Theses of doctoral (PhD) dissertation

Regulation of metabolic processes by nutritional factors in chicken

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Introduction

The need for the production of valuable meat at a reasonable price is growing worldwide, however, animal welfare considerations also have to be taken into consideration. Hence, the use of short chain fatty acids (SCFAs) became more and more common in pig and poultry farming as well, as alternatives of the antibiotics- and hormone-based growth promotion, banned since 2006 in the European Union.

Amongst SCFAs, the most potent and thus the most widely used four-carbon n-butyric acid (in the followings: butyrate) can exert its effects by entering the organism of monogastric species mostly as a feed additive (exogenous origin). The unprotected, free butyrate salts (the most frequently sodium or calcium salts) ensure rapid absorption from the proximal gastrointestinal tract, while the various protected forms are able to reach the hindgut and provide a prolonged butyrate release even in the large intestines. Another source of butyrate (and SCFAs in general) is endogenous, when the soluble non-starch polysaccharide (NSP) content of the diet serves as substrate for the anaerobic microbial fermentation, characteristic in the ceca in avian species. Hence, SCFA, and primarily butyrate production can be enhanced by feeding NSP-rich (rye-, barley- or wheat-based) diet, with concomitant carbohydrase enzyme supplementation in order to diminish the antinutritive effects, due to the higher viscosity of these diets.

Depending on the dose and form of application, butyrate has a wide range of beneficial effects already in the intestines, including stabilization of the microflora, promotion of the differentiation and proliferation of the enterocytes (serving as energy source), modulation of immune responses and incretin production, further, strengthening of gut barrier function. The absorbed portion of butyrate is then delivered to the liver to be consumed for energy production, or to possibly exert its glucose tolerance ameliorating and detoxification modifying actions. Then – forwarded into the systemic circulation –, butyrate is able to reach the extrahepatic tissues, where it can increase the glucose uptake and insulin sensitivity of the cells. In the background of these effects, the dissociation properties, further, the gene expression altering epigenetic and receptor mediated actions of butyrate can be mentioned. Despite that numerous studies have investigated these above listed actions of butyrate, literature data are scarce regarding how this widely used feed supplement influences general metabolic health of chickens, especially in combination with other nutritional factors, and the results are rather contradictory.

Notably higher plasma glucose concentration and lower tissue insulin sensitivity is characteristic for birds, compared to mammals. Hence, glucagon should be considered as the main regulator of blood sugar level in avian species, maintaining the physiologically high blood glucose concentration. The responsiveness of the insulin signaling elements to modifying factors decreases, while that of glucagon signaling pathway increases over time. The improvement of the low insulin sensitivity of chickens is of outstanding importance, as it might

trigger increased protein synthesis via the activation of mammalian (or mechanistic) target of rapamycin (mTOR), which would be desirable especially in the skeletal muscles, possibly leading to better growth performance.

Optimal diet composition might improve growth, and could be practical from environmental and animal welfare point of view as well. With the possibility of industrial amino acid production, ensuring ideal dietary protein and amino acid composition became conceivable, which means reduced dietary crude protein level with limiting amino acid fortification in poultry farming. With this feeding strategy, amino acid supply might meet the real needs of the chicken in the given phase of fattening, without unnecessary charge of the flock and the environment with nondigested and excreted protein fraction or protein degradation products. Reduced dietary crude protein content of the feed with limiting amino acid completion does not impair the growth of the animal, therefore, could be a more advantageous solution than the traditional crude protein concept.

Apart from satisfactory amount and quality of meat, safety of the products is another indispensable criterion for broiler industry. As an opposite, broiler meat has been the main source of human foodborne zoonotic *Campylobacter jejuni* (*C. jejuni*) infection in the European Union for more than a decade. *C. jejuni* colonizes the gastrointestinal tract of practically all broiler flocks and causes general and in some cases, life threatening human infection, if the carcass is getting contaminated during the slaughter process and the end product is not treated properly afterwards. The intervention strategies would aim the reduction of *C. jejuni* colonization already in the live phase, however, the limitations of vaccination and the legal restrictions of use of antibiotics bring other alternative methods, such as acidification of drinking water and application of pro- and prebiotics to the fore. In this aspect, SCFAs and especially butyrate proved to selectively inhibit the growth of pathogenic bacteria, e.g. *Escherichia coli* and *C. jejuni* strains, *Clostridium* and *Salmonella* spp., while supporting the members of the eubiotic microflora.

Aims of the study

Briefly summarized, the main aims of this PhD study were:

Ad 1, to monitor a set of biochemical blood plasma parameters, reflecting the age-related responsiveness of the main processes of the avian intermediary metabolism, evoked by the type of dietary cereal (wheat vs. maize), crude protein content (normal vs. reduced by 15% and fortified with limiting amino acids) and sodium (n-)butyrate supplementation (1.5 g/kg diet vs. no supplementation) in broiler chickens.

Ad 2, to investigate how the gene and protein expression of selected prominent members of hepatic insulin and glucagon signaling are influenced by the above detailed nutritional factors in the phase of intensive growth.

Ad 3, to gain information on the possible changes of carcass traits and the chemical composition of meat induced by butyrate of different types (free sodium (n-)butyrate salt vs. various protected forms), as well as dietary crude protein level (normal vs. reduced by 15% and fortified with limiting amino acids) with maize-based diets.

Ad 4, to test the *in vitro* antibacterial effect of sodium (n)-butyrate against *Campylobacter jejuni* strains.

In order to reach these goals, four studies were designed as follows:

Study No	Type of the Study	Age of the animals (days)	Investigated factor	Investigated parameters
Study I	*in vivo*	7, 21, 42	dietary cereal type dietary CP level unprotected butyrate supplementation	Plasma concentration of TP, albumin, uric acid, glucose, TG, GLP-1, GIP and insulin Plasma activity of AST and CK
Study II	*in vivo*	21	dietary cereal type dietary CP level unprotected butyrate supplementation	Hepatic gene expression and protein abundance of GCGR, IRβ and mTOR
Study III	*in vivo*	42	dietary CP level unprotected butyrate supplementation protected butyrate supplementation	Weight of carcass traits, organs and chemical analysis of the femoral and pectoral muscles
Study IV	*in vitro*	-	unprotected butyrate supplementation pH *C. jejuni* strain-related properties	MIC and MBC values of butyrate Antibiotic resistance of *C. jejuni* strains

CP: Crude protein; TP: Total protein; TG: Triglyceride; GLP-1: Glucagon-like peptide 1; GIP: Glucose-dependent insulinotropic polypeptide; AST: Aspartate aminotransferase; CK: Creatine kinase; GCGR: Glucagon receptor; IRβ: Insulin receptor β subunit; mTOR: Mammalian target of rapamycin; MIC: Minimum inhibitory concentration; MBC: Minimum bactericidal concentration.

Materials and methods

Study I-III were conducted in broiler chickens ***in vivo***, while in Study IV, several *Campylobacter jejuni* strains were subjected to sodium (n-)butyrate treatment *in vitro*.

Effect of dietary cereal type, crude protein and butyrate supplementation on metabolic parameters, in relation with age (Study I)

In **Study I**, two hundred and forty day-old male Ross 308 chicks were randomly classified into eight dietary groups (n = 10 per sampling point per group, n = 30 in total per group). Experimental procedures were conducted in strict accordance with the applicable national and international legislation and guidelines, housing conditions met the recommendations of the breeder, feed and drinking water were available *ad libitum*. Dietary regime followed a 2 x 2 x 2 factorial arrangement: broilers were reared on wheat- or maize-based diet (**WB** vs. **MB** groups), with dietary crude protein content matching the recommendations of the given dietary phase (**NP** groups with 22.7%, 21.4% and 19.1% crude protein in starter, grower and finisher diets) or reduced by 15 % (**LP** groups with 19.1%, 18.0% and 16.0% crude protein, respectively) and with or without sodium (n-)butyrate supplementation (**But** vs. **Ctr** groups) in the commonly used dose in poultry nutrition (1.5 g/kg diet). The wheat-based diet – containing c.a. tenfold more NSPs, than maize – was fed with xylanase and glucanase NSP-degrading enzymes, and LP diet was completed with the four first-limiting amino acids (L-lysine hydrochloride, DL-methionine, L-threonine and L-tryptophan).

In order to monitor the presumed age-dependency, selected markers of nitrogen, glucose and lipid metabolism, as well as insulin homeostasis were tested at the age of 7, 21 and 42 days. Peripheral blood samples of ten randomly selected chickens per group were gained by the puncture of brachial vein at every time point, centrifuged subsequently, shock frozen in liquid nitrogen, then stored at -80 ºC until further processing. Plasma concentration of total protein, albumin, uric acid, glucose and triglyceride, further, aspartate aminotransferase and creatine kinase activities were estimated by spectrophotometric measurements, the amount of glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) incretins and insulin concentrations were determined by sandwich ELISA tests.

Modulation of hepatic insulin and glucagon signaling by nutritional factors, at the age of intensive growth (Study II)

In **Study II**, the liver of 21-day-old animals of Study I was used to test the nutrition-dependent gene expression and protein abundance pattern of glucagon receptor, insulin receptor β (IRβ) and mTOR. The time of sampling was determined based on the results of Study I and literature data, indicating highly responsive metabolism in the phase of intensive growth. Further,

researches have revealed that the glucagon signaling elements show age-dependently increasing sensitivity, while the responsiveness of insulin signaling molecules decreases over time, hence, investigation of the selected members of both cascade processes can be considered adequate on d 21.

Ten animals per group were decapitated in CO_2 narcosis on d 21, the liver was exsanguinated, then liver samples were gained for quantitative polymerase chain reaction (q-PCR) and Western blot analyses. PCR samples were taken and placed into RNA isolation reagent and placed on dry ice, while Western blot samples were shock frozen in liquid nitrogen, then all the samples were stored at -80 ºC until processing.

PCR reactions were performed following mRNA isolation and reverse transcription with random hexamer primers. In case of the Western blot analysis, target proteins were identified and quantified by applying specific antibodies following gel electrophoresis and blotting.

Effects of dietary butyrate supplementation and crude protein level on carcass traits and meat composition (Study III)

In **Study III**, seventy Ross 308 male broilers were used. Chicks were randomly classified into seven dietary groups at day-old (n = 10 per group) and were raised under housing conditions as detailed at Study I. Maize was used as bases of diets for all the seven dietary groups (**MB** diets). Five groups of chickens were fed diets with normal dietary crude protein level of the appropriate dietary phase, while two groups received low-protein, limiting amino-acid supplemented diet ([**NP**] and [**LP**] groups, respectively), as described above at Study I. Further, the feed of two groups was supplemented with unprotected sodium (n)-butyrate (1.5 g/kg diet; **But**), and different forms of protected sodium butyrate were blended into the diet of three NP groups as follows: a highly concentrated, film-coated sodium butyrate (with 90% sodium butyrate content, in the dose of 1.0 g/kg diet; **NP S90** group), and vegetable fat-embedded sodium butyrate products with various butyrate contents (with 40% sodium butyrate content, in the dose of 1.5 g/kg diet; **NP SC40** group, as well as with 30% sodium butyrate content, in the dose of 2.0 g/kg diet; **NP SC30** group). Doses were set according to the manufacturer's instructions. Groups without any form of butyrate supplementation were regarded as controls (**Ctr**).

On d 42, chickens were slaughtered in CO_2 narcosis by decapitation, then carcass weight (including skin and wings, excluding giblets), deboned breast meat yield, femoral muscle weight, and the weights of liver, heart, spleen and abdominal adipose tissue were measured. Additionally, representative samples (60 g tissue from the same anatomic site) were taken from the pectoral (*m. pectoralis major*) and femoral (*m. iliotibialis*) muscle for chemical analysis of meat composition. Muscle samples were minced, freeze-dried in liquid nitrogen, ground and stored at -20 ºC until further processing.

Protein content of muscle samples was determined by the Kjeldahl procedure, lipid content was assessed as ether extract using a Soxhlet apparatus.

In vitro antibacterial efficacy of butyrate on distinct *Campylobacter jejuni* strains (Study IV)

For **Study IV**, all the incubations were performed at 40 ºC under microaerobic conditions.

C. jejuni strains (7 field isolates and 1 reference strain) were gently thawed from -80 ºC and microorganisms were streaked out onto Campylobacter Selective Agar (CSA) plates, incubated for 48 h, then some colonies were picked up and inoculated into Bolton broth, containing Campylobacter selective supplement. *Campylobacter* count of the suspensions was determined after 48 h of culturing, Colony-Forming Unit concentration (CFU/ml) was calculated after further 48 h plating on CSAs by plate counting.

As a next step, solutions containing different concentrations of sodium butyrate in the range of 5 to 100 mmol/l (5, 7.5, 10, 15, 20, 30, 50, 100 mmol/l) were inoculated with $7*10^5$ CFU/ml *C. jejuni* on 96-well plates. The pH value of each solution was set at 6.0 or 7.4 by adding the appropriate amount of concentrated hydrochloric acid, to mimic possible *in vivo* cecal pH range evoked by different dietary strategies. All *C. jejuni* strains were tested on all the eight butyrate concentrations listed and at both pH.

After 48 h incubation of *C. jejuni* strains with different concentrations of butyrate, CFU/ml values were determined by plating in a serial dilution. *Campylobacter* colonies were counted after 48 h of culturing; minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values were determined from *Campylobacter* counts.

Antibiotic sensitivity of the tested strains was assessed with enrofloxacin (5 µg/disk) and ampicillin (10 µg/disk) as well, on CSA plates by conventional agar disk diffusion test. In case of an inhibition zone below 5 mm diameter, the strain was declared resistant against the antibiotic in question.

Statistics

In **Study I** and **III**, multivariate analysis of variance (ANOVA analysis) was used to evaluate the main effects (WB vs. MB diet, LP vs. NP groups and butyrate supplementation vs. no added butyrate), pair wise comparisons of dietary groups were made with post-hoc Tukey-tests. Results of sampling times were analyzed separately, where applicable. Groups receiving maize-based diet with normal protein level, without butyrate supplementation were used to calculate age-dependent changes, by Mann-Whitney test.

In **Study II**, main and interaction effects were evaluated with multivariate ANOVA analysis, the R package *emmeans* was used to perform pairwise comparison of the estimated marginal means, P values and confidence levels were adjusted with Tukey-tests.

In **Study IV**, descriptive statistics were performed, due to the limitations of sample size.

Results were considered statistically significant when $P < 0.05$ in case of all evaluations.

Results and discussion

Effect of dietary cereal type, crude protein and butyrate supplementation on metabolic parameters of broilers, in relation with age (Study I)

In **Study I**, a remarkable age-dependency was found in terms of the metabolic responsiveness to the investigated nutritional factors. The majority of the significant alterations were measured in the period of intensive growth (d 21). Further, dietary cereal type exerted its effects on the indicators of amino acid and protein metabolism mostly, presumably through the intensified endogenous butyrate production, supported by the high NSP content of wheat. Despite the numerous significant effects of the dietary factors, all but one measured values were in the physiological range and even LP diet did not cause growth depression. Plasma creatine kinase enzyme activity – deliberated from muscle cells – exceeded the physiological limit at the age of 42 days, however, no macroscopic signs of muscle damage were seen during dissection. Interestingly, plasma glucose concentration decreased on d 21 without detecting significant changes in plasma insulin or incretin levels. Plasma uric acid concentration decreased, while plasma creatine kinase activity, GLP-1 and insulin concentrations increased over time, independent of any nutritional factors.

Modulation of hepatic insulin and glucagon signaling by nutritional factors in broiler chicken, at the age of intensive growth (Study II)

The gene expression of glucagon receptor increased in WB and LP groups, however, it did not cause the increase of protein abundance. As an opposite, butyrate supplementation reduced the protein abundance of the same parameter without alteration in the gene expression, suggesting possible posttranslational modifications in the background. In case of IRβ, WB diet ameliorated and butyrate supplementation diminished the protein abundance, with several interactions on both gene expression and protein level. The phenomenon highlights that different sources of butyrate might lead to distinct, sometimes opposite actions. The gene expression of mTOR was triggered by WB and LP diet, however, only WB diet managed to maintain this change in protein abundance as well. Regarding that WB diet was able to increase the protein abundance of both investigated members of insulin signaling, this cereal could be suitable for the amelioration of hepatic insulin sensitivity in the phase of intensive growth. This hypothesis might be reinforced by the lowered plasma glucose concentration with unchanged plasma insulin and GLP-1 levels, measured in Study I. Nonetheless, the detected interactions draw the attention on the importance of careful diet formulation in order to reach the desired beneficial effects.

Effects of dietary butyrate supplementation and crude protein level on carcass traits and meat composition of broiler chickens (Study III)

All the tested protected butyrate products and LP diet increased carcass weight at the age of slaughter, but unprotected butyrate could – together with protected butyrate products and lowered crude protein – only increase relative breast meat yield, possibly through its complex action in the intestines and the amelioration of systemic insulin sensitivity, leading to better muscle protein synthesis. The success of LP diet can be explained by the higher amino acid to protein ratio in the LP groups, as the consequence of the limiting amino acid supplementation, compared to control groups. However, the chemical composition of the breast meat remained unchanged, referring to the stability of this type of meat. Contrary, all the tested dietary factors increased the lipid and decreased the protein content of the femoral muscle without affecting its mass, with no any significant effect on the amount of abdominal fat mass. Protected butyrate products proved to be more successful in augmenting carcass weight and relative breast meat yield than unprotected one, which could be explained with the differences in the absorption properties of the two types of supplementation.

In vitro antibacterial efficacy of butyrate on distinct *Campylobacter jejuni* strains (Study IV)

In general, butyrate exerted its antimicrobial properties in much lower concentrations at pH 6.0 (vs. pH 7.4). This was expected based on the fact that at lower pH, non-dissociated butyric acid form of this molecule is characteristic, which is effective against pathogens. For seven strains, the MIC value – that equals with the MBC value on this pH – was calculated as 100 mmol/l concentration at pH 7.4, while MIC was 5 mmol/l and MBC was strain-dependently 5-7.5 mmol/l at pH 6.0. Our results suggest that with 90% confidence the growth of any *C. jejuni* strain can be inhibited by the application of 8 mmol/l butyrate treatment at pH 6.0.

Only one strain showed outstanding resistance against butyrate: in this single case, even the highest butyrate concentration was unable to inhibit the growth of this *C. jejuni* strain at pH 7.4, and as high as 30 mmol/l butyrate concentration was capable to fulfill the criteria of MIC and MBC at pH 6.0. Similarly, this strain proved to resist ampicillin treatment, while all other strains were tested sensitive to ampicillin and all the strains were tested sensitive to enrofloxacin treatment.

Taking into consideration that a pH about 6.0 and the calculated MIC (and MBC) butyrate concentrations are achievable with appropriate diet formulation (e.g. feeding NSP-rich diet) in certain parts of the intestinal tract, butyrate could be an effective tool for the control of *C. jejuni* colonization in broilers.

Conclusions

The results show that the applied cereals, crude protein levels and unprotected butyrate supplementation of the diet seem to be applicable safely, as the assessed diet-associated alterations of blood plasma parameters were measured in the physiological range.

The organism of broilers was found to be the most responsive in the phase of intensive growth, when wheat-based diet was able to increase the amount of the tested hepatic insulin signaling proteins, suggesting improved insulin sensitivity, which could positively influence the muscular protein synthesis and thus growth performance.

Reduced dietary crude protein level with concomitant limiting amino acid supplementation, as well as both unprotected and all tested types of protected butyrate products could increase the relative breast meat yield with unchanged muscle composition and ameliorate the quality of femoral muscle without affecting its weight. Therefore, the aforementioned strategies could be effective in the modification of certain carcass characteristics.

Finally, the pH and butyrate concentration measured effective *in vitro* against the growth of most *C. jejuni* strains can be reached with adequate dietary regime, therefore – not forgetting about the possible *in vivo* interactions with other local circumstances –, butyrate could be a proper candidate to combat *C. jejuni* colonization and to be part of new intervention strategies.

New scientific results

Ad 1,

Dietary cereal type and crude protein content significantly influenced the major metabolic blood parameters of broiler chickens, being the most pronounced on d 21. However, all diet-associated metabolic changes were found within the physiological range.

Ad 2,

In the phase of intensive growth (21 days of age), wheat-based diet – compared to maize-based diet – showed to increase the protein abundance of IRβ and mTOR insulin signaling proteins in the liver of broilers, thus potentially enhance the hepatic insulin sensitivity. Unprotected sodium n-butyrate as feed additive could decrease both hepatic GCGR and IRβ protein expressions.

Ad 3,

The production of breast meat of broiler chickens could be efficiently stimulated by 15% lowered dietary crude protein content of the diet supplemented with limiting amino acids and by the application of either protected or unprotected (n-)butyrate, but its chemical composition remained unchanged. In contrast, the same diets altered the femoral muscle composition without affecting relative thigh yield significantly, but proved to increase carcass weight of broilers.

Ad 4,

Sodium (n-)butyrate exerted antibacterial effects against most *C. jejuni* strains in vitro at pH 6.0 in 5 mmol/ml (MIC) and 5 to 7.5 mmol/l (MBC) concentrations which are reachable by adequate diet formulation in the intestines of live broilers.

Own scientific publications

Publications related to the topic of the present dissertation

Full text papers in peer-reviewed journals

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Full text papers in peer-reviewed journals

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