3. Materials and Methods

The Ethical Committee of the Ministry of Nutrition, Agriculture, Forestry and Fishery of Mecklenburg-Vorpommern approved each animal experiment (approval numbers: LVL M-V/310-4/7221.3-2.1-009/03 and LVL-MV/310-4/7221.3-2.3-017/03).

3.1. Experiment 1 – Evaluation of efficacy of novel technological processing of micro-algae using nitrogen balance studies in rats.

3.1.1. *Chlorella vulgaris*

3.1.1.1 Cultivation of *Chlorella vulgaris*

The unicellular green algae, *Chlorella vulgaris*, was obtained from the Institute for Cereal Processing Ltd. (IGV), Nuthetal, Germany, where it was cultivated in a closed photo bioreactor, PBR 4000, using sunlight (Fig. 2) (Pulz et al., 2000). The C1 strain used originated from IPPAS Moscow. For feeding studies on animals, three differently processed micro algae were used - spray-dried (S-D), electroporated prior to spray drying (ES-D) and ultrasonicated prior to spray drying (US-D).

Figure 2.
Photobioreactor PBR 4000

3.1.1.2. ***Technical processing – electroporation***

The algal biomass used for the tests was injected by a pump to the treatment cell with a delivery rate of 120 L per hour. The electrode gap in the cell was 2 cm. During the treatment of the algal biomass (especially of the algal membranes) an electrical field strength of 3 kV/cm was applied, that caused a specific energy input of 7 kJ/kg algal biomass, there was no significant temperature increase. For the total test time the parameters stayed constant.
After electroporation the *C. vulgaris* biomass was spray-dried and had a residual humidity of 4 – 6%.

3.1.1.3. ***Technical processing – ultrasonication***

At the beginning, several tests were conducted regarding the best cell disruption method. For evaluation of the cell disruption efficiency, the number of particles in the suspension was counted (cells and cell particles). The optimal method was as follows: in the first step *C. vulgaris* biomass with a start temperature of 20°C was pressed through ultrasonic device (model UP 400S, Dr. Hielscher GmbH, Teltow, Germany) and the flow cell was cooled with water by a pump to start temperature. The algal cells were treated with an output between 961-1660 W [depending on the dry matter (DM) content] and a pressure was between 2.0 and 5.6 Ba. The high energy input heated biomass (38.0-39.5°C finally) was collected in a tank and cooled before other treatments took place. The biomass was treated three times in total. Each parameter was kept constant, only the start temperature was adjusted to 20°C.

Immediately after the third treatment the *C. vulgaris* biomass was spray-dried and had a residual humidity of 4 – 6%.

3.1.1.4. Composition of *Chlorella vulgaris*

Representative samples were taken from each of the alga powders and analyzed in the laboratory. The dry matter (DM), crude fiber (cFib), fat (EE) and ash (cA) were determined applying the Weender standard procedure (Naumann & Basler, 1993). The nitrogen estimation was done using Dumas dry combustion technique in an elementary analyzer LECO® CNS 2000 (Leco Instrumente GmbH, Moenchengladbach, Germany, UST-ID: no. DE 129401155). The amino acids content was determined on Biochrom 20 Plus Amino Acid

Analyzer (Biochrom Ltd, Cambridge, UK) applying automated amino acid ion exchange column chromatography (200 x 4.6 mm column) on alkaline, acid and oxidized hydrolysates, according to the specifications of Kreienbring & Wuensche (1974) and Kreienbring (1987). The hydrolisation of samples prior to chromatography allowed exact separation of 18 amino acids. Nucleic acids were isolated from 500 mg algae samples according to the procedure described by Schoenhusen et al. (1988; 2004). The preliminary purification for removal of chlorophyll was accomplished by 10 times extraction with ethanol (95%) and a mixture of ethanol and HCl (10%).

The detailed composition of the components of the algae is shown in Table 15. Table 16 shows the algal content of nucleic acids. The amino acids composition is detailed in Table 17.

Table 15. Crude contents of differentially processed *Chlorella vulgaris*.

Chlorella vulgaris	**% DM**	**% cA /DM**	**% N /DM**	**% cP /DM**	**% cFib/DM**	**% EE/DM**
S-D	95.8	7.3	7.1	44.6	9.2	8.6
ES-D	95.7	12.6	7.0	43.7	11.3	8.8
US-D	96.7	7.9	7.1	44.2	7.6	7.8

DM – dry matter, cA – crude ash, cP – crude protein, cFib – crude fiber, EE – crude fat

Table 16. Nucleic acids of *Chlorella vulgaris*.

C. vulgaris / **Nucleic acids**	**S-D**	**ES-D**	**US-D**
RNA (mg/g of DM)	8.17	8.27	9.53
DNA (mg/g of DM)	4.99	4.92	5.55
RNA + DNA (% of DM)	1.3	1.3	1.5

Table 17. Composition of amino acids of *Chlorella vulgaris* and essential amino acid index (EAAI) compared to casein

Component / *C. vulgaris*	CAS	S-D	ES-D	US-D
Amino Acids (g/16 g N) (total)	103.1	81.3	80.9	83.5
Aspartate (ASP)	7.0	8.0	8.0	8.2
Threonine (THR)*	4.1	3.8	4.1	4.2
Serine (SER)	5.0	2.9	3.3	3.2
Glutamate (GLU)	19.5	9.6	8.5	9.6
Glycine (GLY)	2.0	5.0	4.9	5.2
Alanine (ALA)	2.9	6.8	6.7	7.6
Valine (VAL)*	6.7	5.3	5.0	5.3
Isoleucine (ILE)*	5.4	3.8	3.6	3.8
Leucine (LEU)*	9.3	7.5	7.3	7.6
Tyrosine (TYR)	4.8	2.7	3.0	2.7
Phenylalanine (PHE)*	5.2	4.6	4.6	4.6
Histidine (HIS)*	3.5	1.6	1.5	1.7
Lysine (LYS)*	7.8	5.0	4.7	5.0
Arginine (ARG)*	3.5	5.9	7.4	5.9
Proline (PRO)	11.0	4.2	4.1	4.4
Cysteine (CYS)	0.4	1.5	1.6	1.4
Methionine (MET)*	4.2	2.1	1.8	2.1
Tryptophan (TRP)*	0.9	1.0	0.8	1.1
EAAI (in comparison to casein)**	1.0	1.24	1.24	1.24

* - essential amino acids in monogastric animals

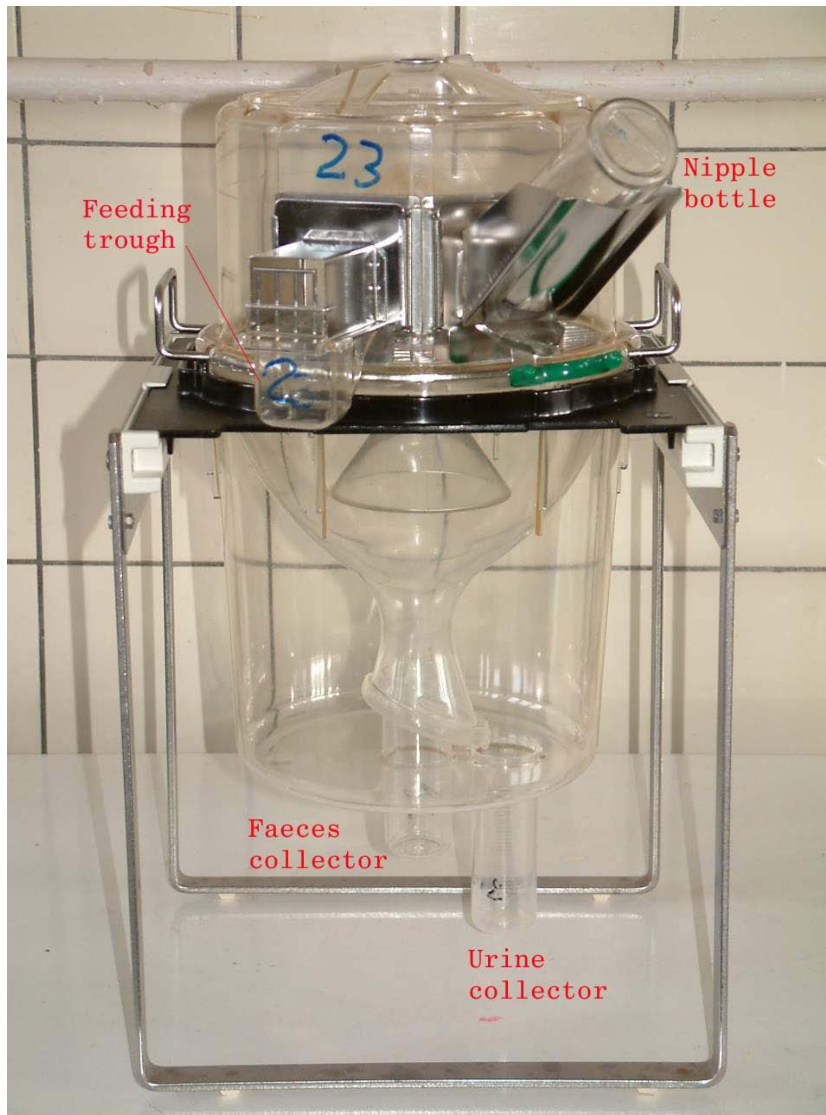
** - essential amino acids index, see point 3.5.1.

3.1.2. Animals

In order to evaluate the protein value of differently processed *Chlorella vulgaris*, 24 male Wistar rats weighing approximately 135 g were obtained from Charles River Laboratories, Germany. The animals were housed in individual metabolism cages (Fig. 3) for 28 days. The humidity of the room air was 50 – 75%, the ambient temperature was held at the

level of 21 ± 1° C, the light regime was 12 hours of light (6.00 am – 6.00 pm) and dark (6.00 pm – 6.00 am). The rats were fed semi-synthetic house diets, which composition is detailed in Table 17. The daily amount of feed was calculated on the basis of body weight (BW) and was equal to 1 g DM per 10 g BW, which resulted in a daily portion of 12 – 14 g DM (13 – 15 g of fresh substance). Tap water was given in nipple bottles *ad libitum*.

Figure 3. Metabolic cage

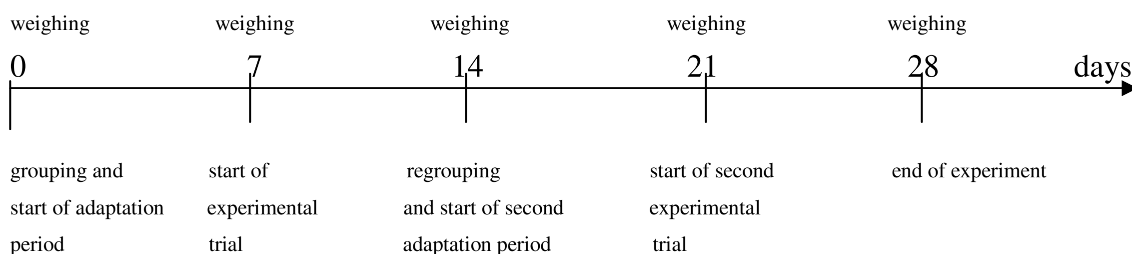


3.1.3. Experimental protocol (grouping, diets, sampling)

The N-balance was according to Thomas-Mitchell method (Mitchell, 1924). Rats were divided into 4 groups; each group consisted of 6 animals. Rats in first group were fed casein as protein source. This was the internal control group to ensure that the whole experiment was processing without any problems and for measurement of growing rats' growth potential. This group will be referred to as "CAS". Rats in the other three groups received experimental diets; these groups will be named "S-DA" (spray-dried algae), "ES-DA" (electroporated and spray-dried algae) and "US-DA" (ultrasonicated and spray-dried algae).

The whole experiment was accomplished in two repetitions. A 7-day adaptation period was followed by 7 days of experimental period. After the first experimental period had finished, all rats were re-grouped into another four groups, in the manner, that no rat belonged to previous group. Next 7 days of adaptation period began, followed by another experimental period of 7 days. In this manner, finally, each group consisted of 12 animals (see Fig. 4).

Figure 4. Experimental approach (Exp. 1).



All rats were fed semi-synthetic house diets, which consisted of N-free base mixture, the experimental protein source and corn starch. The amount of fed diet and its protein content was based on BW of the animals. As rats weighing 100g can eat 10 g DM feed daily, the protein was given in amount of 150 mg N/10 g of DM to cover all protein needs and allow proper growth of rats in this age. Rats from CAS group were fed a diet containing casein enriched with 3% methionine as the protein source. Groups S-DA, ES-DA and US-DA were fed diets containing differently treated algae as a sole protein source (21.0 – 21.4% algae in DM). The composition of all diets is listed in Table 18.

The respective diets were fed to rats in both adaptation and experimental periods. In the first repetition the daily amount of feed given to rats was 12 g of DM (app. 13 g of fresh substance); in the second one the daily amount was 14 g of DM (app. 15 g of fresh substance).

Rats were weighed every 7 days, at the beginning of each period (adaptive and experimental). Leftover food, as well as the feces and urine, were collected during the whole experimental period. 10 to 20 ml of 5%-HCl was added to collected feces and urine to preserve the samples, which were then stored in refrigerator until analyzed.

Table 18. Diet components (%).

Group	**CAS**		**S-DA**		**ES-DA**		**US-DA**	
Repetition	***1***	***2***	***1***	***2***	***1***	***2***	***1***	***2***
N-free mixture*	41.7	50.0	41.7	50.0	41.7	50.0	41.7	50.0
Corn starch	48.5	40.2	37.3	29.0	36.9	28.6	37.1	28.8
Casein + methionine**	9.8	9.8	-		-		-	
S-D *C. vulgaris*	-		21.0	21.0	-		-	
ES-D *C. vulgaris*	-		-		21.4	21.4	-	
US-D *C. vulgaris*	-		-		-		21.2	21.2

*N-free mixture consisted of 18.5% cellulose, 37.1% sugar, 14.8% oil, 7.4% vitamins (Vitamin A - 750 IE, B_1- 1 mg, B_2 - 1 mg, B_6 – 0.5 mg, B_{12} – 0.5 mg, C - 1 mg, D_3 - 25 IE, E – 2.5 mg, K_3 – 0.1 mg, Panthotenic acid - 1 mg, Nicotine acid amide – 2.5 mg, Choline hydrochloride - 100 mg, Folic acid – 0.1 mg, Biotin – 0.01 mg, Inositol – 12.5 mg, p-Aminobenzoic acid - 5 mg, fulfilled ad. 1 g with wheat starch), 14.8% minerals ($CaCO_3$ - 6.86%, Ca-citrate – 30.83%, $CaHPO_4 \cdot H_2O$ – 11.28, K_2HPO_4 – 21.88, KCl – 12.47%, NaCl – 7.71%, $MgSO_4$ 3.83%, $MgCO_3$ – 3.52%, Fe-III ammoncitrate – 1.53%, $MnSO_4 \cdot H_2O$ – 0.02%, $CnSO_4 \cdot H_2O$ – 0.0078%, KJ- 0.0041%, NaF – 0.051%, $AlNH_4(SO_4)_2 \cdot 12\ H_2O$ – 0.009%, $ZnCO_3$ – 0.006%) and 7.4% wheat starch.

**Methionine was added to casein in the amount of 3% casein.

3.1.4. Analyses

Nitrogen content was determined in feed, urine and feces. Dry matter content was measured in feed, leftover and feces. The dry matter determination was accomplished applying the Weender standard procedure (Naumann & Basler, 1993). The nitrogen estimation was done using Dumas dry combustion technique in an elementary analyzer LECO® CNS 2000 (Leco Instrumente GmbH, Moenchengladbach, Germany, UST-ID: no. DE 129401155). Furthermore, the amino acids content in diets and feces was determined and

the analysis was done on Biochrom 20 Plus Amino Acid Analyzer (Biochrom Ltd, Cambridge, UK) applying automated amino acid ion exchange column chromatography (200 x 4.6 mm column). To allow clear chromatographic dividing of 18 amino acids, alkaline, acid and oxidized hydrolysates were done prior to chromatography, according to the specifications of Kreienbring & Wuensche (1974) and Kreienbring (1987).

3.1.5. Nutritional parameters

The dry matter and nitrogen data obtained from sample analyzing were used for estimating the nutritional value of protein of *Chlorella vulgaris*. Calculations were done according to Bock (1966) with some modifications. The parameters and formulas determined for this purpose were as follows:

a. Nitrogen (N) in uneaten feed (leftover - LO)

$$\text{N in LO (mg/day)} = \frac{\text{N in DM of feed (mg/g) x total DM of LO (g)}}{\text{Days of experimental period}}$$

b. N-intake

N-intake (mg/day) = N given in feed (mg/day) – N in LO (mg/day)

c. N- balance

N-balance (mg/day) = N-intake (mg/day) – N-feces (mg/day) – N-urine (mg/day)

d. Daily weight gain (dWG) in experimental period (ep)

dWG (g/day) = [BW at the end of ep (g) – BW at the beginning of ep (g)] / days of ep

e. Daily dry matter intake (dDMI)

$$\text{dDMI (g/day)} = \text{DM-offered (g/day)} - \frac{\text{DM in LO (g)}}{\text{days of ep}}$$

f. Maintenance nitrogen requirement (MNR, metabolic and endogenous nitrogen)

$$\text{MNR (mg/day)} = 0.3705 \times \text{mean BW in ep (g)} + 0.1673 \times \text{mean DMI (g/day)} + 7.4390^{*}$$

* all factors come from a series of N-free experiments done in the FBN on rats according to Bock (1966) with modifications

g. Nitrogen lost from intestines (IN - intestinal nitrogen, metabolic nitrogen)

$$\text{IN (mg/day)} = 0.0708 \times \text{mean BW in ep (g)} + 0.4102 \times \text{mean DMI (g/day)} - 2.2145^{*}$$

* all factors come from a series of N-free experiments done in the FBN on rats according to Bock (1966) with modifications

h. Apparent crude protein digestibility (aPD)

$$\text{aPD (\%)} = \frac{\text{N-intake (mg/day)} - \text{N-feces (mg/day)}}{\text{N-intake (mg/kg)}} \times 100$$

i. True crude protein digestibility (tPD)

$$\text{tPD (\%)} = \frac{\text{N-intake (mg/day)} - \text{N-feces (mg/day)} + \text{IN (mg/day)}}{\text{N-intake (mg/day)}} \times 100$$

j. Biological value (BV)

$$BV\ (\%) = \frac{\text{N-balance (mg/day)} + \text{MNR (mg/day)}}{\text{N-intake (mg/day)} - \text{N-feces (mg/day)} + \text{IN (mg/day)}} \times 100$$

k. Net protein utilization (NPU)

$$NPU\ (\%) = \frac{\text{N-balance (mg/day)} + \text{MNR (mg/day)}}{\text{N-intake (mg/day)}} \times 100$$

l. Productive protein value (PPV)

$$PPV\ (\%) = \frac{\text{N-balance (mg/day)}}{\text{N-intake (mg/day)}} \times 100$$

m. Protein efficiency ratio (PER)

$$PER = \frac{\text{WG (g/day)}}{\text{N-intake (g/day)} \times 6{,}25}$$

n. Apparent amino acid digestibility (aAAD)

$$aAAD\ (\%) = \frac{\text{AA-intake (mg/day)} - \text{AA-feces (mg/day)}}{\text{AA-intake (mg/day)}} \times 100$$

o. Essential amino acids index (EAAI)

$$AAI = \sqrt[n]{\frac{a_1 \text{ in experimental protein}}{a_1 \text{ in compared protein (casein)}} + \frac{a_2}{a_2} + \ldots + \frac{a_n}{a_n}}$$

a = g AA/16g N

3.1.6. Statistics

Statistical analyses were done using software STATISTICA version 6.0. Tukey HSD Test done after ANOVA determined the statistical significance of the data at $p<0.05$.

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

3.2.1. Animals

11 male adult Wistar rats (weighing app. 200 g) obtained from Charles River Laboratories, Germany, were housed in standard plastic laboratory boxes, on sawdust bedding. 2 - 3 rats were kept in each cage. Feed intake and collection of urine and feces was not possible in these cages. The ambient environmental conditions and light regime was as described in *Experiment 1*.

3.2.2. Experimental protocol

Commercial rat chow (Altromin®) was ground and mixed with spray-dried *Chlorella vulgaris* powder obtained from IGV Ltd, in ratio of 20% of the algae and 80% of chow. The prepared ground mixture was then pelleted and dried at 60°C. The food was weighed and offered to rats in amount of 25 g per rat each day. Water was provided in nipple bottles *ad libitum.* Rats had free access to feed and water, and the only restriction in feed intake was the total amount of offered chows. The experiment lasted for 4 weeks.

3.2.3. Blood collection and preparation for analysis

Rats were bled just before start of the experiment, on day 0, then every 7 days for 28 days. Blood was collected from a tail vein under light inhalant anesthesia. For induction of anesthesia rats were kept for several minutes in an induction chamber filled with isofluran vapors (IsoFlo®). Time of induction depended on the individual animal's reaction. The anesthetized rat was checked for stimuli reaction (tail pinching). When this test was positive (no reaction), the tail end was cut off with a scalpel blade and blood was collected into a Multivette ® 600 LH (micro tube of 600 µl volume, containing lithium heparin) (Sarsted AG & Co., Nuembrecht, Germany) (Fig. 5). Blood samples were kept in refrigerator until the collecting procedure from other rats had been finished. All samples were later centrifuged by 2,500 rpm for 10 minutes at 4°C (Varifuge 3.0R, Heraeus Sepatech, Berlin, Germany). Afterwards plasma samples were transferred into Eppendorf mini tubes and frozen at -25°C. Plasma samples were stored frozen until analyzed.

Figure 5. Blood collection in rat applying tail bleeding.

3.2.4. Analyses

Plasma was analyzed in the Office for Veterinary and Foodstuff Examination of the Mecklenburg-Vorpommern (LVL), Rostock, Germany. The samples were analyzed for alanine aminotransferase (ALAT), alkaline phosphatase (ALP), aspartate aminotransferase (ASAT), uric acid, urea and creatinine. For ALAT, ASAT and ALP measurements an optimized standard method was applied, approved by German Association for Clinical Chemistry. For uric acid determination an enzymatic Trinder method was applied. In short, uricase was added to a sample and after 5 minutes of incubation in 37°C the absorbance was measured by 546 nm. Urea was measured with enzymatic UV method, and for creatinine measurement a kinetic Jaffe method was applied. All tests were done using commercial kits specified for each analysis (Labor + Technik GmbH, Germany).

3.2.5. Statistics

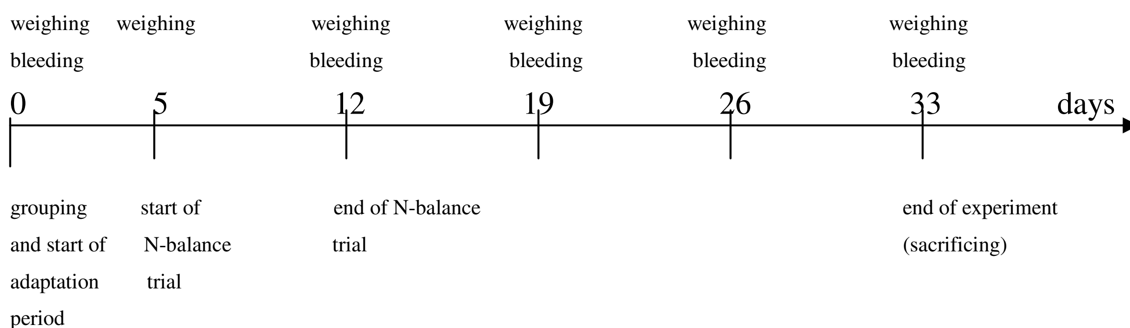
Statistical analyses were done using software STATISTICA version 6.0. Tukey HSD Test done after ANOVA determined the statistical significance of the data at $p<0.05$.

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

3.3.1. Animals and protocol

This experiment was a combination of the two previous experiments. 16 male Wistar rats, weighing app. 75 g were obtained from Charles River Laboratories, Germany, and kept singly in metabolic cages, under ambient conditions described in section 1.2., for 33 days. Rats were divided into two groups, and each group consisted of 8 animals. The feed was prepared on the BW basis as described in *Experiment 1*. The amount of feed was 10g DM / 100g BW, the protein amount was calculated on basis of nitrogen and equaled to 150 mg N / 10g DM. The control group (CAS) was fed diet containing casein as the sole protein source. Water was given in nipple bottles *ad libitum*. In the experimental group, US-DA, the sole protein source was ultrasonicated and spray-dried *Chlorella vulgaris* (this group was adapted as the group US-DA in *Exp. 1*). The adaptation period to new conditions and feed lasted for 4 days. This period was followed by N-balance experimental trial, which lasted for 7 days. After the N-balance trial had finished, the rats were kept in the same metabolism cages and were fed the same diet for another 21 days. A schematic protocol is shown in Figure 6.

Figure 6. Experimental approach (Exp. 3).



3.3.2. Samples

At the beginning of the whole experiment, before the adaptation period began, all rats were weighed. The animals were then weighed at the beginning of N-balance trial (on day 5) and then every seven days (on day 12, 19, 26, 33). Feed and water intake as well as urine production were recorded daily throughout the whole experiment. Urine was also collected daily for further analyses. During the period of N-balance trial feces and leftover food were

collected as well as urine samples. 10 to 20 ml of 5%-HCl was added to urine and feces as preservative and samples were stored in a refrigerator until analyzed.

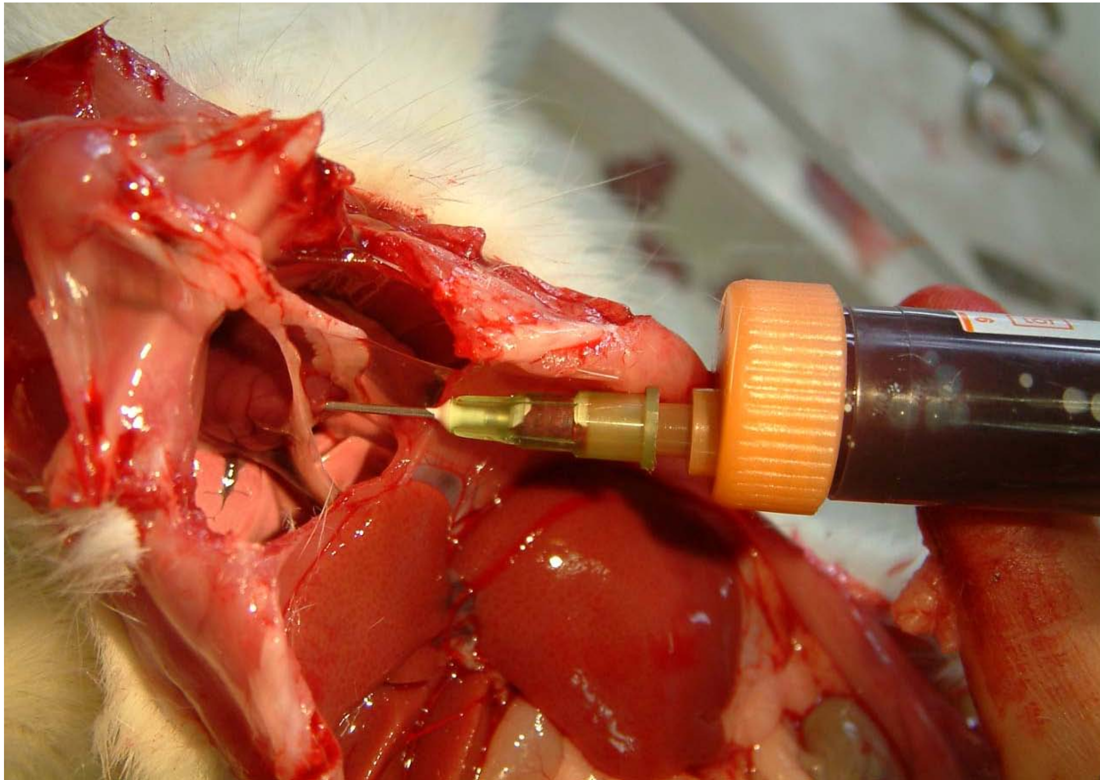
Blood samples were taken via tail bleeding under inhalant anesthesia as previously described in *Experiment 2* on day 0, 12, 19 and 26 (Fig. 5). Blood was collected into mini tubes filled with gel-separator and the samples were stored at 4ºC till further process. Later, samples were centrifuged at 2,500 rpm for 10 minutes at 4°C (Varifuge 3.0R, Heraeus Sepatech, Berlin, Germany); thereafter serum was transfered into Eppendorf mini tubes. Finally, serum was frozen at -25°C and stored under this temperature conditions till analyzed.

On day 33 all rats were sacrificed. The animals were chemically restrained with intramuscular injection of ketamine (10 - 15 mg *in toto*) and xylazine (6 – 8 mg *in toto*) mixture (Fig. 7). Anesthetized rats were exsanguinated by cardiac puncture (Fig. 9). Blood was collected into tubes for serum collection (Monovetten, Sarstedt, Germany) and stored in refrigerator until further processing of serum separation and transfer as described above. An aliquot of blood was collected into mini tubes containing EDTA-K (Multivetten-EDTA, Sarstedt, Germany).

Organs (liver, heart, lungs, kidneys, testicles, stomach, small and large intestines and spleen) were cut out and weighed. The length of stomach (*curvatura major*), of the whole small intestine, caecum, and colon were recorded. Kidneys, livers, and intestines (mid jejunum, caecum and colon) were shock-frozen in liquid N and stored in -80°C until analyzed.

Figure 7. Intra-muscular injection of chemical restraints.

Figure 8. Blood collection from heart



3.3.3. Analyses

Feed and all samples collected in N-balance trial (leftovers, feces and urine) were analyzed as described in *Experiment 1*.

For allantoin determination urine was centrifuged by 4,000 rpm, for 10 minutes at 4°C. After centrifugation, 0.1 ml of urine was taken and diluted to 50 mL with distilled water, to receive a dilution of 500 times. The allantoin concentration in urine was measured according to Borchers (1977). Briefly, urine was alkalized and heated in boiling water for approximately 12-15 minutes. Then 2M HCl with 2,4-dinitrophenylhydrazin was added and the solution was further boiled for 3 minutes. After cooling in cold-water bath, sodium hydroxide was added to alkalize the solution. The absorbance was measured at 520 nm after standing at room temperature for 10 minutes.

For uric acid determination in urine enzymatic Trinder method (Barthelmai et al., 1962; Trinder, 1969) was applied with use of commercial kit (Labor + Technik, Germany). In short, uricase was added to urine and after 5 minutes of incubation in 37°C the absorbance was measured by 546 nm.

For determination of serum allantoin concentration, serum was treated with 10%-TCA, centrifuged at 4,000 rpm for 15 minutes at 4°C, and then was processed in the same way as urine. For uric acid determination in serum the same method was applied as for uric acid detection in urine.

The activities of ALAT, ASAT, ALP and glutamate dehydrogenase (GLDH), and the concentrations of total protein, urea and creatinine were measured in serum. Whole blood was analyzed for red blood cells count (RBC), white blood cells count (WBC), hematocrit (Ht), hemoglobin, lymphocytes and thrombocytes. Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were then calculated. All biochemical and hematological analyses of blood were done at the Office for Veterinary and Foodstuff Examination of the Mecklenburg-Vorpommern (LVL), Rostock, Germany using standard procedures (tests used for biochemical analyses as described in point 3.2.4; whole blood parameters measured on cell counter).

3.3.4. Histological examination

The liver (mid part of right lobe) and kidney (middle section) were sliced on cryotome Leica CM 3050 S (Germany) at chamber and knife temperature of -25 °C; the intestinal samples were sliced by chamber temperature of -24 °C and knife temperature of -20 °C. Frozen sliced were then stained classically with Mayer's haematoxylin and eosin.

Slices (3-5 section/sample) were observed under light microscope (Olympus BX51). Pictures of typical samples were taken under 10- and 20- fold objective with a digital camera SIS Color View 12 and the pictures were saved using software AnalySIS Pro 1.0.

3.3.5. Statistics

Statistical analyses were done using software STATISTICA version 6.0. Tukey HSD Test done after ANOVA determined the statistical significance of the data at $p<0.05$.

3.4. Experiment 4 – Effect of feeding Chlorella vulgaris on reproductive and growth parameters of mice

3.4.1. Composition of diets

A commercial feed (Altromin®, Standard 1310) normally used for rearing mice was chosen. This feed was fed to control mice. The experimental group received the same commercial diet which was supplemented with 1.0 % of spray-dried micro-algae *Chlorella vulgaris* (obtained from Bioproducts Prof. Steinberg Ltd, Kloetze, Germany). The experimental diet was prepared by Altromin® Ltd. 99.0% of standard diet was mixed with 1.0% micro-algae and pelleted. The composition of the Altromin® Standard 1310 diet, as declared by the producer, is shown in Table 19.

Table 19. Composition of Altromin® Standard 1310 diet for rodent rearing

Parameter	Amount (in diet)	Parameter	Amount (in diet)
Water	13.5 %	Vitamin A	15,000.0 IU/kg
Ash	6.5 %	Vitamin E	75.0 mg/kg
Crude protein	22.5 %	Vitamin B1	18.0 mg/kg
Crude fat	0.5 %	Vitamin B2	12.0 mg/kg
Crude fiber	4.5 %	Vitamin B6	9.0 mg/kg
N-free extracts	48.0 %	Vitamin B12	24.0 mg/kg
Energy	12.5 MJ/kg	Nicotin Acid	36.0 mg/kg
K	1.0 %	Folic acid	2.0 mg/kg
Mg	0.2 %	Cholin	600.0 mg/kg
Ca	0.9 %	Vitamin D3	600.0 IU/kg
P	0.7 %	Vitamin K3	3.0 mg/kg
Na	0.2 %	Panthotenic Acid	21.0 mg/kg
Mn	45.0 mg/kg	Biotin	60.0 mg/kg
Cu	13.0 mg/kg	Vitamin C	36 mg/kg
I	1.0 mg/kg		
Fe	160 mg/kg		
Zn	60 mg/kg		

The algal contents were determined in a certified analytical laboratory of The Institute for Cereal Proceedings Ltd, Bergholz-Rehbruecke, Germany. Chemical analyses were done according to specifications of § 35 LMBG (Amtliche Sammlung für Lebensmitteluntersuchungen). The results of these analyses are summarized in Table 20.

Table 20. Composition of spray-dried biomass of *Chlorella vulgaris*

Parameter	**Amount in biomass**	**Fatty acids (FA)**	**% (of lipid extract)**	**Amount in biomass**
Water	3.6 %	C12:0	0.08	2 mg/kg
Ash	9.13 %	C14:0	1.45	31 mg/kg
Crude protein	52.8 %	C16:0	21.26	453 mg/kg
Crude fat	8.1 %	C16:1	2.99	64 mg/kg
Crude fiber	20.8 %	C16:2	0.14	3 mg/kg
Carbohydrates	5.6 %	C16:4	0.47	10 mg/kg
Energy	1291 kJ	C18:0	1.65	35 mg/kg
K	2260 mg/100g	C18:1	12.50	267 mg/kg
Mg	388 mg/100g	C18:2 *n*-6	21.16	451 mg/kg
Ca	581 mg/100g	C18:3 γ-linolenic acid *n*-3	0.38	8 mg/kg
Fe	172 mg/100g	C18:3 α- linolenic acid *n-3*	35.36	754 mg/kg
Cu	2.6 mg/100g	C18:4	0.68	14 mg/kg
Zn	14.5 mg/100g	C20:0	0.43	9 mg/kg
Mn	150 mg/kg	C20:1	0.15	3 mg/kg
I	0.5 mg/kg	C20:2	0.06	1 mg/kg
Carotenoids	0.41 g/100g	C20:3	0.32	7 mg/kg
Vitamin B12	75.4 µg/100g	C20:4	0.41	9 mg/kg
Chlorophyll	2.13 g/100g	C20:5	0.29	6 mg/kg
Polyphenols	0.38 g/100g	C22:0	0.22	5 mg/kg
		total *n-3*	36.32 g/100g	
		total *n-6*	22.48 g/100g	
		Saturated FA	25.08 g/100g	
		Simple unsaturated FA	15.65 g/100g	
		Multiple unsaturated FA	58.80 g/100g	

Both control and experimental diets were additively analyzed in our laboratory for dry matter, crude contents and amino acids applying methods already described in point 3.1.1.4. The determined composition is listed in Table 21.

Table 21. Chemical composition and amino acids of control and experimental (control with 1.0% of spray-dried micro-algae *Chlorella vulgaris*) diet. Essential AA are marked with *.

Parameter	Control diet	Experimental diet
DM (%)	91.0	91.3
% N/DM	4.3	4.6
% cP/DM	26.8	28.4
% cA/DM	7.0	6.7
% cFib/DM	4.2	3.9
% EE/DM	5.9	5.5
AA (g/16g N) - total	83.2	81.9
ASP	8.2	8.2
THR*	3.5	3.4
SER	4.2	4.1
GLU	16.3	15.3
GLY	3.8	3.7
ALA	4.4	4.4
VAL*	4.0	3.9
ILE*	3.5	3.4
LEU*	6.6	6.4
TYR	2.5	2.4
PHE*	4.3	4.2
HIS*	2.3	2.2
LYS*	4.7	4.6
ARG*	5.5	5.4
PRO	5.5	5.2
CYS	1.7	2.2
MET*	1.5	1.9
TRP*	1.0	1.2
EAAI	1.18	1.19

3.4.2. Animals and protocol

Female mice, strain Fzt:DU, were used. They were housed at the Research Institute for the Biology of Farm Animals (FBN), Dummerstorf, Germany. Animals were kept under standardized conditions, with environmental temperature of 22°C, humidity of 55 - 60% and light regime of 12 hours light (6.00 am – 6.00 pm) and 12 hours darkness (6.00 pm – 6.00 am). The sibling mice (generation F_0) were divided into 2 groups, each group consisted of 55 females. First group was fed commercial mouse dry feed, the other one was fed the feed enriched in 1.0% algae powder. The females were kept two per cage and received the experimental diet starting from the 21st day of live. Food was given *ad libitum*. Under conditions applied in this experiment, daily feed intake recording was impossible.

The females were weighed on the 42nd day of life and on the mating day (61st-63rd). Within the groups females were mated at random with a mating ratio of 2:1 avoiding sib mating. After mating all mice were checked for conception by visual inspection for a copulatory plug. All 109 mice from the F_0 generation (54 in control and 55 in algae group) gave birth to their pups (one mouse from control group died before mating). After parturition, number of live males and females (generation F_1), as well as stillborn littermates in all litters, was counted, and all litters were weighed. Litters were then standardized to achieve 9 pups per litter (4 males and 5 females). Litter size and litter weight were recorded on the 10th and 21st day of life. On the 21st day of life, 2 males and 2 females from each litter were weaned and kept separately (females in one cage, males in another) for further observation. They were fed experimental or control diet and were weighed on 21st, 42nd and 63rd day of life. 2 females from each group were then mated with 1 non-littermate male. After mating all mice were checked for conception by visual inspection for a copulatory plug and on the 18th day of pregnancy (or in some cases 1-3 days earlier) 1 female from each cage was slaughtered to examine prenatal fertility traits (57 and 59 in control and experimental group, respectively).

The other females were kept till parturition. After parturition, number of live males and females (generation F_2), as well as the number of dead littermates in all litters was counted, all litters were weighed. Litter size and litter weight were measured on the 10th and 21st day of life. On the 21st day of life, 2 males and 2 females were weaned and kept separately for further observation. They were fed respective feed and were weighed on 21st, 42nd and 63rd day of life. 2 females from each group were then mated with 1 non-littermate male. On the 18th day of pregnancy (or 1-3 days earlier, in some cases), 50 females from each group were sacrificed and the procedure as described above followed. The other females were

kept till parturition; the littermates (males and females, dead pups – generation F_3) were counted and weighed on the parturition day and thereafter on the 10th and 21st day of life, when the experiment finished.

To obtain reliable comparisons regarding fetuses' weight, data obtained from slaughter of pregnant mice on the 16th or 18th day of parturition were used.

3.4.3. Sampling

Pregnant mice (in the 18th day of pregnancy) were bled out under inhalant anesthesia with isoflurane (IsoFlo®). The blood was taken directly from heart into mini-tubes containing Lithium-Heparin (Li-Heparin Multivetten, Sarstedt, Germany) and EDTA (EDTA-Multivetten, Sarstedt, Germany). Afterwards, liver, kidneys, heart and spleen were removed and weighed. The ovaries were taken out of the body and checked for *corpora lutei* under a magnifying glass. The uterus was dissected and live, dead and adsorbed fetuses were counted, separately for each uterine horn. The live fetuses were weighed separately. Gastrointestinal tract was taken out of the body, emptied, flushed with sterile physiologic saline and dissected. Length of major curvature of stomach, length and weight of small intestine, caecum and colon were recorded.

3.4.4. Analyses

Blood samples were analyzed for red blood cells (RBC), white blood cells (WBC) with the differentiation for eosinophiles, basophiles and neutrophiles, packed cell volume (PCV, hematocrit), lymphocytes, monocytes, thrombocytes, and hemoglobin. Mean corpuscular volume (MCV, mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were then calculated. After centrifugation serum was collected and Fe-concentration together with Fe-binding capacity was measured. All biochemical and hematological analyses of blood were done at The Office for Veterinary and Foodstuff Examination of the Mecklenburg-Vorpommern (LVL), Rostock, Germany using standard procedures (as described in section 3.3.3).

3.4.5. Statistics

Statistical analyses were done using software STATISTICA version 6.0. Tukey HSD Test done after ANOVA determined the statistical significance of the data at $p<0.05$.

3.5. Experiment 5 – Balance study for measurement of influence of micro-algal supplementation on nutrients digestibility and utilization in mice.

This experiment was a follow-up study to Experiment 4, where such data as feed intake could not be recorded. Supplementation of the feed with 1.0% *C. vulgaris* could change the palatability of the feed or could influence the digestibility and utilization of the feed, and therefore this balance study was conducted.

3.5.1. Diet

Diets used in this experiment were the diets already described in Exp. 4 (see section 3.4.1.).

3.5.1.1. N-free diet

There is little information available on the nitrogen requirements of mice. To evaluate the daily maintenance nitrogen requirement, needed for calculations of nutritional parameters, a N-free diet was fed to mice. The home made N-free mixture consisted of 18.5% cellulose, 37.1% sugar, 14.8% oil, 7.4% vitamins (Vitamin A - 750 IE, B_1- 1 mg, B_2 - 1 mg, B_6 – 0.5 mg, B_{12} – 0.5 mg, C - 1 mg, D_3 - 25 IE, E – 2.5 mg, K_3 – 0.1 mg, Panthotenic acid - 1 mg, Nicotine acid amide – 2.5 mg, Choline hydrochloride - 100 mg, Folic acid – 0.1 mg, Biotin – 0.01 mg, Inositol – 12.5 mg, p-Aminobenzoic acid - 5 mg, fulfilled ad. 1 g with wheat starch), 14.8% minerals ($CaCO_3$ - 6.86%, Ca-citrate – 30.83%, $CaHPO_4 \cdot H_2O$ – 11.28, K_2HPO_4 – 21.88, KCl – 12.47%, NaCl – 7.71%, $MgSO_4$. 3.83%, $MgCO_3$ – 3.52%, Fe-III ammoncitrate – 1.53%, $MnSO_4 \cdot H_2O$ – 0.02%, $CnS0_4 \cdot H_2O$ – 0.0078%, KI- 0.0041%, NaF – 0.051%, $AlNH_4(SO_4)_2 \cdot 12\, H_2O$ – 0.009%, $ZnCO_3$ – 0.006%) and 7.4% wheat starch.

3.5.2. Animals

In order to evaluate the digestibility, and utilization of nutrients of feed supplemented with 1.0% of spray-dried *Chlorella vulgaris*, 32 female mice strain Fz:Du (24-28 days of age) weighing approximately 16 g (obtained from mouse laboratory of our Institute) were used. The animals were housed in metabolism cages, 2 per cage, for 15 consecutive days. The humidity of the room air was 50 – 75%, the ambient temperature was held at the level of 22 ± 1° C, the light regime was 12 hours of light (6.00 am – 6.00 pm) and dark (6.00 pm – 6.00 am). Mice received feed in daily amount of 8.8 g/cage in the control and 8.4 g/cage in the experimental group. Tap water was given in nipple bottles *ad libitum*.

In order to evaluate the daily maintenance nitrogen requirements of mice in this age group, a feeding trial with N-free mixture was undertaken after the main experimental trial. For this part of the experiment, the same mice were used and housed under the same conditions as described above. All 32 mice in 16 cages were fed N-free mixture in amount of 8.3 g/cage for 5 days, water was given *ad libitum* in nipple bottles.

3.5.3. Experimental protocol

The accomplished experiment was balance study, in principle similar to the Experiment 1 (see point 3.1.3.). Two mice were kept in one cage to obtain enough material for analyses. Mice were divided into 2 groups, control group fed Altromin® 1310 diet (CG) and experimental group fed on diet supplemented with 1.0% of the micro-algae (AG). Daily amount of diet was calculated on dry matter and nitrogen basis. According to NRC (1995), laboratory mice require 2.9 – 3.2% of nitrogen in diet (180 – 200g crude protein/kg diet). As nitrogen content in control feed was 4.2% and taking into consideration, that mouse weighing 15g intakes about 3.5g DM daily, we calculated daily amount of offered control feed to be 4g DM/mouse, to assure covering of all nutritional demands. This amount of feed contained 166 mg N and on this basis the amount of experimental feed was calculated to assure the same nitrogen amount offered to experimental animals. Therefore, experimental diet was offered in daily amount of 3.8 g DM/mouse. These amounts were equal to daily amount of fresh matter per cage offered in CG to be 8.8 g and in AG to be 8.4 g.

Adaptation to conditions and the diet lasted for 4 days (adaptation period) and was followed by experimental period lasting for 6 days. Mice were fed their respective diets throughout both periods. Mice were weighed at the beginning of each period and at the end of

the experimental period. Leftover, urine and feces were collected daily and treated as in Experiment 1 (see point 3.1.3.).

After the end of the experimental trial all 32 mice from 16 cages were fed N-free mixture in amount of 8.3 g/cage for 5 days. First two days were for adaptation of animals to the diet and in this period the gut emptied of nitrogen taken with the previous feed. During the last three days urine and feces were collected and treated in the same way as in experimental period. The collection period could not be longer as the animals fed N-free diet become undernourished and a catabolic phase begins. This affects the recorded data, which becomes unphysiological and not useful.

3.5.4. Analyses

All analyses for dry matter, nitrogen and amino acids determination were accomplished in the same manner as in Experiment 1 and are described in detail in point 3.1.4. Feces were also analyzed for crude ash, crude fiber, crude fat and carbohydrates applying the same methods as for determination of feed components and are already described in point 3.1.1.4.

3.5.5. Nutritional parameters

The dry matter and nitrogen data were used for calculation of the nutritional value of protein of control and experimental diet. The equations are already mentioned in point 3.1.5. Calculations were done for each pair of animals (one cage) to reduce error. However, in this study we have accomplished N-free trial to obtain experimentally the daily elimination of nitrogen from the body. Daily nitrogen content in urine and in feces was determined in urine and fecal samples collected during the N-free trial and obtained data was used for calculation of maintenance nitrogen requirement (MNR). The equation was as follows:

MNR = N excreted in urine (NU) + N lost from intestines with feces (IN)

The MNR value obtained in this matter for each pair of animals (one cage) was then used for further calculations. No essential amino acids index was calculated.

For calculation of apparent digestibility of crude ash (aAD), crude fiber (aFibD) and crude fat (aFD), the equation for apparent crude protein digestibility shown in point 3.1.5.h) was used, where instead of nitrogen, the respective parameter was taken.

3.5.6. Statistics

Statistical analyses were done using software STATISTICA version 6.0. Tukey HSD Test done after ANOVA determined the statistical significance of the data at $p<0.05$.