2 Materials and Methods

2.1 Animal Studies

Three experiments were carried out with growing male Sprague Dawley rats supplied by Charles River GmbH (Sulzfeld, Germany). The animals were housed individually in Macrolon cages, in a room maintained at a temperature of 22±2°C and 50-60% relative humidity with lighting from 06.00 to 18.00 hours. All described experimental procedures were according to the established guidelines for the care and handling of laboratory animals, and were approved by the council of Saxony-Anhalt, Germany. To standardize the feed intake, the diets were fed daily in restricted amounts in between 8 and 9 O'clock. The diets varied in the protein source. Water was provided ad libitum from nipple drinkers.

In experiment 1, 20 rats with an initial body weight (± SD) of 70.2 ± 4.3 g were randomly assigned to two groups of 10 rats each and fed semi-synthetic diet for 21 days with casein or ethanol washed soy protein isolate as protein source.

In experiment 2, 72 rats with an initial body weight (± SD) of 72.3 ± 5.6 g were randomly assigned to one of the six groups (n = 12/group) and fed semi-synthetic diet for 20 days with protein isolated from pork, beef, fish, turkey, casein or ethanol washed soy protein isolate.

In experiment 3, 60 rats with an initial body weight (± SD) of 76.5 ± 5.3 g were randomly assigned to one of 5 groups (n = 12/group) and fed semisynthetic diet for 22 days with fish protein, casein, ethanol washed soy protein isolate, pea or lupin protein.

The rats within one experiment were offered equal rations daily, and the amount fed was adjusted to meet the expected consumption based on the feed intake of the previous day. The body weight of the experimental animals was recorded weekly.

2.2 Characterisation of the Diet

The compositions of the experimental diets used in experiment 1, 2 and 3 are shown in Table 2.1. All diets contained 200 g protein/kg diet. The protein source varied according to the experimental design. All diets contained 100 g lard/kg (Laru, Langensiepen, Ruckebier, Bottrop, Germany) as a proatherogenic fat. The diet contained sufficient amounts of minerals (Table 2.2) and

vitamins (Table 2.3) based on the recommendations by the American Institute of Nutrition (AIN) for rodents (Reeves et al. 1993).

The diets were freshly prepared by mixing 900 g dry substance with 100 g fat and water and subsequent freeze drying (CHRIST, BETA 1-8, freeze dryer, Germany). The residual water content of the diet was <5g/100 g. The diets were stored at 4°C up to the consumption. In all experiments, diets were administered in restricted amounts to standardize the diet intake.

Table 2.1: Diet Components (Experiment 1, 2 and 3)

Components	Amount [g/kg Diet]
Protein[1]	200
Fat (lard)	100
Sugar	200
Cellulose (fibre)	50
Corn starch	389.5
Minerals + Vitamins	60
Cholesterol	0.5

[1]Casein and ethanol washed soy protein isolate in experiment 1.

Pork, beef, fish, turkey protein isolated from meat, casein, and ethanol washed soy protein isolate were used in experiment 2.

Pea, lupin, ethanol washed soy protein isolate, fish protein and casein were used in experiment 3.

In order to meet the AIN recommended amounts in experiment 3, all plant protein diets were supplemented with DL-methionine (Pea diet: 3.6 g/kg; Lupin diet: 4.4 g/kg; Soy protein diet: 3 g/kg). Lupin protein diet was also supplemented with lysine (6.4 g/kg). These amino acids were supplemented on the expense of cellulose.

Table 2.2: Mineral Composition of the diets, used in Experiment 1, 2 and 3

Elements	Components	Amount [mg/kg Diet]
Calcium	Calcium carbonate	5000
Calcium, Phosphorus	Dicalcium phosphate	1561
Magnesium	Magnesium oxide	507
Potassium	Potassium sulphate	3600
Sodium, Chlorine	Sodium chloride	1019, 1571
Iron	Ferrous sulphate	35
Zinc	Zinc oxide	30
Manganese	Manganese oxide	10
Copper	Copper sulphate	6
Selenium	Sodium silinite	0.15
Iodine	Calcium iodate	0.2

Table 2.3: Vitamin Composition of the diets, used in Experiment 1, 2 and 3

Components	Amount [/kg Diet]
Vitamin A	5000 IU
Vitamin D3	1000 IU
Vitamin K	0.75 mg
Thiamin B1	5 mg
Riboflavin B2	6 mg
Pyridoxine B6	6 mg
Nicotinic acid	30 mg
Folic acid	2 mg
Calcium pentathenate	15 mg
Biotin	0.2 mg
Choline chloride	1000 mg
Vitamin E-Acetate	75 IU

2.3 Analysis of the Experimental Diets

For analysis of the fatty acid composition of the complete diet (experiment 2 & 3), total lipids were extracted according to method of Hara and Radin (1978) modified by Eder and Kirchgessner (1994). To extract total lipids, 7.0 g ground diet was dissolved in 30 ml Hexane/Isopropanol (3:2, v/v) solvent. The samples were shaken (Incubator Shaker G 25, Brunswick Scientific, Edison, New Jersey, USA), overnight at ambient temperature, and afterwards centrifuged at 2000 rpm, for 10 min. The clear supernatant was used for the fatty acid analysis. Fatty acids concentrations of the diets were analysed by gas chromatography (Eder and Brandsch 2002).

Table 2.4.a: Fatty Acid Concentrations of the Diets used in Experiment 2

Fatty Acids	Pork diet	Beef diet	Fish diet	Turkey diet	Casein diet	Soy diet
	[g Fatty acid/kg dry matter]					
16:0	25.9	26.1	25.5	25.7	25.7	25.6
16:1 n-7 + n-9	2.5	2.5	2.6	2.5	2.5	2.4
18:0	16.8	16.8	16.0	16.0	16.2	16.2
18:1 n-9 + n-11	37.2	37.3	37.3	37.3	37.6	37.4
18:2 n-6	10.6	10.6	10.7	10.6	10.8	11.5
18:3 n-3	0.9	0.9	0.9	0.9	0.9	1.0
20:1 n-9	0.8	0.8	0.8	0.9	0.9	0.8
20:2 n-6	0.5	0.5	0.5	0.5	0.5	0.5
20:3 n-6	0.1	0.1	0.1	0.1	0.1	0.1
20:4 n-6	0.1	0.1	0.1	0.1	0.1	0.1
20:5 n-3	0.1	0.1	0.1	0.2	0.1	0.1
22:3 n-9	0.2	0.1	0.1	0.1	0.0	0.0
22:5 n-3	0.1	0.1	0.1	0.1	0.1	0.1

22:6 n-3	0.0	0.0	0.0	0.2	0.0	0.0

Conditions:

Column: FUSED FATTY ACID PHASE silica rectifying column
30 m/0.53 mm inside diameter (Macherey and Nagen, Düren)

Feed Gas: Helium, 8.4 ml/min

Quantity: 1µl

Detector: Flame ionization detector

Table 2.4.b: **Fatty Acid Concentrations of the Diets used in Experiment 3**

Fatty Acids	**Fish diet**	**Casein diet**	**Soy diet**	**Lupin diet**	**Pea diet**
	[g Fatty acid/kg dry matter]				
16:0	25.4	25.3	25.4	25.3	25.1
16:1 n-7 + n-9	2.27	2.27	2.27	2.27	2.22
18:0	15.0	15.1	15.1	15.1	14.8
18:1 n-9 + n-11	39.6	39.8	39.9	39.9	39.4
18:2 n-6	10.4	10.4	10.5	10.4	11.3
18:3 n-3	0.87	0.88	0.89	0.88	1.12
20:1 n-9	0.89	0.89	0.89	0.89	0.88
20:4 n-6	0.12	0.12	0.12	0.12	0.12
20:3 n-6	0.03	0.02	0.02	0.03	0.02
20:5 n-3	0.08	0.06	0.05	0.06	0.05

Table 2.4.a shows the fatty acid concentrations of different diets used in experiment 2 and Table 2.4.b shows the fatty acid concentrations of different diets used in experiment 3. Within one experiment, fatty acid concentrations of the different diets were very similar. Only in experiment 3, the pea diet showed a bit higher concentration of linoleic acid (18:2, n-6); and α-linoleic acid (18:3, n-3) than the other diets.

2.4 Processing of the Dietary Proteins

Soy Protein Isolate: Pre-treatment

To minimize possible effects of soy protein associated isoflavones that may partly be responsible for cholesterol reduction by soy protein. The soy protein isolate (90% protein: Protein Technologies International, Ieper, Belgium) used for this study was additionally ethanol washed, a process which has been shown to be an efficient way to remove remaining isoflavones (Fukui et al. 2004). The soy protein isolate was washed twice with 10 volumes of 60% ethanol by stirring for 2 hours at RT. Protein was filtered, air dried and ground to fine powder.

Casein: (Nähr Kasein, Meggle, Wasserburg/Inn, Germany), was used without further processing.

Meat Proteins: Pork, beef, and turkey proteins were isolated from lean meat, and fish protein was isolated from Alaska pollack fillets (Shukla et al. 2006a), which were purchased in a local supermarket. The frozen fish fillets and all kinds of meat were chopped into small pieces, all visible fat and connective tissues were removed. Chopped meat were boiled for 30 minutes and then chilled over night at 4°C. Meat mash was homogenized in a grinder (Ika Universalmühle M20, Staufen, Germany). The crude protein was obtained after freeze drying (CHRIST, BETA 1-8, Germany) and rehomogenization. To remove protein associated lipids, the crude protein powder was treated twice with 10 volumes of acetone and once with 10 volumes of acetone/ethanol (1:1, v/v) solution. The protein was filtered air dried and ground to fine powder.

Pea protein: (PISANE, Cosucra, Belgium), was used without further processing.

Lupin protein: (Lupin Protein Isolate Type E, Fraunhofer Institüt für Verfahenstechnik und Verpackung, Freising, Germany), was used without further processing.

2.5 Analysis of the Dietary Proteins

Dry substances and amounts of raw protein, raw fat and raw ashes of all dietary proteins used are shown in table 2.5.

Dry Matter Analysis

Percent dry matter of each protein was determined by the method of Naumann and Basler (1993).

The glass wares with lid were dried in an oven (105°C, 1h), cooled down in a desssicator and weighed (i.e. weight A). 1.0 g fine homogenized protein sample was added (i.e. weight B), and dried for 3 h at 105°C. The probes were cooled down in desiccators and weighed (i.e. weight C). After an additional drying period of 1 h, process was repeated until constant weight achieved.

$$\%\ \text{dry matter} = \frac{(C - A) \times 100}{(B - A)}$$

Table 2.5: Dry matter and raw nutrients of the dietary Proteins used in Experiment 1, 2 and 3

Dietary proteins		Dry Matter	Protein	Fat	Ashes
		[g/100 g Protein]			
Exp 1	Casein	90.9	91.8	0.88	4.74
	Soy	84.7	93.0	1.24	5.03
Exp 2	Pork	91.6	94.1	1.97	3.19
	Beef	91.1	94.0	1.81	2.94
	Fish	91.6	92.6	0.76	4.21
	Turkey	91.3	95.0	0.66	2.96
	Casein	90.9	91.8	0.88	4.74
	Soy	84.7	93.0	1.24	5.03
Exp 3	Fish	93.3	91.8	0.86	4.69
	Casein	90.9	91.8	0.88	4.74
	Soy	91.8	98.0	0.65	3.65
	Pea	93.7	85.7	8.07	4.81
	Lupin	93.9	96.0	1.06	4.02

Protein Analysis

Protein was determined by the method of Kjeldahl (1883). The samples were digested with a strong acid so that it releases nitrogen which can be determined by the titration technique. Since all the proteins contain about the same amount of nitrogen (16%), the protein concentration was calculated by multiplying the nitrogen content with 6.25.

The samples were weighed and digested by heating in the presence of sulphuric acid, and anhydrous sodium sulphate. Digestion converts nitrogen in the food into ammonia, and other organic matter to CO_2 and H_2O. The ammonia gas liberated from the solution moves into the receiving flask which contains excess of boric acid. The low pH of the solution converts ammonia gas into ammonium ion, and simultaneously converts the boric acid to the borate ion. The nitrogen content was estimated by titration of the ammonium borate formed with standard sulphuric or hydrochloric acid. The concentration of the hydrogen ions (in moles) required to reach the end point is equivalent to the concentration of nitrogen that was in the original sample. Once the nitrogen content has been determined it is converted to the protein content using the appropriate conversion factor:

$$\% \text{ Protein} = 6.25 \times \% \text{ N}$$

Fat Analysis

Extraction of the crude fats was performed with the Soxtec HT 1043 extraction unit (Foss GmbH, Hamburg, Germany) on the basis of return-back principle with the solvent petroleum ether and followed by evaporation of the solvent and collection of the crude fat through weighing extracted material. Before the extraction an acidic hydrolysis was carried out to extract all fat substances which are not extractable by solvents. 1 g of the sample was hydrolysed with 120 ml 0.3 mole/L HCl for 15 min.

Ashes Analysis

The ash fraction contains all the mineral elements jumbled together. It allows calculation of nitrogen free extract (by difference) from dry matter and provides an estimate of contamination.

This method consists of oxidizing all organic matter in a weighed sample of the material by incineration and determining the weight of the ash remaining.

Homogenized samples were weighed (5.0 g) into dried (ignited and tarred) crucibles and placed in a drying oven at 100°C for 24 hours, followed by transferred to a cool muffle furnace and temperature was increased stepwise to 550°C ± 5°C. Temperature was maintained, until a white ash appeared (~8 hours).

$$\text{Ash (\%)} = \frac{(\text{crucible + ash weight}) - (\text{crucible weight}) \times 100}{(\text{crucible + sample weight}) - (\text{crucible weight})}$$

Amino Acid Analysis of the Dietary Proteins

Determination of the amino acid composition was performed with ion exchange chromatography using an amino acid analyser (Biotronic L C 3000**,** Eppendorf, Hamburg, Germany).

In order to determine methionine and cysteine the probes were oxidised: 250 mg diet was mixed with 5 ml of oxidation mixture (0.5 ml 30% H_2O_2, 4.5 ml phenol containing 98% formic acid and 25 mg phenol) and incubated for 24 h at 0°C. The oxidation was stopped by adding 0.9 g sodium disulfide. Afterwards, oxidised samples were hydrolysed with 50 ml of a hydrolysis mixture (492 ml HCl, 1 g phenol, and water to 1 L) for 24 h at 110°C (Bassler and Buchholz 1993). After cooling the probes, NaOH was added until the pH-value reached 2.2. The solution volume was adjusted and an internal standard (20 µmol Norleucin/ml citrate buffer pH 2.2) was added to the probe. An aliquot of this hydrolysate was applied to the amino acid analyzer. The separation of the amino acids was carried out by elution with buffer solutions of different pH-values. Elute was mixed continuously with ninhydrin-reagent and the extinction was read at 440 nm for proline and at 570 nm for all other amino acids. For calculation the peak volume of the amino acids and internal standard were used.

Amino Acid Concentrations of the Diets:

The amino acid concentrations of the diets used in experiment 2 are presented in table 2.6.a. Soy protein had higher concentrations of cysteine, aspartic acid, glycine, alanine, arginine and lower concentrations of serine, glutamic acid, proline, tyrosine, valine methionine and lysine. The ratios of lysine/arginine and mthionine/glycine were also lower in soy protein.

Table 2.6.a: Amino Acid Concentrations of the Diets used in Experiment 1

Amino acid	Soy	Casein
	[g/kg diet]	
Cysteine	1.8	0.8
Aspartamic acid	19.5	13.2
Serine	8.6	10.8
Glutamic acid	29.0	42.2
Proline	7.5	20.2
Glycine	6.9	3.4
Alanine	7.6	5.4
Tyrosine	5.5	8.6
Arginine	11.6	6.4
Methionine	2.5	5.2
Threonine	6.2	7.8
Valine	8.1	12.1
Isoleucine	7.6	8.8
Leucine	14.8	17.6
Phenylalanine	8.6	9.6
Histidine	5.7	6.4
Lysine	10.1	14.6
Lysine/Arginine	0.9	2.3
Methionine/Glycine	0.4	1.5

The amino acid concentrations of the diets used in experiment 2 are presented in table 2.6.b. Soy protein and proteins isolated from meat, pork, beef, turkey and fish protein had higher concentrations of cysteine, aspartic acid, glycine, alanine, arginine and lower concentrations of

serine, glutamic acid, proline, tyrosine, valine, compared to casein. Soy protein had lower concentrations of methionine and lysine compared to casein. Meat proteins and fish protein had similar amino acids concentration only turkey protein had higher concentration of histidine. Ratios of lysine/arginine, and methionine/glycine were highest in the casein.

Table 2.6.b: Amino Acid Concentrations of the Diets used in Experiment 2

Amino acid	Fish	Pork	Beef	Turkey	Soy	Casein
	[g/kg diet]					
Cysteine	1.7	1.7	1.5	1.7	1.8	0.8
Aspartamic acid	20.0	18.5	18.3	18.4	19.5	13.2
Serine	7.8	7.5	7.3	7.0	8.6	10.8
Glutamic acid	30.6	30.4	31.6	29.2	29.0	42.2
Proline	5.6	6.9	6.5	6.0	7.5	20.2
Glycine	7.9	8.9	8.1	8.0	6.9	3.4
Alanine	11.4	11.7	11.6	11.8	7.6	5.4
Tyrosine	5.1	5.0	5.4	4.8	5.5	8.6
Arginine	10.7	11.5	11.5	11.0	11.6	6.4
Methionine	5.7	4.7	4.8	5.1	2.5	5.2
Threonine	8.1	8.5	8.6	8.2	6.2	7.8
Valine	9.3	11.7	8.8	9.4	8.1	12.1
Isoleucine	7.9	7.9	8.1	8.6	7.6	8.8
Leucine	15.4	15.6	15.8	15.6	14.8	17.6
Phenylalanine	6.6	6.5	6.7	6.6	8.6	9.6
Histidine	6.0	7.4	7.0	10.4	5.7	6.4
Lysine	17.4	17.3	17.5	17.3	10.1	14.6
Lysine/Arginine	1.6	1.5	1.5	1.6	0.9	2.3
Methionine/Glycine	0.72	0.5	0.6	0.6	0.4	1.5

Table 2.6.c: Amino Acid Concentration of the Diets used in Experiment 3

Amino Acids	Pea	Lupin	Soy	Fish	Casein
	[g/kg diet]				
Cysteine	1.4	2.0	2.1	1.6	0.8
Aspartamic acid	18.7	19.6	20.9	17.4	13.2
Serine	8.3	9.5	9.3	7.4	10.8
Glutamic acid	29.2	42.9	37.3	27.5	42.2
Proline	7.2	7.4	9.8	5.7	20.2
Glycine	6.1	6.0	7.1	7.5	3.4
Alanine	6.4	5.1	7.2	10.0	5.4
Tyrosine	5.2	8.2	5.9	5.1	8.6
Arginine	14.1	18.3	14.1	11.0	6.4
Methionine	1.4	0.6	2.0	5.0	5.2
Threonine	5.9	5.8	6.5	7.7	7.8
Valine	8.0	6.7	8.5	8.7	12
Isoleucine	7.6	8.8	8.4	7.7	8.8
Leucine	13.9	14.4	14.6	14.2	17.6
Phenylalanine	8.6	7.3	9.6	7.1	9.6
Histidine	4.7	4.2	5.8	5.7	6.4
Lysine	11.4	6.7	10.8	15.7	14.6
Lysine/Arginine	0.8	0.4	0.8	1.4	2.3
Methionine/Glycine	0.2	0.1	0.3	0.7	1.5

In order to meet the recommended amounts in experiment 3, all plant protein diets were supplemented with DL-methionine (Pea diet: 3.6 g/kg; Lupin diet: 4.4 g/kg; Soy protein diet: 3 g/kg). Lupin protein diet was also supplemented with lysine (6.4 g/kg).

The amino acid concentrations of the diets used in experiment 3 are presented in table 2.6.c. The fish protein and casein had higher levels of lysine and methionine compared to plant proteins

(pea, lupin and soy protein). Soy protein had higher concentration of cysteine, aspartic acid, glycine, alanine, arginine compered to casein. Lupin and casein had higher concentrations of glutamic acid and tyrosine. Casein had higher concentrations of proline and valine compared to fish and plant proteins. Ratios of lysine/arginine, and methionine/glycine were lower in fish and plant proteins diets compared to casein.

Genistein Quantification in the Ethanol Washed Soy Protein Isolate

Quantitative determination of genistein, the major isoflavone in soy protein, was used to proof the efficacy of the ethanol extraction process. Genistein was determined by a modification of the method of Bilia et al. (2001).

In brief, soy protein isolate was hydrolyzed with HCl (1mol/L) in 50 % ethanol for 1 h at room temperature. After neutralization with NaOH, isoflavones were extracted three times with 20 ml ethyl acetate. After evaporation of ethyl acetate, isoflavones were dissolved in ethanol and used for a quantitative HPLC analysis of genistein using an ELITE LaChrom HPLC system (Hitachi, Manheim, Germany). The calibration was performed with Genistein.

Conditions:
Eluent A: Phosphoric acid, pH: 3
Eluent B: Acetonitrile
Flow rate: 1 ml/min
Detection wavelength: 329 nm

Sample	Concentration (mg/g)
Soy protein isolate before washing (Exp. 1 & 2)	0.42
Ethanol washed soy protein isolate (Exp. 1 & 2)	0.03
Soy protein isolate before washing (Exp. 3)	0.50
Ethanol washed soy protein isolate (Exp. 3)	0.08

2.6 Sample Preparation

After completions of the feeding period, rats were starved overnight (Experiment 1 and 2) to ensure that all rats had nearly the same interval of starvation until killing and to avoid the contamination of plasma with chylomicrones. While in Experiment 3, rats were killed in non-fasted state. Rats were anesthetized lightly with diethyl-ether and killed by decapitation. Whole blood was collected in heparinized monovettes. The liver was excised, rinsed in ice cold NaCl solution and immediately shock frozen in liquid nitrogen. Small portion of liver was stored at -80°C, for RNA isolation. The remaining organ was stored at -20°C. Faeces were collected from the last week of feeding, freeze dried (CHRIST; BETA 1-8, Germany), weighed and stored at -20°C up to the analysis.

2.6.1 Blood, Plasma and Lipoproteins

For the preparation of plasma, blood was centrifuged (3000 rpm, 4°C, 10 min). Plasma was stored at -20°C up to the analysis. Plasma lipoprotein fractions were separated by step-wise ultracentrifugation (RC-M150, SORVALL 1997), appropriate density cuts commonly used for the measure of rat lipoproteins (Sparks et al. 1998, Giudetti et al. 2003, Sirtori et al. 2004).

Reagents**:**

Solution A**:** 0.195 M Sodium chloride solution

Solution B**:** 0.195 M Sodium chloride solution and 2.44 M Sodium bromide solution

The plasma densities were adjusted with solution A and centrifuged at 150,000 rpm, at 4°C, for 1.5 h (RC-M150, SORVALL 1997). The lipoproteins of very low density (VLDL) were removed as upper phase ($\delta < 1.006$ g/ml). After the separation of VLDL the plasma densities were again adjusted with solution B and centrifugation process was repeated in the same way. Lipoprotein of low density (LDL) deposited as upper phase ($1.006 < \delta < 1.063$ g/ml). The remaining portion contained high density lipoprotein (HDL) fraction ($\delta > 1.063$ g/ml). All the lipoprotein samples were stored at -20°C.

2.6.2 Preparation of Homogenate, Cytosol and Microsomes

Liver homogenate and liver cytosol were prepared by the method of Garg et al. (1998) and Christiansen et al. (1991).

Liver Homogenate: 1.5 g frozen liver was chopped into small pieces, 4 ml of homogenizing buffer (0.1 M phosphate buffer pH 7.4 with 0.25 M sacccharose) was added and homogenized at 4°C in a homogenizer (Potter-S, Brown, Biotech International, Melsungen). The cell debris were separated by centrifugation (3000 rpm, 4°C, and 10 min). The homogenate in the supernatant was stored at -20°C.

Liver Cytosol: Liver homogenate was centrifuged at 26,000 rpm, at 4°C, for 15 min. The supernatant was centrifuged again at 105,000 rpm, at 4°C, for 1 h. The resulting clear cytosol in the supernatant was stored at -20°C.

Liver Microsomes: For preparation of liver microsomes, liver homogenates were centrifuged at 15,000 rpm for 20 min at 4°C. The microsomal pellet was obtained by centrifugation of the 15,000 rpm supernatant at 105,000 rpm for 1 h at 4°C.

2.6.3 Extraction of total Lipids from the Liver and Faeces

The extraction of the total lipid was performed by the method of Hara and Radin (1978) modified by Eder and Kirchgessner (1994). The total lipids from the homogenized sample materials (Liver 400 mg, Faeces 500 mg) were extracted with Hexane/Isopropanol solvent (3/2, v/v), by shaking (Incubator Shaker G 25, Brunswick Scientific, Edison, New Jersey, USA) for 18 h at ambient temperature. The supernatant was removed and used for further analysis.

2.7 Analytical Methods

2.7.1 Cholesterol and Triglyceride Determination

Cholesterol and triglyceride concentration in liver, plasma and lipoproteins (VLDL, LDL, and HDL) were determined using enzymatic reagent kits obtained from Merck (Ecoline 25, MERCK Eurolab, Germany).

Cholesterol and its esters are released from lipoproteins by detergents. Cholesterol esterase hydrolyses the esters. In the subsequent enzymatic oxidation by cholesterol oxidase, H_2O_2 is formed. This is converted into a coloured quinonimine in a reaction with 4-aminoantipyrine and salicylic alcohol catalyzed by peroxidase. The absorbance was measured at 500 nm. Determination of triglycerides was performed after enzymatic splitting with lipoprotein lipase. Indicator was quiononeimine which is generated from 4-aminoantipyrine and 4-chlorophenol by hydrogen peroxide under the catalytic action of peroxidase. The absorbance was measured at 550 nm. The

cholesterol and triglyceride concentrations in the plasma and lipoproteins were measured by directly mixing with reaction solution. For measuring the cholesterol and triglyceride concentration in liver and faeces total lipid extracts (2.6.3) were used. The aliquots were dried in a vacuum centrifuge (RC10.22 JOUAN, France). Lipids were dissolved using Triton X-100 (De Hoff et al. 1978).

The quantification of cholesterol and triglyceride was measured by adequate standards.

$$C_s = \frac{A_s \times C_{st}}{A_{st}}$$

C is the Concentration of the cholesterol or triglyceride in the sample, A_s and A_{st} are the absorbance of the sample and standard respectively, and C_{st} is the concentration of the standard.

2.7.2 Estimation of Apolipoproteins

Separated plasma lipoproteins (VLDL + chylomicrones, LDL, HDL) were subjected to analytical sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and apolipoproteins were estimated after densitometric scanning of Coomassie Blue-stained bands according to a modified method of Karpe and Hamsten (1994). Briefly, polyacrylamide gels were cast by a gradient from 4 to 15% acryl amide. The 4% acryl amide solution contained acryl amide (38.96 g/l), bisacryl amide (1.04 g/l), Tris (0.375 M), SDS (0.1%), TEMED (0.875 µl/ml) and ammonium persulphate (0.05%), whereas the 15% acryl amide solution contained acryl amide (146.1 g/l), bisacryl amide (3.9 g/l), Tris (0.375 M), SDS (0.1%), TEMED (0.875 µl/ml) and ammonium persulphate (0.05%).

Total protein content of lipoproteins was determined by the BCA assay with bovine serum albumin as standard. Lipoproteins (250 µg/ml) were dissolved (1:1) in buffer containing 0.22 M Tris, 41% glycerol, 0.7% SDS, 5% mercaptoethanol and 0.002% bromphenol blue and subsequently denaturated at 95°C for 5 min. After chilling 20 µl of denaturated probes containing 2.5 µg proteins were applied to the gels. Electrophoresis was run at 210 V for 1 h. Gels were stained in 0.25% Coomassie, 40% methanol, 10% acetic acid for 1 h and distained in 12% methanol, 7% acetic acid over night. Gels were scanned with a computer connected gel documentation system (Gel-Pro Analyzer, Intas, Göttingen, Germany). Bands of apo B-100, B-48, E and AI were identified by comparing the R_f values with that of high molecular weight standards (Serva, Heidelberg, Germany).

2.7.3 Amino Acids Analysis in Plasma

Protein precipitation was performed by adding 50 µl of 10% sulfosalicylic acid (4°C) to 200 µl plasma, followed by 30 min incubation at 4°C. After centrifugation (15,000 rpm, 10 min at 4°C), 20 µl of the supernatant was mixed with 20 µl internal standard (Norvalin, SERVA) and 80 µl deionized water. Concentrations of free amino acids in the plasma of rats were measured as isoindole derivatives by high performance liquid chromatography (Hewlett Packard, Waldbronn, Germany) according to Schuster 1988**,** after pre-column derivatization. Isoindole derivatives were detected at an excitation wavelength of 337 nm and emission wavelength of 454 nm.

2.7.4 Protein Determination

The protein concentration was determined by the Bradford assay (1976). The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to the protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible colour change.

To 50 µl of the sample 200 µl of the Bradford reagent was added , incubated for 10 minutes at room temperature and absorbance was measured at 595 nm by spectrophotometer (Spectro flour plus, TECAN, Germany), A standard curve with known protein (bovine serum albumin) concentrations was prepared in parallel with the samples. By that standard curve the concentrations of the samples were determined.

2.7.5 Phospholipid Analysis in Plasma

Phospholipids in the plasma were determined enzymatically by a test kit (Phospholipids enzymatiques PAP 150, bioMerieux, France). Phospholipids (lecithins, lysolecithins and sphingomylin) are hydrolysed by phospholipase D and the liberated choline is measured by the TRINDER reaction.

The phospholipids concentration in the plasma was measured by directly mixing with working solution and incubating at 37°C, for 10 min. The optical density (OD) was measured at the wavelength of 505 nm against the reagent blank (ULTRASPEC 2000). The quantification was done by a standard.

$$C\text{sample} = \frac{OD \text{ sample}}{OD \text{ standard}} \times C\text{standard}$$

The phospholipid concentrations were measured in the plasma of rats fed casein or soy protein in experiment 1. No difference was observed between the rats fed casein or soy protein (1.5 mmol/L vs. 1.6 mmol/L), respectively.

2.7.6 Bile Acid Analysis

Bile acid concentrations in plasma and faeces were analyzed enzymatically (MERCK) by a modified method of Marlett & Fisher (2002). 3-α-hydroxy-bile acids are specifically converted to the corresponding 3-keto derivatives in the presence of NAD^+ and with the aid of 3-α-hydroxysteroid dehydrogenase. The NADH formed reacts with Nitrotetrazolium Blue under the catalytic influence of diaphorase to give a blue formazan derivative, which was measured at 500 nm.

Preparation of Bile Acid Extract

About 0.4 g freeze dried, fine ground faeces were dissolved in 4 ml Toluol/Methanol solvent (1:1, v/v), and shaken (Incubator Shaker, G 25) for 18 hour at ambient temperature, vortexed and centrifuged at 2000 rpm, for 10 min. Supernatant was transferred and resuspended in toluol/methanol solvent (1:1, v/v). Supernatant was pipetted for each probe and corresponding blank and vacuum centrifuged. Afterwards, 20µl triton/chloroform (1:1, v/v) solvent was added to each probe and corresponding blank, vortexed and vacuum centrifuged.

The bile acid concentrations in plasma and faeces were measured by incubation of the sample and their corresponding blanks with sample reaction solution and blank reaction solution. The reaction was stopped by the addition of stop reagent. The absorbance was read at 500 nm and used for quantification. The calculation of bile acid concentration in the samples was performed by means of standard after subtracting the corresponding sample blanks.

2.7.7 Thin layer chromatography

The fractions of free and esterified cholesterol in the liver were separated by thin layer chromatography and were determined densitometrically (Hojnacki et al. 1976).

Running solvent**:** n-Hexane: Diethyl-ether: 100 % acetic acid = 80: 20: 3

Staining solution: 20 % Ammonium sulphate (Isocommerz)

Probe: Liver extracts (2.6.3)

Standard**:** Cholesterol /Cholesterol ester**:** (3 mg/3mg)/1 ml Hexane /Isopropanol (3:2, v/v) solvent

Cholesterol and Cholesterol ester (cholesterol oleate, 98%) obtained from Sigma were mixed in the ratio (1**:**1, w/w), that makes the concentration of each (C/CE) 1.5 mg/ml. Several concentrations (1.5; 3.0; 7 µg/µl) of the above standard were used.

All the samples and standards were spotted on a Thin Layer Chromatography (TLC)-aluminum foil (Kieselgel 60, 20X20 cm, MERCK) with the device LINOMAT 5 (CAMAG, Switzerland). The foil was inserted in the running chamber and the solvent was run on the plate up to ½ cm left from upper side. The foil was air dried, and stained in the staining chamber (30 sec). Brown colour bands appeared after drying in an oven at 200°C for 5 min. The foil was photographed and evaluated by the associated device (Syngene Gene Genious Synopsis, USA). Calculation was performed with a standard curve.

2.7.8 Activity of Lipogenic Enzymes

The activities of the enzymes Glucose 6-phosphate dehydrogenase (G6PDH) and fatty acid synthase (FAS) were determined in liver cytosol (2.6.2). The results were related to the protein concentration.

2.7.8.1 Glucose 6-Phosphate Dehydrogenase

Activity of G6PDH (E.C.1.1.1.49) was measured by the method of Deutsch (1995). Glucose 6-phosphate and $NADP^+$ react in the presence of enzyme G6PDH and form 6-phosphogluconolactone and $NADPH/H^+$. The rate of increase in absorbance at 339 nm corresponds to the increase of $NADPH/H^+$ and therefore to the G6PDH activity.

Reagents:

Phosphate buffer: 0.1 M K_2HPO_4/KH_2PO_4 (pH 7.4)

Test medium**:** 500 mM Tris-(hydroxymethyl)-amino methane
3.8 mM NADP (Na-salt)
63 mM $MgCl_2$
33 mM glucose 6 phosphate

5 mM Malenimide

Liver cytosol was diluted 1:10 by the phosphate buffer and incubated at 25°C. Test medium was also maintained at 25°C and added to the cytosol. After an incubation of 2 minutes the increase in absorption was measured at 339 nm for 1 minute (ULTRASPEC 2000). One blank was also measured simultaneously and the sample reading was corrected by the blank reading.

$$Activity = \frac{V \times \Delta E}{\varepsilon \times d \times v}\left[\frac{nmol\ NAPDH / H^{+}}{mg\ \ \mathrm{Pr}otein \times \min}\right]$$

V→Total volume (1000 µl)

ΔE→Change in the absorption at 339 nm

∈→Molar extinction coefficient of NADPH (6.22 *10^{-3}[mL* $nmol^{-1}$* cm^{-1}])

d→Thickness [cm]

v→ Volume of the sample (50µl)

2.7.8.2 Fatty Acid Synthase

The activity of fatty acid synthase (EC.2.3.1.85) was measured by the method of Nepokroeff et al. (1975). In the presence of FAS malonyl CoA and acetyl CoA oxidize NAPDH into $NADP^{+}$ and n-carbonic acid is formed. The activity of FAS is proportional to the NADPH required.

Reagents:

Preincubation medium:	500 mM K_2HPO_4/KH_2PO_4 (pH 7.0), 5.0 mM Dithiothreitol
Medium 1:	700 mM K_2HPO_4/KH_2PO_4 (pH 7.0), 0.14 mM $NADPH/H^{+}$
	1.4 mM EDTA, 1.4 mM Dithiothreitol
Medium 2:	0.33 mM Acetyl CoA
Medium 3:	1.0 mM Malonyl CoA

Test Medium = 700 µl Medium 1 + 100 µl Medium 2 + 100 µl Medium 3

Liver cytosol diluted with preincubation medium (1:5, v/v) was incubated at 37°C for half an hour. Test mediums were incubated at 25°C and were mixed with cytosol probe in a cuvette, the absorption was measured by spectrophotometer (ULTRASPEC 2000) at 340 nm for 1 minute. One

blank without malonyl CoA was measured; from blank, oxidation of NADPH by FAS was corrected.

$$Activity = \frac{V \times \Delta E}{\varepsilon \times d \times v}\left[\frac{nmol}{mg\ \ \mathrm{Pr}otein \times \min}\right]$$

V→ Total volume (1000 µl)

ΔE→ Change in absorption at 340 nm

∈→ Molar extinction coefficient of NADPH (6.22 *10^{-3}[mL* $nmol^{-1}$* cm^{-1}])

d→ Thickness [cm]

v→ Volume of the sample (20µl)

2.7.9 MTP activity

The microsomal triglyceride transfer protein (MTP) activity was measured in the liver homogenate by a commercially available Fluorescence assay (Chylos, Inc., Woodburg, NY, USA) kit. MTP activity was measured by incubating liver homogenates with donor vesicles containing fluorescence-labeled lipids, and measuring the fluorescence transferred to acceptor vesicles.

Triplicate vesicles were pipetted in a fluorescence microtiter plate. Water and samples were added to the vesicles. Ingredients were allowed to reach room temperature. Reaction was started by adding homogenate to control and test, and isopropanol to the totals. Microtiter plate was incubated at RT for 30 min. Fluorescence units (FU) were measured using excitation wavelengths of 470 nm and emission wavelengths of 550 nm.

% Transfer in controls (C): (Control $_{FU}$ – Blank $_{FU}$) / (Total $_{FU}$ – Blank $_{FU}$) X 100

% Transfer in test samples (D): (Test $_{FU}$ – Blank $_{FU}$) / (Total $_{FU}$ – Blank $_{FU}$) X 100

2.7.10 Expression of Enzymes in the Liver

All expression experiments were based on the principle of reverse transcriptase and polymerase chain reaction (PCR).

2.7.10.1 Isolation of total RNA

Isolation of total RNA was performed according to Guanidinium Thiocyanate method following Chirgwin et al. (1979) with TRIZOL Reagent (Invirogen, Life technologies). About 10 mg frozen liver was homogenized in 1 ml of Trizol in an ice cooled hand homogenizer followed by incubation at 25°C, for 5 min. By adding chloroform and following centrifugation, probes were separated into an aqueous phase and an organic phase. RNA remains in aqueous phase. The RNA in the aqueous upper phase was transferred in a new eppendorf tube. The RNA was recovered by precipitation with isopropyl alcohol. The resulting RNA pellet was washed twice with 75% ethanol, vortexed, centrifuged (9700 rpm, 4°C, 5 min), and air dried. Finally the RNA pellet was dissolved in 50 µl of RNAse free water with slow vibration (Thermal Mixer Comfort, Eppendorf, Hamburg) at 60°C for 5 min. The RNA samples were stored at -80°C.

2.7.10.2 Quality and Concentration of RNA

To determine quality and concentration of RNA, the reaction tubes and solutions to be used were RNAse free, either in suitable form of commercial manufacturers referred or 0.1 % diethylpyrocarbonate (DEPC) treated and autoclaved. All the reagents/buffers needed were prepared with 0.1 % DEPC treated high purity water. The RNA concentration was measured spectrophotmetrically (ULTRASPEC 2000). The RNA was diluted to 1**:**100 and the absorption was measured against water at 260 nm and 280 nm in quartz cuvettes.

$$RNA\left[\mu g/ml\right] = A_{260} \times 40 \times 100$$

A_{260} is the absorption at 260 nm, 40 is the RNA [µg/ml] concentration at A_{260} and 100 is the dilution factor.

The purity estimation of RNA was done by the quotient absorption at 260 nm and absorption at 280 nm. All samples pointed a quotient of 1.6 or up which indicates good quality of RNA.

The quality of RNA was also analzyed by gel electrophoresis.

For the RNA gel electrophoresis following reagents were used:

Gel buffer (pH 7.0)	Running buffer	Loading buffer
200 mM Mops	100 ml gel buffer	4 ml 10 × gel buffer
50 mM Sodium acetate	20 ml Formaldehyde	20 mg Bromophenol blue
10 mM EDTA	880 ml water	80 µl 500 mM EDTA (pH 8.0)
		720 µl Formaldehyde (37%)
		2 ml Glycerol (100%)
		3 ml Formamide

MOPs: 3-[N-Morpholino] propanesulphonic acid; EDTA: Ethylene Diamine Tetra Acetic acid;

The gel was prepared from 0.36 g Agarose (SERVA), 3 ml gel buffer and 26.5 ml DEPC-water. The gel was heated to clear solution (5 min), and cooled down to 65°C. Afterwards, 540 µl formaldehyde and 14 µl ethidium bromide (0.5 µg/ml) were added to the gel. The gel was dried and then equilibriated in the running buffer for 30 min. 10 µl RNA (4 µg) with 4 µl loading buffer was poured in the slots and electrophoresis was ran (1 h, 5-7 V/cm). The gel was photographed under UV light and bands were evaluated with the associated device.

2.7.10.3 cDNA Synthesis

The RNA was copied into cDNA. **(**Omniscript transferase and buffer were purchased from Quigen, Hilden, Germany and dNTPs were purchased from Carl Roth GmbH, Karlsruhe, Germany).

Mater mix was added to the RNA and cDNA was synthesized for 59 minutes at 37°C (Mastercycler, Personal, Cologne, Germany). Reaction was stopped by denaturing at 93°C for 5 min.

Reagents	1× Sample (Master mix)
RNAse free water	10.5µl
10×buffer	2.0µl
Di-oxynucleotide triphosphate (10 mM)	2.0 µl
Oligonucleotides (T 18)	2.0 µl
Omniscript transferase	1.0 µl
RNA (0.4 µg/µl)	5.8 µl

2.7.10.4 Semiquantitative PCR

The gene expression for the enzymes HMG-CoA reductase, FAS, MTP, ACAT-2, SREBP-1c, SREBP-2, APO-B, CYP7A1 and LDL-receptor were determined by semi-quantitative PCR method and compared with the household gene GAPDH which was run in parallel. Tag-polymerase, buffer and $MgCl_2$ were purchased from Promega (MADISON, WI, USA).

The reagents used for the PCR and their ratio:

Reagents	1× Sample (Master mix)
DEPC-Water	10.2 µl
25 mM $MgCl_2$	1.2 µl
10×Buffer	2.0 µl
10mM dNTPs	0.4 µl
Forward primer (5 pmol/µl)	2.0 µl
Reverse primer (5 pmol/µl)	2.0 µl
Tag-polymerase	0.2 µl
cDNA	2.0 µl

Master mix was added to the cDNA and PCR was run, for corresponding gene annealing temperature and cycle numbers (Mastercycler, Personal, Cologne, Germany).

PCR Program:

Reaction cycle	Temperature (°C)	Time
Denaturing	93°C	30 seconds
Annealing	Depending on the gene	30 seconds
Extension	72°C	1 minute

Primer sequences: All the Primers and dNTPs were purchased from Carl Roth GmbH, (Karlsruhe, Germany).

Primer	Sequences (Forward and Reverse)	Product length	Annealing temp	Cycle numbers
HMG CoA Reductase	Forward 5'-AAG GGG CGT GCA AAG ACA ATC -3' Reverse 5'- ATA CGC CAC GGA AAG AAC CAT AGT- 3'	404 bp	56.7°C	30
FAS	Forward 5'-CCT CCC CTG GTG GCT GCT ACA A-3' Reverse 5'-CCT GGG GTG GGC GGT CTT T-3'	224 bp	60°C	30
MTP	Forward 5'-CGC GAG TCT AAA ACC CGA GTG-3' Reverse 5'-CCC TGC CTG TAG ATA GCC TTT CAT-3'	241 bp	57°C	35
SREBP-1c	Forward 5'-GGA-GCC-ATG-GAT-TGC-ACA-TT-3' Reverse 5'-AGG-AAG-GCT-TCC-AGA-GAG-GA-3'	191bp	60°C	35
SREBP-2	Forward 5'-CCG GTA ATG ATG GGC CAA GAG AAA G-3' Reverse 5'-AGG CCG GGG GAGACA TCA GAA G-3'	404 bp	60°C	35
APO-B	Forward 5'-A AAG GGG AGG GAA AAG GTT-3' Reverse 5'-A GGT AGG GGC TCA CAT TAT TGG-3'	286 bp	56°C	24
CYP7A1	Forward 5'-CAA GAC GCA CCT CGC TAT CC-3' Reverse 5'-CCG GCA GGT CAT TCA GTT G-3'	206 bp	60°C	38

LDL Receptor	Forward 5'-ACG GGC TGG CGG TAG ACT GGA-3' Reverse 5'-TGA GGC GGT TGG CAC TGA AAA-3'	474 bp	59°C	35
GAPDH	Forward 5'-A TGG CCT TCC GTC TTC C-3' Reverse 5'-GGG TGG TCC AGG GTT TCT TAC TC-3'	337 bp	60°C	24
PPAR α	Forward 5'-CCCTCTCTCCAGCTTCCAGCCC-3' Reverse 5'-CCACAAGCGTCTTCTCAGCCATG-3'	555 bp	65°C	29
Glutathione-S-Transferase	Forward 5'-AGG GGG AGA ATG CCA AGA AGT TC 3' Reverse 5'-GGG TAG AGC CTG TGG ATG GTA GTC-3'	466 bp	56°C	50
ACO	Forward 5'-CTTTCTTGCTTGCCTTCCTTCTCC-3' Reverse 5' GCCGTTTCACCGCCTCGTA 3'	415 bp	60°C	27
CYP4A1	Forward 5'-CAG AAT GGA GAA TGG GGA CAG C-3' Reverse 5'-TGA GAA GGG CAG GAA TGA GTG G-3'	459 bp	65°C	29
INSIG-1	Forward 5'-ATT TGG CGT GGT CCT GGC TCT GG-3' Reverse 5'-GCG TGG CTA GGA AGG CGA TGG TG-3'	389 bp	62°C	25
INSIG-2	Forward 5'-AAG CGT GGC CCC TAC ATT TCC TC-3' Reverse 5'-GGC CAC GCA GCG CAT AAC AC-3'	303 bp	59°C	28
Δ 6-Desaturase	Forward 5'-TCC CCA CTA TGC CAA GAC ACA ACT T-3' Reverse 5'-CAA AAG CCC TCC CCT CCC TCT G-3'	246 bp	60°C	30

Gel Electrophoresis: The PCR products were evaluated by the Agarose gel electophoresis. The separation of the PCR product developed was performed through Electrophoresis. 2% Agarose (SERVA) gel was prepared in 0.5×TBE buffer, boiled to clear solution and cooled down to 55°C and ethidium bromide was added to the gel. After drying, gel was equilibriated in the running buffer (0.5 ×TBE) for 30 min. 10 µl PCR products (stained with loading buffer) were poured in the wells and electrophoresis was run for 45 min at 80 V. The gels were photographed under the UV light and evaluated with the associated device (SYNGENE).

Regents used for Gel electrophoresis:

Gel buffer	5×Running buffer(TBE)	6×Loading buffer
0.7 g agarose (SERVA)	54 g Tris base	300 µl Glycerol (30%,v/v)
35 ml 0.5×TBE	27.5 g Boric acid	2.5 mg Bromophenol blue (0.25%)
14 µl Ethidium bromide	3.72 g $Na_2EDTA.2H_2O$	700 µl water
(0.5 µg/ml)	1 litre with water	

2.7.10.5 cDNA Array Analysis

For the expression analysis cDNA Macro array ATLAS™ Nylon 1.2 Array (Clontech, Heidelberg, Germany) were used. These Rat 1.2 Nylon membranes contain 1176 protein/genes involved in lipid metabolism and metabolism of carbohydrate, amino acid, xenobiotics, vitamins as well as genes, different receptors, hormones, protein and factors (Translation, Transcription, and Growth) according to the manufacturer's protocol.

For the cDNA arrays, total RNA isolated from the liver (2.7.10.1) was used. The analysis was performed with the RNA of the third experiment rats (Soy, Fish, and Casein group). Equal amounts of the RNA of three rats within the same dietary treatment group were mixed into one pool of 50 µg. Therefore, 4 RNA pools per treatment group were used for a separate hybridization.

Method: 50 µg total RNA (total volume 45 µl) per pool was prepared as described in ATLAS Pure Total RNA Labelling System (Clontech).

The substantial work procedures are described in the following:

Enrichment of the RNA: Total RNA (50 µg) was enriched with Biotinylated Oligo (Deoxy-Thymine) (dT). Streptavidin magnetic beads were added and the magnetic RNA was concentrated on a magnetic particle separator (Promega, Mannheim, Germany).

cDNA synthesis and Radioactive labelling: The cDNA was synthesized as described for ATLAS NYLON 1.2 ARRAYS (Clontech). The non bound fragments were separated by column chromatography. The cDNA was labeled with radioactive Alpha-^{33}Phosphorus [α-^{33}P] Deoxyadenosine triphosphate (dATP) (Perkin Elmer, Boston, USA).

Hybridization: The membranes were placed in hybridizing bottles. Before hybridization the quality of probes were checked by hybridizing them to a blank nylon membrane (supplied). After the addition of radioactivity, labeled samples were hybridized for 18 h at 68°C.

Exposition: Membranes were washed accordingly, afterwards mounted on whatman paper (3 MM) and wrapped in a plastic wrap. The array membranes were exposed to the Phosphorimager screen for 24 h. The array images were visualized using the Bio-Image analyzer Fujifilm BAS-1500 and TINA 2.0, software (Raytest).

Evaluation of the Data: Gene specific signal intensities on the arrays were quantified using ATLAS IMAGE 2.01 software (Clontech) and corrected for background. The allocation of the signals to the genes of the ATLAS rat 1.2 Arrays took place via the adaptation of the appropriate mask to the respective array. The background was the mean signal intensity of the unspotted fields on the array. Genes whose signal intensities were at least 1.5-fold the background level, were considered.

Normalization and comparison of the arrays: The level of the signal intensities of the particular arrays varied due to the quality and quantity of the assigned RNA. In order to be able to compare, the array data were normalized. Normalization of the signal intensities was done by relating them to the mean signal intensities of all genes. In our group prestudies (Sülzle et al. 2004, Ringseis and Eder 2005), this method of normalization yielded a higher reproducibility than normalization by various housekeeping genes. For the comparison of the twelve arrays, the four arrays of the control group (casein) were taken as one control array. But the four arrays of the control group were individually normalized; their relative signal intensities were multiplied by a coefficient. All the 8 treatment arrays were compared from one control array. For the Evaluation of data EXEL 2000 (Microsoft, USA) was used. The gene expression was taken as a relation ship between the signal intensities from treatment to the control array.

2.7.11 Western Blotting

For western blotting, liver microsomal fractions (2.6.2) of the rats fed soy protein or casein containing diets in experiment 3 were used. Samples were dissolved (1**:**4) in a buffer containing 0.22 M Tris, 41% glycerol, 0.7% SDS, 5% mercaptoethanol and 0.002% bromphenol blue and subsequently denaturated at 95°C for 5 min. After chilling 20 µl of denaturated probes containing 40 µg proteins were applied to the gels. For gel electrophoresis, 5% stacking gel and 10% running gel gradients were used. Electrophoresis was run at 210 V for 1 h.

Running gel (10%) Reagents	Volume
Acrylamide/Bisacrylamide (30%)	5 ml
Running Buffer (1.125 M, pH 8.8)	5 ml
SDS (10%)	0.15 ml
DEPC Water	4.85 ml
TEMED	12.5 µl
Ammonium per sulphate (10%)	75 µl

Stacking gel (5%) Reagents	Volume
Acrylamide/Bisacrylamide (30%)	0.72 ml
Stacking Buffer (0.5 M, pH 6.8)	2.576 ml
SDS (10%)	6 µl
DEPC Water	0.98 ml
TEMED	11.4 µl
Ammonium per sulphate (10%)	56.6 µl

Proteins resolved by electrophoresis were transferred from gel to nitrocellulose membrane electrophoretically (95 mA, 90 min). The nitrocellulose membrane which contained the resolved proteins was first incubated with milk protein (5%) for 10 min, to block nonspecific protein binding sites. Then the membrane was incubated with the primary antibody anti HMG CoA R (rabbit polyclonal IgG, Catalog 07-457, UPSTATE, Lake Placid, New York) (diluted 1**:**1000), for 2 h at RT. The unbound primary antibody was washed away with TBST-buffer and the membrane was incubated with the secondary antibody (anti rabbit conjugate, Sigma) (diluted 1**:**5000) for 1 h at RT. Afterwards, the membrane was washed three times with TBST buffer and once with alkaline phosphate buffer. The membrane was stained with p-Nitro-Blue-Tetrazolium chloride (NBT) and 5-Bromo-4-chloro-3-indolylphosphate (BCIP), dissolved in alkaline phosphate buffer, till the brown colour bands develop.

Running buffer for SDS-Page	Blot buffer for SDS-PAGE	5x TBST (pH 7.5)	Alkaline phosphate buffer (pH 9.8)
3.0 g Tris 14.4 g Glycine 1.0 g SDS 1 litre with water	3 g Tris 14.4 g Glycine 0.5 g SDS 200 ml Methanol 1 litre with water	50 mM Tris 750 mM NaCl 0.5 % TWEEN 20	25 ml 1 M Tris/HCl pH 9.8 15 ml 5 M NaCl 2.5 ml 1 M MgCl2 500 ml with water

Evaluation: The membranes were photographed with a computer connected system (Gel-Pro Analyzer, Intas, Göttingen, Germany). Bands of HMG CoA R were identified by comparing with the high molecular weight standards (Serva, Heidelberg, Germany).

2.8 Statistical Analysis

In all the three experiments, treatment effects were analyzed using the MINITAB statistical software with one-factorial analysis of variance (Minitab, State College, PA, USA). For statistical significant F-values, individual mean values were compared by the Fisher's multiple range tests. Treatment effects were considered statistically significant for $p<0.05$. All results are mean ± SD.