Nutritional factors as regulatory molecules of hormonal signalling, metabolic health and inflammatory pathways in chicken

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Final report

I. Introduction

Improving metabolic health is highly important in animal production to maintain intensive and economic growth and to consider animal welfare. As broiler meat is one of the most common, valuable and easily available protein sources in human nutrition, the significance of efficient poultry herding has been highly increased for the last decades. During animal farming, welfare and economic aspects should also be addressed by improving animal health. Since the endocrine system and hormonal signalling are primarily involved in the metabolic regulation, affecting the endocrine pathways by nutritional factors, such as dietary components or feed additives may trigger improvements in metabolic health and productivity of broilers as well.

Since the banning of the application of antibiotics and hormones as growth promoters in the European Union in 2006 (Phillips, 2007), there is a growing interest for alternative, natural feed additives, especially for the widely used short chain fatty acids (SCFA; Michard, 2008). Amongst them, applying the four carbon butyric acid ([n-]butyrate) is primarily common in poultry nutrition (Chamba et al., 2014).

It is proven that butyrate maintains gut health by supporting the gastrointestinal epithelium (Kotunia et al., 2004), by improving the intestinal barrier function and absorptive capacity and by stabilizing the eubiotic intestinal microflora (Hu and Guo, 2007). Further, butyrate as an epigenetically active molecule has the ability to modify gene expression by triggering histone hyperacetylation (Kien et al., 2008; Mátis et al., 2013), presumably leading to alterations in metabolic pathways and their regulation. For instance, the gene expression of hepatic drug-metabolizing cytochrome P450 (CYP) enzymes was modified by orally applied butyrate in chicken (Csikó et al., 2014); however, these alterations were finally not realized on the level of enzyme activity (Mátis et al., 2013).

Recent investigations have also revealed that orally applied butyrate increases pancreatic insulin secretion and systemic insulin sensitivity, further, triggers elevated plasma concentrations of the incretin hormones Glucagon-like Peptide 1 (GLP-1) and Glucose-dependent Insulinotropic Polypeptide (GIP) in mice (Lin et al., 2012). Earlier studies of our research group indicated that butyrate, applied in a daily oral bolus, could influence insulin signalling in chicken by selectively up-regulating insulin receptor β (IRβ) in skeletal muscle (Mátis et al., 2015). However, the age-dependency of butyrate's effect on insulin homeostasis could be hypothesized, increasing fasting plasma insulin and glucose levels of three-week-old broilers (Mátis et al., 2015), but not at the age of 6 weeks (Kulcsár et al., 2016). Some other authors have also described age-related decrease in the sensitivity of insulin signalling proteins to nutritional factors both in mammals (Gupte et al., 2008) and birds (Deng et al., 2014).

Butyrate can be used as a feed additive (exogenous origin), when supplementation of the diet is carried out by adding either free, unprotected butyrate salts (the most common is sodium butyrate) or various protected forms. Unprotected butyrate salts can be absorbed mainly by simple diffusion in non-dissociated form; therefore, the absorption is most intensive in the proximal, acidic section of the gastrointestinal tract (Manzanilla et al., 2006). The other main source of butyrate for broilers is the production by the anaerobe microbial fermentation of carbohydrates in the caeca (endogenous origin), where formation of SCFA can be promoted by providing more substrates for the caecal microflora (Molnár et al., 2015). This can be achieved by increasing the ratio of resistant starch or soluble non-starch polysaccharides (NSP) in the feedstuff (Jamroz et al., 2002). Major components of soluble NSP are beta-glucans and arabinoxylans, the latter is considered as the main contributor to the increased viscosity of digesta of animals fed with NSP-rich diet, such as wheat or barley (Cowan et al., 1996). Nevertheless, the adverse effects of higher viscosity can be partly eliminated by xylanase and glucanase enzyme supplementation (Cowan et al., 1996; Engberg et al., 2004). These NSP-degrading enzymes cleave long-chain polysaccharides to shorter oligosaccharides, resulting in decreased viscosity of the digesta; furthermore, the produced easily fermentable carbohydrates serve as potential substrates for microbial SCFA, primarily butyrate production (Kulcsár et al., 2017).

Among various dietary components, the reduction of crude protein (CP) content of diets applied in broiler nutrition deserves attention as a both economic and environmental issue, providing possibility for diminished nitrogen excretion (Donsbough et al., 2010). There are some experimental evidences that slight reduction of dietary CP content with simultaneous limiting amino acid supplementation would be possible without depression of growth of chickens (Darsi et al., 2012). Nevertheless it is still poorly investigated, how this altered feeding condition influences the metabolism and thus the physiological state of the animals.

II. Aims, overview of the trials

Based on the above mentioned literature data, in the present project our main goal was to investigate the possible effects of certain dietary factors (dietary components and butyrate as feed additive) on the endocrine metabolic regulation of broiler chickens in order to improve animal health and productivity.

(1) At first, some preliminary studies were carried out to assess **(1.1.) the mechanisms of butyrate's effects on hepatic drug-metabolizing CYP enzymes** and to gain some evidence concerning its action **(1.2.) on incretin hormones** affecting insulin homeostasis. These studies were conducted in the Division of Biochemistry, Department of Physiology and Biochemistry, University of Veterinary Medicine, Budapest.

(2) Based on our previous studies and on these pilot trials, the **main animal feeding experiment** was carried out in the Research Institute for Animal Breeding, Nutrition and Meat Science, National Agricultural Research Center, Herceghalom. The following dietary factors were studied:

- **Dietary cereal type: maize- vs. wheat-based diet.** Maize contains only limited amount of soluble NSP, while wheat is especially rich in them. As the latter diet was supplemented with xylanase and glucanase NSP-degrading enzymes, in this case the caecal microbial SCFA production was highly stimulated by providing more substrates for the gut flora.
- **Dietary CP content: adequate ("normal") vs. slightly decreased ("lower") dietary CP level.** Diets were formulated with CP content meeting standard requirements of the appropriate dietary phase ("normal

protein" [NP] groups with 22.0%, 21.1% and 19.0% CP in starter, grower and finisher diets, respectively) or reduced by 15% ("low protein" [LP] groups with 18.7%, 17.9% and 16.1% CP). Low CP diets were fortified with limiting amino acids to avoid any disturbances caused by essential amino acid deficiency.

- **Dietary sodium butyrate supplementation**: broiler diets were supplemented with or without 1.5 g/kg diet unprotected sodium butyrate.

For this animal feeding trial, two hundred and forty newly hatched male Ross 308 broiler chicks were obtained from a commercial hatchery (Gallus Company, Devecser, Hungary) and were randomly allocated to eight dietary groups (n=10 per sampling points per group, n=30 in total per group). The animals were housed in metal pens on wheat straw litter. Environmental conditions were controlled and set according to the requirements of the Ross technology (Aviagen, 2014). Feed and drinking water were provided *ad libitum* thorough the entire study.

The housing and treatment of the animals were conducted in strict accordance with the applicable national and international laws as well as with the institutional guidelines. Experimental procedures were approved by the Government Office of Pest County, Food Chain Safety, Plant Protection and Soil Conservation Directorate, Budapest, Hungary (number of permission: PEI/001/1430-4/2015).

To investigate the above described dietary factors, a treatment regime of a 2 x 2 x 2 factorial arrangement was applied as indicated in **Table 1**.

Table 1. Experimental design of the feeding groups

Group No	Group size/ Slaughtering point[1]	Main carbohydrate source	CP content	Sodium butyrate supplementation
1	10	Maize	adequate	no
2	10	Maize	adequate	1.5 g/kg diet
3	10	Maize	low	no
4	10	Maize	low	1.5 g/kg diet
5	10	Wheat[2]	adequate	no
6	10	Wheat[2]	adequate	1.5 g/kg diet
7	10	Wheat[2]	low	no
8	10	Wheat[2]	low	1.5 g/kg diet

[1]Slaughterings were carried out at 1, 3 and 6 weeks of age. Regarding the slaughtering point of 6 weeks, an additional set of 10 animals/group was applied for studies on carcass composition.
[2]With xylanase-glucanase supplementation

All diets were set to be isocaloric within a phase, formulated to suit the Ross 308 recommendations (NRC, 1994), and were fed in mash form. Compositions and calculated nutrient contents of diets are indicated in **Appendix 1-3** (see at the end of the report).

At 1, 3 and 6 weeks of age, chickens (n=10/group) were slaughtered by decapitation, previously narcotized with carbon dioxide. Several samples were taken from the animals as indicated in **Table 2**.

Table 2. Overview of samples taken from chickens of the feeding trial

Sample	Target of investigation	Method	Place of analysis
2.1. Blood plasma (from brachial vein)	Metabolic and endocrine parameters	ELISA, colorimetric assays	Budapest
2.2. Carcass, pectoral and gastrocnemic muscle (from a separate set of animals)	Carcass composition and meat quality	Chemical analyses	Herceghalom
2.3. Liver	Insulin and glucagon signalling	qRT-PCR, Western blot	Budapest, Germany
2.4. Gastrocnemic muscle	Insulin and glucagon signalling	qRT-PCR, Western blot	Budapest, Germany
2.5. Abdominal adipose tissue	Insulin and glucagon signalling	qRT-PCR, Western blot	Budapest, Germany
2.6. Liver, duodenal and ileal mucosa	CYP enzymes	Luminometric assays	Budapest
2.7. Ileal and caecal ingesta	Genetic analysis of gut microbiota	T-RFLP	Germany

Blood samples were taken from the brachial vein to heparinized tubes on the day prior to slaughtering; freshly separated plasma samples were stored at -80°C until further processing. Tissue samples for Western blotting and CYP analyses as well as ingesta samples were shock-frozen in liquid nitrogen, while those for qRT-PCR were immediately placed into RNA-isolating reagent; all tissue samples were stored at -80°C until the planned analyses. Samples for assessing carcass composition and meat quality (2.2.) were taken at 6 weeks of age from a separate set of animals and were stored at -20°C until subsequent chemical analyses.

The majority of the analyses was carried out in the Division of Biochemistry, Department of Physiology and Biochemistry, University of Veterinary Medicine, Budapest (research group of Dr. Zsuzsanna Neogrády). In the frame of the established international cooperation, certain investigations were conducted in the Research Institute for Animal Breeding, Nutrition and Meat Science, National Agricultural Research Center, Herceghalom (research group of Dr. Hedvig Fébel) and in the research group of Prof. Korinna Huber, Institute of Animal Science, University of Hohenheim, Stuttgart (previous institution of Prof. Huber was the University of Veterinary Medicine, Hannover as indicated in the proposal), mostly by researchers of the Budapest research group.

(3) To study the interaction of metabolic processes and the inflammatory response, ***in vitro*** **studies** were carried out **on primary hepatic cell culture models** in the Division of Biochemistry, Department of Physiology and Biochemistry, University of Veterinary Medicine, Budapest. The specific immunometabolic regulatory role of certain liver cell types, such as that of hepatocytes and Kupffer cells was studied on a **newly established hepatocyte – Kupffer cell co-culture** model of swine and chicken origin.

III. Main results

1.1. Mechanisms of butyrate's modulatory action on hepatic CYP enzymes

It is already known from our previous studies that butyrate can alter the gene expression of hepatic CYP enzymes in chicken both *in vitro* and *in vivo* (Csikó et al., 2014), but this modulatory action is finally not realized on the level of enzyme activity (Mátis et al., 2013). The aim of the present study was to provide some evidence if butyrate can alter the activity of hepatic CYPs in chickens exposed to CYP-inducing xenobiotics. Ross 308 chickens in the grower phase were treated with daily intracoelomal phenobarbital (PB) injection (80 mg/kg BW), applied as a non-specific CYP-inducer, simultaneously with two different doses of intra-ingluvial sodium butyrate boli (0.25 g/kg BW and 1.25 g/kg BW) for 5 days. Activities of CYP2H and CYP3A subfamilies were assessed by specific enzyme assays from isolated liver microsomes.

According to our results, the lower dose of orally administered butyrate significantly attenuated the PB-triggered elevation of both hepatic CYP2H and CYP3A activities (**Fig. 1.A-B**), which might be in association with the partly common signalling pathways of butyrate and CYP-inducing drugs, such as that of PB. Based on these data, butyrate may take part in pharmacoepigenetic interactions with simultaneously applied drugs or other CYP-inducing xenobiotics, having a huge importance on food safety and from pharmacotherapeutic approach as well. However, butyrate was found to be capable to maintain physiological CYP activity by attenuating CYP induction, underlining the safety of butyrate application in poultry nutrition.

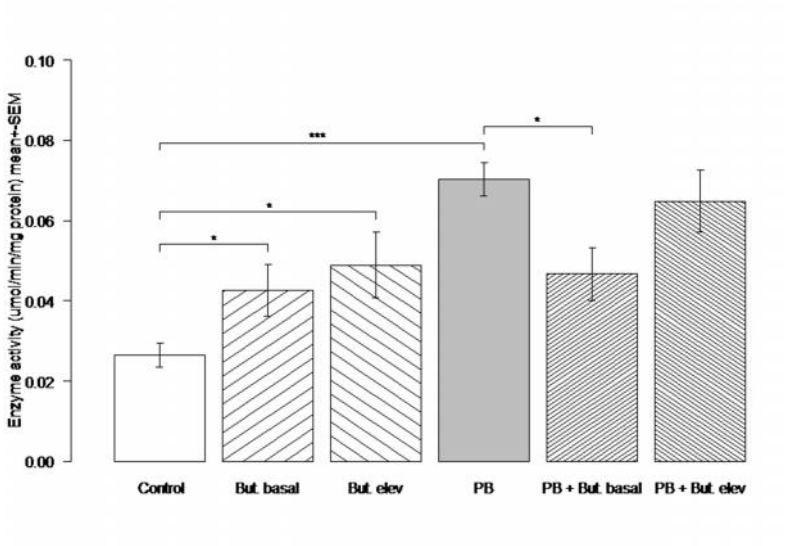

Fig. 1.A

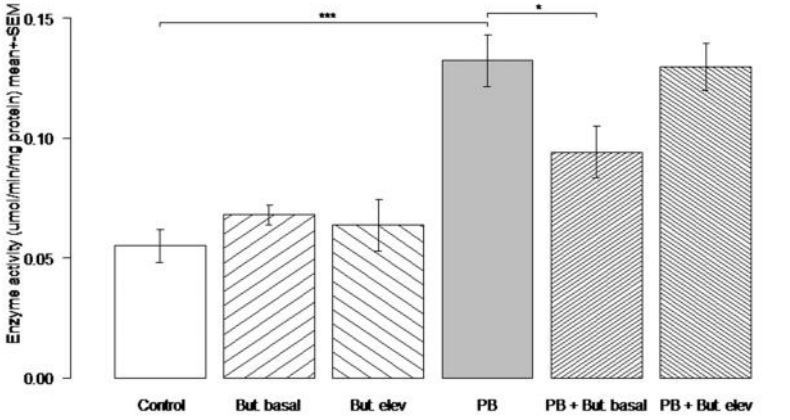

Fig. 1.B

Figure 1. Mean specific activity of hepatic microsomal CYP enzymes of chickens.
A. Average amount of 4-aminophenol produced in aniline hydroxylation assay (µmol/min per mg microsomal protein), indicating the mean specific activity of hepatic microsomal CYP2H subfamily.
B. Average amount of formaldehyde produced in aminopyrine N-demethylation assay (µmol/min per mg microsomal protein), indicating the mean specific activity of hepatic microsomal CYP2H/CYP3A37 enzymes.
Results of enzyme assays, carried out with hepatic microsome fractions of phenobarbital ("PB")-treated (80 mg/kg BW) and non-treated chickens without butyrate addition and with two different doses ("But basal", 0.25 g/kg BW; "But elev", 1.25 g/kg BW) of a daily oral butyrate application on days 20-24 (n=8/group).
Results are expressed as mean ± SEM. Significant differences: *P<0.05, ***P<0.001.

1.2. Effects of butyrate on incretin homeostasis in chicken

The pancreatic secretion of insulin, a key endocrine regulator of metabolism and growth, can be greatly influenced by the gut-derived incretin hormones, namely by GIP and GLP-1. As insulin is a major stimulator of growth, affecting its producion may be of special importance in food-producing livestock. The aim of the present preliminary study was to investigate novel ways of modulating incretin and insulin homeostasis in chickens and rabbits by nutrition, e.g. by oral butyrate application, also studying the mechanisms of incretin action in both species as a comparative approach.

Acute oral butyrate challenge significantly decreased plasma GIP levels by approx. 40% in both species: significant interactions of butyrate exposure and incubation time were found in both chickens (at 30 and 60 min following butyrate ingestion, applying the dose of 1.25 g/kg BW) and rabbits (at 30 and 60 min after butyrate administration, applying the dose of 0.25 g/kg BW), while plasma GLP-1, insulin and glucose concentrations remained unaffected by butyrate in both species over time. Results of plasma GIP, GLP-1, insulin and glucose concentrations gained in chickens are presented in **Fig. 2**.

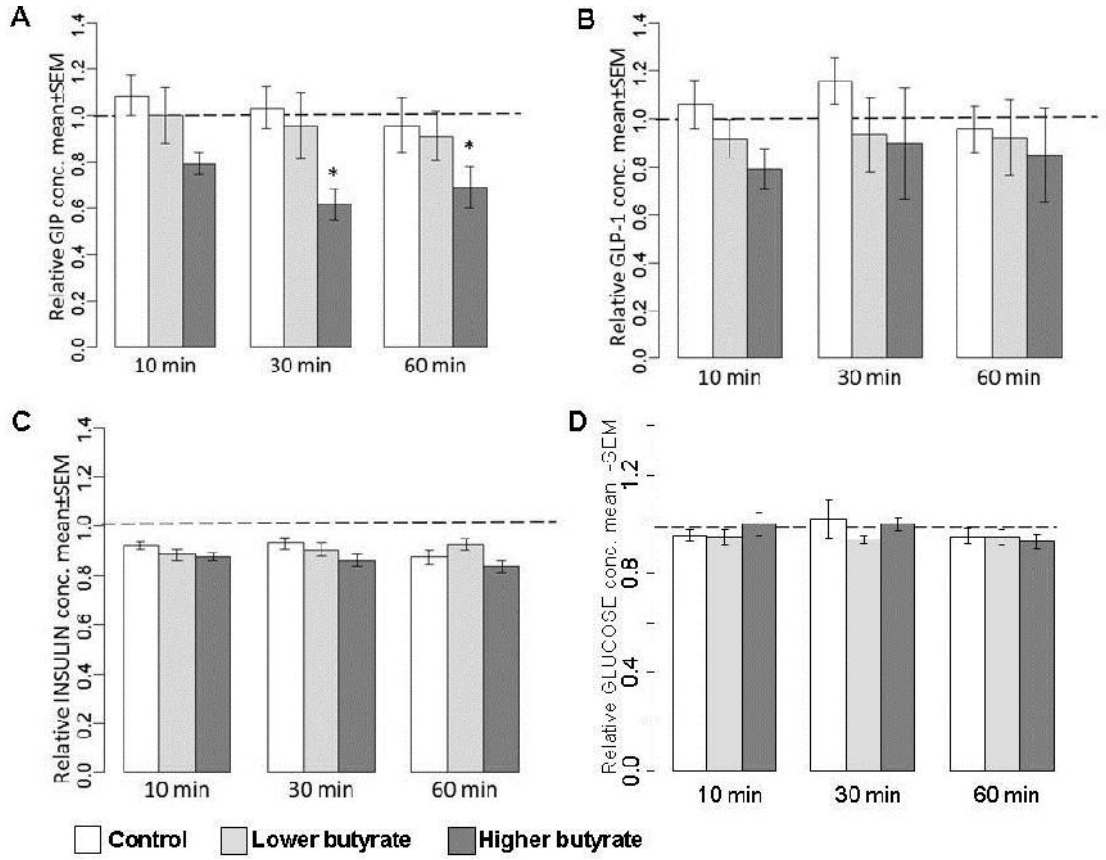

Fig. 2.

Figure 2. Relative concentrations of A. GIP, B. GLP-1, C. insulin and D. glucose in the blood plasma of chickens. Sodium butyrate was applied in the doses of 0.25 g/kg BW (referring to "Lower butyrate") and 1.25 g/kg BW (indicated by "Higher butyrate"). Relative hormone concentrations were calculated by considering the baseline value of each animal at 0 min as 1. Results are expressed as mean ± SEM. Statistical analysis of data was performed by a randomized linear mixed model, asterisks indicate statistical significance compared to the 0 min values of the appropriate group (interaction between time and treatment), $*P < 0.05$.

These results are in contrast to butyrate's stimulating effect on both incretin and insulin secretion in mice, indicating specific, species-dependent differences even among mammalian species. Further, based on the analyzed correlations between the measured endocrine parameters (regardless of the butyrate exposure), it can be assumed that incretins may regulate pancreatic insulin release in rabbits on a partly different way compared to mice, humans and chickens.

2. Production data of the main animal feeding experiment

The animal trial was designed for monitoring certain diet-associated metabolic changes on molecular level, thus the analysis of growth performance parameters was not targeted in the present project. However, the body weight of the animals was recorded throughout the entire study. Considering these data, broilers kept on wheat-based diet had significantly greater body weights at week 1 and 3 when compared to maize-based dietary groups (**Table 3**). Similarly, low protein diet (fortified with limiting amino acids) had an increasing action on body weight gain at all time points (**Table 3**).

The improving action of wheat-based diet may be explained with the beneficial effects of the SCFA, primarily butyrate, intensively produced in the caecal fermentation (the deteriorative effects of the increased viscosity were prevented by the xylanase-glucanase supplementation). Regarding dietary CP levels, a slight reduction seems to be a great tool to improve productivity and decrease nitrogen loss; however, the applied limiting amino acid fortification is suggested to play pivotal role in maintaining animal health and production.

Table 3. Body weight results of the main animal feeding experiment

		Abbreviation of dietary group								
Parameter	**Week**	**MB NP**	**MB NP +But**	**MB LP**	**MB LP +But**	**WB NP**	**WB NP +But**	**WB LP**	**WB LP +But**	**Significant differences**
Body weight (g)	1	171.0 ±8.4	179.8 ±4.9	174.9 ±7.1	179.6 ±8.1	177.6 ±9.4	184.7 ±8.4	196.6 ±9.9	211.4 ±9.9	**WB vs. MB , *LP vs. NP
	3	679.5 ±20.8	638.1 ±34.1	824.3 ±32.9	864.0 ±28.6	826.0 ±37.2	845.6 ±30.7	821.2 ±34.0	811.6 ±33.7	**WB vs. MB, ***LP vs. NP
	6	2234.5 ±97.5	2315.6 ±117.8	2934.7 ±47.9	2635.3 ±97.0	2394.0 ±92.2	2406.5 ±112.7	2810.0 ±73.6	2686.5 ±146.4	***LP vs. NP

MB: Maize-based diet; WB: Wheat-based diet supplemented with NSP-degrading xylanase and glucanase enzymes; NP: “Normal protein” group reared on a diet with crude protein content adequate to the dietary phases; LP: “Low protein” group reared on a diet with crude protein content reduced by 15 % in each dietary phase, supplemented with limiting amino acids; But: Sodium (n-)butyrate supplementation of the diet in the dose of 1.5 g/kg diet.

Results are expressed as mean ± SEM. Statistical analysis of data was performed by multi-way ANOVA test to evaluate main effects. Main effects were determined as follows: WB vs. MB diet, LP vs. NP groups and butyrate supplementation vs. no added butyrate. N=10 per sampling points per group, n=30 in total per group.
*** $p<0.001$; ** $p<0.01$; * $p<0.05$

2.1. Effects of dietary cereal type, crude protein content and butyrate supplementation on key metabolic parameters of broilers

As mentioned earlier, blood plasma samples of Ross 308 broilers (for experimental design please see **Table 1**) were investigated at the age of 1, 3 and 6 weeks. According to our results, total protein (TP) concentration increased in wheat-based and decreased in low CP groups on week 3, while butyrate reduced albumin/TP ratio on week 1. Uric acid level was elevated by wheat-based diet on week 1 and by wheat-based diet and butyrate on week 3, but decreased in low CP groups on week 3 and 6. Aspartate aminotransferase activity was increased by wheat-based diet on week 3, while creatine kinase activity was intensified by low protein diet on week 3 and 6. Blood glucose level was decreased in wheat-based groups on week 3; however, triglyceride concentration augmented in the same groups on week 3. No change of GLP-1, GIP and insulin concentration was observed.

Concluding these data, an age-dependent metabolic responsiveness of broilers to the investigated dietary factors could be found; dietary cereal type was a potent modulator of metabolism; further, limiting amino acid supplemented low crude protein diet might have beneficial impact on growth of chickens.

Results gained by blood plasma analyses can be found in **Table 4-6**.

Table 4. Results of the parameters describing the metabolism of nitrogen containing compounds

		Abbreviation of dietary group								
Parameter	**Week**	**MB NP**	**MB NP +But**	**MB LP**	**MB LP +But**	**WB NP**	**WB NP +But**	**WB LP**	**WB LP +But**	**Significant differences**
Total protein (g/l)	1	22.58 ±1.47	22.90 ±1.42	23.03 ±1.57	23.36 ±1.01	26.34 ±1.51	23.99 ±1.07	24.08 ±1.41	23.24 ±1.25	-
	3	24.58 ±0.84	26.08 ±0.87	24.65 ±0.80	23.48 ±0.75	28.99 ±1.05	29.53 ±0.71	25.80 ±1.48	25.10 ±0.58	***WB vs. MB, ***LP vs. NP
	6	25.12 ±1.36	27.30 ±1.51	27.61 ±0.94	27.46 ±1.21	29.01 ±1.23	27.27 ±1.00	29.79 ±0.95	27.21 ±1.14	-
Albumin/ TP (%)	1	55.92 ±1.74	53.21 ±1.88	55.20 ±1.95	53.90 ±2.18	54.96 ±1.86	51.23 ±1.01	54.48 ±1.65	51.19 ±1.82	*But vs. NoBut
	3	48.00 ±0.97	44.95 ±1.29	46.34 ±1.12	45.68 ±0.57	45.17 ±2.19	45.12 ±1.56	45.25 ±1.88	46.62 ±1.25	-
	6	49.21 ±1.40	47.88 ±1.58	47.78 ±1.71	48.73 ±1.96	48.17 ±1.68	47.92 ±2.51	48.76 ±2.34	49.59 ±1.90	-
Uric acid (micromol/l)	1	418.7 ±36.1	350.1 ±26.1	369.6 ±30.4	387.7 ±28.8	478.4 ±44.7	412.2 ±25.2	412.5 ±32.3	443.9 ±39.6	*WB vs. MB
	3	269.6 ±28.7	310.5 ±20.5	188.3 ±16.6	216.2 ±24.4	308.9 ±23.4	355.9 ±24.9	298.3 ±27.1	312.9 ±19.5	***WB vs. MB, ***LP vs. NP, *But vs. NoBut
	6	194.3 ±15.3	190.8 ±16.8	135.4 ±17.2	182.9 ±13.3	215.0 ±22.3	227.1 ±14.9	160.8 ±11.1	192.0 ±21.0	**LP vs. NP
Aspartate amino-transferase (IU/l)	1	178.8 ±16.6	168.3 ±10.7	183.6 ±12.9	170.6 ±7.1	198.0 ±18.2	174.4 ±9.8	191.4 ±10.3	174.9 ±7.6	-
	3	160.6 ±20.4	165.8 ±5.7	150.1 ±5.4	157.4 ±7.4	164.9 ±9.3	172.4 ±7.4	174.9 ±12.4	185.4 ±14.9	*WB vs. MB
	6	279.9 ±42.1	337.8 ±39.3	339.9 ±21.8	264.6 ±25.9	288.3 ±33.2	237.6 ±24.7	333.9 ±33.6	333.6 ±24.9	-

Creatine kinase (IU/l)	1	1220 ±94	1590 ±287	1541 ±194	1430 ±161	1148 ±87	1330 ±254	1494 ±94	1220 ±92	-
	3	1374 ±160	1142 ±92	1408 ±251	2163 ±351	1437 ±196	1238 ±109	1994 ±189	1436 ±221	**LP vs. NP
	6	15529 ±4770	27735 ±8471	22087 ±3070	14877 ±3187	13753 ±4297	9733± 2778	29729 ±6238	30646 ±5977	*LP vs. NP

MB: Maize-based diet; WB: Wheat-based diet supplemented with NSP-degrading xylanase and glucanase enzymes; NP: "Normal protein" group reared on a diet with crude protein content adequate to the dietary phases; LP: "Low protein" group reared on a diet with crude protein content reduced by 15 % in each dietary phase, supplemented with limiting amino acids; But: Sodium (n-)butyrate supplementation of the diet in the dose of 1.5 g/kg diet; NoBut: Diet with no sodium (n-)butyrate supplementation; TP: Total protein.

Results are expressed as mean ± SEM. Statistical analysis of data was performed by multi-way ANOVA test to evaluate main effects. Main effects were determined as follows: WB vs. MB diet, LP vs. NP groups and butyrate supplementation vs. no added butyrate. N=10 per sampling points per group, n=30 in total per group.

*** $p<0.001$; ** $p<0.01$; * $p<0.05$

Table 5. Results of the parameters describing glucose homeostasis and the metabolism of lipids

		Abbreviation of dietary group								
Parameter	**Week**	**MB NP**	**MB NP +But**	**MB LP**	**MB LP +But**	**WB NP**	**WB NP +But**	**WB LP**	**WB LP +But**	**Significant differences**
Glucose (mmol/l)	1	14.07 ±1.34	11.90 ±0.37	18.58 ±2.03	13.28 ±0.51	12.64 ±0.71	18.69 ±2.14	15.95 ±1.72	13.91 ±1.01	-
	3	15.69 ±1.60	16.81 ±1.71	15.84 ±1.61	15.10 ±1.64	13.34 ±0.53	13.16 ±0.39	13.38 ±0.38	13.23 ±0.40	**WB vs. MB
	6	14.83 ±0.35	14.65 ±0.32	13.81 ±0.38	14.08 ±0.43	14.29 ±0.37	13.50 ±0.44	14.39 ±0.35	14.45 ±0.36	-
Triglyceride (mmol/l)	1	0.752 ±0.047	0.634 ±0.062	0.648 ±0.034	0.738 ±0.057	0.821 ±0.037	0.681 ±0.039	0.746 ±0.077	0.714 ±0.061	-
	3	0.542 ±0.071	0.557 ±0.053	0.836 ±0.073	0.774 ±0.112	0.837 ±0.067	0.866 ±0.090	0.666 ±0.088	0.934 ±0.066	*WB vs. MB
	6	0.792 ±0.076	1.006 ±0.094	0.995 ±0.169	0.995 ±0.110	1.313 ±0.100	1.138 ±0.066	0.951 ±0.131	1.009 ±0.089	-

MB: Maize-based diet; WB: Wheat-based diet supplemented with NSP-degrading xylanase and glucanase enzymes; NP: “Normal protein” group reared on a diet with crude protein content adequate to the dietary phases; LP: “Low protein” group reared on a diet with crude protein content reduced by 15 % in each dietary phase, supplemented with limiting amino acids; But: Sodium (n-)butyrate supplementation of the diet in the dose of 1.5 g/kg diet.
Results are expressed as mean ± SEM. Statistical analysis of data was performed by multi-way ANOVA test to evaluate main effects. Main effects were determined as follows: WB vs. MB diet, LP vs. NP groups and butyrate supplementation vs. no added butyrate. N=10 per sampling points per group, n=30 in total per group.
** $p<0.01$; * $p<0.05$

Table 6. Results of the parameters describing insulin homeostasis

		Abbreviation of dietary group								
Parameter	**Week**	**MB NP**	**MB NP +But**	**MB LP**	**MB LP +But**	**WB NP**	**WB NP +But**	**WB LP**	**WB LP +But**	**Significant differences**
GLP-1 (pg/ml)	1	148.1 ±21.9	101.9 ±16.6	76.1 ±7.4	82.2 ±8.3	119.1 ±14.9	122.2 ±16.6	124.4 ±13.7	139.1 ±10.4	-
	3	290.3 ±31.8	292.0 ±20.4	292.1 ±15.7	276.5 ±23.1	246.5 ±12.9	266.4 ±22.3	273.9 ±16.0	274.9 ±14.7	-
	6	240.5 ±11.8	218.6 ±10.3	218.0 ±11.3	219.6 ±10.0	218.3 ±12.6	233.6 ±10.3	215.4 ±7.4	265.0 ±18.5	-
GIP (pg/ml)	1	147.9 ±32.8	143.4 ±46.2	126.9 ±60.8	123.9 ±36.3	153.1 ±43.7	102.9 ±40.2	260.8 ±97.2	172.4 ±42.8	-
	3	181.0 ±43.9	188.7 ±48.1	178.8 ±43.3	242.9 ±66.8	118.9 ±40.9	112.9 ±53.7	163.1 ±56.4	144.3 ±39.6	-
	6	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	-
Insulin (ng/ml)	1	4.071 ±0.057	4.148 ±0.126	3.811 ±0.026	3.976 ±0.064	3.926 ±0.108	3.831 ±0.046	4.132 ±0.171	3.931 ±0.047	-
	3	8.377 ±0.290	9.099 ±0.465	8.568 ±0.421	8.857 ±0.240	9.169 ±0.554	9.285 ±0.714	9.123 ±0.630	8.237 ±0.241	-
	6	5.209 ±0.283	5.527 ±.413	6.081 ±0.504	5.401 ±0.450	5.688 ±0.272	5.383 ±0.340	4.783 ±0.175	5.403 ±0.326	-

MB: Maize-based diet; WB: Wheat-based diet supplemented with NSP-degrading xylanase and glucanase enzymes; NP: "Normal protein" group reared on a diet with crude protein content adequate to the dietary phases; LP: "Low protein" group reared on a diet with crude protein content reduced by 15 % in each dietary phase, supplemented with limiting amino acids; But: Sodium (n-)butyrate supplementation of the diet in the dose of 1.5 g/kg diet; GLP-1: Glucagon-like Peptide 1 (GLP-1); GIP: Glucose-dependent Insulinotropic Polypeptide.
Results are expressed as mean ± SEM. Statistical analysis of data was performed by multi-way ANOVA test to evaluate main effects. Main effects were determined as follows: WB vs. MB diet, LP vs. NP groups and butyrate supplementation vs. no added butyrate. N=10 per sampling points per group, n=30 in total per group.

2.2. Effects of dietary cereal type, crude protein content and butyrate supplementation on carcass composition and meat quality of broilers

The main aims of this part of the study were to test how unprotected and various protected forms of butyrate can affect carcass parameters and chemical composition of muscles in broilers. Therefore, chickens kept on maize-based diet supplemented with unprotected sodium butyrate (see **Table 1**) were included in this analysis. Further, different forms of protected sodium butyrate were applied to three additional groups according to the followings: a highly concentrated, film coated sodium butyrate (Intest-Plus S90 with 90% sodium butyrate content, 1.0 g/kg diet, indicated as "S90" group); vegetable fat embedded sodium butyrate products with various butyrate contents (Intest-Plus SC40 with 40% sodium butyrate content, 1.5 g/kg diet, indicated as "SC40" group; Intest-Plus SC30 with 30% sodium butyrate content, 2.0 g/kg diet, abbreviated as "SC30" group). Protected butyrate products were obtained from Palital Feed Additives, Velddriel, The Netherlands; doses were set according to the manufacturer's instructions.

Carcass traits were measured, chemical composition of pectoral and femoral muscles were analyzed at the age of 6 weeks. Carcass weight (**Fig. 3**) was significantly increased by all protected butyrate types tested, while relative breast meat yield was significantly higher in both unprotected and protected butyrate supplemented chickens compared to controls. Protein content of femoral muscle was significantly decreased, but its fat content was significantly elevated by all types of butyrate addition, improving meat quality of thighs. However, no changes were detected in the chemical composition of pectoral muscle. In conclusion, breast meat production can be effectively stimulated by unprotected or protected butyrate supplementation, while its chemical composition remains unchanged, in contrast to the femoral muscle. Applying butyrate as a feed additive seems to be a proper tool to improve carcass yield and composition of broilers, contributing to more efficient poultry meat production.

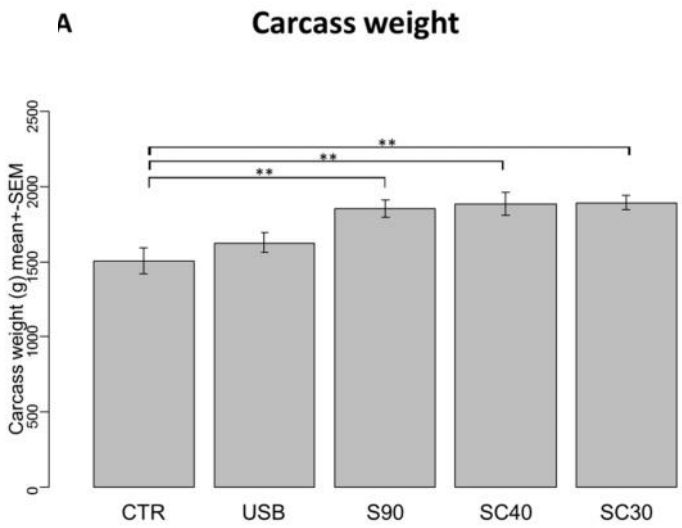

Fig. 3.

Fig. 3. Results of carcass weight measurements. The abbreviations of the experimental groups are as follows: CTR: control diet with no butyrate supplementation; USB: diet with unprotected sodium butyrate supplementation (1.5 g/kg diet); S90: diet film coated protected sodium butyrate supplementation (Intest Plus S90 with 90% sodium butyrate content, 1.0 g/kg diet); SC40: diet with vegetable fat embedded protected sodium butyrate supplementation (Intest Plus SC40 with 40% sodium butyrate content, 1.5 g/kg diet); SC30: diet with vegetable fat embedded protected sodium butyrate supplementation (Intest Plus SC30 with 30% sodium butyrate content, 2.0 g/kg diet).
Results are expressed as mean ± SEM. Significant differences revealed by post-hoc tests are marked with the following asterisks: #P<0.10, *P<0.05, **P<0.01, ***P<0.001.

2.3-2.5. Effects of dietary cereal type, crude protein content and butyrate supplementation on insulin and glucagon signalling of broilers

As described earlier, in the present part of the study we aimed to investigate the effect of maize- and wheat-based diet (referring to lower and higher dietary soluble NSP content) on the insulin and glucagon homeostasis of broiler chicken at different ages. Details of the applied study design and experimental groups have been described earlier (please see **Table 1**). Tissue samples from liver and gastrocnemic muscle were taken at week 1, 3 and 6, and from abdominal adipose tissue on week 3 and 6. Expression of certain key proteins of insulin signalling IRβ, Protein Kinase B (PKB or Akt, phosphorylated and dephosphorylated form), mammalian Target of Rapamycin (mTOR) and Glucagon Receptor (GR) was examined by Western blotting. The gene expressions of the targeted signalling elements were investigated after mRNA isolation and reverse transcription by qRT-PCR. Data were analyzed by two-way ANOVA and pairwise comparison using the R 2.14.0 software.

In the liver, IRβ and mTOR protein expressions were higher in chickens fed with wheat-based diet compared to maize-based dietary groups both at week 3 and 6. Furthermore, the phosphorylation of PKB/Akt was significantly decreased in animals kept on wheat-based

diet compared to the maize-based group at week 3 (**Fig. 4.A**). The GR protein expression in the liver did not differ in wheat- and maize-based groups; however, a strong age effect was detected: GR expression at week 1 was lower than at week 3 and 6. In gastrocnemic muscle, no effect of the diet type was found on the studied protein expressions, but butyrate as a feed additive diminished the phosphorylation state of PKB/Akt (**Fig. 4.B**). In addition, there was a significant age effect in the case of IRβ, PKB and mTOR in muscle: protein expressions were higher at week 1 than at the later ages.

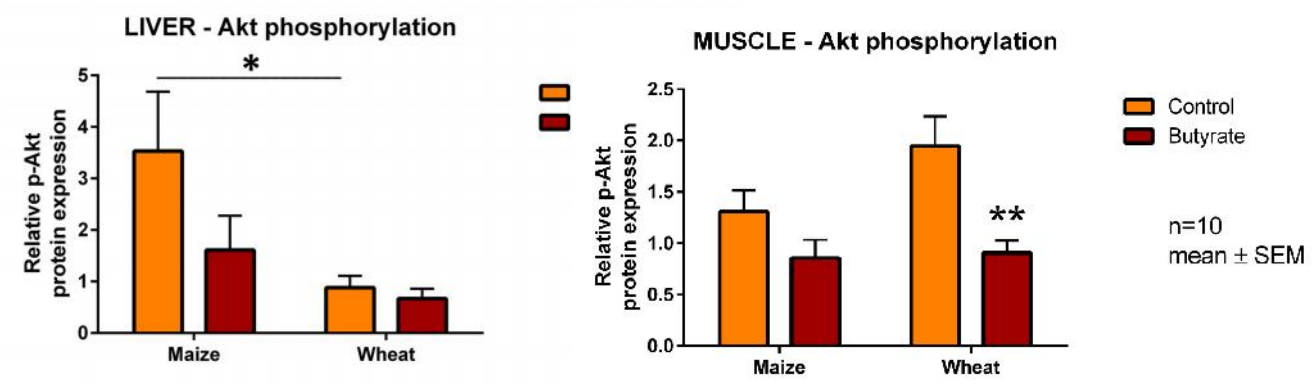

Fig. 4.A-B

Figure 4. Relative expression of p-Akt in (Fig. 4.A) liver and (Fig. 4.B) muscle of chickens on day 21. Maize and wheat indicate the type of cereal used; "Butyrate"=supplementation of the feed with 1.5 g sodium butyrate/kg diet. Results are expressed as mean ± SEM, n=10/group.

According to the PCR trials, on the level of mRNA age had a significant effect on the gene expression of mTOR in the liver: the highest value was measured at the age of 6 weeks. Moreover, in 3-week-old chickens low dietary CP level and wheat-based diet increased the gene expression of mTOR. The GR showed increased gene expression at the age of 3 weeks, triggered by low CP levels and wheat-based diet.

Based on the results obtained, the present study highlights that different dietary cereal types (maize or wheat) are able to influence the expression of certain key members of insulin signalling in broiler chicken in a tissue-specific manner. As the effect of diet type appeared to be most pronounced at week 3, our results suggest that dietary soluble NSP content could have the greatest influence on the tested signalling proteins during the fast growing phase in broiler chicken. Further, our results also indicate that independently from the diet type, the expression of certain regulatory proteins of the insulin and glucagon cascade has remarkable age-dependency.

2.6. Effects of dietary cereal type, crude protein content and butyrate supplementation on hepatic and intestinal drug-metabolizing CYP enzymes of broilers

As butyrate is already known as a modulator of drug-metabolizing CYP enzymes (see also trial 1.1), the aim of this part of the project was to monitor the activity of CYP1A2 enzymes in the liver and small intestines after the applied dietary treatments. Tissue samples from liver, and mucosal scraping samples of duodenum and ileum were taken at week 1, 3 and 6 (n=10/group at all age) from Ross 308 broilers as described earlier. After homogenization of the samples, post-mitochondrial supernatants (so called S9 fractions) were isolated by differential centrifugation. The CYP1A2 activity was determined by luminescent CYP Glo Assays.

In the liver, the activity of CYP1A2 enzymes was significantly higher in the wheat-based dietary group compared to maize-based diet, regardless of age. However, concerning the diet type no significant difference was found in either the duodenum or the ileum. The CYP1A2 activity in the liver did not change with age, but we found a significant increase (at week 6 significantly higher than at week 1) in the duodenum, and a marginally significant ($0.05<P<0.10$) increasing trend (at week 3 and week 6 greater than at week 1) in the ileum.

Our results suggest that the composition of the diet could influence the activity of the hepatic CYP enzymes, thus modify the ability of the liver to metabolize xenobiotics. The drug-metabolizing function of the liver is well-developed already at the age of one week in broiler chicken, but due to the age-dependently increasing quantity and activity of intestinal CYPs, the importance of small intestinal detoxification could also increase with age.

2.7. Effects of dietary cereal type, crude protein content and butyrate supplementation on the gut microbiota of broilers

The genetic analysis of gut microbiota was performed by investigating the ileal and caecal ingesta. For terminal restriction fragment length polymorphism (T-RFLP) analysis, fluorescent labeled amplicons were digested by the restriction enzyme HaeIII. Illumina amplicon sequencing was carried out for the V1-2 region of the 16S rRNA gene.

Comparison of the T-RFLP patterns and amplicon sequences revealed distinct differences by intestinal sections and dietary groups. The overall composition of bacterial communities was significantly affected by the diet type, and similarity analysis depicted a clear grouping by the dietary cereal type with both methods. In caecal digesta, the Shannon diversity was lower in chickens kept on wheat-based diet compared to their maize-based counterparts. The abundance of the *Bacteroides* genus was increased in animals fed with

maize-based diet, while *Lactobacilli* and butyrate-producing members of the genus *Lachnospiraceae* were more abundant in wheat-based dietary groups.

Based on these results, it can be stated that the composition of the diet may affect the overall structure of gut microbiota of broilers. The soluble NSP content of the wheat-based diet as stimulatory substrate for intestinal bacterial fermentation had a stronger stimulus on bacterial communities than butyrate supplementation of the diet. Butyrate-forming bacteria, belonging to *Lachnospiraceae* seemed to be stimulated by the increased amount of NSP in wheat-based diets.

3. *In vitro* studies on primary hepatic cell culture models

Different liver cell types may play specific role in the metabolic adaptation or the inflammatory and stress-associated responses of the organ. The interaction of metabolism, inflammation and stress should be addressed on cellular level by applying hepatic cell culture models. As Kupffer cells, the resident liver macrophages are highly involved in the regulation of hepatic inflammatory response, the main goal of this part of the study was to improve and to characterize a hepatocyte – Kupffer cell co-culture of swine and chicken origin for modeling hepatic inflammation and for testing the possible immunometabolic role of different cell types.

These monolayer co-cultures were prepared from primary isolated hepatocytes and Kupffer cells of 2-month-old pigs or 3-week-old broilers, gained by multi-step perfusion of the liver, including digestion with collagenase and by differential centrifugation. Cell types were co-cultured in the ratio of 6:1 and 2:1, mimicking different states of liver inflammation. The prepared cell cultures (**Fig. 5**) were characterized by immunofluorescent detection of macrophage-specific CD-68 antigen (**Fig. 6**) and by latex phagocytosis assay.

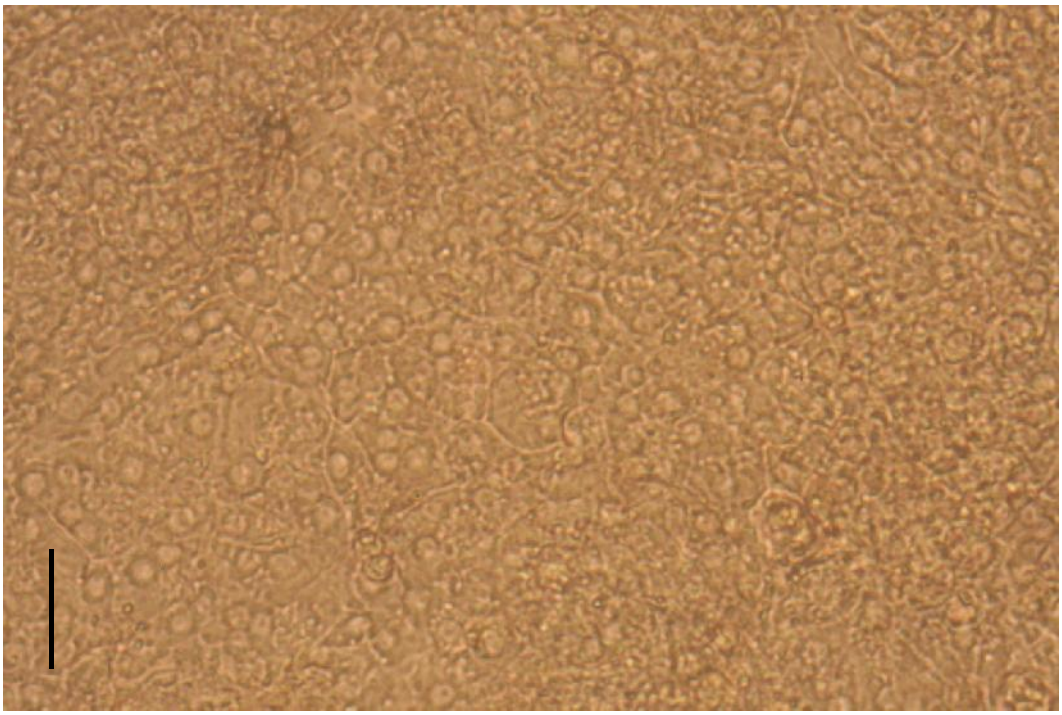

Fig. 5.

Figure 5. Primary culture of chicken hepatocytes after 24 h incubation. Phase contrast microscopy, bar = 50 μm.

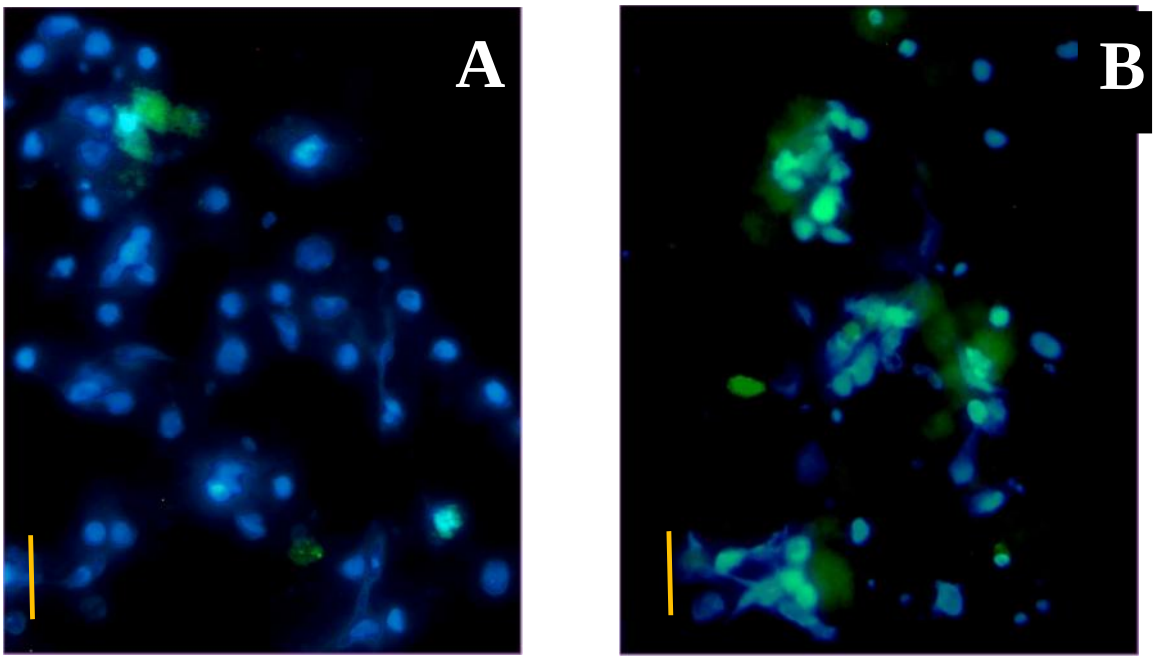

Fig. 6.A-B

Figure 6. Immunohistochemical characterization of hepatocyte-Kupffer cell co-cultures of swine origin. Hepatocyte-Kupffer cell co-culture with the cell ratio of **A.** 6:1 and **B.** 2:1 (hepatocyte to Kupffer cell) after 24 h culturing by immunohistochemical detection of Kupffer cells with the macrophage-specific CD-68 antigen (40x magnification, bar=50 μm). Blue colour indicates cell nuclei visualized by DAPI staining, while green colour refers to CD-68 positive cells detected by FITC-coupled CD-68 specific antibody.

According to our results on porcine cell cultures, lipopolysaccharide (LPS) challenge of both 6:1 and 2:1 co-cultures resulted in elevated interleukin-8 (IL-8) and that of 6:1 co-cultures in increased IL-6 production with a higher extent than on hepatocyte mono-cultures, justifying the key role of Kupffer cells in pro-inflammatory cytokine production. LPS-induced IL-8 production was successfully attenuated by concomitant application of both sodium butyrate and terpinen-4-ol on hepatocyte mono-cultures, but not on co-cultures, demonstrating the importance of the presence of Kupffer cells in cell cultures as inflammatory models. Furthermore, specific inhibitors of the transmembrane serine protease matriptase were also tested on hepatic cell cultures, addressing cell viability, H_2O_2 and hepcidin production.

On hepatic cell cultures of chicken origin, cell viability was significantly decreased by heat stress (44°C vs. 38°C) on both co-cultures and mono-cultures directly after heat exposure. A prolonged deterioration in cell viability was observed following 20 h recovery time only in case of cells exposed to heat stress for the longer, 2 h incubation time. In this case, cellular H_2O_2 production was significantly increased on both cell cultures, but with a remarkably higher extent on co-cultures than on hepatocyte mono-cultures.

Based on these data, the applied primary hepatocyte-Kupffer cell co-culture is suggested to be a proper tool for *in vitro* investigations on liver physiology and hepatic inflammation, and can be used as a useful model mimicking *in vivo* conditions in veterinary research.

IV. Overall discussion

In the frame of the project, the effects of various nutritional factors, such as those of butyrate as a feed additive, dietary NSP and CP content have been investigated on certain metabolic pathways and the productivity of broiler chickens. Based on our results, it can be suggested that **butyrate – either applied as a feed additive or produced in the caecal microbial fermentation – can improve metabolic health by influencing endocrine regulation and energy homeostasis in chickens**.

By addressing the key questions of the study, it was described that the examined nutritional factors – primarily the dietary cereal type (wheat vs. maize) and butyrate supplementation – could influence insulin and glucagon homeostasis by altering the gene and protein expression level of certain members of the cellular signalling pathways. The observed endocrine modulations were also reflected in various alterations of key metabolic parameters in blood plasma. The activity of drug-metabolizing CYP enzymes was also modified by the diet, highlighting the possibility of important feed-drug interactions. Furthermore, it was also shown that diet type is an important factor influencing gut microbiota, contributing to the maintenance of health and production. As a result of the several diet-triggered metabolic alterations, butyrate as a feed additive was found to be a potent stimulator of productivity by increasing carcass weight and altering the chemical composition of meat. In addition, the anti-inflammatory action of butyrate was justified on newly established primary hepatic cell culture models, being proper tools for further studies of the liver function and its adaptation on cellular level.

Most of the described diet-associated metabolic changes were found to be age-dependent: chickens were most sensitive in several cases at the age of 3 weeks, in the phase of the most intensive growth; thus age should be considered as a critical factor when studying metabolic processes in broilers. Further, regardless of the investigated dietary factors, some age-dependent differences of insulin and glucagon signalling or CYP enzymes were also described for the first time in the present project.

As an important outcome, it can be summarized that wheat-based diet – with xylanase-glucanase enzyme supplementation – can be applied in broiler farming without deteriorative effects to improve metabolic health and production. The described beneficial actions of this diet type are supposed to be in association with its higher soluble NSP content, degraded by the supplemented enzymes, thus leading to an increased oligosaccharide load to the intestinal microflora and subsequently stimulating the caecal production of SCFA, primarily that of

butyrate. Similarly, broiler diets with slightly reduced CP content and with limiting amino acid fortification seemed to be a good tool in sustainable and economic poultry production.

In conclusion, certain **dietary factors can be considered as proper tools for improving metabolic health and animal production of broiler chickens**. The innovation of this study is to provide novel data on molecular and cellular level, which highlight new ways in poultry nutrition and may contribute to several practical applications.

The project was carried out in a joint international collaboration of the Division of Biochemistry, Department of Physiology and Biochemistry, University of Veterinary Medicine, Budapest (research group of Dr. Zsuzsanna Neogrády), the Research Institute for Animal Breeding, Nutrition and Meat Science, National Agricultural Research Center, Herceghalom (research group of Dr. Hedvig Fébel) and the Institute of Animal Science, University of Hohenheim, Stuttgart (research group of Prof. Korinna Huber). This cooperation provided especially great possibilities for complex investigations of diet-associated molecular alterations in chickens based on the wide spreading skills of the participating Hungarian and German experts. Beside the numerous new scientific results, this 3-year-project enabled an active international exchange of researchers and was also involved in the education of PhD and graduate students.

V. List of papers published in the frame of the project

1. Papers in peer-reviewed journals:

Gábor Mátis, Patrícia Hatala, Anna Kulcsár, Janka Petrilla, Zsuzsanna Neogrády (2015): **A Kupffer-sejtek szerepe a máj gyulladásos és metabolikus folyamatainak szabályozásában. Irodalmi áttekintés. Role of Kupffer cells in the regulation of hepatic inflammatory and metabolic processes. Literature review.** MAGYAR ÁLLATORVOSOK LAPJA, 137, 477–486.

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2. Papers in conference proceedings:

Gábor Mátis, Anna Kulcsár, Janka Petrilla, Zsuzsanna Neogrády (2015): **Establishment of a porcine hepatocyte – Kupffer cell co-culture as a novel inflammatory model in veterinary research.** 18TH INTERNATIONAL SYMPOSIUM ON CELLS OF THE HEPATIC SINUSOID. Asilomar, USA, 11-13 November 2015

Anna Kulcsár, Gábor Mátis, Janka Petrilla, Máté Mackei and Zsuzsanna Neogrády (2016): **Comparative examinations on the nutritional modulation of incretin and insulin secretion in chicken and rabbit.** DVG CONFERENCE, PHYSIOLOGY AND BIOHEMISTRY, Berlin, Germany, 30 March – 1 April 2016

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Anna Kulcsár, Gábor Mátis, Janka Petrilla, Petra Talapka, Zsuzsanna Neogrády, Korinna Huber (2017): **Effect of maize- or wheat-based diets on the abundance of selected proteins involved in insulin signaling of broiler chicken.** GFE (SOCIETY OF NUTRITION PHYSIOLOGY) CONFERENCE 2017, Göttingen, Germany, 14-16 March 2017

Gábor Mátis, Anna Kulcsár, Janka Petrilla, Petra Talapka, Márton Bardóczy, Máté Mackei, Zsuzsanna Neogrády, Hedvig Fébel (2017): **Investigations on the effects of certain nutritional factors on carcass composition of broiler chickens.** GFE (SOCIETY OF NUTRITION PHYSIOLOGY) CONFERENCE 2017, Göttingen, Germany, 14-16 March 2017

Janka Petrilla, Gábor Mátis, Anna Kulcsár, Petra Talapka, Enikő Bíró, Máté Mackei, Hedvig Fébel, Zsuzsanna Neogrády (2017): **The effect of dietary cereal type, crude protein content and butyrate application on selected markers of metabolism in broiler chickens.** GFE (SOCIETY OF NUTRITION PHYSIOLOGY) CONFERENCE 2017, Göttingen, Germany, 14-16 March 2017

Anna Kulcsár, Gábor Mátis, Janka Petrilla, Petra Talapka, Hedvig Fébel, Zsuzsanna Neogrády, Korinna Huber (2017): **Influencing insulin homeostasis of broiler chicken by maize- or wheat-based diets.** THE XXTH WORLD VETERINARY POULTRY ASSOCIATION CONGRESS, Edinburgh, Scotland, 4-8 September 2017

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Petrilla Janka: **Metabolikus és gyulladásos folyamatok takarmányozási faktorok segítségével történő szabályozása csirkében.** Supervisor: Neogrády Zsuzsanna. Estimated year of defence: 2019.

4. DVM theses:

Hermándy-Berencz Katalin (2015): **Befolyásolja-e a butirát az indukált citokróm P450 enzimaktivitást a csirke májában?** Supervisors: Petrilla Janka, Mátis Gábor

Bardóczy Márton (2016): **Egyes takarmányozási tényezők brojlercsirkék testösszetételére gyakorolt hatásának vizsgálata.** Supervisors: Mátis Gábor, Fébel Hedvig

Bíró Enikő (2016): **Metabolikus paraméterek változásai a takarmánygabona típusa, a takarmány nyersfehérje-tartalma és butirátkiegészítés hatására csirkében.** Supervisors: Petrilla Janka, Neogrády Zsuzsanna

Reinhardt, Vanessa (2016): **Influence of endogenous and exogenous butyrate on protein kinase B (Akt) phosphorylation in broilers.** Supervisors: Kenéz Ákos, Mátis Gábor, Dr. Korinna Huber

Rokonál Patrik (2016): **Matriptáz-2 gátlás hatásainak vizsgálata májsejttenyészeten és májsejt-Kupffer-sejt ko-kultúrán.** Supervisors: Pásztiné Gere Erzsébet, Mátis Gábor

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Appendix 1. Ingredients and calculated nutrient composition of experimental broiler starter diets, without sodium (n-)butyrate supplementation

Ingredients		Maize-based Normal CP	Maize-based Low CP	Wheat-based Normal CP	Wheat-based Low CP
Maize	%	57.60	61.00	0	0
Wheat	%	0	0	54.79	62.60
Extr. soybean meal	%	27.00	28.00	31.00	26.48
PL-68[*]	%	6.50	0	3.00	0
Sunflower oil	%	3.50	3.50	6.00	5.30
Wheat bran	%	0	1.72	0	0
Limestone	%	1.70	1.60	1.70	1.70
MCP	%	1.80	2.00	1.70	1.70
Salt (NaCl)	%	0.40	0.40	0.40	0.40
Lysine	%	0.44	0.58	0.38	0.60
Methionine	%	0.43	0.44	0.41	0.45
Threonine	%	0.09	0.22	0.11	0.26
Tryptophan	%	0.04	0.04	0	0
Vitamin and mineral premix[†]	%	0.50	0.50	0.50	0.50
Axtra XB 201 enzyme[§]	%			0.015	0.015
Total		100	100	100	100
Calculated analysis					
Dry matter	%	89.65	89.32	89.78	89.47
Crude protein	%	22.02	18.65	22.05	18.76
Soluble NSP	mg/kg	506.88	536.80	5133.82	5865.62
ME	MJ/kg	12.65	12.61	12.63	12.62
Ether extract	%	6.54	6.30	7.49	6.62
Crude fiber	%	2.51	2.74	2.88	2.81
Ash	%	6.97	7.23	7.37	7.42
Lysine	%	1.43	1.43	1.44	1.43
Methionine + Cystine	%	1.07	1.05	1.08	1.07
Threonine	%	0.97	0.94	0.94	0.94
Tryptophan	%	0.23	0.25	0.26	0.24
Arginine	%	1.17	1.24	1.34	1.22
Isoleucine	%	0.74	0.78	0.85	0.78
Leucine	%	1.59	1.68	1.52	1.41
Valine	%	0.83	0.88	0.93	0.86
Total Ca	%	1.15	1.15	1.16	1.14
Total P	%	0.79	0.80	0.82	0.80
Available P	%	0.54	0.53	0.56	0.54

CP: Crude protein; MCP: Monocalcium phosphate; ME: Metabolizable energy; NSP: Soluble non-starch polysaccharide.

[*]Protein concentrate, produced by Europrotein Ltd., Hungary.

[†]Composition per kilogram of premix: vitamin A 2.403 IU; vitamin D_3 775.000 IU; vitamin K 651.000 mg; vitamin E 9.300 IU; vitamin B_1 465.000 mg; vitamin B_2 1.488 mg/kg; vitamin B_6 775.000 mg/kg; vitamin B_{12} 3.260 mg/kg; calcium pantothenate 2.790 mg/kg; folic acid 311.000mg/kg; niacin 9.300 mg/kg; choline chloride 100.800 mg/kg; Fe 12.075 mg/kg; Mn 20.000 mg/kg; Cu 2.500 mg/kg; Zn 16.687 mg/kg; Se 83.750 mg/kg; Co 55.000 mg/kg; I 250.00 mg/kg.

[§]1830 U/kg endo-1,4-beta xylanase and 228 U/kg endo-1,3(4)-beta glucanase

Appendix 2. Ingredients and calculated nutrient composition of experimental broiler grower diets, without sodium (n-)butyrate supplementation

Ingredients		Maize-based Normal CP	Maize-based Low CP	Wheat-based Normal CP	Wheat-based Low CP
Maize	%	60.71	65.31	0	0
Wheat	%	0	0	61.30	66.56
Extr. soybean meal	%	22.20	24.54	19.31	20.01
PL-68[*]	%	8.00	1.00	8.50	2.50
Sunflower oil	%	4.80	4.50	6.70	6.50
Wheat bran	%	0	0	0	0
Limestone	%	1.30	1.20	1.35	1.35
MCP	%	1.35	1.60	1.15	1.15
Salt (NaCl)	%	0.40	0.40	0.40	0.40
Lysine	%	0.34	0.41	0.38	0.48
Methionine	%	0.36	0.37	0.35	0.38
Threonine	%	0	0.15	0.05	0.16
Tryptophan	%	0.04	0.02	0	0
Vitamin and mineral premix[†]	%	0.50	0.50	0.50	0.50
Axtra XB 201 enzyme[§]	%			0,015	0,015
Total		100	100	100	100
Calculated analysis					
Dry matter	%	89.72	89.34	89.90	89.55
Crude protein	%	21.12	17.85	21.10	17.89
Soluble NSP	mg/kg	534.25	574.73	5743.81	6236.67
ME	MJ/kg	13.27	13.24	13.24	13.24
Ether extract	%	7.96	7.39	8.45	7.92
Crude fiber	%	2.34	2.48	2.51	2.61
Ash	%	5.78	6.03	6.00	6.13
Lysine	%	1.25	1.22	1.25	1.22
Methionine + Cystine	%	0.96	0.95	0.94	0.95
Threonine	%	0.84	0.84	0.85	0.81
Tryptophan	%	0.21	0.20	0.20	0.21
Arginine	%	1.01	1.11	0.97	1.02
Isoleucine	%	0.65	0.72	0.62	0.65
Leucine	%	1.45	1.58	1.14	1.20
Valine	%	0.74	0.81	0.70	0.74
Total Ca	%	0.92	0.93	0.90	0.90
Total P	%	0.68	0.69	0.71	0.67
Available P	%	0.45	0.45	0.49	0.44

CP: Crude protein; MCP: Monocalcium phosphate; ME: Metabolizable energy; NSP: Soluble non-starch polysaccharide.

[*]Protein concentrate, produced by Europrotein Ltd., Hungary.

[†]Composition per kilogram of premix: vitamin A 2.403 IU; vitamin D_3 775.000 IU; vitamin K 651.000 mg; vitamin E 9.300 IU; vitamin B_1 465.000 mg; vitamin B_2 1.488 mg/kg; vitamin B_6 775.000 mg/kg; vitamin B_{12} 3.260 mg/kg; calcium pantothenate 2.790 mg/kg; folic acid 311.000 mg/kg; niacin 9.300 mg/kg; choline chloride 100.800 mg/kg; Fe 12.075 mg/kg; Mn 20.000 mg/kg; Cu 2.500 mg/kg; Zn 16.687 mg/kg; Se 83.750 mg/kg; Co 55.000 mg/kg; I 250.00 mg/kg.

[§]1830 U/kg endo-1,4-beta xylanase and 228 U/kg endo-1,3(4)-beta glucanase

Appendix 3. Ingredients and calculated nutrient composition of experimental broiler finisher diets, without sodium (n-)butyrate supplementation

Ingredients		Maize-based Normal CP	Maize-based Low CP	Wheat-based Normal CP	Wheat-based Low CP
Maize	%	63.66	70.25	0	0
Wheat	%	0	0	64.69	69.69
Extr. soybean meal	%	24.50	20.29	19.35	19.35
PL-68[*]	%	3.00	0.70	5.00	0
Sunflower oil	%	5.00	4.30	6.96	6.90
Wheat bran	%	0	0	0	0
Limestone	%	1.09	1.09	1.35	1.26
MCP	%	1.40	1.60	1.15	1.15
Salt (NaCl)	%	0.40	0.40	0.40	0.40
Lysine	%	0.19	0.39	0.25	0.32
Methionine	%	0.26	0.33	0.3	0.31
Threonine	%	0	0.13	0.08	0.15
Tryptophan	%	0	0.02	0	0
Vitamin and mineral premix[†]	%	0.50	0.50	0.50	0.50
Axtra XB 201 enzyme[§]	%			0.015	0.015
Total		100	100	100	100
Calculated analysis					
Dry matter	%	89.46	89.21	89.70	89.40
Crude protein	%	19.04	16.13	19.07	16.20
Soluble NSP	mg/kg	560.21	618.2	6061.45	6529.95
ME	MJ/kg	13.41	13.41	13.38	13.44
Ether extract	%	7.96	7.27	8.51	8.17
Crude fiber	%	2.47	2.36	2.56	2.62
Ash	%	5.46	5.65	5.83	5.80
Lysine	%	1.09	1.08	1.07	1.02
Methionine + Cystine	%	0.86	0.87	0.87	0.86
Threonine	%	0.74	0.74	0.79	0.72
Tryptophan	%	0.18	0.18	0.20	0.21
Arginine	%	1.11	0.99	0.99	1.01
Isoleucine	%	0.71	0.64	0.63	0.65
Leucine	%	1.56	1.48	1.16	1.19
Valine	%	0.80	0.74	0.71	0.74
Total Ca	%	0.85	0.87	0.90	0.87
Total P	%	0.66	0.68	0.69	0.66
Available P	%	0.42	0.44	0.46	0.42

CP: Crude protein; MCP: Monocalcium phosphate; ME: Metabolizable energy; NSP: Soluble non-starch polysaccharide.

[*]Protein concentrate, produced by Europrotein Ltd., Hungary.

[†]Composition per kilogram of premix: vitamin A 2.403 IU; vitamin D_3 775.000 IU; vitamin K 651.000 mg; vitamin E 9.300 IU; vitamin B_1 465.000 mg; vitamin B_2 1.488 mg/kg; vitamin B_6 775.000 mg/kg; vitamin B_{12} 3.260 mg/kg; calcium pantothenate 2.790 mg/kg; folic acid 311.000 mg/kg; niacin 9.300 mg/kg; choline chloride 100.800 mg/kg; Fe 12.075 mg/kg; Mn 20.000 mg/kg; Cu 2.500 mg/kg; Zn 16.687 mg/kg; Se 83.750 mg/kg; Co 55.000 mg/kg; I 250.00 mg/kg.

[§]1830 U/kg endo-1,4-beta xylanase and 228 U/kg endo-1,3(4)-beta glucanase