University of Veterinary Medicine, Budapest
Doctoral School of Veterinary Sciences

Epigenetic effects of butyrate in intestinal and extraintestinal tissues of broiler chicken

Ph.D. thesis

Anna Kulcsár

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Supervisor and consultants:

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Dr. Zsuzsanna Neogrády, CSc

Division of Biochemistry, Department of Physiology and Biochemistry

University of Veterinary Medicine, Budapest

Supervisor

.....

Dr. György Csikó, CSc

Department of Pharmacology and Toxicology

University of Veterinary Medicine, Budapest

Co-supervisor

Dr. Korinna Huber, Univ.-Prof.

Institute of Animal Science

University of Hohenheim

Consultant

Dr. Orsolya Farkas, PhD

Department of Pharmacology and Toxicology

University of Veterinary Medicine, Budapest

Consultant

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Kulcsár Anna

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List of abbreviations

4E-BP1	eukaryotic translation initiation factor 4E-binding protein 1
Akt or PKB	protein kinase B
AMP	adenosine monophosphate
AMPK	AMP activated protein kinase
ANOVA	analysis of variance
ATP	adenosine triphosphate
BSA	bovine serum albumin
BW	body weight
cAMP	cyclic adenosine monophosphate
CoA	coenzyme A
CTR	control group
CYPs	cytochrome P450 enzymes
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FAD	flavin adenine dinucleotide
FFA	free fatty acid
FFR	free fatty acid receptor
FMN	flavin mononucleotide
GDP	guanosine diphosphate
GIP	Glucose-dependent Insulinotropic Peptide
GLP-1	Glucagon-like Peptide 1
GLUTs	glucose transporters
GPRs	G-protein coupled receptors
GTP	guanosine triphosphate
H2A	histone 2A
H2B	histone 2B
H3	histone 3
H4	histone 4
HAT	histone acyl tranferase
HDAC	histone deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	horseradish peroxidase
IU	international unit

IGFs	insulin-like growth factors
IRS-1	insulin receptor substrate 1
IRs	insulin receptors
MAPK	mitogen-activated protein kinase
MB	maize-based diet group
MCTs	monocarboxylate transporters
MDR1	multidrug resistance 1 gene
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
n	sample size
NADP	nicotinamide adenine dinucleotide phosphate
NP B	non-protected butyrate group
NRC	Nutrient Requirements of Chicken
NS	not significant
NSP	non starch polysaccharide
P	probability value
PAGE	polyacrilamide gel electrophoresis
PB	protected butyrate group
PBS(T)	phosphate buffered saline (with Tween)
pH	potential of hydrogen
PI3K	phosphatidylinositol-3-kinase
PIP3	phosphatidylinositol triphosphate
pKa	dissociation constant of an acid
PKB or Akt	protein kinase B
PKCζ	protein kinase C zeta
PPARα	peroxisome proliferator-activated receptor alpha
S6K	ribosomal protein S6 kinase
SCFA	short chain fatty acid
SDS	sodium dodecyl sulphate
SEM	standard error of mean
SGLTs	sodium and glucose co-transporters
SMCTs	sodium dependent monocarboxylate transporters
UDP	uridine diphosphate
WB	wheat-based diet group

1. Summary

The four carbon short chain fatty acid butyrate has quite a wide range of biological activity via epigenetic and receptor-mediated pathways. As in monogastric mammals and birds it is mainly produced by bacterial fermentation in the large intestines, it can work as a sensitive messenger molecule between prokaryotic and eukaryotic organisms and maintain a symbiotic balance between the intestinal bacterial communities and the host. Besides its intestinal effects, butyrate can also be absorbed from the gastrointestinal tract, and by reaching certain organs with the portal and systemic circulation it can provoke variable effects. To enhance its beneficial actions, intestinal butyrate production can be stimulated by certain dietary factors, and it is also used as a feed additive in free or protected form in poultry nutrition.

In my PhD work I aimed to study the action of butyrate from a complex point of view. Thus, I examined the **intestinal and extraintestinal effects** of **different application forms of butyrate** in a **feeding study** and also in **model systems**, in **long-, medium- and short-term studies**, to clarify the effects of butyrate that can be practically manifested in broiler industry.

The form and dose of butyrate application can greatly determine the final site, way and extent of its effect, presumably due to the differences in the absorption and distribution of different application forms in chicken. Therefore, in our **long-term – feeding study** we investigated the **absorption of butyrate** using two different doses of non-protected butyrate (lower dose: 1.5 g/kg diet; higher dose: 3.0 g/kg diet) and protected butyrate (0.2 g/kg diet) as a feed additive, applying wheat-based diet, that due to its high non-starch polysaccharide (NSP) content, could be a precursor of endogenous butyrate production. Intestinal content and blood plasma samples were taken after slaughtering the chickens on day 42 of life. According to our results non-protected butyrate should be absorbed from the gastrointestinal tract before the small intestines, as it did not have any effect on the butyrate concentration in duodenum, ileum and caecum either in lower or in higher dose. However, protected butyrate supplementation increased the butyrate concentration in the ileum, and wheat-based diet resulted in elevated butyrate concentration in the caecum. Butyrate concentration in the systemic blood collected from *vena brachialis* was affected by the higher dose of non-protected butyrate supplementation only. Regarding the portal circulation, the higher dose of non-protected butyrate supplementation and the wheat-based diet resulted in higher blood butyrate concentration in *vena gastropancreaticoduodenalis*, however, in *vena mesenterica communis* higher dose of non-protected butyrate supplementation, protected butyrate

supplementation and wheat-based diet also increased blood butyrate concentration. The observed changes in butyrate concentrations in blood samples can be explained by the anatomical location of the investigated veins, draining different segments of the intestinal tract.

Concerning the intestinal effects of butyrate, we studied the effects of different application forms of butyrate on t**he intestinal activity of the cytochrome P450 (CYP) detoxification enzymes** in the same **long-term feeding study** system. Intestinal CYP enzymes play key role in the first pass metabolism of orally ingested xenobiotics, providing a primary metabolic barrier, being of special importance in maintaining animal health and production. CYP1A4/5 and CYP2H2 activity in duodenal endothelial cells were increased by both higher dose of non-protected butyrate supplementation and wheat-based diet. As wheat-based diet did not increase butyrate concentration in the duodenum compared to the maize-based diet, but elevated it in portal blood plasma, we suppose that duodenal endothelial cells might get butyrate stimulus mainly from the surrounding veins.

To demonstrate the extraintestinal effects of butyrate, we examined the **expression of certain insulin signaling proteins** in the previously described **long-term – feeding study** and also in a **medium-term – multiple bolus study**, where butyrate was applied in a daily oral bolus model system, to study its potential molecular effects. Studying the possible influence of butyrate on insulin signaling in chicken has a special importance, as the regulation of carbohydrate metabolism in birds with high fasting blood glucose concentration and moderate insulin sensitivity is not fully elucidated. In the long-term – feeding study dietary cereal type had the most remarkable effect on the insulin signaling: wheat-based diet increased IRβ and mTOR expression in the liver and mTOR and PKCζ expression in the adipose tissue. IRβ expression in the liver was increased by the lower dose of non-protected butyrate as well. However, in the medium-term – multiple bolus study, where chickens received a daily intra-ingluvial bolus of non-protected butyrate (0.25 g/kg body weight) on days 20-24 of life, butyrate had a tissue-selective impact on insulin signaling in chicken. Butyrate bolus application was associated with decreased protein expression of IRβ in liver and adipose tissues, but with elevated IRβ expression in muscle. Hepatic PI3K protein expression was reduced in the butyrate-treated group, while mTOR was down-regulated by butyrate in liver and subcutaneous adipose tissue. However, butyrate had no detectable influence on PKCζ expression in any examined tissues. Our results demonstrate that the application form of butyrate and the age of chicken could remarkably determine the way of butyrate action. The bolus application of butyrate had more pronounced and partly different effects on certain insulin signaling proteins compared to the long-term feeding application. Further, in the phase of intensive growth (day 20-24 of life), when insulin is mostly involved in growth regulation, broilers were more sensitive to butyrate treatment.

The effect of butyrate bolus application on insulin homeostasis was studied on the **insulin secretion** level as well in our **short-term single bolus study**. 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 (Glucose-dependent Insulinotropic Peptide) and GLP-1 (Glucagon-like Peptide 1). In this short-term – single bolus study we also approached the insulin homeostasis from a comparative point of view, the same study design was applied in chicken and rabbit (a bird and a mammalian species, both being potential targets of butyrate application in nutrition). Single intra-ingluvial bolus application of non-protected butyrate decreased plasma GIP levels in both chickens and rabbits after 30, and 60 min following butyrate ingestion. In chickens the higher dose of butyrate application (1.25 g/kg body weight), while in rabbits the lower dose (0.25 g/kg body weight) had significant effect only. Plasma GLP-1, insulin and glucose concentrations remained unaffected by butyrate in both species over time. These results are contradictory 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 analysed correlations between the measured endocrine parameters (regardless of the butyrate exposure), it can be assumed that incretins might regulate pancreatic insulin release in rabbit on a partly different way compared to mouse, human and chicken. In conclusion, it can be suggested that butyrate is a potent effector of incretin production, which may provide new possibilities in the nutritional modulation of incretin and insulin homeostasis and thus influencing the efficacy of animal production.

Our results highlight that butyrate could have great importance in poultry nutrition. Different application forms can determine the site and way of its biological activity; however, we can state that both the altered caecal microbial butyrate production and butyrate as a feed additive have remarkable intestinal and extraintestinal effect in broiler chicken.

2. Introduction

Nutritional factors and feed additives may be potential effectors of animal health and growth by affecting molecular regulatory mechanisms and various metabolic processes. Since the application of antibiotics and hormones as growth promoters is highly restricted or banned in food-producing livestock (Philips, 2007), the use of alternative feed additives, such as the salts of short chain fatty acid (SCFA) (n-)butyrate (in the followings: butyrate), is increasingly common in animal production. Based on butyrate's stimulatory effect on small intestinal development and its improving action on the balance of the gut microbiota, butyrate is widely used as a natural growth promoting feed additive in poultry nutrition as well (Hu and Guo, 2007; Moquet et al 2016). Butyrate salts, primarily sodium butyrate, and several protected forms, e.g. encapsulated butyrate and various butyric acid esters, such as its glycerides are all applied successfully in poultry (Antongiovanni et al., 2007; Chamba et al., 2014). Protected forms avoid rapid butyrate absorption from the crop, stomach or duodenum by providing a prolonged butyrate release mainly in the distal section of the gastrointestinal tract (Moquet et al., 2016). Butyrate is also produced by the bacterial fermentation of carbohydrates in the caecum of birds (Bergman, 1990) that can be stimulated by the increased dietary uptake of resistant starch. Further, dietary non-starch polysaccharides (NSP) supplemented with NSP-degrading enzymes (xylanase, glucanase) can also increase the caecal butyrate production, as these enzymes can degrade NSP into oligosaccharides providing substrates for SCFA producing bacteria (Jamroz et al., 2002).

Concentration of soluble NSP – mainly arabinoxylans – is higher in wheat than in maize. These compounds can only be degraded in animals by microbial fermentation (de Lange, 2000). Soluble NSP – at moderately higher levels – have some adverse effects on digestion by increasing the viscosity of the digesta, decreasing passage rate, thus extending the time for bacteria to thrive (de Lange, 2000). However, they also have prebiotic effects providing substrates for probiotic bacteria (Yazawa et al., 1978). Application of NSP-degrading enzymes (xylanase, glucanase) in the diet can produce more fermentable oligosaccharides, thus improve their prebiotic characteristics against the undesirable effects of soluble NSP-s (de Lange, 2000). More available bypass substrates promote the microbial fermentation in the caecum resulting in higher total SCFA and butyrate production (Molnár et al., 2015).

Beside the bacterially produced butyrate, that is mainly absorbed and acts in the large intestines, several types of butyrate are widely applied in poultry nutrition as a feed additive. In these cases, butyrate can affect the gastrointestinal epithelium at the more proximal part

of the gastrointestinal tract, and depending on the form and dosage of the application, it is absorbed from different sites of the intestines, thus its extraintestinal effects could appear in different ways. Notwithstanding the common use of butyrate, the absorption and kinetics of its different application forms are still unclear.

Intestinal effects of butyrate are quite various. Butyrate was proved to improve gut health by providing energy for the gastrointestinal epithelium (Roediger, 1982), regulating cell proliferation and differentiation (Neogrády et al., 1988; Gálfi and Neogrády, 2001), enhancing the intestinal absorptive capacity and maintaining the eubiotic gut flora (Hu and Guo, 2007), further, increasing the digestibility of certain amino acids such as methionine (Moquet et al., 2017).

As an epigenetically active molecule, butyrate is capable to modify gene expression by causing histone hyperacetylation *in vitro* in cultured cells (Candido et al., 1978; Roediger, 1982) and *in vivo* as well, for instance in the caecum of piglets (Kien et al., 2008) or in the liver of chickens (Mátis et al., 2013 a; b). Further, butyrate also elicits effects via receptor-mediated pathways, by activating primarily the G-protein coupled receptors, GPR41, GPR43 (Brown et al., 2003) and GPR109A (Thangaraju et al., 2009).

Based on its epigenetic (Kien et al., 2008; Mátis et al., 2013 a; b) and receptor-mediated (Brown et al., 2003; le Poul et al., 2003; Thangaraju et al., 2009) effects, butyrate can also influence the function of microsomal cytochrome P450 (CYP) enzymes (Csikó et al., 2014), mainly involved in the oxidative phase I reactions of xenobiotic biotransformation, playing pivotal role in drug metabolism (Anzenbacher and Anzenbacherová, 2001). The CYP enzymes are primarily expressed in the microsomes of hepatocytes; however, they are also localized in the intestinal mucosa to serve as a primary metabolic barrier for xenobiotics taken up orally (Obach et al., 2001), influencing their bioavailability and toxicity (le Poul et al., 2003). CYP enzymes of the enterocytes perform presystemic metabolism, decreasing the systemic uptake of orally applied drugs (le Poul et al., 2003; Csikó et al., 2014). Several xenobiotics are known to undergo substantial intestinal metabolism, such as rifampicin, phenobarbital and glucocorticoids (Zhang and Benet, 2001). In chicken, CYP1A, CYP2H and CYP3A subfamilies are the most important CYPs being responsible for hepatic drug metabolism (Ourlin et al., 2000). The CYP3A subfamily is also expressed with high activity in the epithelium of the small intestines, showing a decreasing trend from the duodenum towards the ileum, being highly involved in enteral xenobiotic metabolism of chicken (Osselaere et al., 2013).

The gene expression of CYP enzymes is under epigenetic regulation and controlled by certain nuclear receptors, thus may be modified by bioactive molecules eliciting epigenetic activity or influencing receptor-mediated signaling pathways (Singh et al., 2007). Concerning our previous results butyrate was found to decrease the gene expression of CYP1A and

CYP3A37 enzymes, while it up-regulated CYP2H1 gene *in vitro*, in primary cultures of chicken hepatocytes (Csikó et al., 2014). Further, hepatic CYP1A and CYP2H1 gene expression levels were elevated in broilers *in vivo* after receiving sodium butyrate as feed additive (Csikó et al., 2014). These effects of butyrate on the level of gene function were finally not realized in altered enzyme activity as butyrate could not modulate the activity of the examined hepatic CYPs either as a feed additive or in a daily bolus (Mátis et al., 2013 a; b). However, oral butyrate application ameliorated the stimulatory effect of the simultaneously administered enzyme-inducer phenobarbital on CYP2H and CYP3A in the liver of chickens (Mátis et al., 2016). In addition, butyrate was capable to modify some pharmacokinetic parameters of concomitantly applied erythromycin (Csikó et al., 2014). So the possibility of feed-drug interactions cannot be neglected, having a huge impact from food safety and pharmacotherapeutic points of view. It should be taken into consideration that intestinal CYP enzymes, as first pass barrier for detoxifying orally ingested xenobiotics, may also be affected by epigenetically active nutritive factors and feed additives, such as butyrate.

The activity of CYPs can also be modulated by the insulin homeostasis in mammals, reflected in diabetes-associated changes in CYP expressions and activities (Sakuma et al., 2001; Shimojo et al., 2013). Insulin was found to be involved in the regulation of small intestinal CYPs as well: enteral CYP3A activity was decreased by streptozotocin-induced diabetes in rat, which effect could be successfully attenuated by insulin application (Borbás et al., 2006).

At the same time, insulin plays a pivotal role in the regulation of carbohydrate and lipid metabolism, while it is also considered as an indispensable stimulator of growth by increasing protein synthesis and affecting the expression of several growth-related genes (Taniguchi et al., 2006). The insulin homeostasis could be greatly influenced by nutrition, such as by butyrate application. It is known that high-fiber diets or supplementation with resistant starch are associated with a reduced risk of diabetes and cardiovascular diseases as well as with reduction of weight gain in obese human patients (Robertson et al., 2003), which is suggested to be also in association with the elevated intestinal SCFA production (Lin et al., 2012). As insulin is one of the most important regulators of carbohydrate and lipid metabolism in broilers as well, the possible effects of SCFA, first of all those of butyrate on insulin production, reception and signaling are of special interest. There is some evidence that butyrate can stimulate the pancreatic insulin secretion (Lin et al., 2012); further, it has been observed that butyrate is capable to increase insulin sensitivity in mice (Gao et al., 2009; Lin et al., 2012). In these mentioned studies, insulin resistance was experimentally induced by a high-fat diet, which was successfully decreased by oral butyrate application. The protective effect of butyrate against insulin resistance was justified by insulin tolerance

test as well as by the enhanced phosphorylation of certain members of the insulin signaling cascade (insulin receptor substrate-1 and Akt) in skeletal muscle (Gao et al., 2009).

In both mammals and birds, pancreatic insulin release is primarily controlled by the gut-derived incretin hormones, e.g. by GIP (Glucose-dependent Insulinotropic Peptide) and GLP-1 (Glucagon-like Peptide 1) as key members of the enteroinsular axis (Creutzfeldt, 1992). GIP is produced by the K cells of the small intestines and stimulates the insulin secretion of pancreatic β cells together with GLP-1, the latter released from L cells, expressed in small and large intestines as well (Baggio and Drucker, 2007; Doyle and Egan, 2007). Further, GLP-1 can enhance insulin synthesis by increasing insulin gene expression and influences differentiation and proliferation of β cells in human (Holst and Gromada, 2004). The way of incretin action is mostly known from model studies with rodents, while only limited data are available with regard on domestic animal species. Since the carbohydrate metabolism of birds differs from that of mammals, featuring decreased plasma insulin level and tissue insulin sensitivity combined with increased plasma glucose concentration (Braun and Sweazea, 2008), some differences do exist between the regulatory role of incretins of mammals and birds. For instance, GLP-1 elicits its insulinotropic action in chicken more likely by influencing the somatostatin production of pancreatic δ cells rather than by direct β cell stimulation (Watanabe et al., 2014).

Notwithstanding that birds have greatly decreased systemic insulin sensitivity compared to mammals, insulin as an important anabolic hormone is one of the major regulators of metabolism and growth performance in chicken as well (Jozefiák et al., 2010). Therefore, feed additives modifying insulin homeostasis, such as butyrate, may be potential effectors of growth via endocrine metabolic regulation.

3. Literature overview

3.1. Butyrate as a biologically active molecule

3.1.1. Butyrate of endogenous and exogenous origin

The one to six carbon unbranched monocarboxylic acids are called short chain fatty acids (SCFA). They are produced by microbial fermentation from indigestible nutrients in the gastrointestinal tract. All six SCFA can be found in the intestine of vertebrates, however, the most abundant (<95%), hence the most important ones are acetic acid (C2), propionic acid (C3) and (n-) butyric acid (C4; Cook and Sellin, 1998). They maintain the active symbiotic metabolic link between the intestinal microbial community and the host, as they are produced by the microbial degradation of carbohydrates, being indigestible by the mammalian and avian enzymes, and they can be absorbed and utilized as substrate or as regulatory molecule by the host cells. Concerning biological activity, salt of n-butyric acid, (n-)butyrate (in the followings: butyrate, regardless of its dissociation state) has the highest significance among SCFAs (Guilloteau et al., 2010).

In ruminants the most important part of the intestinal tract for microbial digestion is the rumen, while in monogastric mammals and birds it takes place mainly in the caecum and proximal colon, where the total concentration of SCFAs is about 70-140 mmol/l (den Besten et al., 2013). SCFA production occurs in the small intestines as well depending on the diet and bacterial composition, however their concentration is only about 10% of that in colon (Smiriky-Tjardes et al., 2003; den Besten et al., 2013). Most of the produced SCFAs are absorbed by the colonocytes, therefore about 5% is excreted in the feces only (den Besten et al., 2013). The molar ratio of acetate, propionate and butyrate is varying from approximately 75:15:10 to 40:40:20 in a healthy animal (Bergman, 1990); however, these proportions can be altered by several factors combined with each other, including diet type, microbial composition and the pH of the ingesta.

The main precursors for SCFA production are the indigestible carbohydrates, like cellulose, hemicellulose, pectin or resistant starch (Flint et al., 2008). As diet provides the substrates for intestinal bacterial metabolism, it determines the microbial community, which regulates the concentration and ratio of SCFAs that have an indirect feedback effect on the bacterial community mainly by changing caecal milieu.

In total, approximately 10^{14} bacterial individuals live in the mammalian intestines. Concerning SCFA production the most remarkable phyla are the Gram-negative *Bacteroidetes* which includes acetate and propionate producing bacteria, and the phylogenetically quite diverse Gram-positive *Firmicutes* that are responsible for the butyrate

production (den Besten et al., 2013). The most important members of *Firmicutes* are related to *Eubacterium rectale/Roseburia* spp. and to *Faecalibacterium prausnitzii* (Louis and Flint, 2009).

During the anaerobe fermentation of nutrients poly- and oligosaccharides are cleaved into monosaccharides, then monosaccharides are degraded into pyruvate mainly in the process of glycolysis (Miller and Wolin, 1996). Pyruvate has three main ways to be converted into one of the SCFAs, as demonstrated in **Fig. 1**. The ratio of these pathways depends on the microbial composition of the gut that is determined by the environmental milieu. Acetate can be produced by the decarboxylation of pyruvate, but some bacteria are able to synthesize it using CO_2 and H_2. Propionate formation can be carried out through three pathways, namely succinate, lactate and propanodiol pathway. Butyrate synthesis starts with the condensation of two acetyl~CoA, but the final enzymatic way of the conversion of butyryl-CoA to butyrate depends on the bacteria producing it (Rios-Covián et al., 2016).

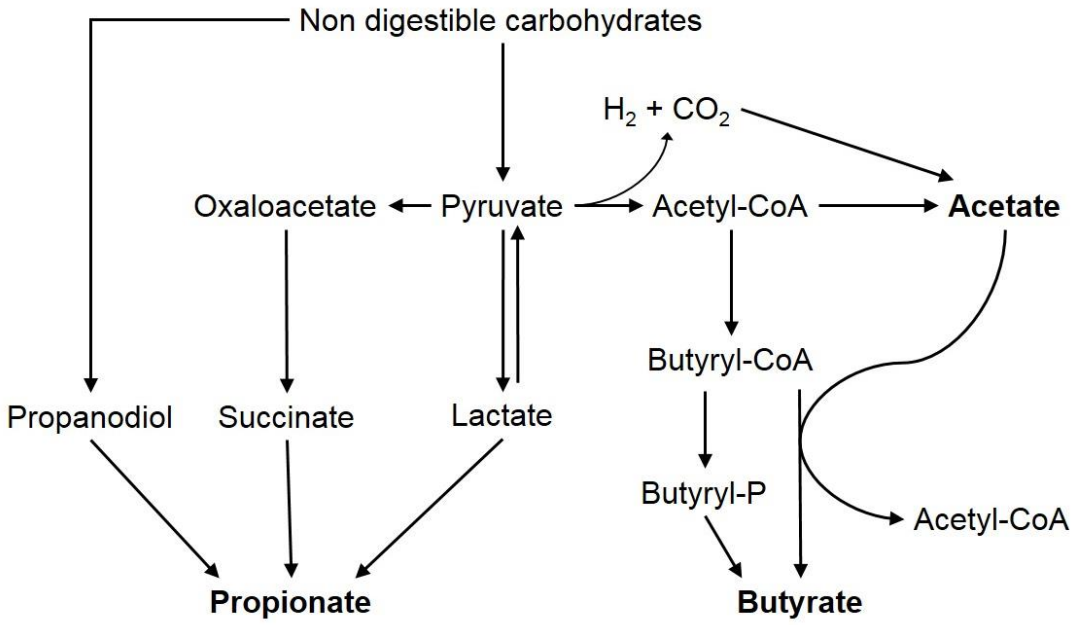

Figure 1. Metabolic pathways of SCFA production (Rios-Covián et al., 2016)

The pH of the ingesta can pivotally influence the bacterial community, hence SCFA production. The more acidic pH 5.5 favors the butyrate producing bacteria, while at pH 6.5 the production of acetate and propionate is dominant (Walker et al., 2005).

Discovering the beneficial physiological properties of SCFA and especially butyrate, livestock industry started to apply it as a feed additive. Since the use of antibiotics as growth promoters has been banned in the European Union by a law passed in 2006, the feed additives of natural origin have come in the view (Phillips, 2007). The growth promoting effect

of butyrate has been demonstrated in several species decades ago (Gálfi and Bokori, 1990; Hu and Guo, 2007) and today it has become a widely used feed additive especially in pig and poultry nutrition.

It can be blended in the fodder in non-protected form, usually as the sodium or calcium salts of butyrate anion, however nowadays the protected forms are becoming more usual in the industry (Chamba et al., 2014). The main goals of the protection are to ensure the prolonged release of butyrate, thus to improve its effect in the small intestines and/or large intestine as well but it also could reduce the characteristic odor, and improve blending properties which facilitate the manufacturing of the product.

There are different methods for the protection of feed additive butyrate. The most current ones are esterification of butyrate (mostly forming glyceride-ester), a special film-coating process, applied with carbohydrate or fat matrix, or combination of these designs.

3.1.2. Absorption of butyrate

The pK_a value of butyrate is 4.82, hence it is presented mostly in undissociated (butyric acid) form in the highly acidic (~pH 1-3) upper part of the gastrointestinal tract (mammals – stomach, birds – proventriculus and gizzard). The cell membrane is permeable for the undissociated forms of SCFA, thus butyrate can be absorbed by simple diffusion, not limited by any transporters (Moquet, 2018). Regarding that the intracellular pH is higher than the pK_a of butyrate, since the undissociated butyric acid is absorbed into the cell, it dissociates to butyrate anion and H^+, for which the membrane is not permeable anymore, therefore butyrate cannot escape from the cell. As these circumstances inhibit the resorption of the molecule, the absorption of butyrate is highly effective in the proximal section of the intestinal tract (Manzanilla et al., 2006). Hence, butyrate applied in non-protected form as feed additive is largely absorbed from the stomach, and only a negligible part can proceed to the small intestines.

Protected forms of butyrate as feed additives, which can reach the small intestines, and especially microbially produced butyrate molecules in the large intestines are released at a higher pH value (~pH 5-8) compared to the upper gastrointestinal tract, thus most of them can be found in dissociated form (Sellin, 1999). Dissociated butyrate is unable to cross the semipermeable membrane with passive transport, so in these cases the principal way of the absorption of butyrate is the active transport. Although the active transport requires energy, its effectiveness is quite high, 95% of SCFA in the intestines are absorbed into the intestinal cells (den Besten et al., 2013).

Three transporter proteins take part in the absorption of butyrate and other SCFAs from the gastrointestinal content into the intestinal epithelial cells. The first one is an SCFA/ HCO_3^-

exchanger (Mascolo et al., 1991; Harig et al., 1996). The existence and relevance of this transporter have been confirmed, however, the clear identity of it is not discovered yet. The second type of transporters belongs to the family of monocarboxylate transporters (MCTs), which import SCFA anions simultaneously with a H^+, thus maintaining decrease of the intracellular pH (Hadjiagapiou et al., 2000). The last type is the sodium dependent monocarboxylate transporters (SMCTs), where the cotransport of a Na^+ and $SCFA^-$ takes place (Takabe et al., 2005; Teramae et al., 2010). The same types of transporters perform the transport of the non-metabolised butyrate from the enterocytes into the portal blood.

3.1.3. Molecular mechanisms of butyrate action

Effects based on the dissociation degree

The widespread beneficial actions of butyrate are mostly based on its selective antimicrobial effect. Butyrate can inhibit the growth of certain pathogenic bacteria, such as enterotoxic *Escherichia coli* strains, *Clostridium* or *Salmonella* spp. in the gastrointestinal tract (Fernández-Rubio et al., 2008).

This selective antimicrobial effect on enteral pathogens is traditionally explained by the different permeability of cell membrane for undissociated and dissociated forms of butyrate, mentioned above. As the ionized, anionic form cannot be transported through the membranes by passive diffusion, it is being accumulated in the bacterial cell, while dissociating protons acidify the cytoplasm. Since most enteral pathogens are especially sensitive to reduced intracellular pH, increased intensity of pumping out the accumulated protons will result in cellular ATP depletion. Elevated cytoplasmic proton concentration can increase the sodium transport as well by enhancing the Na^+/H^+ antiport mechanism, elevating the turgor of the cell. As the pH of the caecum (pH 5-6) is close to the pK_a of butyrate, the proportion of the undissociated form, thus the effectiveness of butyrate against pathogenic bacteria remarkably depends on the environmental pH.

Unlike many pathogens, most fermentative bacteria (such as *Lactobacillus* spp. and *Streptococcus bovis*), being part of the eubiotic enteral microflora, are less sensitive to the decrease of the intracellular pH, so they are protected from anion accumulation (Gálfi and Neogrády, 1996). Due to its antibacterial effect on most enteral pathogens, butyrate improves the balance of the intestinal microflora (Candela et al., 2010).

Energy source: ATP production

Butyrate absorbed from the gastrointestinal tract as a simple fatty acid molecule can easily be engaged in the metabolic processes of the cells, thus it primarily serves as an energy source for enterocytes (Roediger, 1982). Colonocytes prefer butyrate over other

SCFAs for energy production: it covers 60-70% of energy requirement of epithelial cells in this gut section (Roediger, 1982). Even considering the total energy supply of the body, butyrate could provide 10% of daily caloric requirement in human (Bergman, 1990).

In catabolic biochemical processes butyrate can be oxidized into acetyl~CoA via β-oxidation which can enter the citrate cycle and further be oxidized to CO_2, while reduced hydrogen carrier molecules are produced that transport hydrogen to the respiratory chain for ATP production. In certain circumstances, acetyl~CoA can be involved in the production of ketone bodies and the cholesterol synthesis as well. However, in adequate energy supply butyrate can also be the precursor of lipid synthesis (Guilloteau et al., 2010). As butyrate is the main source of energy, most of it is utilized by the enterocytes and only small quantities pass forward to the portal circulation (Guilloteau et al., 2010).

Epigenetic and receptor mediated regulation

The main significance of butyrate comes from the fact, that it is not simply a substrate for biochemical processes, but also can regulate intermediary metabolism in several ways.

Epigenetic effect

Butyrate is considered as an epigenetically active molecule, as it is able to modify the transcriptional pattern of certain genes. Histone proteins (H2A, H2B, H3, H4), wrapped around by DNA, form the nucleosome, that is the basic unit of the eukaryotic chromatin (Arents et al., 1991). The compactness of the chromatin structure depends on the electrostatic attraction between the negatively charged phosphate groups of the DNA and the positively charged amino acids on the N-terminal tails of the histone proteins. The more compact form, called heterochromatin, is transcriptionally inactive, while in case of euchromatin (the loose form), transcription factors can bind to the DNA more easily, thus the gene expression is activated. The transcriptional pattern of certain genes can be influenced by the DNA methylation as well. Attachment of methyl groups to the promoter region of certain genes can prevent the binding of the transcriptional factors, leading to gene silencing.

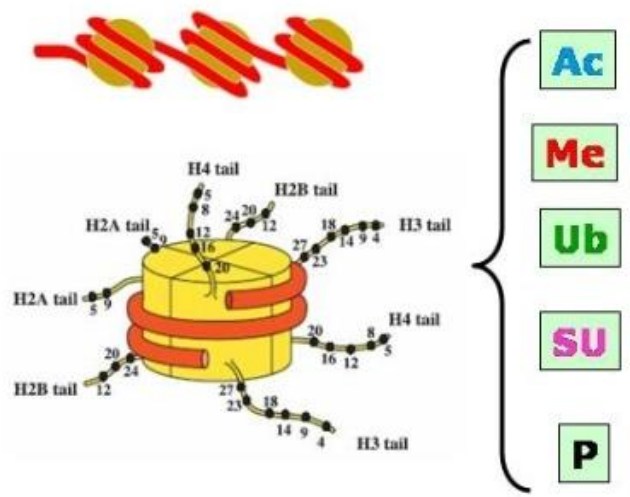

Figure 2. The most important posttranslational modifications of histone proteins. Ac: acetylation, Me: methylation, Ub: ubiquitination, Su: sumoylation (integratedhealthcare.eu)

The compactness of the chromatin can be loosened by the modification of histone proteins. Acetylation, ubiquitination, phosphorylation or sumoylation of histones could hide their positive charge, hence the binding of DNA to histones can be weakened (**Fig. 2**). Acetyl groups are linked on the lysine side chain by histone acetyl tranferase (HAT) enzyme, while the opposite process (the removal of the acetyl group) is catalyzed by the histone deacetylase (HDAC) enzyme (Rada-Iglesias et al., 2007). The two enzymes together can maintain a stable acetylation balance of the histone proteins.

Butyrate could have a double effect on the transcriptional pattern of DNA. In one hand, among other epigenetically active molecules, it is able to inhibit HDAC (Davie, 2003), consequently histones get hyperacetylated that can lead to the more intensive expression of certain genes. However, in the other hand, through hypermethylation of DNA (Cho et al., 2009) gene expression could also be modified.

Receptor mediated effect

The activity of some enzyme molecules, thus the intensity of some metabolic processes can be modified through signaling pathways that always start by the binding of a ligand (signaling molecule) to a receptor. G-protein coupled receptors (GPRs) form the largest cell surface receptor family. Binding of an extracellular signaling molecule to a GPR changes its spatial structure, and usually this linkage causes the binding of G protein to the receptor. G proteins consist of three subunits: α, β, γ. In non-stimulated state GDP is bound to the α subunit of the inactive G protein. The activated GPR induces the α subunit to release

GDP and attach GTP. Due to the binding of GTP the α subunit detaches the β-γ subunit complex which is consequently activated too. The two activated parts of G protein can connect to other compounds, activate them and start signaling processes primarily through cAMP and phosphoinosytol pathways. Signaling molecules of the GPRs could be polypeptides, amino acids, free fatty acids or photons as well (Boldogkői, 2013).

Butyrate as a SCFA acts mainly on GPR41 and GPR43. As these receptors have been described to be activated by free fatty acids they are also called FFR3 (free fatty acid receptor 3) and FFR2, respectively (Brown et al., 2003; Le Poul et al., 2003; Stoddart and Smith, 2008). The gene of GPR43 is mainly expressed in immune cells, but can also be found in white and brown adipose tissue, pancreas and in the large intestines (Regard et al., 2008). GPR41 has a wider distribution than GPR43, but the most important expression sites are the nervous system and the intestines. Butyrate is the signaling molecule of GPR109a as well, which is highly expressed in colonocytes and immune cells and may play a role in the maintenance of gut health through anti-inflammatory signaling (Thangaraju et al., 2009).

3.1.4. Intestinal effects of butyrate – gut health

Butyrate application in livestock nutrition aims mainly to improve feed conversion efficiency. Due to its distribution and absorption pattern, orally applied and microbially produced butyrate both have the highest impact on the intestinal tract. This is the site of the body where butyrate could exert its effect in the most diverse ways.

Based on the dissociation degree, butyrate can influence the microbial community of intestines (Moquet et al., 2016). This effect is based on the different sensitivity of pathogenic and symbiotic bacteria to the intracellular pH changes. Further, butyrate as an epigenetically active molecule may also influence bacterial gene expression, for instance in *Salmonella* spp., where butyrate declined the expression of *Salmonella* pathogenicity island gene, responsible for colonization and virulence of the bacteria (Gantois et al., 2006). In addition, most fermentative bacteria can utilize butyrate as an energy source as well.

Beside the enteral microbial balance, the health and absorption efficiency of the intestinal wall also have an outstanding importance. Butyrate produced endogenously or ingested primarily acts as an energy source for enterocytes. This is particularly true in the large intestines, where butyrate with other SCFAs covers 60-70% of energy supply of colonocytes.

As a biologically active molecule, butyrate has an improving effect on intestinal mucosa as well. It can enhance the barrier function of the intestinal epithelial cells by increasing the expression of tight junction proteins (Wang et al., 2012), and also induce the expression of early response genes, which are in connection with cell division, growth,

differentiation and apoptosis. Further, proglucagon expression can be stimulated by butyrate as well, being responsible for cellular proliferation in the intestines (Andreaopoulou et al., 2014). The efficiency of nutrient absorption can also be developed by the histomorphological effects of butyrate: it was reported to increase villus height (Pelicano et al., 2005; Adil et al., 2010) and crypt depth (Antogiovanni et al., 2007).

Intestinal immune system is of particular relevance, and also has a special role, as due to the dense and diverse bacterial community in the gastrointestinal tract, it must remain relatively hyposensitive (Chang, 2014). Butyrate was found to enhance mucin production and anti-inflammatory properties of small intestines (van Immersel et al., 2010) by modulating the function of macrophages (Chang et al., 2014), increase IgY natural antibody level in the duodenum and jejunum (Moquet, 2018) and induce regulatory T cell production in the colon (Arpaia, 2013; Furusawa, 2013; Smith, 2013).

Before reaching the liver, the most important organ performing detoxification is the intestinal epithelium. Orally ingested xenobiotics all have to be transported through the intestinal epithelium, which functions as a first metabolic barrier. Most drug metabolizing enzymes (mainly cytochrome P450 enzymes) of the liver can be found in the small intestines as well. Due to their function they are inducible enzymes, consequently their expression can highly be modified by dietary factors such as orally applied or microbially produced butyrate, through epigenetic and receptor mediated pathways.

3.1.5. Extraintestinal effects of butyrate

From the intestines, the non-metabolised, absorbed butyrate is transported to the liver via the portal circulation. Hepatocytes can gain energy from the oxidation of butyrate as well, or it can be the precursor of fatty acid, ketone body and cholesterol synthesis (den Besten et al., 2013).

Hepatocytes contain high concentration of CYP enzymes that mainly accomplish the detoxifying function of the liver. Orally applied butyrate is able to modify the gene expression of certain CYP enzymes presumably through epigenetic and receptor mediated pathways (Csikó et al., 2014).

The liver plays a central role in carbohydrate and lipid metabolism as well. As butyrate is delivered directly to the liver from the intestines through the portal veins, it could have a more remarkable effect on the liver compared to the extrahepatic tissues. By the binding of butyrate to GPR41 and GPR43 receptors (Brown et al., 2003), the hepatic AMPK (AMP activated protein kinase) is phosphorylated, that enhances the expression of PPARα (peroxisome proliferator-activated receptor alpha) target genes participating in fatty acid oxidation, lipogenesis, gluconeogenesis, and glycogenesis (Canfora et al., 2015).

Intravenous treatment with butyrate has been detected to decrease lipogenesis, and increase glucose tolerance (den Besten., 2015).

Although the butyrate concentration in the systemic circulation is usually much lower than in the portal blood (Egorin et al., 1999; Knudsen et al., 2005), it has been confirmed that orally applied butyrate is able to reach the skeletal muscle and adipose tissues as well (Gao et al., 2009).

In the muscle, via AMPK mechanism, butyrate increases fatty acid oxidation, the glucose uptake of cells through GLUT4 (Glucose transporter 4) transporter and glycogenesis, further, improves insulin sensitivity (Canfora et al., 2015).

In the adipose tissue butyrate decreases lipolysis probably via the regulation of hormone sensitive lipase (Ge et al., 2008), while in brown adipose tissue it can affect thermogenesis by the activation of uncoupling protein 1 expression (Gao et al., 2009). Butyrate is also able to influence inflammatory processes in adipose tissues, as it reduces secretion of proinflammatory cytokines and chemokines (Canfora et al., 2015).

3.2. The role of intestinal mucosa in the metabolism of xenobiotics

3.2.1. The role of cytochrome P450 enzymes in the biotransformation of xenobiotics

Exogenous substances (xenobiotics) taken into the body can have different origin. Polluting chemicals, pesticides, food and feed additives all belong to the group of xenobiotics. However, in clinical practice, drugs, which are administered directly into the body, may have the highest relevance, therefore studying the bioavailability of them is remarkable also from practical point of view.

Biotransformation along with transport processes result in the excretion of xenobiotics. In one hand this provides an essential protection of the body; however, it may alter the effect of certain drugs used in the therapy (Handschin and Meyer, 2003).

Biotransformation of xenobiotics occurs in two main steps. In phase 1 reactions the mostly apolar compounds are converted into a polar form that is suitable for conjugation. These reactions are mainly catalyzed by monooxygenases (e.g. cytochrome P450 [CYP]), and to a lesser extent by epoxide hydrolases, esterases, alcohol and aldehyde dehydrogenases, and flavin monooxygenases as well (Singh, 2007).

In phase 2 metabolism the intermediary products, gained from phase 1 reactions, are conjugated with certain endogenous compounds increasing their water solubility, then they can easily be eliminated from the body. Amongst others, the most important conjugation enzymes are UDP-glucuronyl transferases, glutathione-S-transferases, methyltransferases and N-acetyltransferases (Temesváry, 2012).

Cytochrome P450 enzymes play an important role in phase 1 metabolism. The members of the CYP enzyme superfamily are haemoproteins. The name “cytochrome P450” derives from the phenomenon that the complex of their reduced form with carbon monoxide has an absorption maximum at 450 nm. Within the cell they are localized primarily in the membranes of the endoplasmic reticulum, therefore, they can be found in the microsomal fraction after differential centrifugation.

Their function is associated with the microsomal electron transport. CYP enzymes convert xenobiotics into a form ready for conjugation in phase 2 metabolism, catalysing reactions such as hydroxylation, N-dealkylation or oxidative deamination. The most common reaction is hydroxylation, when CYP enzymes incorporate a hydroxyl group into a lipophilic molecule by binding molecular oxygen. Electrons are provided for the reaction by

NADPH+H$^+$. The reduction is catalyzed by the FAD and FMN containing enzyme NADPH+H$^+$ cytochrome P450 reductase (Anzenbacher and Anzenbacherová, 2001) (**Fig. 3**).

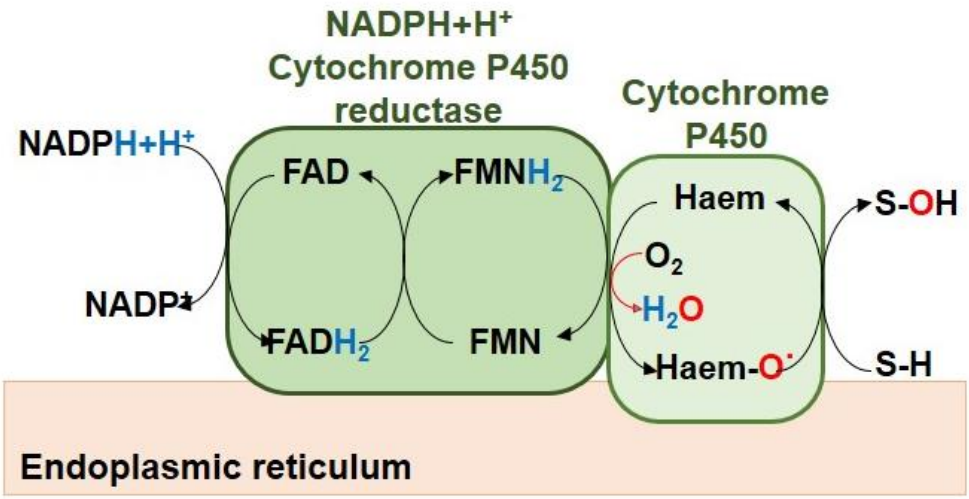

Figure 3. Principle of operation of cytochrome P450 (CYP) enzymes (based on: http://watcut.uwaterloo.ca/webnotes/Metabolism/drugsCypActionMode.html)

The CYP superfamily is currently divided into 11 clans in vertebrates, but the number of families within clans is growing steadily owing to the novel scientific results (Nelson, 2013). Out of several CYP families the first three (CYP1, CYP2 and CYP3 families) take part in drug metabolism mostly (Guengerich et al., 1986). Families can be divided into subfamilies and isoenzymes, based on their substrate specificities.

The members of the CYP1 family can be found predominantly in extrahepatic tissues, mainly in lungs, but after appropriate induction, they can be detected in the liver and the proximal section of the gastrointestinal tract as well (Doherty and Pang, 1997; Temesváry, 2012). CYP2 enzymes are expressed in the liver in large quantities; they include the most subfamilies. From the drug metabolism point of view, CYP3 family, especially CYP3A subfamily has the highest relevance. The highest CYP3A activity can be measured in the liver; however, it can be detected in the small intestinal mucosa as well (Doherty and Pang, 1997). Notwithstanding that the role and significance of different CYP families are nearly identical in various animal species (including human), remarkable interspecific differences could be detected in the expression and activity of certain subfamilies (Martignoni et al., 2006).

The presence and role of CYP enzymes have been mainly studied in mammals (in most cases human or rodents); however, little data are available on CYP enzymes in avian species. Some subfamilies have been characterized in chicken, and some homologies with mammalian CYPs have already been described (Osseleare et al., 2013). For example, avian

CYP1A4 and CYP1A5 can be equivalent to mammalian CYP1A1 and CYP1A2, respectively. In the case of CYP2 family, similarity was proven between chicken CYP2H1, CYP2H2 and human CYP2C (Antonovic and Martinez, 2011), while chicken CYP2C45 has a homology of 56% with human CYP2H1 (Baader et al., 2002). Human CYP3A4, the most important enzyme of CYP3 family in human is of 60% homologous to chicken CYP3A37 (Ourlin et al., 2000).

The action of CYP enzymes can be influenced on several levels. On transcriptional level, the gene expression of CYP1 enzymes is mostly regulated by the aryl hydrocarbon receptor, in the case of CYP2 and CYP3 families it happens mainly by certain nuclear receptors (Pavek and Dvorak, 2008). On posttranslational level, they could be acetylated, glycosylated or deaminated (Benkő, 2008).

3.2.2. The role of the intestinal tract in biotransformation

Liver is known to be the most important site of the first-pass metabolism of xenobiotics, which determines the quantity of them being able to enter the systemic circulation; however, apart from the liver, small intestinal mucosa could have a great significance as well (Pavek and Dvorak, 2008).

As compounds absorbed from the gastrointestinal tract are transported to the liver via portal circulation, the biotransformation activity of these two organs (gut and liver) can complete each other (Lin et al., 1999). Although, the total CYP enzyme activity (thus the drug metabolizing capacity) of liver is higher, intestinal mucosa as a first metabolic barrier can determine the quantity of xenobiotics reaching the liver (Peters and Kremers, 1989; Lin et al., 1999) (**Fig. 4**).

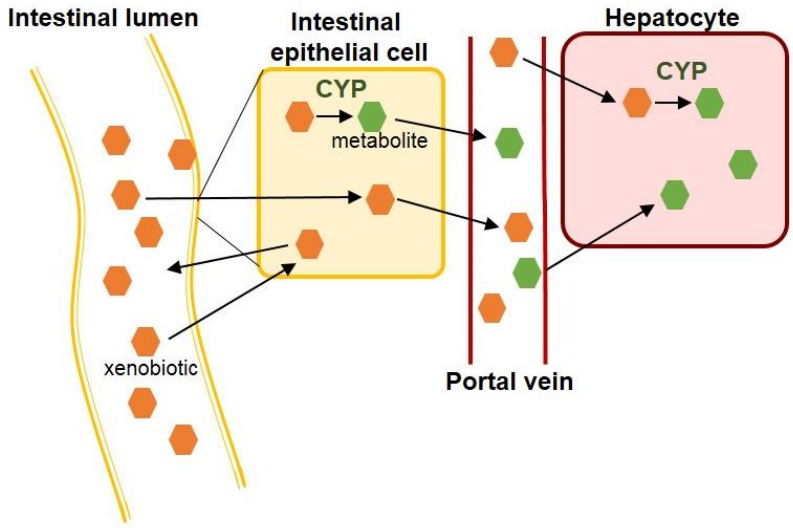

Figure 4. The role of intestinal epithelium in biotransformation (based on: Page et al., 2005)

The primary role of small intestine is the absorption of nutrients; however, the intracellular brush border enzymes are able to metabolize some of the xenobiotics getting into the enterocytes from the lumen (Doherty and Pang, 1997). Therefore, they can basically influence the bioavailability of certain molecules (Doherty and Pang, 1997; Lin et al., 1999).

Due to the limited quantity of the transport proteins in the intestinal mucosa, they can also determine the bioavailability of orally consumed xenobiotics (Tsui and Tamai, 1996). The intestinal absorption occurs via paracellular or transcellular routes paralelly. Paracellular processes are more dominant in case of large amount of intestinal contents, and transcellular processes are more prevalent when the intestines are empty (Zhou, 1999). Concerning bioavailability, transcellular way has higher relevance, as enzymes taking part in the metabolism of xenobiotics are localized inside the epithelial cells, so they only can exert their action if the substrate enters the cell.

Besides the role of intestinal transport proteins affecting the absorption in the gastrointestinal tract, xenobiotics can also be excreted into the lumen (efflux), especially by active transport. The most important efflux transport proteins are the p-glycoproteins, which are expressed on the MDR1 (multidrug resistance 1 or ATP binding cassette subfamily B member 1 [ABCB1]) gene (Goldstein, 1992). They are localized primarily in the apical membrane of the villi in the brush border. The abundance of them increases along the intestine from the duodenum towards the ileum (Hebert, 1997; Lin et al., 1999). P-glycoproteins are able to pump certain molecules from the intestinal epithelial cells back to the lumen, generating recirculation. Consequently, drugs (or other xenobiotics) can enter the epithelial cell again, increasing the chance of enzymatic transformation (Korals et al., 1992). Although the activity of detoxifying enzymes is lower in the small intestines than in the liver, this can be compensated by the recirculation of the substrates, thereby the role of the gastrointestinal tract in the regulation of bioavailability could be comparable to the role of liver, especially in the case of orally administered compounds (Benet et al., 1996). Nevertheless, not only the bioavailability of the orally applied drugs could be affected by the biotransformation system of the intestinal mucosa (Doherty and Pang, 1997; Lin et al., 1999), but also the intraperitoneally and even intravenously administered drugs can reach the metabolizing enzymes of small intestines through the systemic circulation (Bonkowsky et al., 1985).

In the intestinal mucosa xenobiotics are primarily metabolized by the CYP enzymes associated to the endoplasmic reticulum of the epithelial cells. CYP enzymes, similarly to the p-glycoproteins, are expressed in the apical part of the villi (Hoensch et al., 1976; Murray et al., 1988).

CYP enzymes and p-glycoproteins work coordinately, interacting with each other (Benet et al., 1996). Expression rate of CYP enzymes is not homogenous in the intestines, their expression decreases from the duodenum toward the ileum. However, the expression of p-glycoproteins increases along the intestine from the duodenum toward the ileum (Peters and Kremers 1989; Paine et al 1997; Lin et al., 1999), therefore CYP enzymes and p-glycoproteins can influence each other to varying extent. As in the distal intestinal regions there is a lower quantity of CYP enzymes, they can be easily saturated by the substrate. In this case, despite of the intensive circulation maintained by the greater amount of p-glycoproteins, the intensity of biotransformation cannot rise above a given level. Consequently, the proximal region of the small intestine has a major role in the regulation of bioavailability. Even though the activity of intestinal CYP enzymes and p-glycoproteins seem to be closely related, until now there is no study which confirms the correlation between the expressions of the two protein families (Lown et al., 1997; Richter et al., 2004).

The majority of the hepatic CYP isoenzymes can be found in the small intestinal mucosa as well (Paine et al., 2006), but in most cases they are expressed in a smaller quantity (Lin et al., 1999). Concerning recent studies, the results are partly contradictory. For example, CYP1A1 was detected from the duodenum in rat, but from none of the intestinal regions it human (Lin et al., 1999). CYP2C and CYP2D play a pivotal role in the liver and can also be found in the intestinal mucosa of both rat and human; however, with much less protein concentration (Lin et al., 1999). The most important and most expressed isoenzyme of the intestinal biotransformation is CYP3A (Watkins et al., 1987; McKinnon et al., 1995). In some studies – carried out mainly in rat or in human – its protein expression level was lower in the intestine than in the liver (Lin et al., 1999), while some other experiments had opposite results (Watkins et al., 1987). Its metabolic activity (primarily in case of oral xenobiotic administration) was also remarkable, in some cases it may be of the same magnitude as the hepatic activity (van Herwaarden et al., 2009; Leoni et al., 2012). In addition to the differences mentioned above, interestingly the regulation of hepatic and intestinal CYPs seem to differ from each other. In a human erythromycin breath test, while changes in the protein expression and the enzyme activity of CYP3A in small intestines were strongly correlated, such a relationship could not be detected in the liver (Lown et al., 1994).

3.2.3. Influencing the intestinal biotransformation

Bioavailability of drugs, absorbed from the gastrointestinal tract, can be influenced by several factors. The environment of the xenobiotics in intestinal lumen, i.e. the intestinal content, plays a significant role: bile acids, which enter the small intestine, can affect the absorption by changing the water solubility of fat; further, large amount of bicarbonate anions

can also control it by influencing the pH of the intestinal lumen (Doherty and Pang., 1997). The transit time depending on the peristalsis of the intestinal tract, the permeability of the epithelial cell membrane and the nature of the transported molecule can also influence absorption. There is always a constant, non-moving aqueous layer along the brush border (Lin et al., 1999). In the case of lipophilic molecules, the limiting factor is the thickness of this aqueous layer, while in the case of hydrophilic molecules the permeability of the membrane-forming lipid bilayer influences absorption (Komiya et al., 1980). If, as a result, the absorption of drugs is modified, these above-mentioned factors may also have an indirect effect on their metabolism. The gut microbiota can influence the permeability of the intestinal wall by bacterial toxins as well, and as most bacteria of the gut microflora are able to metabolize some xenobiotics, they could have an impact on the biotransformation too (Bezirtzoglou, 2012).

Drug metabolism can also be affected by the blood supply of the mucosa (Lin et al 1999). High blood flow rates result in more intensive transport, so as the transit time shortens, the metabolizing enzymes have reduced access to the drugs, thereby their bioavailability increases (Granger et al., 1980). Circulation of the intestinal tract is greatly influenced by its fullness. After feeding, when the amount of ingesta grows, intestinal circulation is accelerated, which decreases the efficacy of biotransformation in the small intestine (Bond and Levitt, 1979). Although the intensity of blood flow can remarkably affect the biotransformation activity of the intestinal mucosa, it needs to be taken into consideration that only 60-70 % of the total amount of circulating blood can reach the enterocytes (Micflikier et al., 1976; Mailman, 1978).

Similarly to the liver, the expression and activity of CYP enzymes that play a primary role in the metabolism of xenobiotics, can be controlled by several factors in the intestinal mucosa as well. In most cases they can be stimulated by the xenobiotics as substrates themselves (Doherty and Pang, 1997), but numerous other dietary factors may also have an impact on their biotransformation activity. Continuous consumption of grapefruit in human leads to a relevant decrease in the expression of enteric CYP3A4 and CYP3A5 proteins (Lown et al., 1997), and some flavonoids affect the activity of small intestinal CYP1 enzymes (Sergent et al., 2009). Thus, dietary supplements (such as herbs, vitamins, minerals, amino acids) may also influence drug metabolism (Ohnishi and Teruyoshi, 2004), or concomitant medicines can significantly alter the efficacy of each other (Pavek and Dvorak, 2008).

Given that the amount of CYP enzymes is lower in the small intestine compared to the liver, they could be saturated even at a lower substrate concentration. If the administered dose of the drug exceeds this concentration, the intestinal mucosa will not be able to metabolize more substrates, thus they will be forwarded to the liver by the portal flow. Therefore, while at low doses intestinal epithelial cells can metabolize a significant proportion

of xenobiotics, at higher doses the role of hepatic biotransformation becomes pivotal (Bonkovsky et al., 1985; Lin et al., 1999). The route of administration could also have great importance. Inducers administered intravenously affect mainly the activity of hepatic enzymes, while orally applied agents influence mostly the activity of intestinal enzymes. In a study of McDanell and McLean (1984) in rats, orally applied β-naphthoflavone, a CYP inducer commonly used in research, increased intestinal CYP1A activity both at lower and higher doses, while only the higher dose had a detectable effect in liver (Lin et al., 1999). However, the same activator had an identical effect in both organs during intraperitoneal administration (Zhang et al., 1997).

Due to its multiple function, the role of the intestine in terms of the bioavailability of certain compounds is difficult to determine exactly, as in addition to the metabolic activity of the epithelial cells, absorption and efflux have a great importance as well. This could explain the significant interindividual, and even intraindividual temporal differences, especially in case of orally administered xenobiotics (Doherty and Pang, 1997). The biotransformation activity of small intestines can be influenced on several levels by different factors; therefore, although it is very difficult to determine the extent of its effects, it can be never negligible when studying the bioavailability of certain drugs.

3.3. Carbohydrate metabolism and insulin homeostasis in birds

Carbohydrate metabolism of birds differs from that of mammals in many aspects. It is well known that the blood sugar level of birds – with interspecific variance – is significantly higher than what is typical in mammalian species; further the extrahepatic tissues are less sensitive to the blood glucose reducing effect of insulin compared to the mammals (Dupont et al., 2004). Decreased insulin sensitivity, which occurs primarily in skeletal muscle and adipose tissues, could be a direct explanation for the physiologically higher blood sugar concentration at a molecular level; however, the functional relevance of hyperglycemia is not yet fully understood (Braun and Sweazea, 2008). The phenomenon was traditionally explained with flying lifestyle; however, it is contradicted by the fact that oxidation of glucose derived from the blood or glycogen store covers the energy demand of short-haul flights only. In the case of extended flight, the β-oxidation of fatty acids and in some species the degradation of nitrogen-free carbon chains of amino acids provide the required amount of energy (Jenni and Jenni-Eiermann, 1998; Klaassen et al., 2000). Accordingly, migratory birds accumulate fat stores before migration, and during the extended flight a high degree of lipid mobilization can be observed (Davis et al., 2005), which is often associated with ketone body production (Jenni-Eiermann et al., 2002).

3.3.1. The absorption and turnover of glucose

Similarly to the processes in mammals, glucose uptake of avian cells happens by facilitated passive transport using the glucose transporter (GLUT) transmembrane proteins (Braun and Sweazea, 2008). Concerning GLUT proteins, the most important trait in mammalian species is that the insulin dependent GLUT-4, which is expressed in the heart muscle, the skeletal muscle and the adipose tissues of mammals, cannot be found in birds (Dupont et al., 2004). The other main type of transporters, which is required for glucose turnover, is the group of the sodium and glucose co-transporters (SGLTs) that performs secondary active transport. These proteins are also of great importance in birds, especially SGLT-1, which is expressed on the apical membrane of enterocytes.

Absorption of glucose from the gastrointestinal tract occurs along two pathways in both birds and mammals: mainly by the SGLT-1 and GLUT 2 transporter proteins expressed in the enterocytes, and in a lesser extent by paracellular diffusion through the intercellular spaces. The SGLT-1 can be found on the apical side of the intestinal epithelial cells, where, determined by the electrochemical gradient of sodium, it transports glucose into the cell

coupled to two sodium ions. The electrochemical gradient of sodium is maintained by the sodium-potassium ATPase which is localized on the basolateral side of the cells, meanwhile GLUT-2 (on the same basolateral side) transmits glucose from enterocytes to portal capillaries. SGLT-1 has been detected from chicken small and large intestines (Garriga et al., 1999), in the latter its function is presumably the resorption of glucose not resorbed by the kidneys. More than 30% of the absorbed glucose is converted into lactic acid through anaerobic glycolysis in the enterocytes, and enters the circulation in this form (Braun and Sweazea, 2008).

In most bird species, just like in mammals, liver is the main organ for maintaining blood sugar level. If glucose absorption from the intestine is reduced, the liver gains the required amount of glucose (necessary to maintain the physiological blood glucose concentration) from the degradation of glycogen store and from gluconeogenesis. Thus, in case of starvation or carbohydrate-free diet, the glycogen content of the liver is reduced, but the high blood sugar level does not change (Tinker et al., 1986; Braun and Sweazea, 2008).

The most important glucose transporter in the liver is GLUT-2. In addition, GLUT-1, GLUT-3 and GLUT-8 proteins have been detected at mRNA levels, but only in small amounts, so their role is presumably negligible (Carver et al., 2001; Braun and Sweazea, 2008).

Among the extrahepatic tissues, the skeletal muscle utilizes a great amount of glucose, and plays major role in the glucose homeostasis of birds. Glucose serves as an energy source in muscle cells, it can enter glycogenesis, and it can also be the precursor of the amino acid production that is essential for intensive protein synthesis for rapid muscle mass gain (Braun and Sweazea, 2008). Avian skeletal muscle contains less glycogen compared to mammals, however it is still significant, and its degradation increases in case of starvation or physical stress (Tinker et al., 1986; Braun and Sweazea, 2008).

In the glucose uptake of muscle cells in birds, just like in mammals, GLUTs play the primary role. However, a significant difference is that the insulin-dependent GLUT-4 transporter, which has a dominant function in mammals, is missing in birds. Its role is presumed to be partially fulfilled by the recently discovered, also insulin-sensitive GLUT-12 in chicken (Coudert et al., 2015). Nevertheless because of the high rate of expression of the insulin independent GLUT-1 and GLUT-3, insulin-stimulated glucose uptake is of minor importance overall (Braun and Sweazea, 2008). The skeletal muscle of birds can be considered insulin-resistant in comparison to mammals, due to certain specificities of the insulin signaling and the different basal activity of some members of the pathway (Dupont et al., 2004; 2009).

The adipose tissue plays an important but a less significant role in the regulation of glucose turnover compared to skeletal muscle. Due to the lack of GLUT-4 the adipocytes

transport glucose through GLUT-1 and GLUT-3 (Braun and Sweazea, 2008), that serves as a precursor for fatty acid and lipid synthesis. Similarly to the muscle, the insulin sensitivity of adipose tissue is remarkably lower than that in mammals (Dupont et al., 2012), but glucose uptake can be slightly increased by insulin stimulation (Braun and Sweazea, 2008).

Chicken erythroblast cells have been shown to express GLUT-1 in large quantities, but this ability disappears during the maturation to erythrocyte. In addition, the quantity of GLUT-3 also decreases during maturation, so the exact mechanism of glucose uptake of circulating red blood cells is not fully understood (Johnstone et al., 1998; Braun and Sweazea, 2008). In heart muscle, similarly to the skeletal muscle and adipose tissue, there is no detectable GLUT-4 expression (Carver et al., 2001; Seki et al., 2003), but the increased expression of GLUT-1 (and the presence of GLUT-3 and GLUT-8 in lower extent) can compensate it, ensuring the continuous glucose supply of myocardial cells (Braun and Sweazea, 2008).

Like in mammals, energy demand of avian nervous system is largely covered by the aerobic oxidation of glucose taken up from blood (Sepherd and Kahn, 1999). Accordingly, GLUT-1 and GLUT-3 transporters are highly expressed in the brain and provide continuous insulin-independent glucose uptake of neurons (Carver et al., 2001). Most bird species have an organ called *corpus gelatinosum* located in the rhomboidal sinus on the dorsal side of the lumbosacral spine (Möller and Kummer, 2003; Braun and Sweazea, 2008). This organ of special position and function stores significant amount of glycogen, the glucose needed for its synthesis is taken up by the GLUT-1 protein, so the glycogenesis in this site cannot be stimulated by insulin. The mobilization of its glycogen store is also not affected by insulin, the mechanism of regulation is still unclear. Due to the anatomical position of the *corpus gelatinosum*, it breaks the blood-brain barrier and is presumed to play a significant role in the regulation of glucose turnover in the central nervous system, however its exact function has not been clarified yet (Möller and Kummer, 2003; Braun and Sweazea, 2008).

Despite the high glucose concentration in plasma, the urine of birds physiologically does not contain any glucose, as the glucose resorption capacity of the proximal tubules is particularly significant, about 4-5 times higher than in mammals (Braun and Sweazea, 2008). The role of GLUTs-1, -2, and -3 in the tubular resorption of glucose has been confirmed in chicken, but the expression of SGLT and the exact subcellular localization of certain transporters have not been studied yet (Braun and Sweazea, 2008).

Overview of expression and insulin dependence of glucose transporters in mammals and birds can bee seen in **Table 1.**

Because of high degree of renal gluconeogenic activity, kidneys can be considered as an important source of blood sugar in birds. In case of starvation up to 30% of blood glucose derives from the renal gluconeogenesis (Tinker et al., 1986).

Table 1. Overview of expression and insulin dependence of glucose transporters in mammals and birds

	Mammals	**Birds**
GLUT-1	brain, erythrocyte,	brain, adipose tissue, *Corpus gelatinosum*
GLUT-2	liver, kidney, enterocyte	liver, kidney, enterocyte **Partly insulin dependent**
GLUT-3	brain, placenta	brain **Partly insulin dependent**
GLUT-4	heart muscle, skeletal muscle, adipose tissue **Insulin dependent**	Does not exist
GLUT-8	adipose tissue	adipose tissue
GLUT-12	heart muscle, skeletal muscle, **Insulin dependent**	heart muscle, skeletal muscle, **Insulin dependent**

3.3.2. The endocrine regulation of carbohydrate metabolism

In the regulation of carbohydrate metabolism, pancreatic hormones play the most important role. The pancreas of birds contains α, β, δ and F islands. The α and β cells produce glucagon and insulin, the δ cells are responsible for the production of somatostatin and the F-cells for the production of avian pancreatic polypeptide (Braun and Sweazea, 2008). Concerning the tissue of pancreas, it can be stated that the concentration of insulin is significantly lower than that of glucagon.

Most studies in birds have shown that insulin has little effect on changes in blood sugar level and has quite limited impact on the glucose uptake of tissues (Tokushima et al., 2005). Plasma insulin concentration in birds is about 10% of that in mammals which can be in connection with the lower number of β cells and lower concentration of insulin in the pancreas (Dupont et al., 2004). Insulin production and release in the pancreas of birds in contrast to mammals is quite resistant to the stimulating effect of glucose (Hazelwood, 1973). However, tolbutamide increases the insulin concentration and decreases the glucose concentration of blood plasma even in birds. This molecule is known to be an agonist of the voltage-dependent potassium channels that is a key protein for insulin delivery in mammals, so based on its corresponding final effects, the mechanism of pancreatic insulin delivery is presumably similar in birds to that in mammals (Danby et al., 1982; Tinker et al., 1986).

Glucagon concentration in the pancreas is about 8-10 times higher in chicken, than in mammals per unit weight. Like in mammals, exogenous glucose stimulus significantly

reduces glucagon-release from the pancreas (Ruffier et al., 1998). In contrast to insulin, chicken is very sensitive to increased blood glucagon concentrations and responds with increased blood glucose, triglyceride, glycerol and free fatty acid concentrations. Glucagon provokes hyperglycemia by the stimulation of glycogenolytic and gluconeogenic activity of liver also in birds (Hazelwood, 1973).

Somatostatin inhibits the secretion of both glucagon and insulin in birds by a similar mechanism to that in mammals (Sakurai et al., 1974; Braun and Sweazea, 2008). Based on studies in chicken, it is very likely, that the inhibitory effect of glucose on glucagon release occurs via the local effect of somatostatin (Braun and Sweazea, 2008).

The main function of avian pancreatic polypeptide is the stimulation of lipolysis and the inhibition of insulin release. Further, the glycogen stores of the liver are depleted upon release, suggesting that this hormone plays an important role in the regulation of glucose metabolism indirectly (Hazelwood, 1973).

Adrenalin, produced in the adrenal medulla, stimulates glycogenolysis in the skeletal muscle and in the liver, thus it increases blood glucose concentration in birds (Picardo and Dicson, 1982). However, according to certain results, its hyperglycaemic effect is less pronounced than in mammals, hence glucagon could be the main mediator of stress induced blood glucose elevation in birds (Thurston et al., 1993).

Glucocorticoids secreted by the cells of *zona fasciculata* of the adrenal cortex, increase the intensity of gluconeogenesis in chicken, which results in prolonged increase in plasma glucose concentration, similarly to mammals, however no such effect of glucocorticoids was detectable in turkey (Scanes and Braun, 2013).

Growth hormone has a particular importance in the endocrine system of the intensively growing poultry. In broilers, it stimulates the incorporation of amino acids into proteins and simultaneously inhibits gluconeogenesis. In addition, it is considered to be one of the main stimulators of lipolysis, but it only slightly elevates blood sugar level (Scanes and Braun, 2013).

Furthermore, incretin hormones produced by the enteroendocrine L and K cells, namely GIP (glucose-dependent insulinotropic peptide, gastric inhibitory polypeptide) and GLP-1 (glucagon-like peptide 1) also play an essential role in the endocrine regulation of carbohydrate metabolism and in the stimulation of pancreatic hormone secretion in birds (Litwack, 2010). Both hormons bind to the receptors on the surface of β cells, thus they increase the proliferation of β cells in the pancreas and stimulate insulin release (Baggio and Drucker 2007). However, Watanabe et al. (2014) found that in chicken, GLP-1 receptors are expressed on the surface of somatostatin producing δ cells of the pancreas instead of the β cells. Based on these findings, it can be supposed, that GLP-1 stimulates insulin release through a signaling pathway different in chicken compared to mammals. Incretins can

influence other tissues as well. GIP increases fat storage and stimulates ossification, and both GIP and GLP-1 decelerate the emptying of the stomach, reduce the secretion of hydrochloric acid and stimulates the feeling of repletion (Baggio and Drucker 2007).

3.3.3. Insulin homeostasis and the insulin signaling pathway

Although avian tissues exhibit much lower insulin sensitivity compared to mammals, insulin can be considered as a major regulator of carbohydrate metabolism and anabolic processes also in birds (Scanes and Braun, 2013). Its secretion and plasma concentration are influenced by a number of factors, including age, nutrition and genetic background (Jozefiák et al., 2010). From a production physiological point of view, it is of paramount importance that insulin, with insulin-like growth factors (IGF-1 and -2) together stimulates growth and improves animal feed utilization efficiency (Gao et al., 2007; Braun and Sweazea, 2008).

The multiple effects of insulin are exerted through the cascade of insulin signaling pathway, which is well described in mammals (**Fig. 5**). The signaling pathway begins by the ligation of insulin receptor α subunit (IRα) with insulin, whereby insulin receptor β subunit (IRβ) is getting autophosphorylated (White and Kahn, 1994). Due to its tyrosine kinase activity, insulin receptor substrate 1 (IRS-1) is phosphorylated as well, and activates phosphatidylinositol-3-kinase (PI3K), which is responsible for the production of phosphatidylinositol triphosphate (PIP_3). Increasing intracellular concentration of PIP_3 leads to the activation of a variety of protein kinases such as protein kinase B (PKB or Akt) and protein kinase C (PKC). PKC is involved in the translocation of GLUT-4 containing intracellular vesicles to the plasma membrane, hence it stimulates GLUT-4 mediated glucose uptake. Akt activates mTOR, which subsequently increases the phosphorylation of its targets, such as that of the initiation factor 4E-binding protein (4E-BP1) and S6 kinase (S6K), being involved in the enhancement of protein synthesis (Dupont et al., 2009). Another pathway, that is regulated by Akt and mediated by phosphodiesterase and protein-kinase is responsible for enhancing glycogenesis, glycolysis and lipogenesis (White and Kahn, 1994). Growth promoting effect of insulin and IGFs, in addition to the mTOR pathway, is primarily achieved through various transcription factors, such as MAP kinase pathway, or directly by the Akt internalized into the nucleus (White and Kahn, 1994).

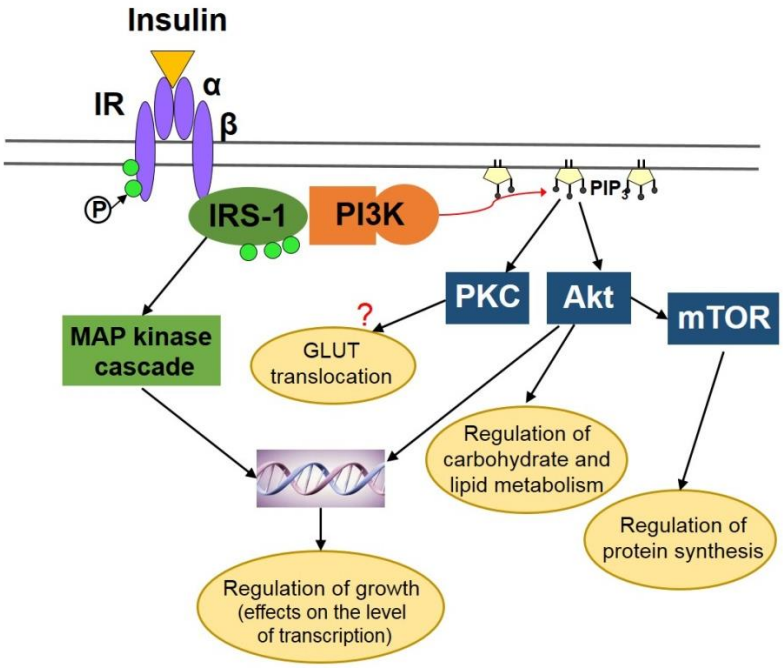

Figure 5. Brief summary of the insulin signaling pathway (Mátis et al., 2014)

The structure and function of insulin receptors in chicken are quite similar to those of mammals (Dupont et al., 2009). In chicken skeletal muscle, the number of IR proteins is the same, however the amount of IRS-1 and PI3K proteins is significantly higher than in rat (Dupont et al., 2004). In contrast to the skeletal muscle, the physiological protein levels of IR and IRS-1 are found to be lower in the adipose tissues of chicken than in rat (Dupont et al., 2012). The phosphorylation ratio of IRβ in chicken muscle cells is twice as high as in rat under the same conditions; however, there is no difference in the degree of tyrosine phosphorylation of IRS-1 between the mentioned species (Dupont et al., 2004). Furthermore, the physiological activity of PI3K is about thirty times higher in chicken skeletal muscle than in rat (Dupont et al., 2004). Due to the physiologically increased phosphorylation level of IRβ and the higher basal activity of PI3K in avian skeletal muscle, the insulin signaling pathway could be considered as it is physiologically in an activated form. Presumably this feature could be the explanation for the relative resistance of avian muscle to insulin, as the activation of the phosphorylation cascade with physiological insulin plasma concentrations is not possible (Dupont et al., 2004). It might be in connection with these findings that in chicken skeletal muscle and adipose tissue the degree of phosphorylation of IRβ and IRS-1 is not affected by fasting, but in chicken liver and in all the tissues of rat the phosphorylation of these proteins significantly decreased after fasting (Dupont et al., 2009; 2012). In addition, the alteration in plasma insulin concentration has no impact on the PI3K activity of the muscle cells but affects it in the hepatocytes in chicken (Dupont et al., 2009). PKC is responsible for the GLUT-4 translocation, hence the regulation of glucose uptake in

mammals. Due to the lack of GLUT-4 the role of PKC in the insulin signaling pathway is not fully understood in chicken. The recently described GLUT-12 expressed in the skeletal muscle of chicken is also insulin-dependent (Coudert et al., 2015); however, it has not been confirmed yet whether the regulation of this transporter is also mediated by PKC.

3.3.4. Significance of insulin homeostasis in growth performance of chicken

Although, due to the described differences in the insulin signaling pathway, the extrahepatic tissues of birds exhibit reduced physiological insulin sensitivity, insulin is considered to be one of the main regulators of growth in chicken (Jozefiák et al., 2010). This effect of insulin can be manifested in several ways. In one hand, it enhances protein synthesis by the mediation of mTOR, on the other hand, stimulates the MAP kinase cascade together with IGF-1 and IGF-2 growth factors. Since the PI3K-induced Akt can enter the nucleus as a transcription factor, this pathway may also affect growth. It has been reported that Akt stimulates proliferation of myoblasts, but inhibits their differentiation, thus contributing to the selective increase in muscle mass in chicken. Further, for this reason, insulin given to day-old chicks improved future growth performance of animals through regulation of the MyoD gene expression (Sato et al., 2012).

Increasing the insulin sensitivity of certain tissues, but primarily that of skeletal muscle, can lead to better production parameters, more efficient feed utilization and more intense muscle mass development. It has been confirmed by Jozefiak and coworkers (2010) that the simultaneous utilization of carbohydrase and phytase enzymes as feed additives significantly enhances the expression of insulin receptors and improves feed utilization in broiler chicken.

Based on the mentioned results, it can be stated that influencing insulin homeostasis and the insulin signaling pathway in chicken by various dietary factors can effectively improve growth performance and feed utilization. Thus, investigation of carbohydrate metabolism, and especially the insulin signaling pathway, is not only of comparative physiological importance, but also of great practical relevance.

4. Significance and aims of the study

Butyrate is a commonly used growth promoter in poultry nutrition. Certain aspects of its wide biological activity are in the focus of recent studies. However, the way and the intensity of its effects may depend on the intestinal and plasma concentration of butyrate, that could be determined by the microbial butyrate production in the caecum and the way, dose and time of oral butyrate application. Therefore, the main purpose of the current PhD work was to study the effects of butyrate in chicken from a complex point of view, from the absorption of butyrate of either endogenous or exogenous origin and including its intestinal and extraintestinal influences. Our results highlight that butyrate of different origin can elicit various biological activities. However, our findings can raise further questions about the exact molecular mechanisms of butyrate action, and thus regarding its effective application in poultry nutrition.

The present PhD work approaches butyrate action partly from practical point of view: to study the effects that could be manifested during the application in poultry farming, and partly from theoretical point of view: to describe the potential mechanisms of butyrate action in a model system To fulfill the above mentioned goals, three main experimental studies were carried out: a long-term – feeding study (Study I.), a medium-term – multiple bolus study (Study II.), and a short-term – single bolus study (Study III.).

In the long-term – feeding study (Study I.) the effects of different application forms (non-protected and protected forms) and doses of butyrate as feed additive were aimed to be compared with those of enhanced microbial caecal butyrate production in broiler chicken. The latter was reached with elevated soluble NSP (non-starch polysaccharide) content and NSP-degrading enzyme supplementation of the wheat-based diet by providing more substrates for the short chain fatty acid producing microbiota. The aim of Study I. was to investigate the intestinal availability, the absorption and the distribution of the various forms of butyrate in the body. In addition, in accordance with the distribution of different application forms, their potential effects on intestinal CYP (cytochrome P450) enzymes and insulin signaling proteins in different organs of chickens were also aimed to be studied. Concerning some earlier results of our research group, butyrate could have an impact on certain hepatic CYP enzymes, however assessing the butyrate-associated changes of small intestinal first-pass drug metabolism, by the monitoring of intestinal CYP activity, may also reflect the possibility of certain pharmacoepigenetic feed-drug interactions, being highly important for food safety and maintenance of animal health as well

Insulin is one of the most important regulatory hormones of the body, as via insulin signaling pathway – beside the regulation of carbohydrate and lipid metabolism – it can influence protein synthesis, growth and proliferation as well. Monitoring the alterations of insulin signaling after dietary butyrate supplementation could highlight the underlying mechanisms beyond growth promoting activity of butyrate. In order to analyse the butyrate-provoked changes in carbohydrate metabolism and insulin homeostasis, plasma glucose and insulin levels were also monitored. In Study I. the effects of butyrate as a feed additive and enhanced microbial caecal butyrate production – that applications can be used in poultry nutrition as well – were studied. Samples were taken from six week old broiler chickens to evaluate the manifested effects of butyrate treatment at the age of slaughtering, to approach the action of butyrate from a practical point of view.

In the medium-term – multiple bolus study (Study II.) broiler chickens, were treated once daily with an intraingluvial non-protected sodium butyrate bolus following overnight feed deprivation for five days. This type of study design in Study II. was aimed to analyse the potential effects of oral butyrate bolus application on insulin signaling in different organs of chickens. Since changes in insulin signaling and carbohydrate metabolism could have the highest impact on the body gain of chicken during the growing period, in Study II. this model system was used at intensively growing three week old broiler chickens to approach butyrate action from a theoretical point of view.

In the short-term – single bolus study (Study III.) a model system was used also: the influence of a single butyrate bolus application with two different doses was investigated. The mechanisms of insulin and - related to it - incretin homeostasis was studied not only in broiler chicken, but in other species too, which could have great importance from comparative physiological point of view as well. Therefore, the main goal of Study III. was to assess the immediate (within one hour) effects of a single intraingluvial non-protected sodium butyrate bolus treatment following overnight feed deprivation on the incretin and insulin secretion in broiler chicken and rabbit. These two species were selected as being potential targets of butyrate administration as a feed additive and also serving as experimental models for birds and mammals. Studying the action of butyrate on the incretin homeostasis may also provide new data regarding the regulatory role of incretins on pancreatic insulin release in different species.

Summarized, the most important aims of this PhD study were:

Ad 1, to study the intestinal availability and absorption of butyrate – originated either from dietary supplementation or produced endogenously by the caecal microflora in chicken.

Ad 2, to investigate the long-term effect of butyrate as dietary supplementation and that of endogenously produced butyrate on the activity of intestinal CYP enzymes in chicken.

Ad 3, (a) to evaluate the long-term effect of butyrate as dietary supplementation and that of endogenously produced butyrate on the expression of certain key protein of the insulin signaling pathway.

(b) to study the medium-term effect of multiple butyrate bolus application on the expression of certain key proteins of the insulin signaling pathway in chicken.

Ad 4, to investigate the short-term effect of single butyrate bolus application on the production of incretin hormones in chicken in comparison with rabbit.

5. Materials and Methods

5.1. Ethic statement

All animal procedures reported herein were carried out in strict accordance with the international and national laws as well as with the institutional guidelines and approved by the Animal Welfare Committee of the University of Veterinary Medicine, Budapest, Hungary (number of permission: 22.1/4719/003/2008). (During the studies, the University of Veterinary Medicine operated under the name of Faculty of Veterinary Science, Szent István University.) Husbandry, experimental procedures and euthanasia were approved by the County Food Chain Safety and Animal Health Directorate of Zala, Hungary (ZAI/100/1361-009/2013). Prior to tissue sample collection, chickens were slaughtered by decapitation in general anaesthesia induced by carbon dioxide or narcotized by an intracoelomal injection of xylazin and pentobarbital mixture to minimize pain.

5.2. Chemicals

All chemicals were purchased from Sigma-Aldrich (Munich, Germany) except when otherwise specified.

5.3. Animals, treatments and sampling – Study I. (Long-term – Feeding study)

Summary of the study design and measured parameters in Study I.

Study design

Groups	n	Study
MB / CTR	22	I/A, B, C
MB / NP B lower	22	I/A, B, C
MB / NP B higher	22	I/A, B, C
MB / PB	22	I/A, B, C
WB / CTR	22	I/A, B, C
WB / NP B lower	22	I/A, B, C
WB / NP B higher	22	I/A, B, C
WB / PB	22	I/A, B, C

Measured parameters

Parameter	n/group	Study
Body weight	22	I/A, B, C
Intestinal butyrate conc.	10	I/A
Plasma butyrate conc.	6	I/B
Plasma glucose, insulin conc.	6	I/C
Intestinal CYP activity	6	I/C
Insulin signaling protein expr.	6	I/C

MB: maize-based diet. **WB:** wheat-based diet supplemented with NSP-degrading enzymes. **CTR:** control group. **NP B lower:** lower dose (1.5 g/kg diet) of non-protected butyrate feed supplementation. **NP B higher:** higher dose (3.0 g/kg diet) of non-protected butyrate feed supplementation. **PB:** protected butyrate (Butipearl® 0.2 g/kg diet) feed supplementation.
A, B, C: subgroups for certain samplings

Day old male Ross 308 broiler chickens (n=176 in total), obtained from a commercial hatchery (Gallus Company, Devecser, Hungary) were housed on wheat straw litter in metal pens at a stocking density of 10 chickens/m^2 under controlled environmental conditions over the entire trial at the Georgikon Faculty of Pannonia University, Keszthely, Hungary. Housing and climatic conditions were adjusted according to the recommendations of the breeder company (Aviagen, 2014): chickens received an artificial lighting regimen starting with 24 h of light period at day 1 of life, then light hours were gradually decreased to 20 hr until Day 8 of life and 16 hr of light were set from Day 9 until Day 42 of life. Temperature was maintained at 33°C for the first 5 days, after gradually reduced until reaching a temperature of 21°C on day 21 of life.

Two different basal diets were applied in all three dietary phases (starter, grower and finisher) of the experiment: a maize-based (**MB**) and a wheat-based (**WB**) diet, the latter with non-starch polysaccharide (NSP)-degrading enzyme (mixture of xylanase and glucanase) supplementation (Axtra XB 201 by Du Pont, Delaware, USA). The soluble arabinoxylan (NSP) contents of maize and wheat were 0.88 and 9.37 mg/g, respectively (measured in the Centre for Agricultural Research, Hungarian Academy of Sciences based on the protocol of Douglas [1981]). The WB diet with higher NSP content supplemented with NSP-degrading enzymes was aimed to provide substrates for the caecal bacterial fermentation in order to enhance caecal butyrate production. All diets were isocaloric, isonitrogenous and were

formulated according to the requirements of broiler chickens (Aviagen, 2014). Ingredients and calculated nutrient contents of the diets are shown in **Table 2**.

Both types of basal diets (MB and WB) were supplemented with two different doses of non-protected butyrate (sodium salt, 1.5 g/kg diet – lower dose or 3.0 g/kg diet – higher dose) or with protected butyrate (Butipearl®, micro encapsulated form, Kemin Industries, Des Moines, Iowa, USA, 0.2 g/kg diet). The lower concentration of non-protected butyrate was set according to the average dose applied in poultry nutrition (Mátis et al., 2013a), while the higher concentration was administered to test the dose-dependency of butyrate activity. Dosage of protected butyrate was determined according to the manufacturer's recommendations. No butyrate was added to the diet of control MB and WB groups.

Based on the feeding regime described above, animals were randomized into eight experimental groups (n=22/group): MB and WB diets with various forms and doses (lower and higher dose of non-protected butyrate [**NP B lower**; **NP B higher**]; protected butyrate [**PB**]) or without butyrate supplementation (controls [**CTR**]). All diets were fed from day 1 to 42, feed and water were provided *ad libitum* during the entire study.

Growth performance of the animals was assessed by recording the body weights of individual chickens on day 1, 10, 24, 35 and 42, respectively; feed intake was measured per pen. Birds were in good health, no signs of illness were observed during the study in any of the experimental groups.

Animals (n=16/group) randomly chosen from experimental groups were slaughtered by decapitation on day 42 in carbon dioxide anaesthesia without any feed deprivation before sampling.

From 10 of these chickens (**I/A**), samples of ingesta were taken from various sections (duodenum, ileum, caecum) of the gastrointestinal tract (n=10/group, **I/A**; **Fig. 6**) and kept at -80°C until further gas chromatographic analysis of intestinal butyrate concentrations.

Blood samples were drawn from the brachial vein of six chickens per group (**I/C**) into heparinized tubes prior to slaughtering on day 42; blood plasma was separated by immediate centrifugation (1000g, 10 min), shock frozen in liquid nitrogen and stored at -80°C until measurement of plasma glucose and insulin levels. Duodenal mucosa, liver, gastrocnemius muscle and subcutaneous adipose tissue samples were also collected from the same animals as used for the blood sampling described above (n=6/group, **I/C**). Regarding the intestinal mucosa samples, the small intestine was removed and cut close to the pylorus and flushed by 20 ml of ice-cold PBS. The proximal duodenum was longitudinally opened 5 cm long on the antimesenteric site, and the mucosa layer was gently scraped with a sterile glass microscope slide. All tissue samples were shock-frozen in liquid nitrogen and kept at -80°C until further examinations on intestinal CYP (cytochrome P450) activity or insulin signaling, respectively.

Another six animals per each experimental group (**I/B**) were narcotized by intracoelomal injection of xylazin (10 mg/kg body weight [BW]) and pentobarbital (40 mg/kg BW) for the assessment of the blood concentration of butyrate also without any feed deprivation before sampling. (A separate set of animals had to be sampled for this part of the trial, because the applied narcotics could have influenced the effects of butyrate on intestinal CYP activity.) Blood samples were drawn from the brachial vein and, after opening the coelom, from different vessels (*v. gastropancreaticoduodenalis* and *v. mesenterica communis*) of the hepatic portal system (**Fig. 6**). Freshly separated plasma samples were shock frozen in liquid nitrogen and stored at -80°C for the measurement of plasma butyrate concentrations.

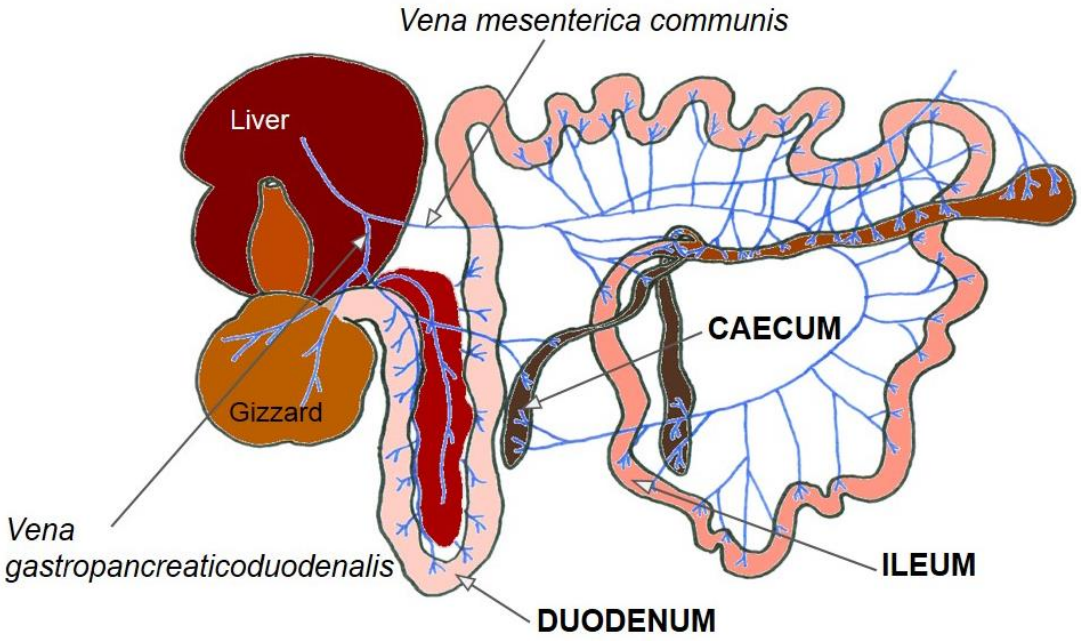

Figure 6. Overview of the hepatic portal system draining the gastrointestinal tract in chicken

Table 2. Ingredients and calculated nutrient composition of experimental diets of chickens in various phases of Study I.

	Starter		Grower		Finisher	
Ingredient (g/kg diet)	Maize-based	Wheat-based	Maize-based	Wheat-based	Maize-based	Wheat-based
Maize	456	172	519	138	564	88
Wheat	0	300	0	400	0	500
Extracted soybean meal	351	272	309	204	333	202
Fullfat soybean	99	162	79	164	4	109
Sunflower oil	45	45	50	50	60	60
L-Lysine	1	2	1	2	0	2
DL-Methionine	4	4	3	3	2	3
L-Threonine	1	1	1	1	0	0
Limestone	18	18	15	16	15	15
MCP	16	15	14	13	13	12
NaCl	2	2	2	2	2	2
$NaHCO_3$	2	2	2	2	2	2
Vitamin-mineral premix*	5	5	5	5	5	5
Axtra XB 201 TPT**	0	0.15	0	0.15	0	0.15
Total	1000	1000	1000	1000	1000	1000
Calculated nutrient composition						
AME_n (MJ/kg)	12.6	12.6	13.0	13.0	13.3	13.3
Crude protein (g/kg)	220.0	220.0	200.0	200.0	190.0	190.0
Crude fibre (g/kg)	31.5	32.4	30.6	31.2	28.7	29.5
Ether extract (g/kg)	85.0	92.6	85.0	97.2	84.5	96.3
Lysine (g/kg)	14.3	14.3	12.4	12.4	11.2	11.2
Methionine (g/kg)	7.2	7.2	6.2	6.2	5.4	5.4
Methionine + Cysteine (g/kg)	11.4	11.3	9.9	10.1	8.9	9.1
Calcium (g/kg)	10.5	10.5	9.0	9.0	8.5	8.5
Available phosphorus (g/kg)	5.0	5.0	4.5	4.5	4.2	4.2

Abbreviations used in Table 2

AME_n: Apparent Metabolizable Energy; MCP: Monocalcium phosphate;

* Vitamin-mineral premix was supplied by UBM Company (Pilisvörösvár, Hungary). The active ingredients of the premix were as follows (per kg of diet):

Vitamin-mineral premix in starter and grower diet - Vitamin A – $2.9x10^6$ IU, Vitamin D_3 – $1x10^6$ IU, Vitamin E – $2x10^4$ mg, Vitamin K_3 – $4.9x10^2$ mg, Thiamin – $9x10^2$ mg, Riboflavin – $2.1x10^3$ mg, Pyridoxin – $1.5x10^3$ mg, Cobalamin – 16,0 mg, Niacin – $8.3x10^3$ mg, Pantothenic acid – $3x10^3$ mg, Folic acid – $2.64x10^2$ mg, Biotin – 30 mg, Betain – 1.34 $x10^5$ mg, Phytase NP – $3x10^5$ mg, Monensin-Na – $2.2x10^4$ mg (only grower), Narasin – $1x10^4$ mg (only starter), Nicarbasin – $1x10^4$ mg (only starter), Antioxidant – $5x10^5$ mg, Zn – $2.5x10^4$ mg, Cu – $4x10^3$ mg, Fe – $1.5x10^4$ mg, Mn – $2.5x10^4$ mg, I – $2.7x10^2$ mg, Se – 54 mg;

Vitamin-mineral premix in finisher feed - Vitamin A – $2x10^6$ IU, Vitamin D_3 – $7.7x10^5$ IU, Vitamin E – $1x10^4$ mg, Vitamin K_3 – $5.4x10^2$ mg, Thiamin – $3.8x10^2$ mg, Riboflavin – $1x10^3$ mg, Pyridoxin – $6.4x10^2$ mg, Cobalamin – 3.8 mg, Niacin – $5.7x10^3$ mg, Pantothenic acid – $2x10^3$ mg, Folic acid – $2.64x10^2$ mg, Biotin – 28 mg, Vitamin C – $8x10^3$ mg, Betain – 3.86 $x10^4$ mg, Phytase NP – $3x10^5$ mg, Antioxidant – $5x10^5$ mg, Zn – $1.92x10^4$ mg, Cu – $1.92x10^3$ mg, Fe – $5.8x10^3$ mg, Mn – $5.8x10^3$ mg, I – $2.4x10^2$ mg, Se – 70 mg.

** The active enzyme ingredients contained in Axtra XB 201 TPT Enzyme comlex were as follows (per kg of diet): endo-1,4-beta-xylanase – 1830 IU, endo-1,3(4)-beta-glucanase – 228 IU. 5.4.

5.4. Animals, treatments and sampling – Study II. (Medium-term – Multiple Bolus study)

Summary of the study design and measured parameters in Study II.

Study design

Groups	n	Study
CTR	10	II
NP B lower	10	II

Measured parameters

Parameter	n/group	Study
Body weight	10	II
Plasma butyrate conc.	10	II
Plasma glucose, insulin conc.	10	II
Insulin signaling protein expr.	10	II

CTR: control group. **NP B lower:** lower dose (0.25 g/kg BW) of non-protected butyrate as a daily oral bolus on days 20-24.

Day old broiler chickens of the Ross 308 strain (mixed gender, n=20 in total) obtained from a commercial hatchery (Bábolna Tetra Ltd., Uraiújfalu, Hungary) were housed on wheat straw litter in metal pens at a stocking density of 10 chickens/m^2 under controlled environmental conditions over the entire trial at the Department of Physiology and Biochemistry, University of Veterinary Medicine, Budapest, Hungary. Housing and climatic circumstances were set according to the Ross technology over the entire trial (Aviagen, 2014). Feed and water were provided *ad libitum*; the applied diet was formulated based on the requirements of the breed (Aviagen, 2014): chickens received an artificial lighting regimen starting with 24 h of light period at day 1 of life, then light hours were gradually decreased to 20 hr until Day 8 of life and 16 hr of light were set from Day 9 until Day 24 of life. Temperature was maintained at 33°C for the first 5 days, after gradually reduced until reaching a temperature of 21°C on day 21 of life. Ingredients and calculated nutrient composition of the diet is shown in **Table 3**.

The body weights of individual chickens were recorded on day 1, 7, 14, 20 and 24, respectively; feed intake was measured per group. Birds were in good health, no differences could be observed regarding growth parameters of different groups during the whole length of the experiment.

On days 20-24, ten animals were treated once daily following overnight feed deprivation with an intraingluvial non-protected sodium butyrate bolus (sodium salt, 0.25 g/kg BW – referring to the average dose used in poultry nutrition; applied in a solution of 0.1 g/ml, 2.5 ml/kg BW, administered by a crop tube **[NP B lower]**. Distilled water (2.5 ml/kg BW) was applied for ten chickens under the same conditions as a control group **[CTR]**. All animals were fasted for additional 2 h after each treatment to enhance butyrate absorption. This already validated model provides a fast short-term butyrate exposure, being suitable for the assessment of *in vivo* metabolic action of butyrate as a descriptive approach (Mátis et al., 2013b).

Animals were slaughtered in carbon dioxide anesthesia by decapitation on day 24, 2 h after the last treatment. Blood samples were drawn from the brachial vein into heparinized tubes directly before decapitation; blood plasma was separated by immediate centrifugation (1000g, 10 min), shock frozen in liquid nitrogen and stored at -80°C until analysis. Tissue samples were taken from the liver, subcutaneous and abdominal adipose tissue, and the gastrocnemius muscle. All tissue samples collected were shock frozen in liquid nitrogen and stored at -80°C until further examinations on insulin signaling.

Table 3. Ingredients and calculated nutrient composition of experimental diets of chickens in Study II.

Ingredient (g/kg diet)	**Value**
Maize	593.7
Extracted soybean meal	310.0
Corn gluten meal	50.0
Limestone	15.0
MCP	18.5
L-Lysine	1.8
DL-Methionine	1.0
NaCl	4.0
Vitamin-mineral premix*	6.0
Total	1000
Calculated nutrient composition	
AME_n (MJ/kg)	11.9
Crude protein (g/kg)	212.0
Crude fibre (g/kg)	25.3
Ether extract (g/kg)	29.4
Ash (g/kg)	65.9
Lysine (g/kg)	11.9
Methionine (g/kg)	4.9
Methionine + Cysteine (g/kg)	8.6
Calcium (g/kg)	11.6
Available phosporus (g/kg)	4.5

Abbreviations used in Table 3

AME_n: Apparent Metabolizable Energy; MCP: Monocalcium phosphate;

* Vitamin-mineral premix was supplied by UBM Company (Pilisvörösvár, Hungary). The active ingredients of the premix were as follows (per kg of diet):

Vitamin A – $2.9x10^6$ IU, Vitamin D_3 – $1x10^6$ IU, Vitamin E – $2x10^4$ mg, Vitamin K_3 – $4.9x10^2$ mg, Thiamin – $9x10^2$ mg, Riboflavin – $2.1x10^3$ mg, Pyridoxin – $1.5x10^3$ mg, Cobalamin – 16,0 mg, Niacin – $8.3x10^3$ mg, Pantothenic acid – $3x10^3$ mg, Folic acid – $2.64x10^2$ mg, Biotin – 30 mg, Betain – 1.34 $x10^5$ mg, Phytase NP – $3x10^5$ mg, Monensin-Na – $2.2x10^4$ mg (only grower), Narasin – $1x10^4$ mg (only starter), Nicarbasin – $1x10^4$ mg (only starter), Antioxidant – $5x10^5$ mg, Zn – $2.5x10^4$ mg, Cu – $4x10^3$ mg, Fe – $1.5x10^4$ mg, Mn – $2.5x10^4$ mg, I – $2.7x10^2$ mg, Se – 54 mg;

5.5. Animals, treatments and sampling - Study III. (Short-term – Single Bolus study)

Summary of the study design and measured parameters in Study III.

Study design

Groups	n	Study
Chicken / CTR	7	III/A
Chicken / NP B lower	7	III/A
Chicken / NP B higher	7	III/A
Rabbit / CTR	7	III/B
Rabbit / NP B lower	7	III/B
Rabbit / NP B higher	7	III/B

Measured parameters

Parameter	n/group	Study
Body weight	7	III/A, B
Plasma glucose, insulin conc.	7	III/A, B
Plasma GIP, GLP-1 conc.	7	III/A, B

CTR: control group. **NP B lower:** lower dose (0.25 g/kg BW) of non-protected butyrate as a single oral bolus. **NP B higher:** higher dose (1.25 g/kg BW) of non-protected butyrate as a single oral bolus
A: chicken experiment, **B:** rabbit experiment

Chicken experiment (III/A)

Day old male Ross 308 broiler chickens (n=21 in total, **III/A**), obtained from a commercial hatchery (Bábolna Tetra Ltd., Uraiújfalu, Hungary), were randomized into 3 experimental groups and were housed on wheat straw litter in metal pens at a stocking density of 10 chickens/m^2 under controlled environmental conditions over the entire trial at the Department of Physiology and Biochemistry, University of Veterinary Medicine, Budapest, Hungary; housing and climatic circumstances were set according to the Ross technology over the entire trial (Aviagen, 2014): chickens received an artificial lighting regimen starting with 24 h of light period at day 1 of life, then light hours were gradually decreased to 20 hr until Day 8 of life and 16 hr of light were set from Day 9 until Day 24 of life. Temperature was maintained at 33°C for the first 5 days, after gradually reduced until reaching a temperature of 21°C on day 21 of life. Feed and water were provided *ad libitum*; the applied diet was formulated

based on the requirements of the breed (Aviagen, 2014). Ingredients and calculated nutrient composition of the diet is shown in **Table 4/A**.

Individual body weights were measured on day 1, 7, 14, 20 and 24, respectively; feed intake was measured per group. Birds were in good health, no differences could be observed regarding growth parameters of different groups during the whole length of the experiment.

On day 24, seven animals per group were treated following overnight feed deprivation with a single intraingluvial non-protected sodium butyrate bolus in two different doses, administered by a crop-tube. A randomly selected group of chickens received a dose of 0.25 g non-protected sodium butyrate/kg BW **[NP B lower]** (referring to the average dose used in poultry nutrition; applied in a solution of 0.1 g/ml, 2.5 ml/kg BW), while a higher dose of sodium butyrate was given to another group of seven animals **[NP B higher]** (1.25 g/kg BW, ingested in a solution of 0.5 g/ml, administered as 2.5 ml/kg BW) to test the dose-dependency of butyrate's action. Physiological saline solution was applied for seven chicks under the same conditions as a control group **[CTR]** (2.5 ml/kg BW).

Blood samples were drawn from the brachial vein of chickens into heparinized tubes as follows: prior to the butyrate exposure (0 min); 10, 30 and 60 min after the bolus treatment. Following immediate centrifugation (1000g, 10 min), plasma samples were shock frozen in liquid nitrogen and stored at -80°C until further processing.

Rabbit experiment (III/B)

Six-week-old male Pannonian Giant rabbits (n=21 in total, **III/B**;obtained from Anas Ltd., Nagyhajmás, Hungary) were randomized into 3 experimental groups and were housed on wheat straw litter in metal pens at a stocking density of 10 rabbits/m^2 at the Department of Physiology and Biochemistry, University of Veterinary Medicine, Budapest, Hungary under controlled environmental conditions according to the requirements of the breed: during the whole experiment rabbits received an artificial lighting regimen 8 h of light period per day. Temperature was maintained at 21°C. Animals had free access to the supplied pelleted diet (**Table 4/B**), hay and drinking water.

The body weights of the animals were measured on the day of arrival, and before treatment. Animals were in good health, no differences could be observed regarding growth parameters of different groups within the whole length of the experiment.

At the age of 7 weeks, after overnight feed deprivation, seven rabbits per group were exposed to a single sodium butyrate bolus given by a gastric tube with the same dosage as administered in the chicken experiment (0.25 g/kg BW **[NP B lower]** and 1.25 g/kg BW **[NP B higher]**), while seven control animals were treated with physiological saline solution (2.5 ml/kg BW **[CTR]**).

Blood samplings were conducted similarly to the chicken study; samples were taken from the marginal ear vein before butyrate ingestion (0 min) and 10, 30 and 60 min after the bolus exposure. Following immediate centrifugation (1000g, 10 min) plasma samples were shock frozen in liquid nitrogen and stored at -80°C until analysis.

Table 4/A. Ingredients and calculated nutrient composition of experimental diets of chickens in Study III/A.

Ingredient (g/kg diet)	**Value**
Maize	593.7
Extracted soybean meal	310.0
Corn gluten meal	50.0
Limestone	15.0
MCP	18.5
L-Lysine	1.8
DL-Methionine	1.0
NaCl	4.0
Vitamin-mineral premix*	6.0
Total	1000
Calculated nutrient composition	
AME_n (MJ/kg)	11.9
Crude protein (g/kg)	212.0
Crude fibre (g/kg)	25.3
Ether extract (g/kg)	29.4
Ash (g/kg)	65.9
Lysine (g/kg)	11.9
Methionine (g/kg)	4.9
Methionine + Cysteine (g/kg)	8.6
Calcium (g/kg)	11.6
Available phosporus (g/kg)	4.5

Table 4/B. Ingredients and calculated nutrient composition of experimental diet of rabbits in Study III/B.

Ingredient (g/kg diet)	Value
Maize grain	320
Wheat bran	200
Soybean meal	180
Wheat straw	120
Lucerne hay	50
Rice bran	50
Linseed straw	28
Sunflower meal	25
Lime stone	20
DL-Methionine	1
NaCl	3
Vitamin-mineral premix*	3
Total	1000

Calculated nutrient composition	
AME_n (MJ/kg)	18.2
Crude protein (g/kg)	170.0
Crude fibre (g/kg)	154.9
Ether extract (g/kg)	29.0
Ash (g/kg)	98.0
Lysine (g/kg)	6.0
Methionine (g/kg)	4.1
Calcium (g/kg)	13.0
Available phosporus (g/kg)	8.6

Abbreviations used in Table 4

AME_n: Apparent Metabolizable Energy; MCP: Monocalcium phosphate;

* Vitamin-mineral premix was supplied by UBM Company (Pilisvörösvár, Hungary). The active ingredients of the premix were as follows (per kg of diet):

Vitamin A – $2.9x10^6$ IU, Vitamin D_3 – $1x10^6$ IU, Vitamin E – $2x10^4$ mg, Vitamin K_3 – $4.9x10^2$ mg, Thiamin – $9x10^2$ mg, Riboflavin – $2.1x10^3$ mg, Pyridoxin – $1.5x10^3$ mg, Cobalamin – 16,0 mg, Niacin – $8.3x10^3$ mg, Panthotenic acid – $3x10^3$ mg, Folic acid – $2.64x10^2$ mg, Biotin – 30 mg, Betain – 1.34 $x10^5$ mg, Phytase NP – $3x10^5$ mg, Monensin-Na – $2.2x10^4$ mg (only grower), Narasin – $1x10^4$ mg (only starter), Nicarbasin – $1x10^4$ mg (only starter), Antioxidant – $5x10^5$ mg, Zn – $2.5x10^4$ mg, Cu – $4x10^3$ mg, Fe – $1.5x10^4$ mg, Mn – $2.5x10^4$ mg, I – $2.7x10^2$ mg, Se – 54 mg;

5.6. Measurement of intestinal butyrate concentrations by gas chromatography (I/A)

Gas chromatographic measurement of intestinal butyrate concentrations was carried out at the Department of Animal Sciences and Animal Husbandry, Georgikon Faculty, University of Pannonia, Keszthely, Hungary.

Intestinal ingesta samples were prepared for gas chromatographic measurements according to the method of Atteh et al. (2008) with slight modifications. Duodenal and ileal samples (1400 µl) were homogenized with 300 µl of 8% HCl solution containing 1 mg/ml pivalic acid inner standard, while 600 µl of 2% HCl (containing 0.1 mg/ml pivalic acid inner standard) was added to 250 µl caecal ingesta. Butyrate concentrations were determined by gas chromatography (TRACE 2000, Thermo Scientific, USA) using a 30 m (0.25 mm inner diameter) fused silica column (Nukol column, Supelco Inc., Bellefonte, PA, USA). The detector type was FID with a split injector (1:50), the injection volume was set as 1µl at 220°C and the detection was performed at 250°C. Helium was used as a carrier gas with the pressure of 83 kPa. A standard mixture of short chain fatty acids, comprised of acetate, propionate, n-butyrate, i-butyrate, n-valerate and i-valerate (1, 4, 8 and 20 mmol/l) as external standards was applied for calibration. The lower limit of quantification was 0.0001 µmol/g ingesta.

5.7. Measurement of plasma butyrate concentrations by gas chromatography (I/A, II)

Gas chromatographic measurement of plasma butyrate concentrations was carried out in the Research Institute for Animal Breeding, Nutrition and Meat Science, National Agricultural Research Center, Herceghalom, Hungary.

0.5 ml of blood plasma samples were supplemented with 0.1 ml of 25% metaphosphoric acid and i-valerate inner standard (1 mg/ml) prior to analysis. Butyrate was separated and quantified by gas chromatography (Shimadzu GC 2010, Japan), using a 30 m (0.25 mm inner diameter) fused silica column (Nukol column, Supelco Inc, Bellefonte, PA, USA). The detector type was FID with a split injector (1:50), the injection volume was set as 1µl at 220°C and the detection was performed at 250°C. Helium was used as a carrier gas with the pressure of 83 kPa. A standard mixture of short chain fatty acids (20 mmol/l), comprised of acetate, propionate, n-butyrate, i-butyrate, n-valerate and i-valerate as external standards was applied for calibration. The lower limit of quantification was 0.037 µmol/l plasma.

5.8. Assessment of intestinal CYP activity (I/C)

Microsomes of small intestinal samples were isolated by multi-step differential centrifugation according to the modified protocol of Stohs et al. (1976). Briefly, duodenal mucosa samples were homogenized in 10 ml of a solution containing 0.25 mol/l sucrose and 5 mmol/l EDTA with Tri-Rhomogenizer (Tri-R Instruments,. Inc., Rockville Centre, USA). Following the centrifugation at 600 g for 1 min for separating the cell nucleus fraction, the supernatant was centrifuged at 1,500 g for 10 min to sediment and discard the brush border fraction. After centrifuging the upper phase at 12,000 g for 15 min to separate the mitochondria, microsomes were subsequently isolated by ultracentrifugation (Beckman Optima xl 90 ultracentrifuge, USA) at 105,000 g for 60 min from the supernatant. Finally, the sediment was resuspended in a 0.1 mol/l phosphate buffer, containing 0.1 mmol/l EDTA and 20% glycerol and sonicated for 10 sec. All homogenization and resuspendation steps were performed on ice, while the centrifugations were done at +4°C. Microsomal total protein concentration was determined with a Bicinchoninic Acid Protein Assay Kit (Fisher Scientific, Rockford, IL, USA) on a microplate in triplicates, using bovine serum albumin (BSA) as a standard.

The activity of small intestinal CYP1A4/5 (equivalent to human CYP1A2), CYP2H2 (equivalent to human CYP2C9) and CYP3A37 (equivalent to human CYP3A4) enzymes was monitored by P450-Glo assays (Promega, Madison, Wisconsin, USA) from isolated duodenal microsomes following the manufacturer's instructions. Equal microsomal total protein contents were ensured for the assay by appropriate dilutions according to the previously determined protein concentrations. The P450-Glo™ assay uses CYP enzyme substrates that are derivatives of beetle luciferin [(4S)-4,5-dihydro-2- (6´-hydroxy-2´-benzothiazolyl)-4-thiazolecarboxylic acid]. The derivatives are converted by CYP enzymes to luciferin products, finally D-luciferin is formed and detected in a second reaction with the Luciferin Detection Reagent. The amount of luminescent light produced in the second reaction is proportional to CYP activity. The luminometric measurements were carried out by Victor X2 reader (Perkin Elmer, Waltham, Massachusetts, U.S.)

5.9. Measurement of plasma glucose (I/C, II, III/A, B), insulin (I/C, II, III/A,B), GIP (III/A,B) and GLP-1 (III/A,B) concentrations

Plasma glucose concentrations were determined by the colorimetric Fluitest Glucose Assay kit (Analyticon, Lichtenfels, Germany). The applied wavelength was 546 nm measured by Multiscan Go ELISA reader (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.).

Insulin, GIP (glucose-dependent insulinotropic peptide) and GLP-1 (glucagon-like peptide 1) concentrations of blood plasma samples were assessed by chicken- and rabbit-specific sandwich ELISA kits, purchased from MyBioSource (San Diego, CA, USA), according to the manufacturer's instructions.

Insulin assays employ the competitive inhibition enzyme immunoassay technique. The microtiter plate has been pre-coated with an antibody specific to insulin. Apart from the standards or samples biotin-conjugated insulin is added to the wells. A competitive inhibition reaction is launched between insulin (standards or samples) and biotin-conjugated insulin with the pre-coated antibody specific for insulin. The more amount of insulin is in the samples, the less antibody is bound by biotin-conjugated insulin. Finally, avidin-conjugated horseradish peroxidase (HRP) and substrate solution is added to the wells and the color develops, where the intensity is opposite to the amount of insulin in the sample.

GIP and GLP-1 assays use double-sandwich ELISA. The pre-coated antibody is a chicken-specific GIP/GLP-1 monoclonal antibody, and the detecting antibody is a polyclonal one with biotin labeling. Samples and biotin labeled antibodies were added into ELISA plate wells and washed out. Then avidin-peroxidase conjugates and substrate were added to the wells. Absorptions were measured by Multiscan Go ELISA reader (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.) on 450 nm. The color depth and the measured target hormone concentrations in the samples are positively correlated.

5.10. Western blot analyses of insulin signaling proteins (I/C, II)

The protein expression of key members of insulin signaling pathway, e.g. that of insulin receptor β (IRβ; I/C, II), atypical protein kinase C zeta (PKCζ; I/C, II), mammalian target of rapamycin (mTOR; I/C, II) and phosphatidyl-inositol-3-kinase (PI3K; II), was assessed from the liver (I/C, II), skeletal muscle (I/C, II), subcutaneous (I/C, II) and abdominal (II) adipose tissue samples by semiquantitative Western blotting in duplicates.

Approximately 50-60 mg tissue samples were ground and homogenized in 1 ml lysis buffer [50 mmol/l HEPES (Carl Roth GmbH, Karlsruhe, Germany), 4 mmol/l ethylene glycol-bis(2-aminoethylether)-N,N,Nc,Nc-tetraacetic acid, 10 mmol/l EDTA, 0.1% Triton X-100, 100 mmol/l β-glycerol phosphate, 15 mmol/l sodium pyrophosphate, 5 mmol/l sodium orthovanadate, 2.5 mmol/l sodium fluoride, protease inhibitor (cOmplete, Mini; Roche Diagnostics GmbH, Mannheim, Germany)]. Protein concentration in the supernatants was measured using Bradford reagent (Serva Electrophoresis GmbH, Heidelberg, Germany). Protein extracts were diluted to 1.5 mg/ml (liver), 0.5 mg/ml (adipose tissues), and 2 mg/ml (muscle) in loading buffer [50 mmol/l Tris-HCl, 10% glycerol, 2% SDS (Serva Electrophoresis GmbH), 0.1% bromophenol blue, and 2% mercaptoethanol; final concentrations] and processed with (mTOR, PI3K) or without (IRβ and PKCζ) heat denaturation at 95°C, for 5 min. Twenty µg protein per lane were separated on a 5% stacking and 8.1% separation polyacrylamide gel by PAGE (60 V for 35 min and 120 V for 90 min, respectively). After tank blotting (Trans-Blot Turbo Transfer System, Bio-Rad, USA; 25V for 20 min), membranes were blocked in 10% (IRβ and PKCζ) or 5% (mTOR, PI3K) fat-free milk-containing PBST (phosphate buffered saline with Tween 20) for 90 min at room temperature. An overnight incubation was performed at 4°C with the following primary antibodies at the given concentrations: IRβ (Santa Cruz Biotechnology, CA, USA), 1:100 in 5% fat-free milk/PBST; PKCζ (Santa Cruz Biotechnology, CA, USA), 1:200 in 5% fat-free milk/PBST; mTOR (Cell Signaling, Frankfurt, Germany), 1:250 in 5% BSA/PBST; PI3K (Cell Signaling, Frankfurt, Germany), 1:200 in 1% BSA/PBST. Primary antibodies were detected by an anti-rabbit secondary antibody coupled with horseradish peroxidase (1:2,000 in 5% fat-free milk/PBST) for 1 h at room temperature. Finally, chemiluminescence was generated with the SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, IL, USA) in case of IRβ and PI3K, whereas with Lumiglo Chemiluminescent Substrate (KPL, Gaithersburg, MA, USA) for PKCζ and mTOR. Chemiluminescence was detected by a ChemiDoc XRS+ system (Bio-Rad Laboratories GmbH., München, Germany). Bands were quantified by densitometry using Image Lab 4.0 software (BioRad Laboratories GmbH, München, Germany), trace quantities were standardized to the Indian Ink stained bands.

5.11. Statistical analyses

Statistical analyses of data were performed with R 2.14.0 software.

In Study I. two-way analysis of variance (ANOVA) was applied to evaluate the main effect (i.e. an effect that is not conditional on other variables) of WB diet compared to MB diet and the main effect of butyrate supplementations. Means of lower dose of non-protected butyrate,

higher dose of non-protected butyrate, and protected butyrate groups were compared to control groups within the two basal diet types (MB and WB) by pairwise comparison. If there were no significant interactions between diet type and butyrate supplementation, P values of the main effects are presented in the text, in case of significant interactions P values of the post hoc tests are presented.

In Study II. one-way ANOVA was used for comparison of results of the treated group with those of controls.

In Study III. relative hormone concentrations (considering the baseline value of each animal at 0 min as 1) were compared by a randomized linear mixed model. The possible interactions between treatment and incubation time were assessed by the model; significant interactions indicated that the appropriate butyrate treatment significantly influenced the time course of the measured parameter at a given time point compared to that of controls. Correlations between the measured parameters were analyzed by Pearson's correlation test. As correlations were calculated from individual data of all time points and groups together, the obtained results were confirmed by further tests (randomized linear mixed model in case of GIP/GLP-1 – insulin correlation and by analyzing time points separately in case of GIP – GLP-1 correlation).

Level of significance was considered as $P < 0.05$. Results are expressed as mean ± SEM.

6. Results

6.1. Study I. Long-term – Feeding Study

6.1.1. Growth performance

Birds were in good health, no signs of illness were observed during the study in any of the experimental groups. None of the orally applied butyrate treatments (non-protected butyrate in lower or higher dose, protected butyrate) had any significant effect on the final body weight of chickens. However, body weights of chickens kept on WB (wheat based) diet were significantly higher compared to MB (maize-based) diet on day 24-36-42 (**Table 5**).

Table 5. Effect of the diet type on mean body weight and mean feed intake

	Maize-based diet	**Wheat-based diet + NSP-degrading enzymes**	**Significance**
Body weight (g)	mean ± SEM		
Day 1	40.5 ± 0.02	39.4 ± 0.02	*
Day 10	244 ± 0.5	262 ± 0.4	NS
Day 24	1139 ± 1.7	1261 ± 1.6	**
Day 36	2229 ± 2.8	2433 ± 2.9	**
Day 42	2728 ± 3.3	3068 ± 3.5	***
Mean feed intake (g/bird/day)			
Day 1-9	27	30	-
Day 10-23	96	102	-
Day 24-35	164	172	-
Day 36-42	149	175	-

Body weight was measured individually on day 1, 10, 24, 36 and 42. Data are expressed as mean ± SEM. Feed intake was measured per pen, and the mean daily feed intake was calculated as the feed intake at a given period divided by the number of days between the two measurements and the number of animals in the diet group (MB, WB). N=172 totally. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$ NS: not significant

6.1.2. Intestinal butyrate concentrations

There were no significant differences among any experimental groups concerning butyrate concentration in duodenal content (main effect of non-protected butyrate – lower and higher dose and that of protected butyrate: P = 0.226, P = 0.183, P = 0.151, respectively; **Fig. 7/A**). Butyrate concentration in the ileum was significantly increased by the protected butyrate treatment only compared to controls (main effect: P < 0.001; **Fig. 7/B**). Butyrate concentration in the caecal ingesta was significantly elevated by WB diet compared to chickens kept on MB diet (main effect: P = 0.003; **Fig. 7/C**).

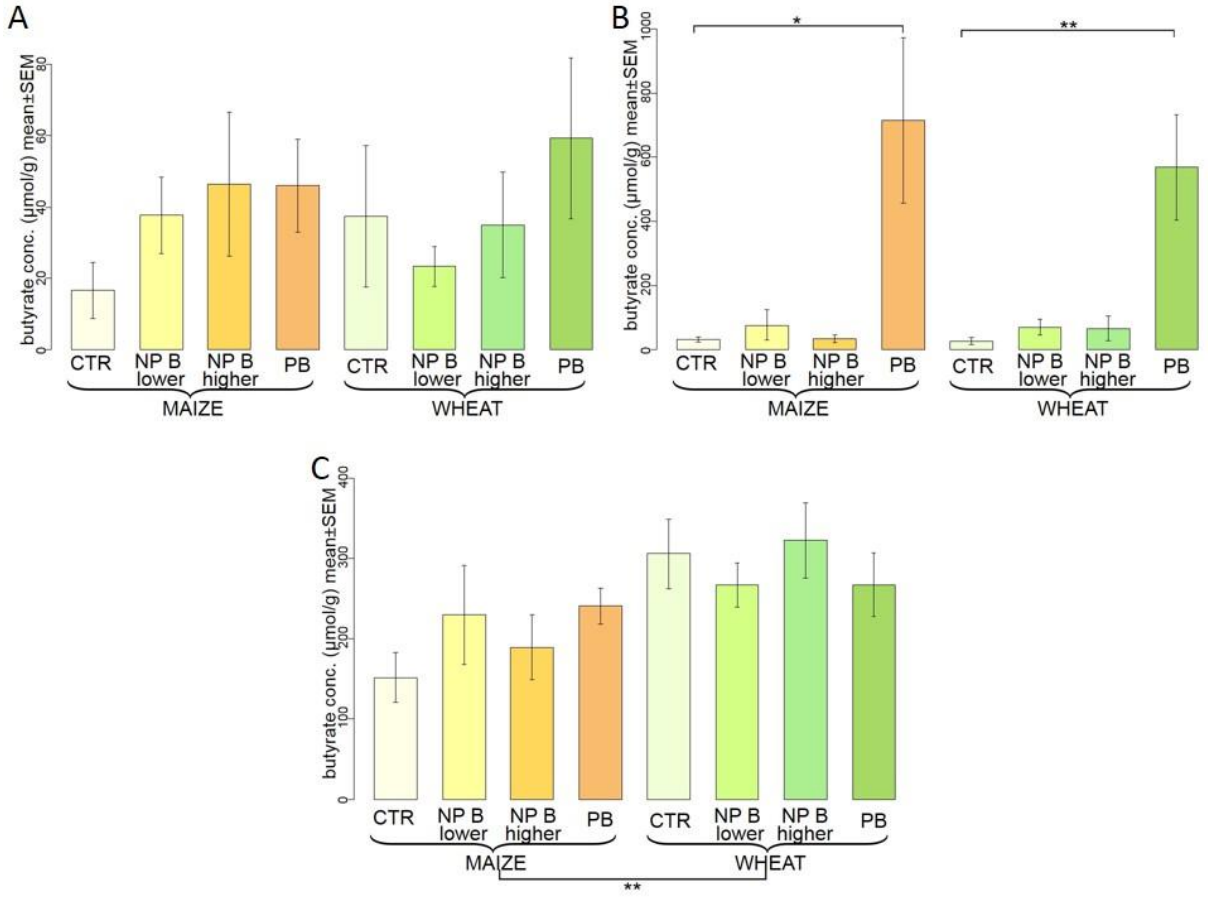

Figure 7. Effect of different cereal types in the diet and butyrate supplementations on the intestinal butyrate concentrations in broiler chickens

A. Duodenum **B.** Ileum **C.** Caecum

MAIZE: maize-based diet. **WHEAT:** wheat-based diet supplemented with NSP-degrading enzymes. **CTR:** control group. **NP B lower:** lower dose (1.5 g/kg diet) of non-protected butyrate feed supplementation. **NP B higher:** higher dose (3 g/kg diet) of non-protected butyrate feed supplementation. **PB:** protected butyrate (Butipearl® 0.2 g/kg diet) feed supplementation. Asterisks at the columns indicate the significances of post hoc tests, asterisks at the braces indicate the significances of the main effect of diet. ** P < 0.01; * P < 0.05

6.1.3. Plasma butyrate concentrations

In *v. gastropancreaticoduodenalis*, the higher dose of non-protected butyrate supplementation resulted in significantly higher plasma butyrate concentrations compared to controls (main effect: P < 0.001, **Fig. 8/A**). However, WB diet, the higher dose of non-protected butyrate and protected butyrate supplementation all significantly increased plasma butyrate concentration in *v. mesenterica communis* (main effect: P < 0.001 in all three cases, **Fig. 8/B**). Plasma butyrate concentration in *v. brachialis* was affected by the higher dose of non-protected butyrate application only, where significant elevation was detected (main effect: P < 0.001, **Fig. 8/C**).

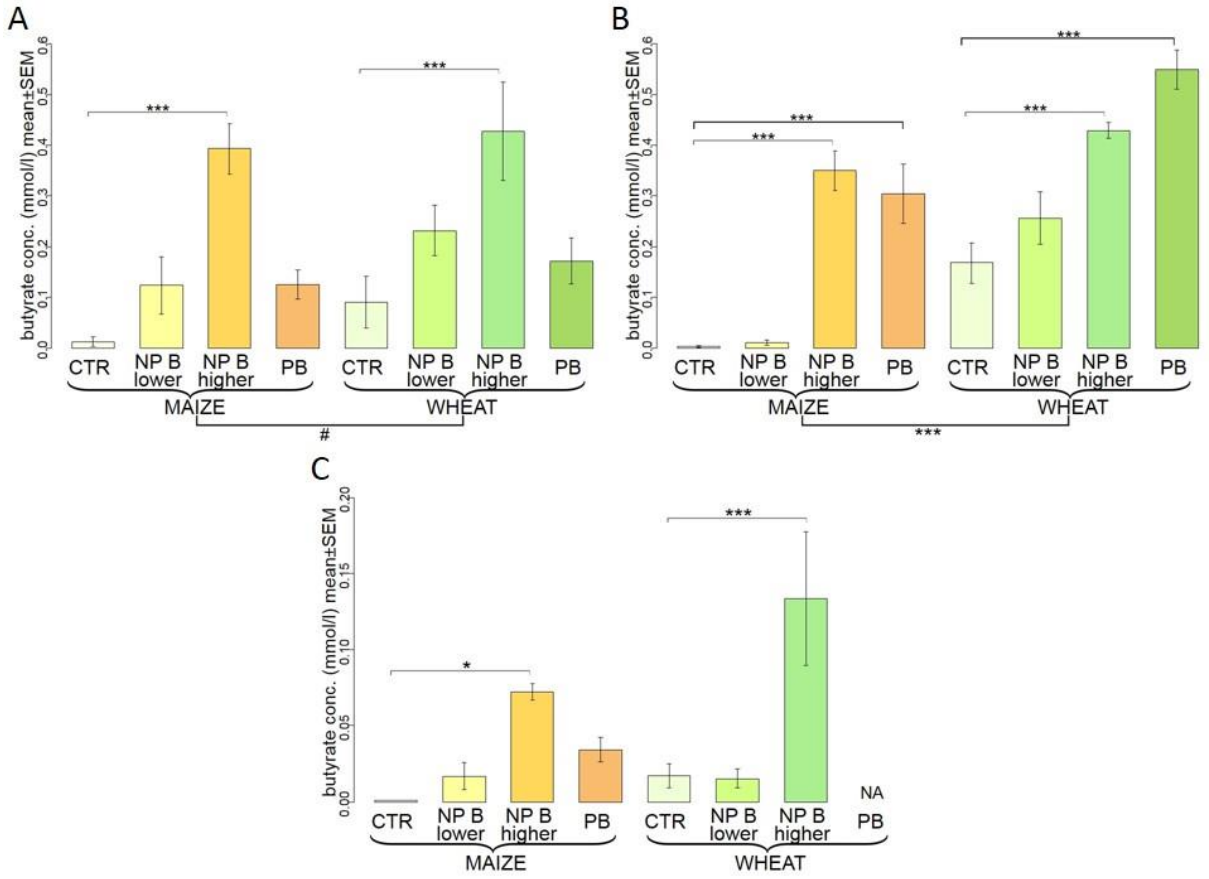

Figure 8. Effect of different cereal types in the diet and butyrate supplementations on the plasma butyrate concentrations in broiler chickens

A. *Vena gastropancreaticoduodenalis* **B.** *Vena mesenterica communis* **C.** *Vena brachialis*

MAIZE: maize-based diet. **WHEAT:** wheat-based diet supplemented with NSP-degrading enzymes. **CTR:** control group. **NP B lower:** lower dose (1.5 g/kg diet) of non-protected butyrate feed supplementation. **NP B higher:** higher dose (3.0 g/kg diet) of non-protected butyrate feed supplementation. **PB:** protected butyrate (Butipearl® 0.2 g/kg diet) feed supplementation. Asterisks at the columns indicate the significances of post hoc tests, asterisks at the braces indicate the significances of the main effect of diet. *** P < 0.001; * P < 0.05; # P <0.1

At Fig. 8/C for technical reason data of WHEAT PB group are not available.

6.1.4. Intestinal CYP activity

Luminescent measurement of CYP (cytochrome P450) enzyme activities in duodenal mucosa revealed that the activity of all CYP enzymes was significantly increased by the WB diet compared to MB diet (main effects: CYP1A4/5: P < 0.001, CYP2H2: P = 0.043, CYP3A37: P < 0.001; **Fig. 9**). However, in case of CYP3A37 the difference was of negligible extent. Higher dose of non-protected butyrate supplementation significantly increased CYP1A4/5 (P=0.002) and CYP2H2 (P=0.002) activity compared to controls only within animals fed with MB diet (interactions between diet type and butyrate supplementation were as follows: CYP1A4/5: P < 0.007, CYP2H2: P = 0.027, CYP3A37: NS, **Fig. 9**).

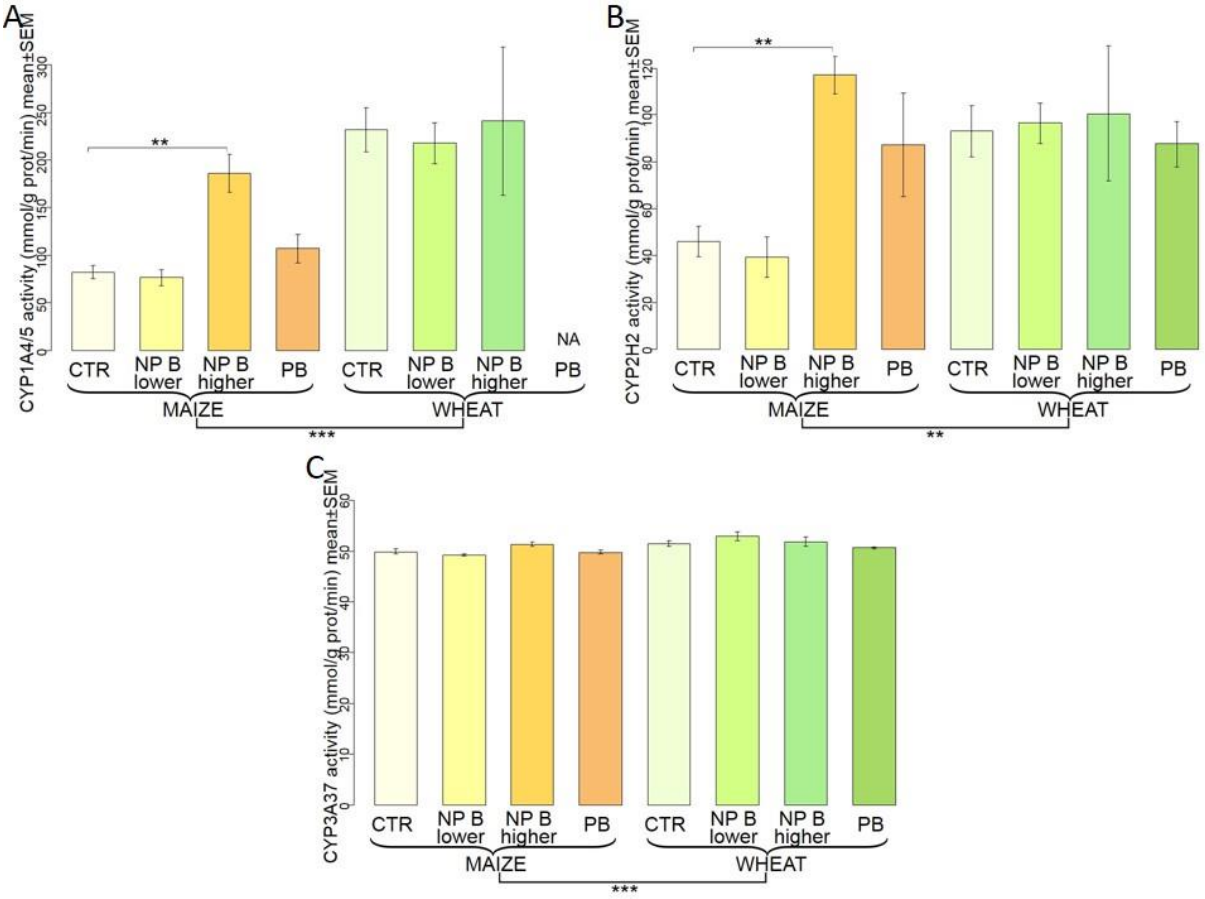

Figure 9. Effect of different cereal types in the diet and butyrate supplementations on the intestinal CYP activity in broiler chickens

A. CYP1A4/5 **B.** CYP2H2 **C.** CYP3A37

MAIZE: maize-based diet. **WHEAT:** wheat-based diet supplemented with NSP-degrading enzymes. **CTR:** control group. **NP B lower:** lower dose (1.5 g/kg diet) of non-protected butyrate feed supplementation. **NP B higher:** higher dose (3.0 g/kg diet) of non-protected butyrate feed supplementation. **PB:** protected butyrate (Butipearl® 0.2 g/kg diet) feed supplementation. Asterisks at the columns indicate the significances of post hoc tests, asterisks at the braces indicate the significances of the main effect of diet. *** P < 0.001; ** P < 0.01

At Fig. 9/A for technical reason data of WHEAT PB group are not available.

Plasma glucose and insulin concentrations

Glucose concentration of the blood plasma did not differ significantly among any treatment groups (mean value: 12.5 ± 0.3 mmol/l); however, insulin concentrations were significantly decreased by WB diet compared to MB diet (MB: 12.4 ± 0.4 mIU/l, WB: 10.3 ± 0.3 mIU/l; main effect: P = 0.001).

6.1.5. Insulin signaling proteins

Representative bands of the studied proteins of insulin signaling pathway in different tissues can be seen on **Fig. 10**.

The protein expression of IRβ (insulin receptor β) was significantly increased by WB diet compared to MB diet in the liver (main effect: P = 0.034, **Fig. 11/A**), similarly to that of mTOR (mammalian target of rapamycin) in the liver (main effect: P = 0.003, **Fig. 12/A**) and in the subcutaneous adipose tissue (main effect: P = 0.006, **Fig 12/C**). Further, PKCζ (protein kinase C ζ) expression was significantly greater in the subcutaneous adipose tissue of WB-fed chicks compared to animals kept on MB diet (P = 0.006, **Fig. 13/C**). Oral butyrate supplementation affected the expression of IRβ in the liver only: it was significantly up-regulated by the lower dose of non-protected butyrate within the WB dietary group (P = 0.003, **Fig. 11/A**).

Notwithstanding that WB diet had relevant effects on both CYP enzymes and insulin signaling proteins, no correlation could be found between them.

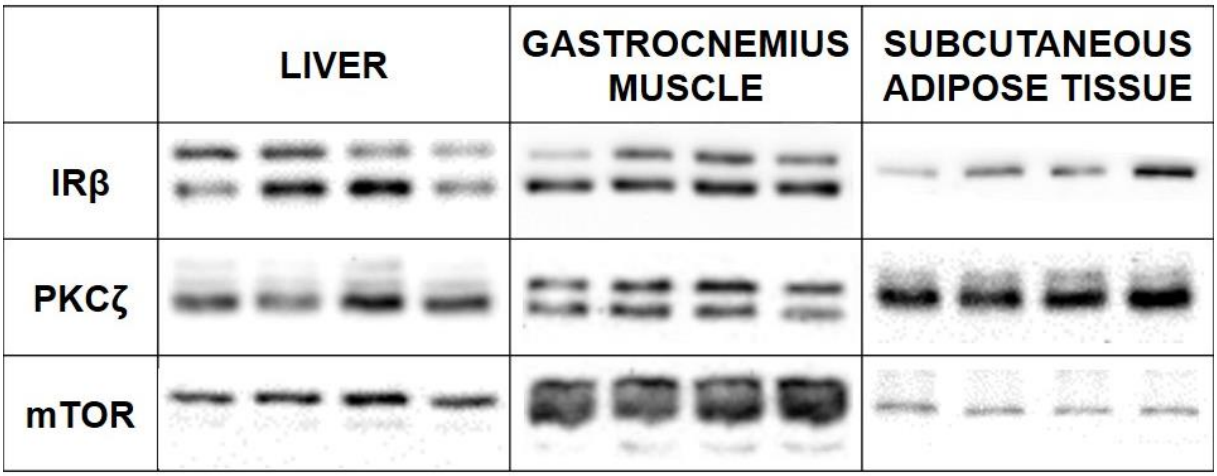

Figure 10. Representative bands of the studied proteins of insulin signaling pathway in different tissues, obtained by Western blotting in Study I/C

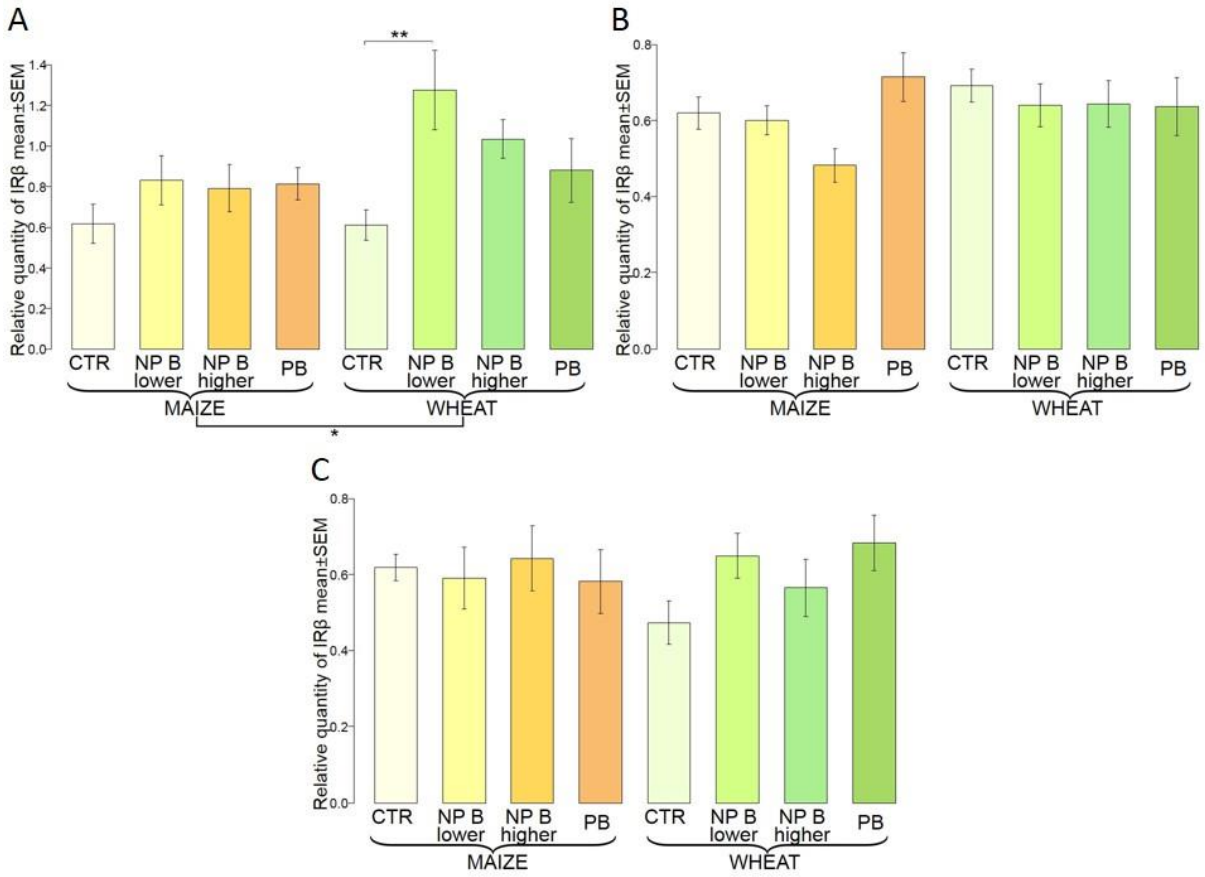

Figure 11. Effect of different cereal types in the diet and butyrate supplementations on the protein expression of insulin receptor β (IR β) in broiler chickens

A. Liver **B.** Gastrocnemius muscle **C.** Subcutaneous adipose tissue

Vertical (y) axis presents the standardized trace quantity values.

MAIZE: maize-based diet. **WHEAT:** wheat-based diet supplemented with NSP-degrading enzymes. **CTR:** control group. **NP B lower:** lower dose (1.5 g/kg diet) of non-protected butyrate feed supplementation. **NP B higher:** higher dose (3.0 g/kg diet) of non-protected butyrate feed supplementation. **PB:** protected butyrate (Butipearl® 0.2 g/kg diet) feed supplementation. Asterisks at the columns indicate the significances of post hoc tests, Asterisks at the braces indicate the significances of the main effect of diet. ** $P < 0.01$; * $P < 0.05$

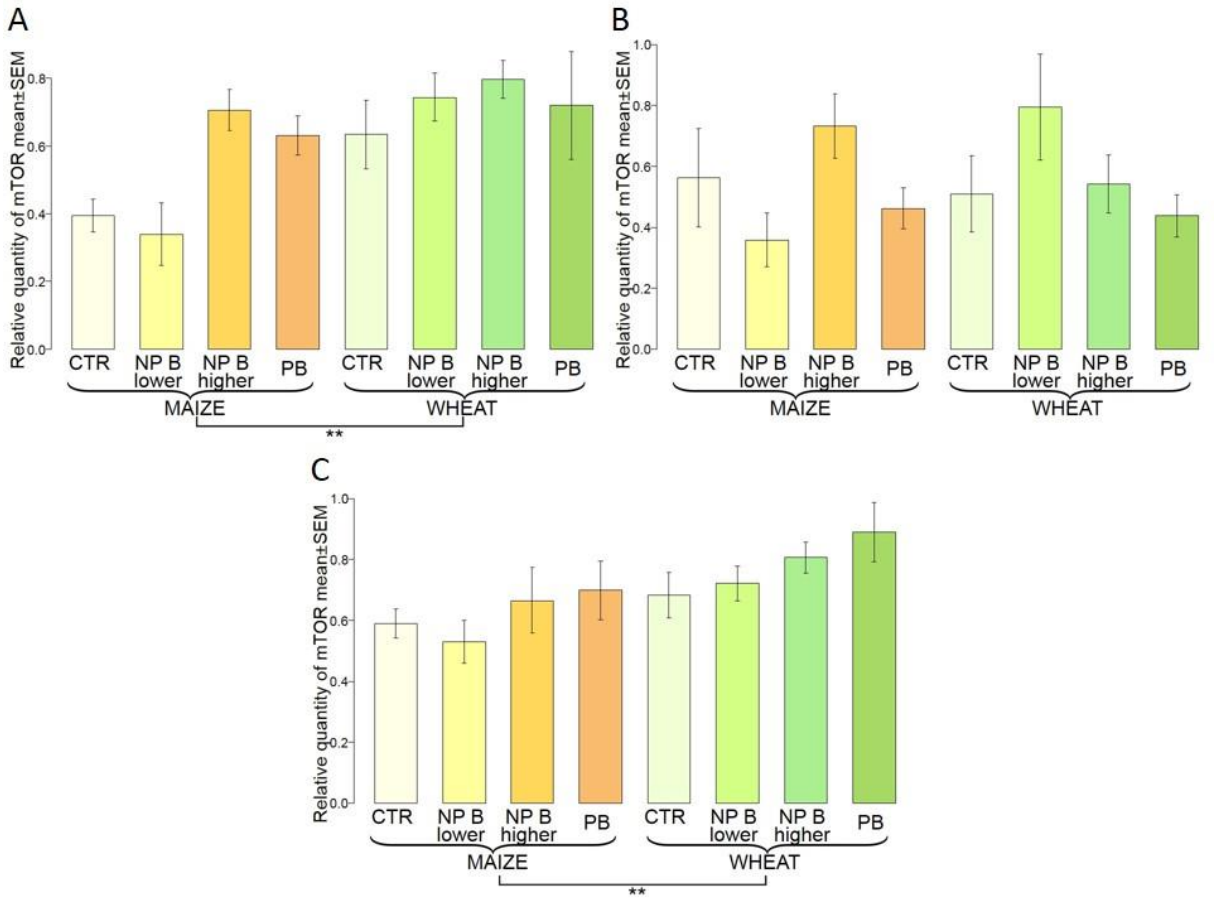

Figure 12. Effect of different cereal types in the diet and butyrate supplementations on the protein expression of mammalian target of rapamycin (mTOR) in broiler chickens

A. Liver **B.** Gastrocnemius muscle **C.** Subcutaneous adipose tissue

Vertical (y) axis presents the standardized trace quantity values.

MAIZE: maize-based diet. **WHEAT:** wheat-based diet supplemented with NSP-degrading enzymes. **CTR:** control group. **NP B lower:** lower dose (1.5 g/kg diet) of non-protected butyrate feed supplementation. **NP B higher:** higher dose (3.0 g/kg diet) of non-protected butyrate feed supplementation. **PB:** protected butyrate (Butipearl® 0.2 g/kg diet) feed supplementation. Asterisks at the columns indicate the significances of post hoc tests, Asterisks at the braces indicate the significances of the main effect of diet. ** $P < 0.01$

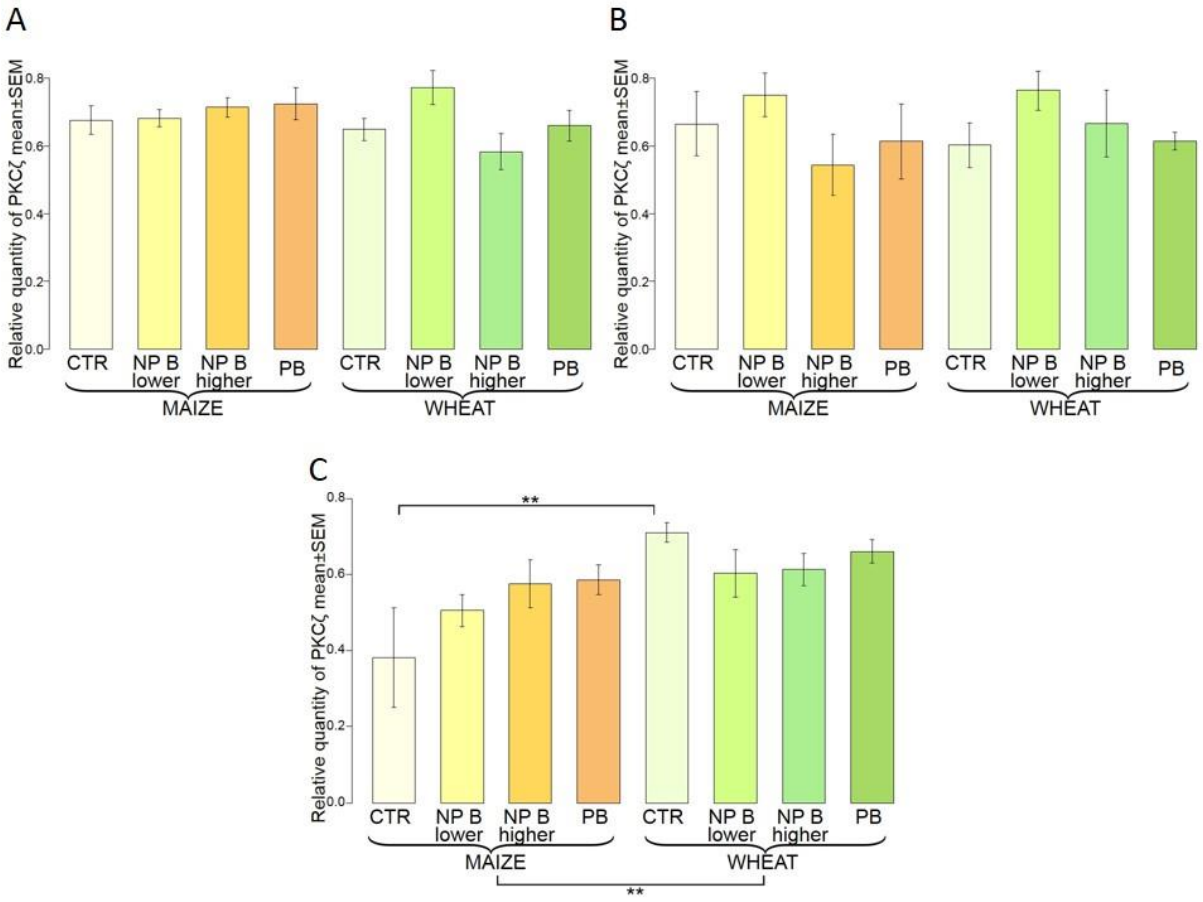

Figure 13. Effect of different cereal types in the diet and butyrate supplementations on the protein expression of protein kinase C ζ (PKCζ) in broiler chickens

A. Liver **B.** Gastrocnemius muscle **C.** Subcutaneous adipose tissue

Vertical (y) axis presents the standardized trace quantity values.

MAIZE: maize-based diet. **WHEAT:** wheat-based diet supplemented with NSP-degrading enzymes. **CTR:** control group. **NP B lower:** lower dose (1.5 g/kg diet) of non-protected butyrate feed supplementation. **NP B higher:** higher dose (3.0 g/kg diet) of non-protected butyrate feed supplementation. **PB:** protected butyrate (Butipearl® 0.2 g/kg diet) feed supplementation. Asterisks at the columns indicate the significances of post hoc tests, Asterisks at the braces indicate the significances of the main effect of diet. ** $P < 0.01$

6.2. Study II. Medium-term – Multiple Bolus study

6.2.1. Growth performance

No differences could be observed regarding growth parameters of different groups compared to controls within the whole experiment (**Table 6**).

Table 6. Effect of intraingluvial butyrate bolus application on mean body weight and feed intake

	CTR	NP B lower	Significance
Body weight (g)	mean ± SEM		
Day 1	43.6 ± 0.6	44.1 ± 0.8	NS
Day 7	220.2 ± 3.9	212.6 ± 6.3	NS
Day 14	471.7 ± 6.5	475.0 ± 14.0	NS
Day 20	855.0 ± 11.4	850.6 ± 24.7	NS
Day 24	1072.4 ± 14.3	1106.0 ± 15.3	NS
Mean feed intake (g/bird/day)			
Day 1-6	27.8	29.3	-
Day 7-13	60.5	61.5	-
Day 14-19	87.7	86.6	-
Day 20-24	99.0	99.9	-

CTR: control group (intraingluvial bolus application of distilled water on days 20-24). **NP B lower:** lower dose (0.25 g/kg BW) of non-protected butyrate intraingluvial bolus application on days 20-24.
Body weight was measured individually on day 0, 7, 14, 20 and 24. Data are expressed as mean ± SEM. Feed intake was measured per pen (10 chicken/pen), and the mean daily feed intake was calculated as the feed intake at a given period divided by the number of days between the two measurements and the number of animals in the experimental group. NS: not significant

6.2.2. Plasma butyrate concentrations

Orally applied butyrate caused more than a 2-fold increase ($P < 0.001$) in plasma concentration of butyrate in the treated chickens (38.38 ± 3.81 µmol/l) compared to the control animals (16.67 ± 3.35 µmol/l).

6.2.3. Plasma glucose and insulin concentrations

Oral butyrate application was associated with increased ($P < 0.001$) plasma concentration of fasting glucose in broilers: 13.88 ± 0.32 mmol/l glucose was measured in treated chickens versus 11.93 ± 0.27 mmol/l in control animals. Concomitantly, plasma concentration of insulin was greater ($P < 0.001$) in butyrate-treated chicks (8.79 ± 0.06 mIU/l) compared to the control ones (8.28 ± 0.12 mIU/l).

6.2.4. Insulin signaling proteins

Western blot analysis concerning key members of insulin signaling revealed that butyrate treatment was associated with decreased protein expression of IRβ in the liver ($P < 0.008$) and in both examined adipose tissues (abdominal: $P < 0.003$, subcutaneous: $P< 0.001$), but with its increased expression in the skeletal muscle ($P < 0.045$) (**Fig. 15/A**). Hepatic PI3K (phosphatidylinositol-3-kinase) expression was reduced by butyrate ($P < 0.007$), but no changes could be observed in other organs (**Fig. 15/B**). The protein expression of mTOR was downregulated by butyrate in both the liver ($P < 0.001$) and the subcutaneous adipose tissue ($P < 0.038$) (**Fig. 15/D**). Nevertheless, the protein expression of PKCζ was not influenced significantly by oral butyrate administration in any of the examined tissues of chickens (**Fig. 15/C**). Representative bands obtained by Western blotting are shown in **Fig. 14**.

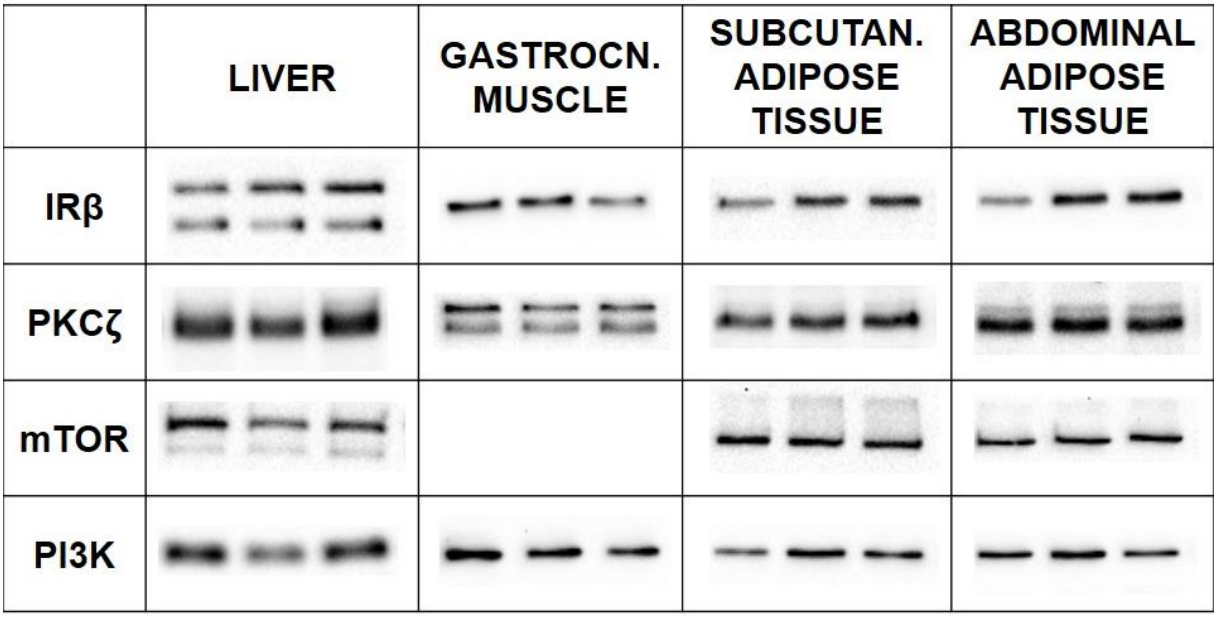

Figure 14. Representative bands of the studied proteins of insulin signaling pathway in different tissues, obtained by Western blotting in Study II.

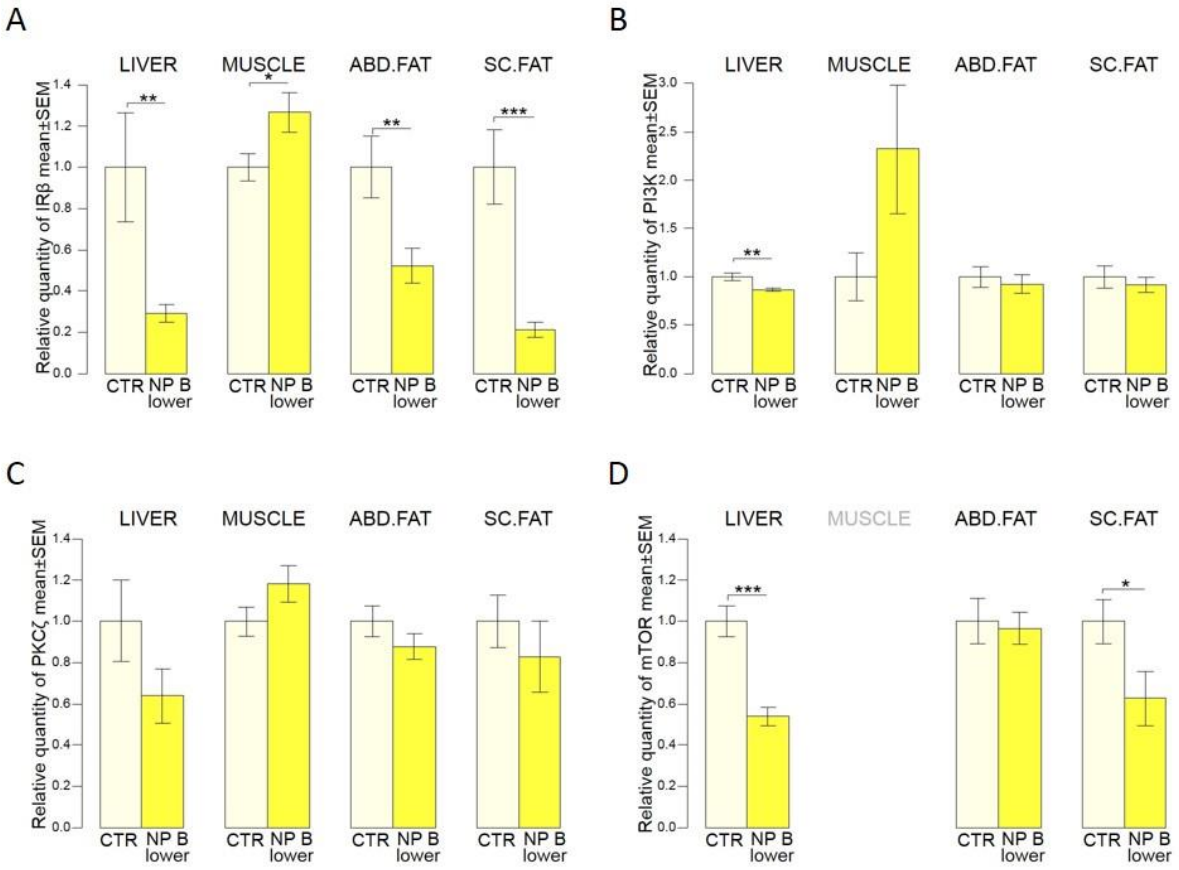

Figure 15. Effect of intraingluvial butyrate bolus application on the protein expression of insulin signaling proteins in broiler chickens

A. IRβ **B.** PI3K **C.** PKCζ **D.** mTOR

Vertical (y) axis presents the standardized trace quantity values.

CTR: control group (intraingluvial bolus application of distilled water). **NP B lower:** lower dose (0.25 g/kg BW) of non-protected butyrate intraingluvial bolus application. Asterisks at the columns indicate the significant differences.

*** P < 0.001; ** P < 0.01; * P < 0.05

6.3. Study III. Short-term – Single Bolus study

6.3.1. Growth performance

No differences could be observed regarding growth parameters of different groups compared to controls within the whole experiment in either chickens or rabbits (**Table 7, 8**).

Table 7. Effect of the diet type on mean body weight and feed intake of chickens

	CTR	NP B lower	NP B higher	Significance
Body weight (g) mean ± SEM				
Day 1	41.6 ± 0.5	43.2 ± 0.2	42.4 ± 0.3	NS
Day 7	221.2 ± 3.9	215.1 ± 5.6	225.1 ± 6.6	NS
Day 14	485.2 ± 7.2	473.8 ± 10.6	479.3 ± 7.8	NS
Day 21	894.6 ± 13.6	888.0 ± 19.2	902.5 ± 12.4	NS
Day 24	1160.3 ± 14.7	1136.8 ± 13.7	1197.4 ± 17.5	NS
Mean feed intake (g/chicken/day)				
Day 1-6	27.6	28.4	28.8	-
Day 7-13	60.7	59.7	59.8	-
Day 14-20	89.3	88.9	91.2	-
Day 21-24	102.4	104.2	103.7	-

CTR: control group (intraingluvial bolus application of physiological saline). **NP B lower:** lower dose (0.25 g/kg BW) of non-protected butyrate intraingluvial bolus application. **NP B higher:** higher dose 1.25 g/kg BW) of non-protected butyrate intraingluvial bolus application.

Body weight was measured individually on the day of arrival (day 1) and on the day of treatment (day 24). Data are expressed as mean ± SEM. Feed intake was measured per pen (7 animals/pen) and the mean daily feed intake was calculated as the feed intake at a given period divided by the number of days between the two measurements and the number of animals in the experimental group. NS: not significant

Table 8. Effect of the diet type on mean body weight and feed intake of rabbits

	CTR	NP B lower	NP B higher	Significance
Body weight (g) mean ± SEM				
Day 42	1517 ± 16.9	1558 ± 14.1	1465 ± 9.9	NS
Day 49	1562 ± 16.4	1660 ± 9.4	1553 ± 15.4	NS
Mean feed intake (g/rabbit/day)				
Day 42-49	104.3	97.9	104.9	-

CTR: control group (intragastric bolus application of physiological saline). **NP B lower:** lower dose (0.25 g/kg BW) of non-protected butyrate intragastric bolus application. **NP B higher:** higher dose 1.25 g/kg BW) of non-protected butyrate intragastric bolus application.

Body weight was measured individually on the day of arrival (day 4) and on the day of treatment (day 49). Data are expressed as mean ± SEM. Feed intake was measured per pen (7 animals/pen) and the mean daily feed intake was calculated as the feed intake at a given period divided by the number of days between the two measurements and the number of animals in the experimental group. NS: not significant

6.3.2. Plasma GIP, GLP-1, insulin and glucose concentrations

Considering the effect of the time after bolus treatment (10, 30, 60 min) on the measured endocrine parameters, GIP (glucose-dependent insulinotropic peptide) and GLP-1 (glucagon-like peptide 1) concentrations were not influenced significantly by time (**Fig. 16/A**, **B**; **Fig. 17/A**, **B**). As an opposite, plasma insulin level significantly decreased with time in chickens and rabbits as well ($P = 0.002$ and $P = 0.016$, respectively; **Fig. 16/C**; **Fig. 17/C**) compared to the 0 min baseline values. Blood glucose concentrations of chickens were not altered by time (**Fig 16/D**), but increased with time ($P = 0.002$) in case of rabbits (**Fig. 17/D**).

Concentration of GIP in blood plasma of chickens was affected by butyrate exposure: significant interactions were detected between the higher dose (1.25 g/kg BW) of butyrate and incubation time ($P = 0.038$ and $P = 0.034$ for 30 and 60 min, respectively). These interactions are reflected by the butyrate-associated decrease of plasma GIP levels at 30 and 60 min with approx. 40% compared to 0 min values (**Fig. 16/A**). Similar significant interactions ($P = 0.036$ and $P = 0.039$ for 30 and 60 min, respectively) could be observed in rabbits, where plasma GIP concentrations were lowered 30 and 60 min after ingestion of the lower dose of butyrate (0.25 g/kg BW) with approx. 45% when compared to the initial baseline values (**Fig. 17/A**).

Regarding the GLP-1, insulin and glucose concentrations no significant interactions were observed between butyrate exposure and incubation time so according to our results

butyrate had no significant effects on GLP-1, insulin and blood sugar levels either in chickens or in rabbits (**Fig. 16/B, C, D; Fig. 17/B, C, D**).

Time zero concentrations of all hormones and those of glucose are indicated in **Table 9** for chickens and in **Table 10** for rabbits.

Table 9. Concentration of GIP, GLP-1, insulin and glucose in the blood plasma of chickens at 0 min.

	GIP (pg/ml)	**GLP-1 pg/ml**	**Insulin mU/l**	**Glucose mmol/l**
CTR	46.56 ± 6.44	9.62 ± 1.63	10.05 ± 0.15	11.49 ± 0.35
NP B lower	35.41 ± 2.24	9.20 ± 0.90	9.70 ± 0.16	10.54 ± 0.28
NP B higher	36.21 ± 3.79	7.05 ± 1.29	9.56 ± 0.28	11.20 ± 0.51

CTR: control group (intraingluvial bolus application of physiological saline). **NP B lower:** lower dose (0.25 g/kg BW) of non-protected butyrate intraingluvial or intragastric bolus application. **NP B higher:** higher dose (1.25 g/kg BW) of non-protected butyrate intraingluvial or intragastric bolus application. Results are expressed as mean ± SEM

Table 10. Concentration of GIP, GLP-1, insulin and glucose in the blood plasma of rabbits at 0 min.

	GIP (pg/ml)	**GLP-1 pg/ml**	**Insulin mU/l**	**Glucose mmol/l**
CTR	58.85 ± 12.69	96.65 ± 10.07	68.58 ± 17.00	5.53 ± 0.16
NP B lower	75.50 ± 11.30	86.78 ± 4.29	33.72 ± 4.90	6.01 ± 0.18
NP B higher	55.86 ± 10.08	99.76 ± 8.31	36.60 ± 2.02	5.80 ± 0.12

CTR: control group (intragastric bolus application of physiological saline). **NP B lower:** lower dose (0.25 g/kg BW) of non-protected butyrate intraingluvial or intragastric bolus application. **NP B higher:** higher dose (1.25 g/kg BW) of non-protected butyrate intraingluvial or intragastric bolus application. Results are expressed as mean ± SEM

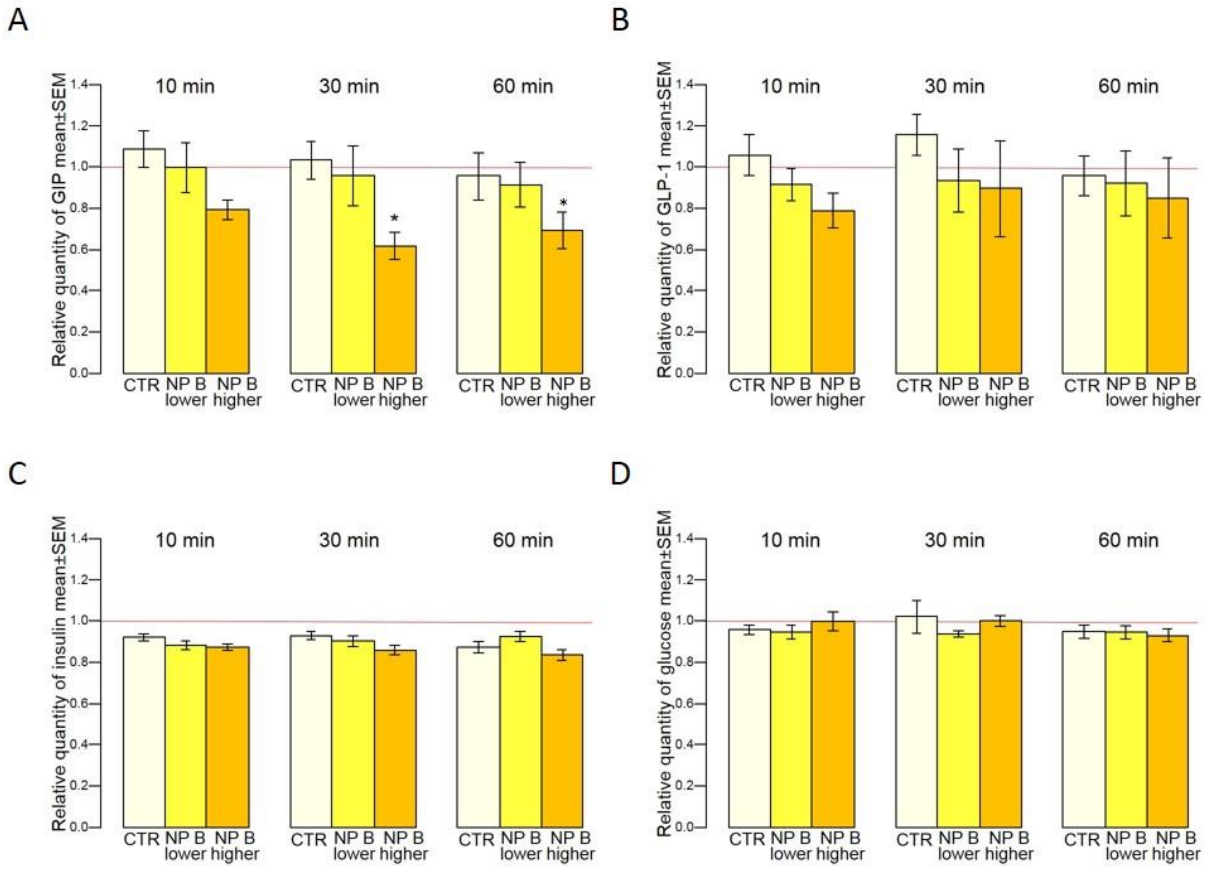

Figure 16. Relative concentrations of **A.** GIP **B.** GLP-1 **C.** insulin and **D.** glucose in the blood plasma of broiler chickens

CTR: control group (intraingluvial bolus application of physiological saline). **NP B lower:** lower dose (0.25 g/kg BW) of non-protected butyrate intraingluvial bolus application. **NP B higher:** higher dose (1.25 g/kg BW) of non-protected butyrate intraingluvial bolus application.

Relative hormone concentrations were calculated by considering the baseline value of each animal at 0 min as 1. Asterisks at the columns indicate statistical significance compared to the 0 min values of the appropriate group (interaction between time and treatment). * $P < 0.05$

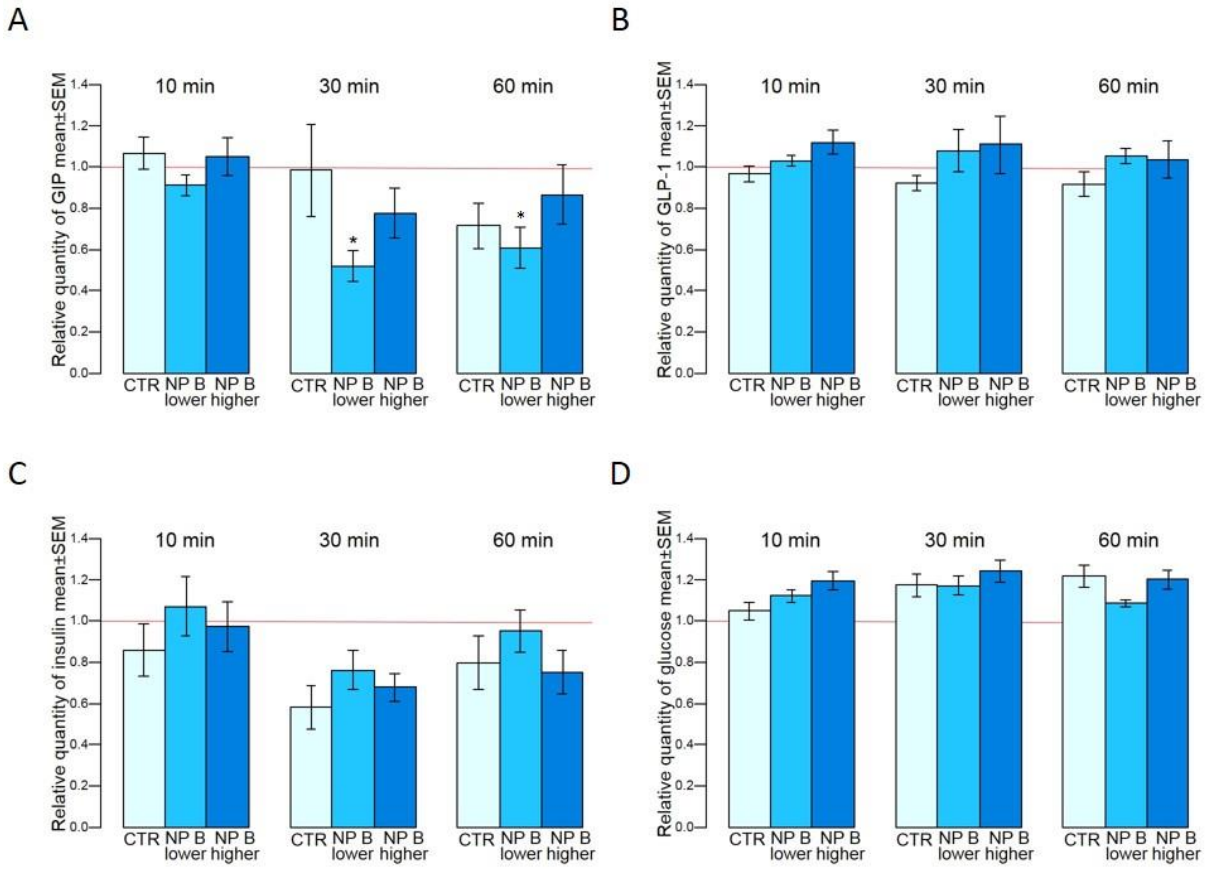

Figure 17. Relative concentrations of **A.** GIP **B.** GLP-1 **C.** insulin and **D.** glucose in the blood plasma of rabbits

CTR: control group (intragastric bolus application of physiological saline). **NP B lower:** lower dose (0.25 g/kg BW) of non-protected butyrate intragastric bolus application. **NP B higher:** higher dose (1.25 g/kg BW) of non-protected butyrate intragastric bolus application.

Relative hormone concentrations were calculated by considering the baseline value of each animal at 0 min as 1. Asterisks at the columns indicate statistical significance compared to the 0 min values of the appropriate group (interaction between time and treatment). * $P < 0.05$

6.3.3. Correlations between the measured endocrine parameters

Concerning the correlations between the measured endocrine parameters in chickens (regardless of the butyrate exposure) highly significant (P < 0.001) positive correlations were found between plasma GIP and GLP-1 values (**Fig. 18/A**), GIP and insulin levels (**Fig. 18/B**) and GLP-1 and insulin concentrations (**Fig. 18/C**). In rabbits a significant negative correlation was observed between plasma GIP and GLP-1 levels (P = 0.010; **Fig. 19/A**), while there was no significant correlation between GIP and insulin values (P = 0.180; **Fig. 19/B**). However, plasma GLP-1 and insulin concentrations positively correlated on a significant manner (P = 0.007; **Fig. 19/C**) in rabbits as well. We found no significant correlation between plasma insulin and glucose concentration either in chickens or in rabbits. All correlation results were justified by the approved confirmatory tests as well; however in case of GIP – GLP-1 correlation of rabbits no significant correlation was found when time points were analyzed separately.

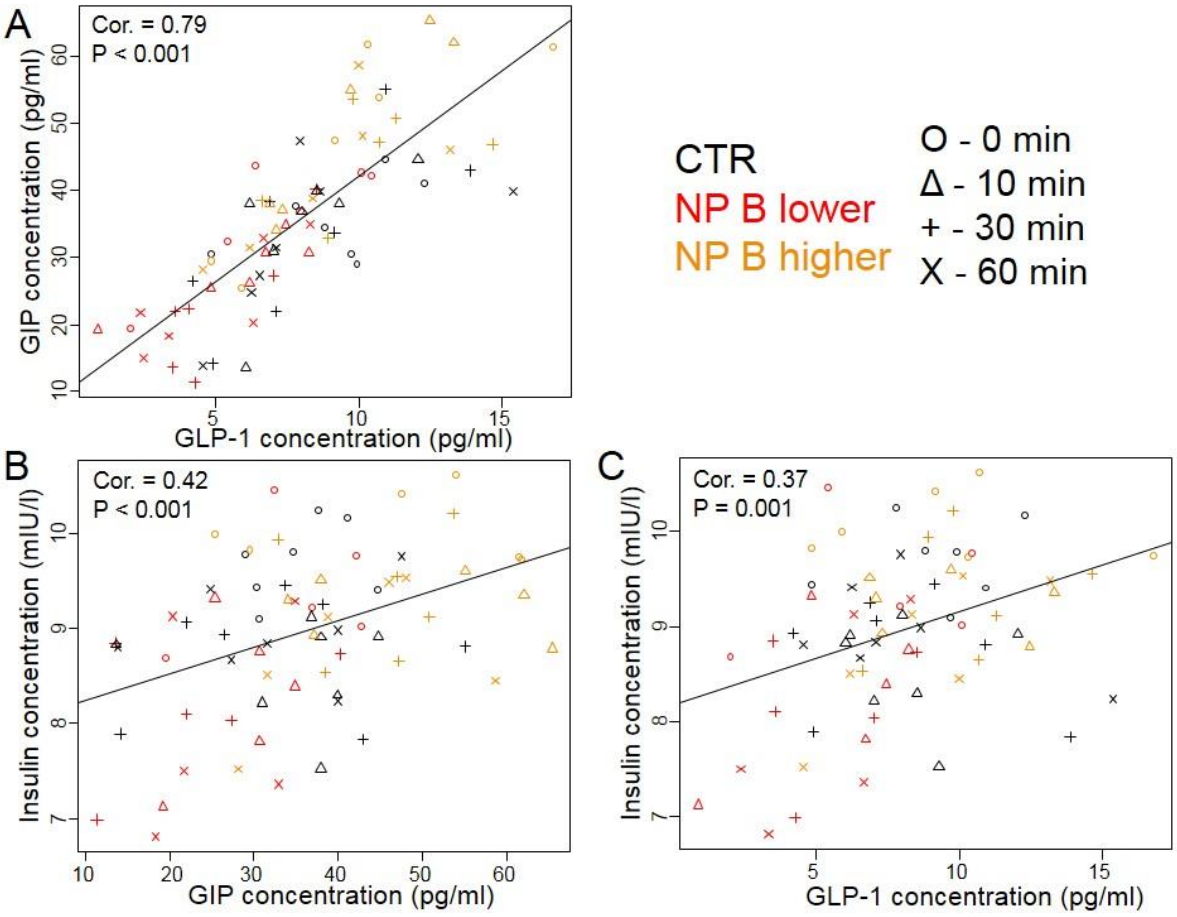

Figure 18. Correlation between **A.** GIP and GLP-1 **B.** GIP and insulin **C**. GLP-1 and insulin concentrations in the blood plasma of broiler chickens

Each dot refers to an individual animal according to its blood plasma hormone concentrations indicated on the axes.

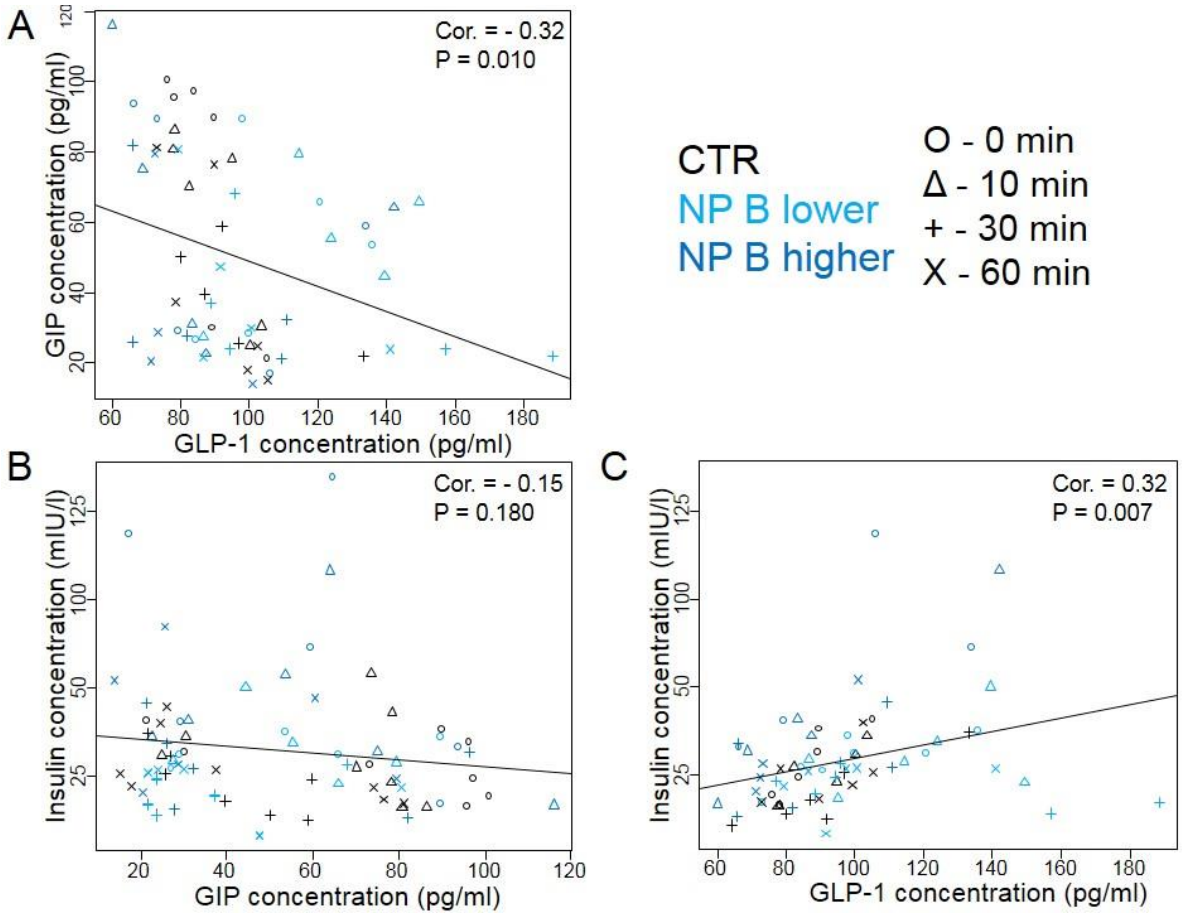

Figure 19. Correlation between **A.** GIP and GLP-1 **B.** GIP and insulin **C.** GLP-1 and insulin concentrations in the blood plasma of rabbits

Each dot refers to an individual animal according to its blood plasma hormone concentrations indicated on the axes.

7. Discussion

7.1. Butyrate (of exogenous and endogenous origin) as growth promoter

Butyrate is widely used as a growth promoter in poultry nutrition (Panda et al., 2009; Zhang et al., 2011) in different application forms. However, results concerning its effect on growth performance are not always consistent. Some studies have found that oral non-protected butyrate treatment increased body weight gain and improved carcass quality (Leeson et al., 2005; Hu and Guo, 2007), whereas others observed that it had no effect on the body weight gain (Shahir et al., 2013). Data about the benefits of the protected form of butyrate are also quite diverse. In some cases the beneficial effect on the gastrointestinal tract was confirmed without growth promoting activity (Mahdavi and Torki, 2007), but its influence on body weight gain has also been described (Mallo et al., 2012; Chamba et al., 2014).

In our **long-term – feeding study** (study I.) no significant differences were found in the final body weight of chickens fed by diets supplemented with either non-protected or protected butyrate compared to control. In contrast, elevated final body weight was observed in the WB (wheat based) dietary groups compared to MB (maize-based) groups. Our results regarding the growth promoting effect of xylanase and glucanase supplementation of soluble NSP (non starch polysaccharide) enriched diet, in connection with the enhanced caecal butyrate production are in accordance with earlier studies (Mathlouthi et al., 2012; Shahir et al., 2013), however the effect of additional differences between the two diet type cannot be excluded.

Nevertheless, we have to state, that this long-term – feeding study was not designed to be a performance study (concerning either the sample size or the grouping design), thus the body weight results have to be handled carefully, only as a background of the main goals of the research.

In the following **medium-term and short-term – single bolus study** (study II. and study III.), the way of butyrate application (daily intraingluvial bolus) was designed to clarify the potential effects of orally applied butyrate on insulin and incretin homeostasis, thus the recorded growth parameters of butyrate-treated chickens cannot be extrapolated to poultry farming.

Therefore, growth performance data should not be discussed in details, but we have to underline that body weight and feed intake values of birds met the Ross standard (Aviagen,

2014), and there were no significant differences between groups, which could have influenced the main results of the studies.

7.2. Butyrate absorption and distribution

Regarding the absorption of different application forms of butyrate in our **long-term – feeding study** (study I./A), non-protected butyrate supplementation (either in lower or higher dose) failed to significantly increase butyrate concentration in any of the studied intestinal sections. Butyrate can be absorbed by the epithelial cells from the lumen of the gastrointestinal tract, partly by active transport through monocarboxylate transporter 1 (MCT-1) and mainly by simple diffusion in non-dissociated form. Therefore, absorption of non-protected butyrate should be the most intensive in the proximal, acidic section of the gastrointestinal tract (Manzanilla et al., 2006, Moquet et al 2017), e.g. in the crop and gizzard, where the non-dissociated form is of the highest proportion. In contrast, micro-encapsulated butyrate is protected from the early absorption so released and absorbed in the distal parts of the gastrointestinal tract only (Chamba et al., 2014). Our results confirm this hypothesis, as protected butyrate application increased butyrate concentration in the ileum only. Thus it can improve gut-health in the small intestines, and it can exert its epigenetic effects when absorbed through the intestinal wall. Butyrate concentration in the caecum was influenced by WB diet only. This is in association with the higher soluble NSP content of the WB diets, which, following its degradation to oligosaccharides by the supplemented xylanase and glucanase, serves as a substrate of the intensive microbial butyrate production in the large intestine (Hübener et al., 2002; Jamroz et al., 2002; Guilloteau et al., 2010).

It is known that absorbed SCFA have a high rate of first-pass hepatic clearance (Steliou et al., 2012). Studies have shown that approximately 75% of acetate, 90% of propionate and 95% of butyrate is eliminated from the portal blood by the active hepatic metabolism (Peters et al., 1992); therefore peripheral plasma butyrate concentrations were not in close correlation with the increased portal SCFA levels (Cummings et al., 1981).

To assess the fate and absorption of different forms of butyrate beyond the gastrointestinal tract, in our **long-term – feeding study** (study I./B) blood plasma samples were analyzed taken from the *vena gastropancreaticoduodenalis*, *vena mesenterica communis* and *vena brachialis*. Butyrate concentration in the *vena gastropancreaticoduodenalis* was significantly increased by the higher dose of non-protected butyrate application. These results are in accordance with the anatomical distribution of areas drained by the examined hepatic portal veins (**Fig. 6**). The *vena gastropancreaticoduodenalis* collects blood mainly from the gizzard and the duodenum, the suggested site of the absorption of non-protected butyrate. The *vena mesenterica communis*

collects blood primarily from the distal part of the small intestines and from the large intestine (Nickel et al., 1977). Such origin of this vein may explain our results as protected butyrate treatment (released mostly in the ileum) and WB diet supplemented with NSP-degrading enzymes (increased caecal butyrate production) resulted in a higher mesenteric butyrate concentration. The higher dose of non-protected butyrate treatment also increased the butyrate concentration in this vein. However, it must be mentioned that due to anatomical and sampling conditions, the streaming of minor amount of blood from the *vena gastropancreaticoduodenalis* into the vena *mesenterica communis* during sampling could not be excluded. As the dietary butyrate concentration was considerably high in these experimental groups (3 g/kg diet), this issue during the sampling procedure may have resulted in a detectable increase in butyrate concentration of the mesenteric vein. The higher dose of non-protected butyrate treatment had the strongest influence on butyrate concentration in both studied portal veins.

Owing to the high rate of hepatic butyrate metabolism (Guilloteau et al., 2010), in the **long-term – feeding study** (study I./B) effects of WB diet and protected butyrate supplementation did not result in detectable elevation of butyrate concentration in peripheral plasma samples (*vena brachialis*); it was significantly increased by the pronounced effect of non-protected butyrate in higher dose only. Similar phenomenon was found in pigs, where caecal microbial butyrate production resulted in elevated butyrate concentration in the portal, but not in the peripheral circulation (Egorin et al., 1999; Knudsen et al., 2003).

Regarding the results of our **medium-term – multiple bolus study** (study II.), where chickens were treated with a more intensive bolus application of butyrate in the dose of 0.25 g/kg BW once daily for five days, we found that although butyrate is partly metabolized in the liver, a certain amount passed through to the systemic circulation, causing significant increase in its plasma concentration. Similar increase of plasma butyrate concentration was found in mice (Gao et al., 2009), but applying a higher dose of butyrate.

The described absorption and distribution properties of orally applied (non-protected or protected) and bacterially produced caecal butyrate in our long-term – feeding study, and bolus application of non-protected butyrate in our medium-term – multiple bolus study are of special relevance by determining the biological action of butyrate in the gastrointestinal tract and beyond the intestines as well.

7.3. Intestinal action: Effects of butyrate on intestinal CYP activity

Orally added or microbially produced butyrate could modify the intestinal CYP (cytochrome P450) activity, presumably via its epigenetic and receptor-mediated effects. In addition to the detoxifying function of the liver, small intestine also has an important role in the first pass metabolism of drugs and other xenobiotics (Lin et al., 1999; Obach et al., 2001), providing an enormous absorption and contact surface (Lin et al., 1999). The intestinal detoxifying mechanisms are mostly related to the CYP enzymes, being responsible for the Phase I metabolism of drugs such as phenacetin, mephenytoin, omeprazol, proguanyl and certain barbiturates. In birds CYP1A4/5 was found to play a pivotal role in T2 toxin metabolism (Shang et al., 2013). These enzymes can be found in the small intestines as well; showing a decreasing trend from the duodenum towards the ileum, however, their activity in the gastrointestinal tract is usually lower than in the liver (Peters and Kremers, 1989; Lin et al., 1999).

Concerning our results in the **long-term – feeding study** (study I./C), butyrate had a remarkable impact on the activity of the studied CYP1A4/5 and CYP2H2 enzymes in the duodenal mucosa; being significantly increased by the higher dose of non-protected butyrate within MB diet and by butyrate produced microbially in the caecum (WB diet). Lower dose of the non-protected butyrate had no significant effect on any studied CYP enzymes, as it may have not reach the critical concentration in the intestinal epithelial cells.

Our previous studies have have revealed that butyrate do have an impact on hepatic CYP enzymes in chickens. *In vitro*, in primary culture of chicken hepatocytes, butyrate affected the gene expression of all CYP1A, CYP2H and CYP3A (Csikó et al., 2014). In *in vivo* feeding studies, orally added non-protected butyrate application increased the gene expression of hepatic CYP1A and CYP2H, but not that of CYP3A; however, such effects were not detected at the level of enzyme activity (Mátis et al., 2013a; Csikó et al., 2014). The more pronounced effect of orally applied butyrate on intestinal CYP activity could arise from the fact that duodenal CYPs may be exposed to dietary butyrate supplementation more directly than hepatic CYP enzymes. However, it cannot be excluded that intestinal CYPs could be more inducible compared to hepatic isoenzymes.

Wheat based diet (that stimulates microbial butyrate production in the caecum) exerted the most pronounced CYP inducing effect in the duodenum. Notwithstanding that several other parameters of wheat based diet (SCFAs, amino acids, long chain fatty acids) could be involved in the effect of the diet type, this finding could presume that butyrate, absorbed from the large intestine and transported to the proximal intestinal sections via the portal and systemic circulation, may also activate intestinal CYP enzymes from the basolateral side of

the enterocytes. Similarly, studies with rats have shown that intravenous CYP inducer treatment influenced the expression and activity of intestinal CYP enzymes; furthermore, this effect was less pronounced than following oral administration of the same agent (Bonkovsky et al., 1985; Zhang et al., 1997; Lin et al., 1999). However, additional indirect effects of butyrate mediated by the autonomic nervous system cannot be excluded (Frankel et al., 1994; Kien et al., 2007).

Concluding the results of the long-term – feeding study, novel intestinal effects of butyrate administration have been described and compared among various application forms and sources in association with their absorption sites and concentrations in the portal and systemic circulation of broiler chickens. According to our results, the activity of small intestinal drug-metabolizing CYP enzymes could be modulated by nutrition-associated factors, such as by WB diet with NSP-degrading enzyme supplementation in connection with caecal microbial butyrate production, and by oral non-protected butyrate application. Based on the key role of duodenal CYPs as a first pass metabolic barrier against orally ingested xenobiotics, all efforts altering their function could be of high importance from food safety point of view.

7.4. Extraintestinal action: insulin homeostasis

7.4.1. Effects of butyrate on insulin signaling proteins

Studying insulin homeostasis in our **long-term – feeding study** (study I./C) in **six-week-old** broiler chickens, a remarkable response to the diet type could be detected. Concerning the feeding regime applied, caecal total SCFA and butyrate concentrations were significantly, nearly twofold increased by the WB diet compared to the chickens kept on the MB diet. This finding might be in association with the different soluble NSP content of the applied cereals mainly. However, it should be taken into consideration that maize and wheat also greatly differ in some other parameters (such as amino acid and fatty acid profile), thus the impact of certain other dietary factors in the observed diet-associated changes cannot be excluded.

In this part of the long-term – feeding study expression of key insulin signaling proteins were quantified by Western blotting and found to be influenced by the diet type and butyrate application in certain tissues of six-week-old chickens.

The IRβ (insulin receptor β), as an initial, thus a highly important member of the insulin signaling pathway, was significantly up-regulated in the liver of chickens kept on WB diet compared to MB groups. Dietary butyrate supplementation also affected IRβ in the liver, where lower dose of non-protected butyrate application increased protein expression in

animals fed with WB diet, compared to the WB control (without butyrate supplementation) animals. In agreement with our results in the long-term – feeding study, it was described already in 1987 that *in vitro* butyrate treatment could increase insulin receptor expression in mouse embryonic fibroblast NIH 3T3 cells, previously transfected with human kidney insulin receptors (Whittaker et al., 1987). Similarly, in another *in vitro* study, butyrate was found to be capable to increase the expression of both α and β subunits of insulin receptor in one type of Burkitt lymphoma cells, associated with morphological differentiations (Newman et al., 1989).

Protein expression of mTOR (mammalian target of rapamycin), which is most commonly known to take part in the regulation of protein synthesis (Dupont et al., 2009), was up-regulated by WB diet both in the liver and subcutaneous adipose tissue. It was found recently that mTOR contributed to the mediation of butyrate's inhibitory effect on the proliferation of cultured mouse embryonic fibroblast cells *in vitro* (Kochetkova et al., 2013).

The higher NSP content of diet (WB diet) significantly increased the protein expression PKCζ (protein kinase C ζ) in the subcutaneous adipose tissue only. Among other atypical PKCs, PKCζ is involved in the translocation of GLUT-4 containing intra-cellular vesicles to the plasma membrane in mammals (Dupont et al., 2009). As GLUT-4 is functionally replaced by GLUT-12 in chicken (Coudert et al., 2013), the exact mechanism regulating this recently described transporter is not totally clear yet, thus the role of avian PKCζ should be investigated in further studies.

In our long-term – feeding study, none of the examined proteins were significantly influenced by any type of butyrate treatment in skeletal muscle. Further, significant alterations of protein expression levels were mainly associated to the cereal type, presumably in connection with the enhanced caecal SCFA production (including butyrate) in WB groups.

Regarding the investigated blood plasma parameters in this long-term – feeding study, no significant differences were found in the blood glucose concentration among experimental groups. Although birds are known to be less insulin sensitive than mammals (Braun and Sweazea, 2008), our results may suggest that the increased expression levels of insulin signaling proteins in the liver of chicken kept on WB diet could play a role in the maintenance of the constant blood glucose level even at the WB diet-associated decreased plasma insulin concentration.

As all significantly affected insulin signaling proteins were up-regulated by the WB diet or by the oral butyrate exposure in our long-term – feeding study, the insulin sensitivity of chicken is supposed to be stimulated by the application of wheat as the main carbohydrate source (presumably mediated by the NSP-triggered caecal SCFA release including butyrate), and by applying butyrate as a feed additive as well. Since insulin plays pivotal role in the

maintenance of growth, inducing insulin sensitivity in chicken – being physiologically much less insulin responsive than mammals – could be of special importance by improving growth performance and metabolic health.

Concluding our results, our long-term – feeding study describes new ways of influencing insulin homeostasis of chicken by nutrition, such as by butyrate as a feed additive and by applying various cereals as dietary carbohydrate sources with different NSP levels. It should be stressed out that the application of WB diet (with higher soluble NSP levels), may have a strong influence on insulin homeostasis by stimulating the intestinal SCFA production including butyrate. Based on these findings, the role of SCFA as potent effectors of the endocrine metabolic regulation, primarily that of butyrate, was highlighted by comparing different application forms and by describing some underlying molecular mechanisms. Concerning the data obtained, applying higher dietary soluble NSP levels and/or butyrate as a feed additive can be a promising tool in poultry farming to influence insulin homeostasis and thus improving metabolism, growth and animal health.

In our **medium-term – multiple bolus study** (study II.), butyrate treatment in a daily bolus (0.25g/kg BW for 5 days) altered insulin sensitivity in **three-week-old** broilers in a tissue-specific manner.

In contrast to the long-term – feeding study and mentioned literature (Whittaker et al., 1987; Newman et al., 1989), oral butyrate treatment decreased the IRβ expression in the liver and adipose tissues of chickens; however, IRβ was up-regulated in skeletal muscle in the medium-term – multiple bolus study. These results suggest that butyrate can influence the cellular insulin-dependent glucose uptake in a tissue-specific manner, thus glucose is being shifted from the liver and adipose tissue to the muscles. The butyrate-triggered selectively enhanced glucose uptake of skeletal muscles may stimulate the metabolic activity of striated muscle cells, possibly also having an influence on muscle protein synthesis and carcass composition. This may confer relevant advantages in meat-producing animals.

In the medium-term – multiple bolus study PI3K (phosphatidylinositol-3-kinase) was also evaluated. This member of the insulin signaling plays a key role via the activation of Akt (protein kinase B) in the metabolic actions of insulin, such as regulating glucose uptake, stimulating glycogenesis, glycolysis and especially in liver, lipid synthesis (Cheatham, 1994). Therefore, it can be suggested that the ability of orally applied butyrate to decrease hepatic PI3K protein expression can be of special importance for the moderation of hepatic lipidosis in growing broilers.

Concerning our results, orally applied butyrate was not able to alter significantly the expression of PI3K on protein level in the examined extrahepatic tissues of chicken, but reduced it in the liver. This can be explained either with the suspected different sensitivity of various organs to butyrate or with the kinetic properties of butyrate: the gut-derived high

amount of butyrate primarily reaches liver, while only smaller quantity is transported to the peripheral tissues.

The butyrate-induced decrease in mTOR expression of the liver and subcutaneous adipose tissue, reported in the medium-term – multiple bolus study, can be associated with the lower IRβ expression and may cause suppressed protein synthesis in these organs. Interestingly, abdominal adipose tissue was not involved in the butyrate-treatment associated alteration of mTOR, while mTOR was under the detection level in the muscle.

No significant changes were found in the quantity of PKCζ on protein level in any examined tissues induced by the butyrate bolus application in the medium-term – multiple bolus study.

In general, liver was mostly involved in butyrate-triggered changes of the insulin signaling, which may be explained by its direct butyrate exposure from the intestines via the hepatic portal system. However, butyrate could be also transported to the extrahepatic tissues, reflected by elevated plasma butyrate concentration in medium-term – multiple bolus study, so it had the potential to act as a biologically active molecule. The observed data suggest that butyrate bolus application can influence the metabolic state of skeletal muscle in a completely different manner: butyrate treatment was associated with up-regulation of the insulin receptor beta expression and not significantly tended to stimulate ($P<0.10$) the PI3K and PKCζ signaling pathways, hence insulin sensitivity of muscle cells seemed to be increased by butyrate.

Possibly as a consequence of the detected butyrate-associated changes in insulin signaling, fasting blood glucose concentration significantly increased in butyrate-treated animals compared to controls. Since IRβ was down-regulated in the liver and in the adipose tissues, blood glucose and in response, insulin levels were consecutively elevated. However, skeletal muscle exclusively showed increased insulin receptor expression in association with butyrate application. Nonetheless, this glucose shifting into muscles was not capable to prevent the increase in plasma glucose and subsequently, the elevation in plasma insulin.

Elevated plasma insulin concentration was also reported in mice following oral butyrate supplementation (Lin et al., 2012). Since butyrate treatment increased insulin sensitivity of extrahepatic tissues in mice (Gao et al., 2009), these changes of insulin level in mammals are suggested to be more likely related to butyrate's ability to stimulate pancreatic insulin gene expression and secretion (Lee et al., 1994).

Based on our results regarding fasting glucose and insulin levels, the systemic insulin sensitivity seemed to decrease in chickens after five days of daily butyrate bolus treatment compared to controls. The special metabolic response to oral butyrate treatment in chicken might be related to the avian peculiarities of insulin homeostasis and carbohydrate metabolism (Sweazea, 2006), such as the presence of the glycogen body. As birds

preferentially use fatty acids as energy source (Jenni-Eiermann, 2002), butyrate, as a substrate of energy yielding, can contribute to maintaining insulin resistance. However, the exact mechanism, by which butyrate affects insulin signaling in chicken, should be investigated in further studies.

In addition to the the various sources and application forms of butyrate, the partly contradictory results of the long-term – feeding and medium-term – multiple bolus studies could also be in association with the different age of broilers (three weeks vs six weeks). The arising hypotheses may recommend further studies on age-, application form- and origin-dependency of butyrate's efficacy. Considering the age-dependent differences, the effects of butyrate on insulin homeostasis of broilers may be less pronounced at the age of 6 weeks, because the phase of intensive growth, when insulin as an anabolic hormone is mostly involved in growth regulation, is already completed. It was previously described that sensitivity of insulin signaling proteins decreased with age in both mammal (Gupte et al., 2008) and chicken (Deng et al., 2014).

7.4.2. Effects of butyrate on incretins

It has been described that orally applied butyrate as a potent effector of carbohydrate and lipid metabolism can increase both insulin secretion and insulin sensitivity in rat (Gao et al., 2009), mediated partly by the stimulation of intestinal incretin secretion (Lin et al., 2012). Influencing both the pancreatic production and the cellular signaling of insulin may greatly improve its efficacy in the regulation of metabolism and growth. However, in our medium-term – multiple bolus study butyrate significantly decreased the expression of key insulin signaling proteins in the liver of chickens, but exclusively up-regulated IRβ in skeletal muscles, revealing the species- and tissue-dependency of butyrate's action on insulin homeostasis. Based on these findings, the aim of the **short-term – single bolus study** (study III.) was to provide more data concerning the effects of orally applied butyrate on insulin secretion and its major mediators, the incretins in chicken and rabbit as avian and mammalian target species of butyrate application. In addition, comparing the butyrate-associated changes in the endocrine regulatory mechanisms may help to get a better insight towards the species-related differences of incretin action.

Our results regarding the interaction of butyrate exposure and incubation time showed that orally applied butyrate affected plasma GIP (glucose-dependent insulinotropic peptide) concentrations in both chickens and rabbits, but no significant effects could be detected with regard on the GLP-1 (glucagon-like peptide 1) and insulin levels. The direction, extent and time course of the butyrate-associated changes seemed to be similar in both examined species as an approx. 40% decrease in plasma GIP concentration at the sampling points of

30 and 60 min following butyrate exposure (compared to sampling at 0 min). Even these intense alterations of GIP levels were not realized in significant changes of the plasma insulin concentration, confirming that GIP plays only a partial role in the complex neuroendocrine regulation of insulin release.

The observed butyrate-associated reductions in plasma GIP levels are in contrast with the findings of previous studies in mice, where similarly applied butyrate significantly increased the plasma concentrations of both incretins and insulin (Lin et al., 2012). Notwithstanding that further studies are needed to explain the observed differences between mice and the examined species of our trial, the phenomenon might be in association with some species-related differences in carbohydrate metabolism. There are clear and remarkable differences between the insulin homeostasis of mammals and birds, but in this case various mammalian species (mouse and rabbit) responded to butyrate in a different manner, while chicken and rabbit on a similar way.

However, it should be also highlighted that 3-month-old mice (considered as young adults) were used in the trial of Lin et al. (2012), while both rabbits and chickens of the present study were in the phase of intensive growth. According to the species-specific dietary requirements, rabbit diets always contain higher amount of dietary fibers than those of mouse or chicken, providing more substrate for the microbial SCFA production in the large intestines. Therefore, a relevant amount of butyrate is produced in the caecum of rabbit (Combes et al. 2011), possibly making the tissues more adapted and being less sensitive to butyrate compared to mouse and chicken. Chicken and rabbit were chosen in our present study, because they are both target species of oral butyrate supplementation as a feed additive (Carraro et al. 2005; Hu and Guo 2007); further, they can also serve as avian and mammalian models in metabolic studies. Applying rabbits instead of the most common rodents as models provides more possibilities for inter-species comparisons (newly gained data of rabbit can be compared to those of previous rat and mouse studies and not only to the present results of chicken). However, the intense caecal bacterial digestion in rabbit should be also addressed as a limitation when considering the inter-species differences and applying rabbit as a model species.

The effects of SCFA on incretin homeostasis can also be diverse even in the same species. Continuous, chronic dietary exposure to SCFA significantly decreased plasma incretin levels in mice (Frassetto et al. 2011) and feeding a fiber-rich diet (stimulating intestinal SCFA production) could also diminish plasma GIP concentration in rat (Tuohi and Del Rio 2014). Based on these data, it should be hypothesized that the intestinal expression of SCFA receptors (such as FFR2 and FFR3), the way of SCFA application (acute or chronic challenge, stimulating intestinal fermentation) may also contribute to the observed differences of incretin response (Frassetto et al. 2011; Bolognini et al. 2016). It was also

considered that the age might play a role in the changes of incretin and insulin secretion. Therefore, both chickens and rabbits were investigated in our study in the phase of intensive growth to get a reliable comparability.

When examining the effects of oral butyrate exposure on plasma GIP concentrations in chicken and rabbit, it can be seen that the higher dose of butyrate (1.25 g/kg BW) was required to cause significant alterations in chicken, while only the lower dose (0.25 g/kg BW) was capable to similarly reduce GIP levels in rabbits. This difference might be explained by the mentioned intensive intestinal butyrate production of rabbits (Combes et al., 2011). The high amount of endogenously produced gut-derived butyrate together with the orally ingested one results in much more elevated plasma and tissue butyrate concentrations in rabbit than in chicken. It is also known that especially high concentrations of butyrate may have no or opposite, adverse effects on several metabolic processes compared to lower doses, already stated in numerous *in vitro* and *in vivo* studies. For instance, oral bolus application of butyrate affected the acetylation rate of hepatic histones in chickens in a dose-dependent manner (Mátis et al., 2013b). The acetylation of histone H3 was stimulated by the higher dose (1.25 g/kg BW) of butyrate only, while exclusively the lower dose (0.25 g/kg BW) could increase the acetylation state of histone H4 (Mátis et al., 2013b). Similarly, butyrate applied orally in the dose of 0.25 g/kg BW significantly ameliorated the enzyme inducing action of phenobarbital on hepatic CYP enzymes in chickens, but this effect was absent when given in the dose of 1.25 g/kg BW (Mátis et al., 2016). The lack of butyrate's action at higher concentrations was also found in certain bacterial strains, such as in *Rhodopseum faecalis* RLD-53, where butyrate supplied at lower levels increased hydrogen production, but not at elevated concentrations (Ren et al., 2018). Dose-associated alterations of butyrate's action may be in connection with its dose-dependent epigenetic effects on histone deacetylase and histone acetyltransferase enzymes influencing histone acetylation, playing central role in the mediation of butyrate's major actions (Rada-Iglesias et al., 2007).

The allocation of the animals to experimental groups was carried out randomly, but controlled for the body weight ensuring no significant differences between any groups. The baseline (0 min) plasma concentration of the measured parameters was not taken into account for the allocation, but no significant differences were observed between any groups at 0 min concerning all measures except one. Plasma glucose concentration of rabbits exposed to the lower dose of butyrate was significantly higher than that of controls at 0 min (6.01 ± 0.18 mmol/l vs. 5.53 ± 0.16 mmol/l; $P = 0.011$), but this difference cannot be considered as a relevant one and suggested to play no role in the observed endocrine alterations. The further observable numerical differences (e.g. GIP and insulin in rabbits) did not reach the level of statistical significance. Plasma glucose concentrations increased with time in rabbits, independently of the butyrate exposure. This can be in association with the

stress-sensitivity of rabbits, despite of the minimized pain and stress during repeated blood samplings.

Concerning the correlations between the measured endocrine parameters in short-term – single bolus study, some major differences were found between chickens and rabbits. GLP-1 and insulin were positively correlated in both chickens and rats, justifying the expected stimulatory role of GLP-1 on pancreatic insulin secretion. However, GIP and insulin showed a positive correlation in chickens, but this correlation was completely missing in rabbits. Furthermore, a possible negative correlation, indicating a slight anti-insulinotropic action, cannot be excluded in the latter species between plasma GIP and insulin levels based on the obtained correlation results. In chickens, there was a strong positive correlation between GIP and GLP-1, which was lacking in rabbits.

These findings highlight that there might be species-specific differences in the action of incretins on pancreatic insulin secretion, being important from comparative physiological approach. In chickens, the detected positive correlations confirm the presumed synergistic inducing action of both GIP and GLP-1 on β cell insulin release. However, it can be assumed that GIP does not play major insulinotropic role in rabbit, unlike in chicken, mouse and human (Miyawaki et al., 1999; Gault et al., 2002).

As a conclusion, in the present short-term – single bolus study, it is justified that butyrate has a significant role in influencing insulin homeostasis in both chickens and rabbits, which is suggested to be partly mediated by incretins. It can be stated that butyrate may have different effects on incretin and insulin secretion in various species, presenting differences even among mammalian species. Therefore, it is suggested that the nutritional modulation of insulin secretion should be specifically investigated in each target species, and results from model studies may be extrapolated to other species with strong limitations only. In addition, based on the analyzed correlations it can be assumed that incretins may regulate pancreatic insulin release on a different way in rabbits compared to other examined mammals and chickens: the major stimulatory action of GIP on insulin secretion may be questionable in rabbits according to the lacking correlation of GIP and insulin and the negative correlation between the two incretin hormones.

7.4.3. Effect of butyrate on plasma insulin and glucose concentration

We studied the effect of butyrate treatments on insulin homeostasis on 3 different ways of butyrate exposure: long-term – feed additive, medium-term – multiple bolus application, and short-term – single bolus application.

The impact of butyrate on insulin homeostasis could be reflected by the changes in insulin and glucose plasma concentrations as well. In our studies, a significant effect of butyrate treatment was found in the medium-term study only, where both insulin and glucose

concentrations were increased by the butyrate bolus treatment, and in the long-term – feeding study, where insulin concentration was decreased due to the application of WB diet.

In the **long-term – feeding study** blood plasma insulin concentration was decreased by WB diet, but we found no changes in blood plasma glucose concentration. Among the monitored elements of the insulin signaling pathway, the protein expression of IRβ increased in the liver, mTOR was up-regulated in the liver and subcutaneous adipose tissue, and PKCζ protein expression was stimulated in the subcutaneous adipose tissue by WB diet. As the expression of the mentioned insulin signaling proteins increased by the application of WB diet, the decreased plasma insulin concentration could be considered as a kind of compensation to maintain the constant glucose concentration for a longer time.

In the **medium-term – multiple bolus study** both insulin and glucose concentrations were highly increased due to the daily 0.25 g/kg BW butyrate bolus treatment for five days. Among the investigated insulin signaling proteins, the expression of all IRβ, PI3K and mTOR was decreased in the liver, IRβ was down-regulated in the adipose tissues; however, IRβ protein expression was selectively increased in the skeletal muscles. The medium-term bolus application of butyrate was able to influence the expression of certain signaling proteins, hence this intense effect could affect the balance of the homeostasis, as both insulin and glucose concentrations were highly increased due to the butyrate treatment.

In the **short-term – single bolus study** we found no effect of butyrate bolus treatment on plasma insulin and glucose concentrations even when applied in a higher dose (1.25 g/kg BW). Among the investigated incretin hormones as mediatory factors of insulin secretion, the concentration of GIP in blood plasma was significantly decreased by the higher dose of butyrate in case of chicken.

Although the concentration of GIP as a stimulator of pancreatic insulin production was influenced by butyrate treatment, and we also found a significant positive correlation between both GIP – insulin and GIP – glucose concentrations, during this short, one hour exposure time the effect of butyrate treatment was not detectable on the level of insulin and glucose concentrations of the blood plasma.

8. New scientific results

Ad 1, Different application forms of dietary butyrate can be absorbed from different sections of the gastrointestinal tract of broiler chicken. Protected butyrate supplementation (0.2 g/kg diet) elevates butyrate concentration in the ileum, while wheat-based diet with NSP degrading enzyme supplementation (associated with higher microbial butyrate production in the large intestines) increases it in the caecum. Portal plasma butyrate concentration is increased by all the higher dose of non-protected butyrate (3.0 g/kg diet), the protected butyrate supplementation and the wheat-based diet. However, systemic plasma butyrate concentration is increased by the higher dose of non-protected butyrate supplementation and daily bolus application of non-protected butyrate (0.25 g/kg body weight for 5 days).

Ad 2, Both dietary and endogenous butyrate (produced in the large intestines) alter the activity of certain duodenal cytochrome P450 (CYP) enzymes. The CYP1A4/5 and CYP2H2 activities of six-week-old broiler chickens are increased by butyrate supplementation (non-protected, 3 g/kg diet) and by wheat-based diet with NSP degrading enzyme supplementation (associated with higher microbial butyrate production).

Ad 3, Butyrate is able to influence insulin homeostasis in broiler chicken. Wheat-based diet with NSP degrading enzyme supplementation (associated with higher microbial butyrate production) increases IRβ and mTOR expression in the liver as well as mTOR and PKCζ expression in the adipose tissue of six-week-old chickens. IRβ expression in the liver is stimulated also by the lower dose of non-protected butyrate (1.5 g/kg diet) in 6-week-old chickens. At the age of 3 weeks, daily butyrate bolus application (non-protected, 0.25 g/kg body weight for 5 days) decreases IRβ, PI3K and mTOR expression in liver and IRβ and mTOR expression in the adipose tissues, but increases IRβ expression in the muscle.

Ad 4, Butyrate can be a potent effector of incretin hormones in both chicken and rabbit. Single bolus application of non-protected butyrate decreases plasma GIP concentration of 3-week-old broiler chickens in higher dose (1.25 g/kg body weight), and of 6-week-old rabbits in lower dose (0.25 g/kg body weight).

9. References

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10. Own scientific publications

10.1. Publications related to the topic of the present dissertation

Full text papers in peer-reviewed journals:

Gábor Mátis, Anna Kulcsár, Máté Mackei, Janka Petrilla, Zsuzsanna Neogrády

Comparative study on the modulation of insulin and incretin homeostasis by butyrate in chickens and rabbits

PLOS ONE 13(10): e0205512, 2018.

Anna Kulcsár, Gábor Mátis, Andor Molnár, Janka Petrilla, László Wágner, Hedvig Fébel, Ferenc Husvéth, Károly Dublec Zsuzsanna Neogrády

Nutritional modulation of intestinal drug-metabolizing cytochrome P450 by butyrate of different origin in chicken

RESEARCH IN VETERINARY SCIENCE 113 pp 25-32, 2017.

Anna Kulcsár, Gábor Mátis, Andor Molnár, Janka Petrilla, Ferenc Husvéth, Korinna Huber, Károly Dublecz, Zsuzsanna Neogrády

Effects of butyrate on the insulin homeostasis of chickens kept on maize- or wheat-based diets

ACTA VETERINARIA HUNGARICA 64:(4) pp. 482-496, 2016.

Kulcsár Anna, Mátis Gábor, Kulcsárné Petrilla Janka és Neogrády Zsuzsanna

A bélnyálkahártya szerepe a xenobiotikumok metabolizmusában, különös tekintettel a citokróm P450 enzimrendszerre. Irodalmi áttekintés (The role of intestinal mucosa in the metabolism of xenobiotics with particular regard to the cytochrome P450 enzyme system. Literature review)

MAGYAR ÁLLATORVOSOK LAPJA 138:(4) pp. 243–250, 2016.

Gábor Mátis, Anna Kulcsár, Vanessa Turowski, Hedvig Fébel, Zsuzsanna Neogrády, Korinna Huber

Effects of oral butyrate application on insulin signaling in various tissues of chickens

DOMESTIC ANIMAL ENDOCRINOLOGY 50: pp. 26-31, 2015.

Gábor Mátis, Péter Lengyel, Anna Kulcsár, Janka Kulcsárné Petrilla, Zsuzsanna Neogrády

A szénhidrát-anyagcsere és az inzulin-homeosztázis sajátosságai csirkében. Irodalmi összefoglaló (Special characteristics of carbohydrate metabolism and insulin homeostasis in chicken. Literature review)

MAGYAR ÁLLATORVOSOK LAPJA 136:(6) pp. 342–349, 2014.

Oral and poster presentations on international conferences

Anna Kulcsár, Gábor Mátis, Janka Petrilla, Máté Mackei and Zsuzsanna Neogrády

Comparative examinations on the nutritional modulation of incretin and insulin secretion in chicken and rabbit

DVG Conference, Berlin, Németország, 2016.

Gábor Mátis, Anna Kulcsár, Andor Molnár, Janka Petrilla, László Wágner, Károly Dublecz, Zsuzsanna Neogrády

Butyrate of different origin affects intestinal drug-metabolizing cytochrome P450 enzymes in chicken

XXV World's Poultry Science Congress, Peking, Kína, 2016

Anna Kulcsár, Gábor Mátis, Andor Molnár, László Wágner, Hedvig Fébel, Korinna Huber, Zsuzsanna Neogrády

The effect of different application forms of (n-)butyrate on the intestinal activity of cytochrome P450 enzymes in chicken

GfE (Society of Nutrition Physiology) Conference, Gottingen, Germany, 2015.

Anna Kulcsár, Gábor Mátis, VanessaTurowski, Zsuzsanna Neogrády, Korinna Huber

Effects of oral butyrate application on insulin signaling in various tissues of chickens

VIIIth International Congress on Farm Animal Endocrinology, Billund, Dánia, 2015.

Gábor Mátis, Anna Kulcsár, Janka Petrilla, Zsuzsanna Neogrády

Nutritional modulation of insulin and incretin homeostasis by butyrate in broiler chickens

GfE (Society of Nutrition Physiology) Conference, Gottingen, Germany, 2015.

Gábor Mátis, Anna Kulcsár, Vanessa Turowski, Zsuzsanna Neogrády, Korinna Huber

The effect of orally applied butyrate on the insulin signaling pathway in various tissues of broiler chickens

GfE (Society of Nutrition Physiology) Conference, Gottingen, Germany, 2014.

Gábor Mátis, Anna Kulcsár, VanessaTurowski, Zsuzsanna Neogrády, Korinna Huber

Oral butyrate application affects insulin signaling in chicken

XXI. Tagung der Fachgruppe Physiologie und Biochemie der Deutschen Veterinärmedizinischen Gesellschaft, Zürich, Svájc, 2014.

Oral presentations on Hungarian national conferences

Kulcsár Anna, Mátis Gábor, Petrilla Janka, Mackei Máté és Neogrády Zsuzsanna

Az inkretin és inzulin szekréció eltérő alakulása csirkében és nyúlban: összehasonlító vizsgálatok

MTA Akadémiai Beszámolók, Budapest, Hungary, 2016.

Kulcsár Anna, Mátis Gábor, Molnár Andor, Kulcsárné Petrilla Janka, Farkas Orsolya, Wágner László, Fébel Hedvig és Neogrády Zsuzsanna

Enterális CYP enzimek aktivitásának változása a (n-)butirát különböző alkalmazási formáinak hatására csirkében

MTA Akadémiai Beszámolók, Budapest, Hungary, 2015.

Mátis Gábor, Kulcsár Anna, Kulcsárné Petrilla Janka, Lengyel Péter, Mackei Máté és Neogrády Zsuzsanna

Az inzulin- és az inkretin-homeosztázis befolyásolása nutritív faktorokkal csirkében

MTA Akadémiai Beszámolók, Budapest, Hungary, 2015.

Kulcsár Anna, Mátis Gábor, Kenéz Ákos, Neogrády Zsuzsanna, Korinna Hube

Az inzulin jelpálya egyes fehérjéinek vizsgálata csirkék különböző szöveteiben

MTA Akadémiai Beszámolók, Budapest, Hungary, 2014.

10.2. Publications not related to the topic of the present dissertation

Full text papers in peer-reviewed journals:

Janka Petrilla, Gábor Mátis, Anna Kulcsár, Petra Talapka, Enikő Bíró, Máté Mackei, Hedvig Fébel, Zsuzsanna Neogrády

Effect of dietary cereal type, crude protein and butyrate supplementation on metabolic parameters of broilers.

ACTA VETERINARIA HUNGARICA, 66(3):408-52, 2018.

Kurucz Ádám, Nagy Csaba, Kulcsár Anna, Neogrády Zsuzsanna, Mátis Gábor

Méregtelenítő folyamatok vizsgálata vadon élő állatfajokban. Investigations of detoxifying processes in wild animal species

MAGYAR ÁLLATORVOSOK LAPJA, közlésre benyújtva, 2017.

Gábor Mátis, Anna Kulcsár, Janka Petrilla, Petra Talapka, Zsuzsanna Neogrády

Porcine hepatocyte-Kupffer cell co-culture as an in vitro model for testing the efficacy of anti-inflammatory substances

JOURNAL OF ANIMAL PHYSIOLOGY AND ANIMAL NUTRITION, 101, pp. 201-207, 2017.

Gábor Mátis, Anna Kulcsár, Janka Petrilla, Katalin Hermándy-Berencz, Zsuzsanna Neogrády
Feed-drug interaction of orally applied butyrate and phenobarbital on hepatic cytochrome P450 activity in chickens
JOURNAL OF ANIMAL PHYSIOLOGY AND ANIMAL NUTRITION 100, pp. 637–642, 2016.

Ákos Kenéz, Anna Kulcsár, Franziska Kluge, Idir Benbelkacem, Kathrin Hansen, Lena Locher, Ulrich Meyer, Jürgen Rehage, Sven Dänicke, Korinna Huber
Changes of Adipose Tissue Morphology and Composition during Late Pregnancy and Early Lactation in Dairy Cows
PLOS ONE 10:(5) e0127208, 2015.

Mátis Gábor, Hatala Patrícia, Kulcsár Anna, Kulcsárné Petrilla Janka, Neogrády Zsuzsanna
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
MAGYAR ÁLLATORVOSOK LAPJA 137:(9) pp. 569-575, 2015.

György Csikó, Gábor Nagy, Gábor Mátis, Zsuzsanna Neogrády, Anna Kulcsár, Ákos Jerzsele, Krisztina Szekér, Péter Gálfi
Effects of dietary sodium butyrate on hepatic biotransformation and pharmacokinetics of erythromycin in chickens
JOURNAL OF VETERINARY PHARMACOLOGY AND THERAPEUTICS 37:(4) pp. 406-412, 2014.

Orsolya Farkas, Gábor Mátis, Erzsébet Pászti-Gere, Orsolya Palócz, Anna Kulcsár, Janka Petrilla, György Csikó, Zsuzsanna Neogrády, Péter Gálfi
Effects of Lactobacillus plantarum 2142 and sodium n-butyrate in LPS-triggered inflammation: comparison of IPEC-J2 and primary hepatocyte mono-cultures with a porcine enterohepatic co-culture system
JOURNAL OF ANIMAL SCIENCE 92:(9) pp. 3835-3845, 2014.

Erzsébet Pászti-Gere, Gábor Mátis, Orsolya Farkas, Anna Kulcsár, Orsolya Palócz, György Csikó, Zsuzsanna Neogrády, Péter Gálfi
The effects of intestinal LPS exposure on inflammatory responses in a porcine enterohepatic co-culture system
I**NFLAMMATION** 37:(1) pp. 247-260, 2014.

Gábor Mátis, Zsuzsanna Neogrády, György Csikó, Anna Kulcsár, Ákos Kenéz, Korinna Huber
Effects of orally applied butyrate bolus on histone acetylation and cytochrome P450 enzyme activity in the liver of chicken – a randomized controlled trial
NUTRITION & METABOLISM 10: p. 12, 2013.

Mátis Gábor, Csikó György, Jemnitz Katalin, Veres Zsuzsanna, Fébel Hedvig, Kulcsár Anna, Petrilla Janka, Neogrády Zsuzsanna

A takarmányba kevert butirát citokróm P450 enzimekre gyakorolt hatásának vizsgálata patkány májban: Investigation of the effect of butyrate supplementation of the diet on hepatic cytochrome P450 enzymes in rats

MAGYAR ÁLLATORVOSOK LAPJA 135:(2) pp. 109-116, 2013.

Gábor Mátis, Zsuzsanna Neogrády, György Csikó, Péter Gálfi, Hedvig Fébel, Katalin Jemnitz, Zsuzsanna Veres, Anna Kulcsár, Ákos Kenéz, Korinna Huber

Epigenetic effects of dietary butyrate on hepatic histone acetylation and enzymes of biotransformation in chicken

ACTA VETERINARIA HUNGARICA 61:(4) pp. 477-499, 2013.

Veronika Bókony, Anna Kulcsár, Zoltán Tóth, András Liker

Personality traits and behavioral syndromes in differently urbanized populations of house sparrows (Passer domesticus)

PLoS One 7:(5), e36639, 2012.

Veronika Bókony, Anna Kulcsár, András Liker

Does urbanization select for weak competitors in house sparrows?

OIKOS 119:(3) pp. 437-444, 2010.

András Liker, Veronika Bókony, Anna Kulcsár, Zoltán Tóth, Krisztián Szabó, Balázs Kaholek, Zsolt Pénzes

Genetic relatedness in wintering groups of house sparrows (Passer domesticus)

MOLECULAR ECOLOGY 18:(22) pp. 4696-4706, 2009.

Veronika Bókony, András Liker, Ádám Zoltán Lendvai, Anna Kulcsár

Risk-taking and survival in the House Sparrow Passer domesticus: are plumage ornaments costly?

IBIS 150, pp. 139-151, 2008.

Oral and poster presentations on international conferences

Anna Kulcsár, Dénes Dudás, Gábor Mátis, Patrícia Hatala, Hedvig Fébel, Zsuzsanna Neogrády

The effect of age and diet type on the hepatic and intestinal CYP activity in broiler chicken

XVth European Poultry Conference, Dubrovnik, Horvátország, 2018.

Gábor Mátis, Anna Kulcsár, Patrícia Hatala, Máté Mackei, Zsuzsanna Neogrády

Investigations on the effects of heat stress on hepatic cell culture models of chicken origin

XVth European Poultry Conference, Dubrovnik, Horvátország, 2018.

Anna Kulcsár, Gábor Mátis, Janka Petrilla, Petra Talapka, Hedvig Fébel, Zsuzsanna Neogrády, Korinna Huber

Influencing insulin homeostasis of broiler chicken by maize- or wheat-based diets

XXth World Veterinary Poultry Association Congress, Edinburgh, Skócia, 2017.

Anna Kulcsár, Gábor Mátis, Janka Petrilla, Petra Talapka, Zsuzsanna Neogrády, Korinna Huber

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, Göttingen, Németország, 2017.

Gábor Mátis, Anna Kulcsár, Janka Petrilla, Petra Talapka, Márton Bardóczy, Máté Mackei, Zsuzsanna Neogrády, Hedvig Fébel

Investigations on the effects of certain nutritional factors on carcass composition of broiler chickens

GfE (Society of Nutrition Physiology) Conference, Göttingen, Németország, 2017.

Janka Petrilla, Gábor Mátis, Anna Kulcsár, Petra Talapka, Enikő Bíró, Máté Mackei, Hedvig Fébel, Zsuzsanna Neogrády

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, Göttingen, Németország, 2017.

Gábor Mátis, Anna Kulcsár, Janka Petrilla, Kata Orbán, Zsuzsanna Neogrády

Porcine hepatocyte – Kupffer-cell co-cultures as in vitro models for testing the efficacy of anti-inflammatory molecules

DVG Conference, Physiology and Biohemistry, Berlin, Németország, 2016.

Gábor Mátis, Anna Kulcsár, Janka Petrilla, Zsuzsanna Neogrády

Establishment of a porcine hepatocyte – Kupffer cell co-culture as a novel inflammatory model in veterinary research

XVIIIth International Symposium on Cells of the Hepatic Sinusoid, Asilomar, Amerikai Egyesült Államok, 2015.

Anna Kulcsár, Kinoshita Asako, Ákos Kenéz, Jürgen Rehage, Korinna Huber

Changes in triglyceride concentration of liver, retroperitoneal and subcutaneous adipose tissue around transition period in dairy cows

XXI. Tagung der Fachgruppe Physiologie und Biochemie der Deutschen Veterinärmedizinischen Gesellschaft, Zürich, Svájc, 2014.

Katalin Hermándy-Berencz, Gábor Mátis, Janka Petrilla, Anna Kulcsár, Korinna Huber, Zsuzsanna Neogrády

How can butyrate modify the induced cytochrome P450 enzyme activity in the liver of

chicken?

GfE (Society of Nutrition Physiology) Conference, Göttingen, Németország, 2014.

Gábor Mátis, Anna Kulcsár, Janka Petrilla, Ákos Kenéz, György Csikó, Zsuzsanna Neogrády, Korinna Huber

Epigenetic consequences of oral butyrate application in chicken

GfE (Society of Nutrition Physiology) Conference, Göttingen, Németország, 2013.

Gábor Mátis, Erzsébet Pászti-Gere, Orsolya Farkas, Anna Kulcsár, Orsolya Palócz, Janka Petrilla, György Csikó, Zsuzsanna Neogrády, Péter Gálfi

Effects of LPS challenge and the role of probiotics in IPEC-J2 cell monoculture and a novel porcine enterohepatic co-culture system

International Scientific Conference on Probiotics and Prebiotics, Košice, Szlovákia, 2013.

Gábor Mátis, György Csikó, Katalin Jemnitz, Zsuzsanna Veres, Mónika Szabó, Anna Kulcsár, Ákos Kenéz, Péter Gálfi, Zsuzsanna Neogrády

Effects of dietary butyrate supplementation on hepatic microsomal cytochrome P450 activity in chicken and rat

Febs 3+ Meeting, Opatija, Horvátország, 2012.

Gábor Mátis, Ákos Kenéz, Anna Kulcsár, György Csikó, Zsuzsanna Neogrády, Korinna Huber

Trophic and epigenetic effects of dietary butyrate supplementation in chicken

GfE (Society of Nutrition Physiology) Conference, Göttingen, Németország, 2012.

Veronika Bókony, Anna Kulcsár, Ádám Zoltán Lendvai, Olivier Chastel, Barbara Kanizsai, Diána Lázár, Polett E. Gulyás, Gábor Seress, András Liker

Behavioral and physiological coping styles in house sparrows

XXVth International Ornithological Congress, Campos do Jordão, Brazil, 2010.

Anna Kulcsár, Veronika Bókony, András Liker, Zoltán Tóth

Neophobia and risk-taking of urban and rural house sparrows (Passer domesticus)

Xth Behavioural Ecology Meeting, Cluj Napoca, Románia, 2009.

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Oral and poster presentations on Hungarian national conferences

Kulcsár Anna, Sebők Csilla, Mátis Gábor, Talapka Petra, Hatala Patrícia, Petrilla Janka, Fébel Hedvig, Neogrády Zsuzsanna

Az inzulin és a glukagon jelpálya különböző takarmányozási faktorok segítségével történő szabályozása brojlercsirkében

MTA Akadémiai Beszámolók, Budapest, Hungary, 2018.

Mátis Gábor, Kulcsár Anna, Hatala Patrícia, Tóth Adrienn, Mackei Máté, Neogrády Zsuzsanna

A T-2 toxin sejtkárosító hatásainak összehasonlító vizsgálata csirke primer bélhámsejt- és májsejttenyészeten

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Kulcsár Anna, Mátis Gábor, Kulcsárné Petrilla Janka, Talapka Petra, Neogrády Zsuzsanna, Korinna Huber

A kukorica és búza alapú takarmány hatása az inzulin-homeosztázisra brojlercsirkében

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Kulcsárné Petrilla Janka, Mátis Gábor, Kulcsár Anna, Talapka Petra, Bíró Enikő, Mackei Máté, Fébel Hedvig, Neogrády Zsuzsanna

Metabolikus paraméterek változásai a takarmánygabona típusa, a takarmány nyersfehérje-tartalma és butirátkiegészítés hatására csirkében

MTA Akadémiai Beszámolók, Budapest, Hungary, 2017.

Mátis Gábor, Kulcsár Anna, Kulcsárné Petrilla Janka, Talapka Petra, Bardóczy Márton, Mackei Máté, Neogrády Zsuzsanna, Fébel Hedvig

Egyes takarmányozási tényezők brojlercsirkék testösszetételére gyakorolt hatásának vizsgálata

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A hő-stressz sejtszintű hatásainak vizsgálata csirke májsejt – Kupffer-sejt ko-kultúrán

MTA Akadémiai Beszámolók, Budapest, Hungary, 2017.

Mátis Gábor, Kulcsár Anna, Kulcsárné Petrilla Janka, Orbán Kata, Neogrády Zsuzsanna

Terpinen-4-ol és nátrium n-butirát gyulladáscsökkentő hatásának vizsgálata májsejt – Kupffer-sejt ko-kultúrákon

MTA Akadémiai Beszámolók, Budapest, Hungary, 2016.

Mátis Gábor, Kulcsár Anna, Kulcsárné Petrilla Janka, Hatala Patrícia, Kővágó Csaba, Neogrády Zsuzsanna

Bakteriális lipopoliszacharidok által kiváltott gyulladás vizsgálata sertés hepatocyta – kupffer-sejt ko-kultúra modellen

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Mátis Gábor, Kulcsár Anna, Kulcsárné Petrilla Janka, Hatala Patrícia, K_vágó Csaba, Csikó György, Neogrády Zsuzsanna

A kupffer-sejtek arányának meghatározása sertés primer májsejttenyészeten

MTA Akadémiai Beszámolók, Budapest, Hungary, 2014.

Mátis Gábor, Kulcsárné Petrilla Janka, Kulcsár Anna, Hermándy-Berencz Katalin, Csikó György, Neogrády Zsuzsanna

Befolyásolja-e a butirát az indukált citokróm P450 aktivitást a csirke májában

MTA Akadémiai Beszámolók, Budapest, Hungary, 2014.

Palócz Orsolya, Farkas Orsolya, Csikó György, Pásztiné Gere Erzsébet, Mátis Gábor, Kulcsár Anna, Petrilla Janka, Neogrády Zsuzsanna, Gálfi Péter

Probiotikumok hatása LPS kiváltotta gyulladás mellett in vitro bélhám – májsejt ko-kultúra modellen

MTA Akadémiai Beszámolók, Budapest, Hungary, 2014.

Kulcsár Anna, Molnár Andor, Mátis Gábor, Petrilla, Janka, Pál László, Jerzsele Ákos, Neogrády Zsuzsanna, Dublecz Károly

A különböző koncentrációban adott butirát gatló hatásának *in vitro* vizsgálata Campylobacter jejuni törzsekre

MTA Akadémiai Beszámolók, Budapest, Hungary, 2013.

Farkas Orsolya, Palócz Orsolya, Csikó György, Pásztiné Gere Erzsébet, Mátis Gábor, Kulcsár Anna, Petrilla Janka, Neogrády Zsuzsanna, Gálfi Péter

Oxidatív stress és gyulladás hatásának vizsgálata *in vitro* bélhám- és májsejt ko-kultúra modellen

MTA Akadémiai Beszámolók, Budapest, Hungary, 2013.

Mátis Gábor, Kenéz Ákos, Kulcsár Anna, Csikó György, Neogrády Zsuzsanna, Huber Korinna

A bólusban adott butirát májsejtek hiszton-acetilációjára gyakorolt hatásának vizsgálata brojlercsirkében

MTA Akadémiai Beszámolók, Budapest, Hungary, 2013.

Mátis Gábor, Csikó György, Kulcsár Anna, Petrilla Janka, Pleva Dániel, Neogrády Zsuzsanna, Gálfi Péter

Máj citokróm P450 enzimek aktivitásanak vizsgálata bólusban adott butirátkezelést követően brojlercsirkében

MTA Akadémiai Beszámolók, Budapest, Hungary, 2013.

Mátis Gábor, Csikó György, Kulcsár Anna, Petrilla Janka, Farkas Orsolya, Palócz Orsolya, Neogrády Zsuzsanna, Gálfi Péter

Bakteriális lipopoliszacharidok által kiváltott oxidatív stressz hatása sertés primer májsejttenyészetre

MTA Akadémiai Beszámolók, Budapest, Hungary, 2013.

Matis Gábor, Kenéz Ákos, Kulcsár, Anna, Csikó György, Neogrády Zsuzsanna, Huber Korinna

A takarmánykiegészitőként adott butirát epigenetikus hatásának vizsgálata brojlercsirkében

MTA Akadémiai Beszámolók, Budapest, Hungary, 2012.

Mátis Gábor, Csikó György, Kulcsár Anna, Kenéz Ákos, Jemnitz Katalin, Veres Zsuzsanna, Neogrády Zsuzsanna

A takarmánykiegészitőként adott butirát máj citokróm P450 izonezimekre gyakorolt hatásának vizsgálata brojlercsirkében és patkányban

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A papilloma vírusfertőzés kapcsolata színezeti jellegekkel házi verébnél

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Bókony Veronika, Liker András, Kulcsár Anna

A színezet kapcsolata a predációs kockázat-vállalással házi verebeknél

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10.3. Supervising of DVM theses

Dudás Dénes: A takarmánygabona és a nyersfehérje-tartalom intesztinális méregtelenítő enzimekre gyakorolt hatásának vizsgálata csirkében
Szakdolgozat, supervisor: Kulcsár Anna, 2017.

Sebők Csilla: Egyes takarmányozási faktorok inzulin jelpályára gyakorolt hatásának vizsgálata brojlercsirkében
TDK dolgozat, supervisors: Kulcsár Anna, Hatala Patrícia, 2017.

Karsai Szófia Ludmilla: A butirát enterális citokróm P450 méregtelenítő enzimekre gyakorolt hatásának vizsgálata csirkében
TDK dolgozat, supervisor: Kulcsár Anna, 2014.

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TDK dolgozat, supervisors: Mátis Gábor, Kulcsár Anna, 2013.

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