difference between groups.

Statistical evaluation of the data showed significant differences that are to follow in Table 35, but none of these were differences between groups. Only in case of ALP the differences between groups were significant. It should be noted that the activities of enzymes and concentrations of total protein and metabolites measured in the group fed with micro-algae did not exceed the ranges measured in the group fed casein and so they were within normal physiological ranges.

Table 35. Values of biochemical parameters measured in serum collected from rats fed with casein or ultrasonicated and spray-dried *C. vulgaris* in weekly intervals (mean ± SD; n=8/group)

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

[a-c] – values in column marked with different letters show statistical significance ($p<0.05$)

4.3.6. Hematology

For evaluation of hematological parameters blood was collected from rats' hearts at the end of experiment. In 2 cases in casein group and in 3 cases in algae group blood samples clotted and analyses were impossible. All determined values are summarized in Table 37.

An obvious and significant difference was the number of thrombocytes in blood collected from rats fed for 33 consecutive days with ultrasonicated *C. vulgaris* ($1672 \pm 261 \times 10^3/\mu L$) compared to the number of platelets in blood collected from rats fed casein ($1144 \pm 115 \times 10^3/\mu L$).

Hemoglobin concentration was significantly lower in blood from rats fed the micro-algae. Other parameters did not differ between groups, and the erythrocytes number was almost identical in both groups ($5.73 \pm 0.41 \times 10^6/\mu L$ in casein group vs. $5.72 \pm 0.44 \times 10^6/\mu L$ in algal group).

None of the obtained values pointed to any disturbance of hemopoiesis and they all remained within physiological ranges.

Table 36. Thrombocytes count and hematological parameters of rats fed with casein or ultrasonicated and spray-dried *C. vulgaris* (mean ± SD)*

Group	**Ht** (%)	**RBC** (10^6/µL)	**Hb** (g/100mL)	**MCV** (10^{-15}L)	**MCH** (10^{-9}g)	**MCHC** (g/100 mL)	**TBC** (10^3/µL)	**WBC** (10^3/µL)	**NI** (%)	**NM** (%)	**Lymph.** (%)	**Mon.** (%)
CAS, n=6	34.7 ± 2.0	5.7 ± 0.4	11.7 ± 0.7 ^{a}	60.8 ± 5.9	20.4 ± 0.9 ^{a}	33.8 ± 2.9	1144 ± 115 ^{a}	2.8 ± 0.6	2.8 ± 2.3	32.5 ± 21.0	61.2 ± 21.9	5.2 ± 3.2
US-DA, n=5	31.6 ± 6.2	5.7 ± 0.4	10.7 ± 0.5 ^{b}	55.8 ± 13.6	18.7 ± 1.1 ^{b}	34.9 ± 7.5	1672 ± 261 ^{b}	3.1 ± 0.5	0.8 ± 0.8	22.8 ± 6.7	73.8 ± 7.7	2.6 ± 2.4

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

$^{a-b}$ – values marked with different letters differ statistically, $p<0.05$

*Count of basophiles and eosinophiles was 0 in both groups and therefore is omitted in the table

4.3.7. Histology

Histological evaluation of investigated organ samples (small and large intestines, liver and kidneys) revealed no differences between groups. There were no microscopically seen abnormalities of organs' structure. No cell swallowing, no leucocytes infiltrations, no lipid stores could be observed. There is insufficient space to show microphotographs from all samples, examples are shown in Fig. 17 – 22.

Figure 17. Jejunum from rat fed casein

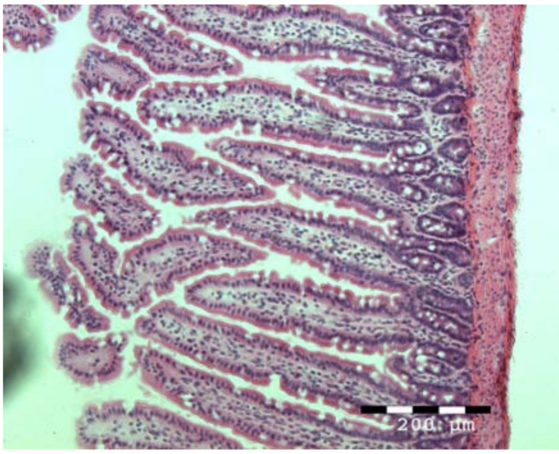


Figure 18. Jejunum from rat fed *C. vulgaris*

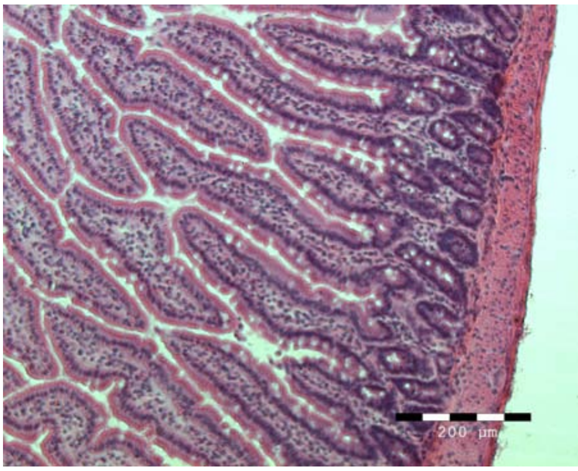


Figure 19. Kidney (cortex) from rat fed casein

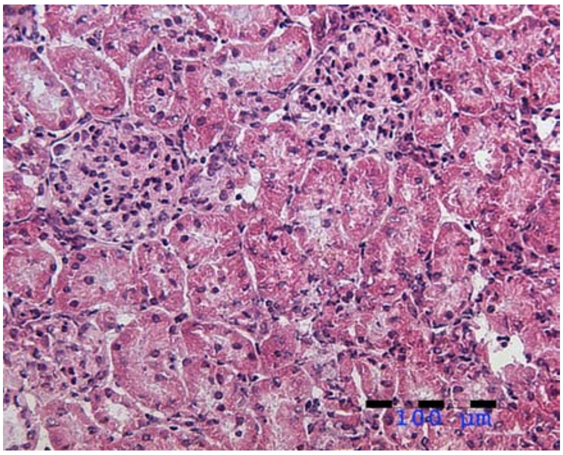


Figure 20. Kidney (cortex) from rat fed *C. vulgaris*

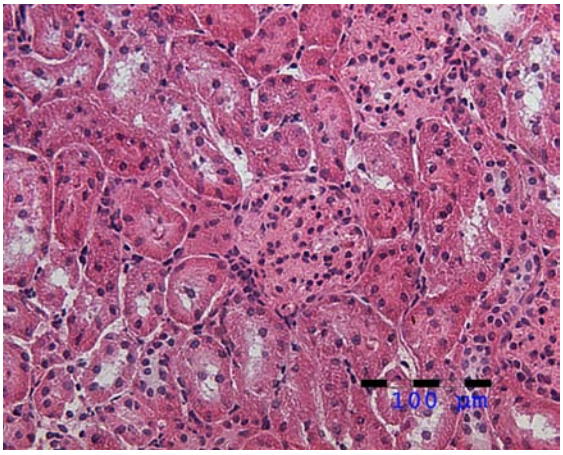


Figure 21. Liver from rat fed casein

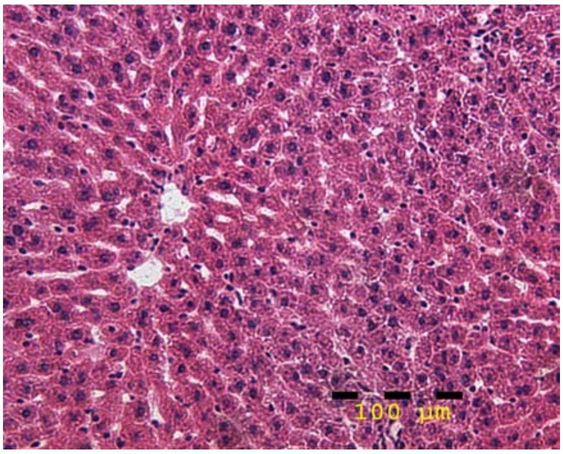
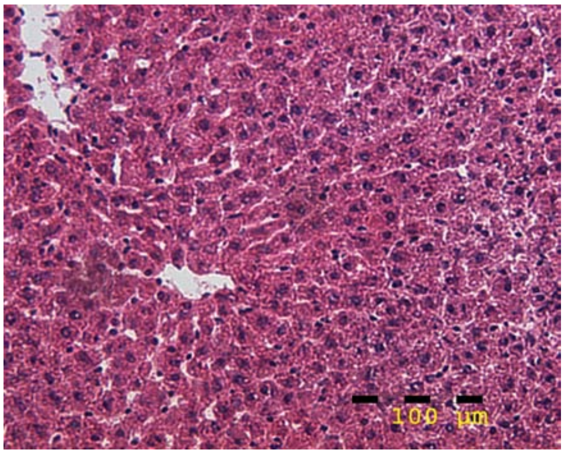


Figure 22. Liver from rat fed *C. vulgaris*



4.4. Experiment 4 – Effect of feeding on C. vulgaris on reproduction and growth of mice

110 female mice from the starting population (F_0) were weaned onto the control or test diet. One mouse from the control group died. All other mice (54 from control and 55 from algal group) gave birth to their pups. Their offspring (F_1) was kept by mothers for 21 days, and so had standing contact to respective feed. After weaning, 2 females and 2 males from each litter were further reared and fed the same diet as the parental generation. The females were mated and half the animals from each group were killed to obtain prenatal traits and half of animals gave birth to next pups generation – F_2; the offspring was reared in the same way as the generation F_1. Again, half of the females were killed and the other half gave birth to pups of next generation – F_3. This was the last generation of the experiment and was kept only till the weaning (21 days).

4.4.1. Growth gain of females

Female mice from the F_0 generation, fed pellets supplemented with 1.0% w/w spray-dried *C. vulgaris*, weighed slightly more then the control mice on the day 42, but on the day 63 the mean weight of the mice was practically equal in both groups (Table 37). A similar pattern is also seen in next generations of female mice (Table 37). Interestingly, the only significant differences were determined between generations within groups, but no significant difference between groups within one generation appeared.

Table 37. Influence of feeding mice with feed supplemented with 1.0% *C. vulgaris* on weight of adult females (generations F_0 – F_2) (g, mean ± SD, n – number of females)

Group	Gen	Weight - day 42	Weight - day 63
Control	F_0	24.78 ± 2.38 (n=55) ^{a}	28.41 ± 1.91 (n=54) ^{a}
	F_1	26.18 ± 2.20 (n=121) ^{bcd}	30.37 ± 2.79 (n=121) ^{b}
	F_2	25.65 ± 2.11 (n=104) ^{ab}	30.18 ± 2.49 (n=106) ^{b}
Algae	F_0	25.57 ± 2.32 (n=55) ^{ad}	28.72 ± 2.35 (n=55) ^{a}
	F_1	26.83 ± 2.51 (n=122) ^{c}	30.89 ± 3.22 (n=122) ^{b}
	F_2	26.13 ± 2.50 (n=93) ^{bcd}	30.70 ± 3.32 (n=93) ^{b}

$^{a-d}$ – values within one column marked with different letter differ significantly, $p<0.05$

4.4.2. Litters

As mentioned above, 109 females from the F_0 generation (54 from control and 55 from algal group) gave birth to their pups. From the F_1 generation 57 females from the control group and 58 females from the experimental group gave birth to their pups, 57 and 59 (respectively) were sacrificed. 47 and 51 females from respective groups from the F_2 generation gave birth to their pups, 50 from each group were sacrificed. The number of live-born pups and the number of pups that survived up to the weaning are shown in the Table 38. Table 39 shows the numbers of young rats that died between birth and the weaning. Generally, there was no difference between groups in the number of live-born mice, but surprisingly, in the groups fed algae there were slightly more stillborn pups per litter, independent of the generation. During the first 10 days a slightly fewer pups from the algae groups died, but number of dead pups on day 21 was similar in both groups.

Litters weights are listed in Table 40. 49 litters from control group and 51 litters from experimental group (generation F_1) were standardized (9 pups, 5 males and 4 females per litter). In this generation, after standardization, 3 litters from the control group and 1 litter from the algae group were lost. The pups in litters from females fed the *C. vulgaris* diet were slightly heavier at weaning than those from the control group (99.86 ± 20.10 g and 96.04 ± 24.16 g, respectively). This difference was not significant. Litters in both other generations were not standardized, but the same tendency was also seen. The litters from algae groups were always slightly heavier than litters from control groups.

From Table 40 one can also see that there exists a big natural diversity between generations, independent of the type of feed fed to mice. Three generations were investigated and during the time of the experiment, litters from next generation within one group were always heavier than litters from the previous generation.

The mean weights of single youngsters are shown in Table 41. There was no difference between groups or generations at the birth. At weaning, the littermates from litters of F_1 and F_2 from mice fed the *C. vulgaris* diet were slightly (but not significantly) heavier than the pups from control group. Even though there was practically no difference at the birth, the youngsters from the algae groups grew a bit better and were heavier at the weaning.

After weaning, two males and two females from each litter were further reared and weighed every 21 days. Recorded weights are summarized in Table 42. As there were not enough litters to obtain the expected number of animals for mating in generation F_1, in several cases more females and males were taken from one litter. Nevertheless, the animals were always kept in pairs, 2 females and 2 males per cage. For this reason number of males and females shown in Table 42 is two times higher than the number of litters on day 21 listed in Table 40.

The females and males from the experimental (algae) group were slightly but significantly heavier at the weaning, but the difference disappeared with time. On the 63rd day of live the weights of males and females were in both groups almost the same. Different sex did neither lead to any differences within or between groups.

Table 38. Influence of feeding mice with feed supplemented with 1.0% *C. vulgaris* on number of life- and stillborn pups and on number of life pups in litters until weaning (mean ± SD per mouse, n – number of litters)

Gen. of pups	**Group**	**Live pups on day 0**	**Dead pups on day 0**	**Pups in standardized litters**	**Pups on day 10**	**Pups on day 21**
F_1	Control	10.7 ± 2.6 (n=52)	0.1 ± 0.3 (n=52)	9	8.0 ± 2.4 (n=50)	7.6 ± 2.8 (n=50)
	Algae	10.9 ± 2.1 (n=53)	0.2 ± 1.0 (n=53)	9	8.1 ± 1.7 (n=51)	8.1 ± 1.7 (n=51)
F_2	Control	11.6 ± 2.2 (n=57)	0.1 ± 0.3 (n=57)	-	10.1 ± 2.8 (n=57)	10.0 ± 2.7 (n=57)
	Algae	10.9 ± 3.2 (n=58)	0.3 ± 1.5 (n=58)	-	9.6 ± 3.3 (n=58)	9.2 ± 3.3 (n=58)
F_3	Control	11.5 ± 3.3 (n=47)	0.2 ± 1.1 (n=47)	-	9.8 ± 4.2 (n=46)	9.5 ± 4.2 (n=46)
	Algae	10.7 ± 4.4 (n=51)	0.5 ± 1.5 (n=51)	-	10.2 ± 3.7 (n=48)	9.0 ± 4.4 (n=48)

Table 39. Influence of feeding mice with feed supplemented with 1.0% *C. vulgaris* on survival rate of pups from birth till weaning (difference between number of pups on day 10-21 and day 0) (mean ± SD, n – number of litters)

Gen. of pups	**Group**	**Live pups on day 0**	**Dead pups till day 10**	**Dead pups till day 21**
F_1	Control	9 ± 0 (n=52)	1.0 ± 2.4 (n=50)	1.4 ± 2.8 (n=50)
	Algae	9 ± 0 (n=53)	0.9 ± 1.7 (n=51)	0.9 ± 1.7 (n=51)
F_2	Control	11.6 ± 2.2 (n=57)	1.5 ± 2.5 (n=57)	1.5 ± 2.5 (n=57)
	Algae	10.9 ± 3.2 (n=58)	1.3 ± 2.0 (n=58)	1.8 ± 2.5 (n=58)
F_3	Control	11.5 ± 3.3 (n=47)	1.9 ± 3.8 (n=46)	2.2 ± 3.9 (n=46)
	Algae	10.7 ± 4.4 (n=51)	1.1 ± 2.1 (n=48)	2.3 ± 4.3 (n=48)

Table 40. Influence of feeding mice with feed supplemented with 1.0% *C. vulgaris* on weight of litters (mean ± SD, n – number of litters)

Generation of pups	**Group**	**Litter weight (g)**			
		Day 0	Day 0 – stand.	Day 10	Day 21
F_1	Control	18.69 ± 3.74 (n=51)	15.50 ± 1.12 (n=49)	51.04 ± 9.46 (n=47)	96.04 ± 24.16 (n=46)
	Algae	18.87 ± 3.68 (n=53)	15.65 ± 1.28 (n=51)	49.93 ± 10.19 (n=50)	99.86 ± 20.10 (n=50)
F_2	Control	20.04 ± 3.70 (n=57)	-----------------	55.70 ± 8.97 (n=55)	100.09 ± 16.05 (n=55)
	Algae	19.07 ± 5.21 (n=58)	-----------------	55.87 ± 10.49 (n=55)	101.17 ± 24.49 (n=55)
F_3	Control	20.75 ± 4.55 (n=46)	-----------------	59.92 ± 13.39 (n=42)	110.70 ± 28.55 (n=42)
	Algae	20.06 ± 5.97 (n=48)	-----------------	58.70 ± 13.89 (n=46)	112.09 ± 28.86 (n=43)

Table 41. Influence of feeding mice with feed supplemented with 1.0% *C. vulgaris* on mean weight of single littermates/litter (mean ± SD, n – number of litters per group)

Generation of pups	**Group**	**Littermate weight (g)**			
		Day 0	Day 0 – stand.	Day 10	Day 21
F_1	Control	1.72 ± 0.13 (n=50)	1.72 ± 0.12 (n=49)	5.94 ± 0.61 (n=47)	11.50 ± 1.46 (n=46)
	Algae	1.74 ± 0.15 (n=53)	1.74 ± 0.14 (n=51)	6.01 ± 0.68 (n=50)	12.10 ± 1.43 (n=50)
F_2	Control	1.74 ± 0.12 (n=57)		5.4 ± 0.72 (n=55)	9.91 ± 1.92 (n=55)
	Algae	1.75 ± 0.15 (n=57)		5.69 ± 1.00 (n=55)	10.79 ± 2.45 (n=55)
F_3	Control	1.79 ± 0.18 (n=46)	-----------------	5.81 ± 1.16 (n=42)	11.19 ± 2.97 (n=42)
	Algae	1.78 ± 0.14 (n=48)	-----------------	5.67 ± 1.13 (n=46)	11.52 ± 2.53 (n=43)

Table 42. Influence of feeding mice with feed supplemented with 1.0% *C. vulgaris* on weights of males and females after the weaning (F_1 and F_2) (g, mean ± SD, n – number of mice)

Group	**Sex**	**Gen**	**Weight - day 21**	**n**	**Weight - day 42**	**n**	**Weight - day 63**	**n**
Control	♂	F_1	11.96 ± 1.79 [a]	122	31.21 ± 3.49	122	38.19 ± 4.32	122
		F_2	10.44 ± 2.07 [b]	110	30.67 ± 2.80	102	37.59 ± 3.55	105
	♀	F_1	11.81 ± 1.41 [c]	122	26.19 ± 2.20	121	30.37 ± 2.79 [a]	121
		F_2	10.29 ± 1.89 [d]	109	25.65 ± 2.11	104	30.18 ± 2.49	106
Algae	♂	F_1	12.51 ± 1.37 [a]	124	31.69 ± 2.39	122	38.07 ± 3.09	122
		F_2	11.18 ± 2.54 [b]	94	31.56 ± 2.69	94	37.85 ± 3.02	92
	♀	F_1	12.33 ± 1.40 [ce]	124	26.83 ± 2.51	122	30.89 ± 3.22 [a]	122
		F_2	11.25 ± 2.18 [de]	93	26.13 ± 2.50	93	30.70 ± 3.32	93

[a-e] – values in columns marked with **the same** letter differ significantly, $p<0.05$

4.4.3. Fetuses

The number of live, dead and absorbed fetuses, as well as number of *corpora lutei* is shown in the Table 43. There is no difference in the number of fetuses between the groups, nor between the generations within groups.

Table 44 summarizes weights of whole litters and weights of single fetuses, recorded on days 16 and 18 of the pregnancy. Because in several cases the mice were sacrificed on other days of pregnancy, as the 16th or 18th, the total number of females per group mentioned in Table 44 is not equal to the number of females that were considered for counting the fetuses in Table 43.

There was no big difference between the weights of the fetuses from both groups, although the fetuses from the algae group were slightly heavier on the 16th day of pregnancy. The difference disappeared on the 18th day of pregnancy. This tendency was seen in both investigated generations.

The number of *corpora lutei* was lower in all cases than the number of live, dead and absorbed fetuses counted together, independent from the group and generation. There was no difference in the number between groups or generations. Therefore, the number of twin pregnancies was similar in both groups.

Table 43. Influence of feeding mice with feed supplemented with 1.0% *C. vulgaris* on number of live, dead and absorbed fetuses, and on the number of *corpora lutei* in mice (mean ± SD, n – number of females)

Generation of females	**Group**	**Live fetuses**	**Dead fetuses**	**Absorbed fetuses**	***Corpora lutei***
F_1	Control, n=57	11.7 ± 2.4	0.1 ± 0.3	0.7 ± 1.0	11.4 ± 2.9
	Algae, n=59	11.6 ± 2.6	0.2 ± 0.4	0.9 ± 1.0	11.7 ± 2.9
F_2	Control, n=50	11.4 ± 3.8	0.1 ± 0.4	0.7 ± 0.9	11.3 ± 2.0
	Algae, n=50	11.6 ± 3.6	0.1 ± 0.4	0.7 ± 1.0	11.0 ± 3.0

Table 44. Influence of feeding mice with feed supplemented with 1.0% *C. vulgaris* on the weight of fetuses during the pregnancy (g, mean ± SD, n – number of females)

Generation of females	Group	Day 16		Day 18	
		Total litter weight	**Fetus weight/litter**	**Total litter weight**	**Fetus weight/litter**
F_1	Control	3.8 ± 1.6 n=11	0.4 ± 0.2 n=11	11.7 ± 4.4 n=43	1.0 ± 0.3 n=43
	Algae	5.5 ± 2.0 n=10	0.5 ± 0.1 n=11	12.3 ± 3.0 n= 45	1.0 ± 0.2 n=45
F_2	Control	4.7 ± 2.1 n=9	0.4 ± 0.2 n=9	12.8 ± 3.2 n=39	1.1 ± 0.1 n=39
	Algae	5.6 ± 2.6 n=11	0.5 ± 0.2 n=11	12.3 ± 3.7 n=35	1.0 ± 0.2 n=35

4.4.4. Blood and serum analyzes

Blood taken at sacrificing was investigated for hematological profile and thrombocytes (platelets) count. Serum was used for determination of iron concentration together with iron binding capacity.

The results of hematological analyses are summarized in Table 45. The numbers of basophils, eosynophils and monocytes were omitted, mostly their percentage was 0 or up to 2, with no differences between groups.

Because of technical problems with the cell counter, not all of blood samples could be analysed for platelets. Moreover, in few cases the blood samples clotted before analysis, this is why the number of investigated samples differs between parameters.

The number of thrombocytes was lower in algae group, both on 16th and on 18th day of pregnancy, independent of the generation. The difference was not significant because of the large standard deviation, but it was a consistent tendency.

Hemoglobin concentration and hematocrit (PCV) measured on 16th and 18th day of pregnancy in mice from F_1 generation were higher in the algae group compared to the control, but there were no differences between the concentrations in the F_2 generation. All other parameters did not differ between groups.

All blood parameters were in physiological ranges for healthy pregnant mice.

Because of the reasons mentioned above, serum could have not been collected from all blood samples and so the iron concentration and iron binding capacity was measured only in some of the blood samples.

Iron concentration was higher in serum collected from algae-fed mice; this difference was observed in both generations, but did not reach statistical significance. By contrast, the iron binding capacity was lower in serum collected from algae-fed animals. Only on the 16th day of pregnancy the obtained concentrations were reversed (Table 46).

Table 45. Hematological parameters in blood of pregnant mice fed commercial feed or feed supplemented with 1.0% *C. vulgaris* (mean ± SD)*

Gen. of fem.	Group	PD	Ht (%)	RBC (10^6/µL)	Hb (g/100 mL)	MCV (10^{-15}L)	MCH (10^{-9}g)	MCHC (g/100 mL)	TBC (10^3/µL)	WBC (10^3/µL)	NI (%)	NM (%)	Lymph. (%)
F_1	Control	16	39.3 ± 2.1 n=8	6.9 ± 0.6 n=8	14.7 ± 0.9 n=8 [a]	57.0 ± 2.6 n=8	21.3 ± 1.3 n=8 [a]	37.4 ± 1.2 n=8	1321 ± 114 n=3	3.1 ± 1.2 n=8	3.2 ± 1.8 n=6	18.5 ± 10.7 n=6	75.3 ± 12.8 n=6
		18	31.7 ± 2.5 n=43 [a]	7.03 ± 0.6 n=43	13.2 ± 1.2 n=43 [ac]	53.7 ± 3.9 n=43	18.8 ± 1.4 n=43 [a]	35.0 ± 2.7 n=43	1508 ± 216 n=27	2.3 ± 1.0 n=43 [a]	3.2 ± 2.8 n=40 [a]	20.1 ± 7.1 n=40	75.4 ± 8.0 n=40
	Algae	16	42.3 ± 3.6 n=10	7.5 ± 0.7 n=10	16.0 ± 1.3 n=10 bd	56.7 ± 4.3 n=10	21.5 ± 2.9 n=10	37.9 ± 3.5 n=10	1298± 290 n=5	3.0 ± 1.8 n=10	1.3 ± 1.3 n=10	16.5 ± 6.3 n=10	78.4 ± 8.9 n=10
		18	38.4 ± 2.6 n=45 [a]	7.3 ± 0.6 n=45	13.7 ± 1.1 n=45 [b]	52.8 ± 4.5 n=45	18.9 ± 01.4 n=45	35.9 ± 2.7 n=45	1265 ± 318 n=22	2.5 ± 1.1 n=45	3.6 ± 2.6 n=37 [a]	19.2 ± 7.2 n=37	76.1 ± 6.7 n=37
F_2	Control	16	41.1 ± 2.5 n=9 [b]	7.3 ± 0.5 n=9	14.2 ± 1.1 n=9	56.4 ± 2.4 n=9 [a]	19.5 ± 0.9 n=9 [b]	34.6 ± 1.8 n=9 [a]	1325 ± 228 n=9	2.0 ± 0.7 n=9	5.3 ± 2.8 n=9	14.7 ± 4.8 n=9	78.4 ± 3.7 n=9
		18	40.0 ± 2.7 n=41 [b]	7.4 ± 0.5 n=41	14.3 ± 0.8 n=41 [c]	54.2 ± 2.9 n=39	19.7 ± 2.0 n=41 [b]	35.7 ± 1.8 n=39	1261 ± 384 n=41	2.1 ± 1.0 n=41 [a]	5.3 ± 2.8 n=41	15.3 ± 5.8 n=41 [a]	78.4 ± 6.7 n=41
	Algae	16	41.5 ± 3.4 n=12	7.3 ± 0.7 n=12	14.3 ± 1.5 n=12 [d]	57.0 ± 3.7 n=12 [a]	19.6 ± 1.5 n=12	34.4 ± 1.5 n=12 [a]	1123 ± 124 n=12	2.6 ± 1.6 n=12	4.2 ± 2.8 n=12	22.0 ± 19.7 n=12 [b]	77.5 ± 9.3 n=12
		18	40.1 ± 3.4 n=36	7.4 ± 0.5 n=37	14.3 ± 1.2 n=37	54.5 ± 3.3 n=36	19.3 ± 1.5 n=37	35.9 ± 2.2 n=36	1198 ± 378 n=37	1.8 ± 1.0 n=37	5.1 ± 2.7 n=37	13.5 ± 5.4 n=37 [ab]	79.7 ± 6.9 n=37

*For clarity, basophils, eosynophils and monocytes were omitted in the table, as their count was 0-2 and did not differ between groups.

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

[a - d] - values with **the same letter** differ significantly, $p<0.05$

Table 46. Iron levels and iron binding capacity in serum from pregnant mice fed commercial feed or feed supplemented with 1.0% *C. vulgaris* powder (µmol/L, mean ± SD)

Generation of females	Group	Day of pregnancy	Fe	Fe-binding capacity
F_1	Control	16	26 ± 17, n=8	55 ± 16, n=6
		18	14 ± 9, n=32	83 ± 43, n=25
	Algae	16	21 ± 12, n=8	72 ± 43, n=6
		18	19 ± 12, n=29	73 ± 53, n=25
F_2	Control	16	18 ± 12, n=8	61 ± 14, n=8
		18	19 ± 9, n=38	61 ± 17, n=35
	Algae	16	26 ± 13, n=12	55 ± 15, n=9
		18	22 ± 10, n=36	54 ± 15, n=32

4.4.5. Internal organs

The internal organs were collected from two generations of mice at the end of pregnancy. Weights and lengths were recorded. There were no differences observed between groups. Colon weights recorded in the generation F_2 were significantly heavier than colon taken from mice of generation F_1, but no feed influence could be observed on colon's weight. Length of gastrointestinal tract did not differ between groups; the only differences recorded were for stomach, which was longer in algal group of generation F_2. These observed differences were most probably incidental. Table 48 and Table 49 show recorded weight and length data.

Table 47. Weights of internal organs of mice fed commercial feed without or with 1.0 % spray-dried *C. vulgaris* (g, mean ± SD).

Organ	**Control F_1 n=57**	**Algae F_1 n=59**	**Control F_2 n=50**	**Algae F_2 n=50**
Stomach	0.30 ± 0.05	0.28 ± 0.05	0.30 ± 0.06	0.30 ± 0.04
Small intestine	2.52 ± 0.39	2.55 ± 0.33	2.65 ± 0.37 ^{a}	2.44 ± 0.34 ^{b}
Large intestine:				
Caecum	0.32 ± 0.05	0.33 ± 0.08	0.33 ± 0.07	0.32 ± 0.05
Colon	0.54 ± 0.13 ^{a}	0.55 ± 0.09 ^{a}	0.67 ± 0.14 ^{b}	0.69 ± 0.10 ^{b}
Liver	3.06 ± 0.42	3.07 ± 0.44	3.15 ± 0.43	3.19 ± 0.46
Spleen	0.20 ± 0.08	0.19 ± 0.07	0.19 ± 0.07	0.21 ± 0.06
Heart	0.20 ± 0.03	0.19 ± 0.02	0.20 ± 0.02	0.20 ± 0.02
Kidney – left	0.23 ± 0.03	0.23 ± 0.03	0.22 ± 0.03	0.23 ± 0.02
- right	0.23 ± 0.03	0.24 ± 0.03	0.23 ± 0.02	0.24 ± 0.03

$^{a-b}$ – values with different letters differ significantly, p<0.05

Table 48. Length of the gastrointestinal tract of mice fed feed with or without 1.0% spray-dried *C. vulgaris* (cm, mean ± SD)

Organ	**Control F_1 n=57**	**Algae F_1 n=59**	**Control F_2 n=50**	**Algae F_2 n=50**
Stomach	2.20 ± 0.37 ^{a}	2.03 ± 0.34 ^{bc}	1.95 ± 0.22 ^{b}	2.13 ± 0.32 ^{ac}
Small intestine	55.26 ± 5.00	56.67 ± 5.04	55.39 ± 4.16	54.68 ± 5.69
Large intestine:				
Cecum	3.46 ± 0.38	3.55 ± 0.51	3.50 ± 0.39	3.53 ± 0.48
Colon	10.22 ± 1.43	10.26 ± 0.96	10.46 ± 1.20	10.71 ± 1.37

$^{a-c}$ – values with different letters differ significantly, p<0.05

4.5. Experiment 5 – Feeding trial for measurement of influence of micro-algal supplementation on feed's crude components digestibility.

This experiment was undertaken to obtain data for measurement of crude components' digestibility of the commercial diet supplemented with 1.0 % spray-dried *C. vulgaris*. Two mice were kept in each cage to assure enough material for analysis and to minimize the analytical error. Therefore the results of nitrogen intake and excretion, N-balance, daily feed intake, daily weight gain and MNR are shown per two mice per day.

Because of the higher N-content in feed supplemented with 1.0 % *C. vulgaris*, N-intake in this group was significantly higher than in control group (301.6 ± 9.3 mg/2 mice/day vs. 274.1 ± 23.6 mg/2 mice/day in respective groups). But the nitrogen excretion with feces and urine was also higher in the experimental group and finally led to slightly lower daily N-balance (40.7 ± 7.0 mg/2 mice/day) compared to control group (42.4 ± 4.7 mg/2 mice/day) (Table 49).

Table 49. Daily nitrogen intake and nitrogen excretion in Experiment 5 [mg/day, n=8 cages (16 mice), mean ± SD]. All values are given for 2 mice.

Group	N-intake	N in feces	N in urine	N-balance
Control	274.12 ± 23.61 [a]	73.99 ± 7.56	157.74 ± 20.25 [a]	42.38 ± 4.74
Algae	301.55 ± 9.29 [b]	77.62 ± 5.72	183.26 ± 10.17 [b]	40.67 ± 7.03

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

Maintenance nitrogen requirement was measured in an N-free trial and was determined to be 28.14 ± 4.02 mg N/2 mice/day (Table 50). Feed intake in both groups was similar [6.6 ± 0.6 g DM/2 mice/day in the controls and 6.8 ± 0.2 g DM/2 mice/day in the algal group (Table 51)]. As these were the same diets as in Experiment 4, where feed intake could not be measured, we therefore confirmed, the feed intake in Experiment 4 did not differ between groups.

Table 50. Daily nitrogen excretion and daily maintenance nitrogen requirement of growing mice measured in N-free trial (mg/2 mice/day, n=32)

NU	IN	MNR
18.77 ± 3.39	9.40 ± 1.62	28.14 ± 4.02

Daily weight gain was slightly better in the control group and was equal to 1.4 ± 0.3 g/2 mice/day compared to the algal group, where it was equal to 1.2 ± 0.2 g/2 mice/day. Digestibility of crude protein and nutritional parameters used for protein value determination did not differ between groups, with the exception of net protein utilization and biological value, which were significantly higher in the control group.

There was no difference seen for the digestibility of crude ash, crude fiber and crude fat. Weight gain and parameters of nutritional value of protein are listed in Table 51. In Table 52 digestibility of other crude components is summarized.

Table 51. Weight gain and parameters of nutritional value of protein of feed with or without 1.0 % supplementation of *C. vulgaris* (mean ± SD). Weight gain is shown for 2 mice; n = 8 cages (16 mice).

Group	dWG (g)	aPD (%)	tPD (%)	NPU (%)	BV (%)	PPV (%)	PER
Control	6.60 ± 0.57	73.00 ± 1.81	76.44 ± 1.84	25.95 ± 3.35 ^{a}	33.93 ± 4.13 ^{a}	15.61 ± 2.60	0.80 ± 0.17 ^{a}
Algae	6.81 ± 0.21	74.25 ± 1.93	77.36 ± 1.91	22.83 ± 2.34 ^{b}	29.50 ± 2.84 ^{b}	13.49 ± 2.33	0.63 ± 0.11 ^{b}

$^{a\text{-}b}$ – values marked with different letters differ significantly, $p<0.05$

Table 52. Apparent digestibility of crude components of feed with and without 1.0 % *C. vulgaris* [%, n = 8 cages (16 mice), mean ± SD]. The apparent crude protein digestibility is already shown in Table 51.

Group	aAD	aFibD	aFD
Control	46.42 ± 3.06	15.67 ± 2.76	86.42 ± 3.31
Algae	46.63 ± 3.24	12.89 ± 3.34	87.09 ± 3.43

Generally, the apparent digestibility of amino acids decreased with feed plus with 1.0 % *C. vulgaris* was fed. However, the difference was significant only for serine, glutamate, valine, isoleucine, proline and cysteine. The values of aAAD are summarized in Table 53.

Table 53. Apparent digestibility of amino acids of feed without and with 1.0% *C. vulgaris* [%, n = 8 cages (16 mice), mean ± SD]

Group/trial aAAD	Control	Algae
ASP	81.31 ± 2.12	79.73 ± 2.13
THR*	74.56 ± 3.12	71.67 ± 2.61
SER	81.80 ± 2.00 ^{a}	78.56 ± 3.46 ^{b}
GLU	88.89 ± 1.24 ^{a}	86.79 ± 1.55 ^{b}
GLY	77.10 ± 2.59	75.86 ± 2.37
ALA	70.81 ± 3.23	68.48 ± 3.09
VAL*	77.78 ± 2.94 ^{a}	74.49 ± 2.15 ^{b}
ILE*	79.11 ± 2.48 ^{a}	76.55 ± 2.27 ^{b}
LEU*	82.73 ± 2.15	80.63 ± 2.10
TYR	82.01 ± 2.72	80.27 ± 3.20
PHE*	84.32 ± 2.11	83.06 ± 2.41
HIS*	85.29 ± 1.79	83.82 ± 2.11
LYS*	75.31 ± 2.86	73.56 ± 3.05
ARG*	89.56 ± 1.68	88.88 ± 1.64
PRO	86.79 ± 1.64 ^{a}	85.03 ± 1.27 ^{b}
CYS	81.21 ± 2.07 ^{a}	84.25 ± 1.29 ^{b}
MET*	87.47 ± 1.68	88.91 ± 1.07
TRP*	85.18 ± 1.94	84.56 ± 1.49
Total AA	82.91 ± 2.22	80.86 ± 1.71

$^{a-b}$ – values marked with different letters differ significantly, $p<0.05$

*essential amino acids in monogastric animals