Szent István University
Postgraduate School of Veterinary Science
Budapest, Hungary

Effects of butyrate on hepatic epigenetics and microsomal drug-metabolizing enzymes in chicken

PhD thesis

By:
Gábor Mátis, DVM

2013

Szent István University
Postgraduate School of Veterinary Science
Budapest, Hungary

Supervisors and consultants:

.....
Dr. Zsuzsanna Neogrády, CSc
Department of Physiology and Biochemistry
Faculty of Veterinary Science, Szent István University
supervisor

.....
Dr. György Csikó, CSc
Department of Pharmacology and Toxicology
Faculty of Veterinary Science, Szent István University
co-supervisor

Dr. Korinna Huber, Univ.-Prof.
Department of Physiology
University of Veterinary Medicine, Hannover
external supervisor

Dr. Katalin Jemnitz, PhD
Institute of Molecular Pharmacology
Research Centre of Natural Sciences, Hungarian Academy of Sciences
consultant

Dr. Krisztina Szekér, PhD
Department of Pharmacology and Toxicology
Faculty of Veterinary Science, Szent István University
consultant

Copy …….of eight.

.....
Dr. Gábor Mátis

”The scientist does not study nature because it is useful; he studies it because he delights in it, and he delights in it because it is beautiful. If nature were not beautiful, it would not be worth knowing, and if nature were not worth knowing, life would not be worth living.”

(Jules Henri Poincaré)

„Nem a cél adja meg a kívánt boldogságot, hanem az érte való küzdelem!”

(Madách Imre)

Table of contents

List of abbreviations

ANOVA	analysis of variance
ANCOVA	analysis of covariance
ATP	adenosine triphosphate
AUC	area under the curve
bp	base pairs
BSA	bovine serum albumin
BW	body weight
CAR	constitutive androstane receptor
cDNA	copy deoxyribonucleic acid
C_{max}	maximum plasma concentration
CoA	coenzyme A
Cy3	cyanine 3
CYP	cytochrome P450
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
E. coli	*Escherichia coli*
dNTP	deoxyribonucleoside triphosphate
EBSS	Earle's Balanced Salt Solution
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene glycol tetraacetic acid
EU	European Union
FAD	flavinadenine dinucleotide
FBS	fetal bovine serum
FCR	feed conversion ratio
FMN	flavinmononucleotide
GALT	gut associated lymphoid tissue
H2A	histone 2A
H2B	histone 2B
H3	histone 3
H4	histone 4
HAT	histone acetyltransferase
HBSS	Hanks' Balanced Salt Solution
HDAC	histone deacetylase
HPLC	high performance liquid chromatography

IM	intramuscular
IU	international unit
K_M	Michaelis-Menten's constant
LPS	lipopolysaccharide
MCT-1	monocarboxylate transporter 1
MRT	mean residence time
NAD^+	nicotinamide adenine dinucleotide
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NCP	nucleosome core particles
OAT	organic anion transporter
OCT	organic cation transporter
PB	phenobarbital
PBS(T)	phosphate buffered saline (with Tween)
PCR	polymerase chain reaction
pK_a	dissociation constant of an acid
PXR	pregnane X-receptor
qRT-PCR	quantitative real-time polymerase chain reaction
RNA	ribonucleic acid
RNase	ribonuclease
RXR	retinoic acid X-receptor
SCFA	short chain fatty acids
SDS-PAGE	sodium dodecyl sulphate polyacrilamide gel electrophoresis
SEM	standard error of mean
TBE	tris-borate-EDTA
$T_{half\text{-}abs}$	absorption half-life
$T_{half\text{-}el}$	elimination half-life
T_{max}	time to maximum plasma concentration
V_{max}	maximal velocity of the enzymatic reaction

1. Summary

The short chain fatty acid butyrate is one of the major end products of the anaerobic microbial fermentation of carbohydrates in the forestomachs of ruminants and in the large intestine of monogastric mammals, birds and humans. Butyrate is also a widely used feed additive as an alternative growth promoter due to its beneficial effects on growth performance, first of all in poultry and pig nutrition. This is of special importance due to the banning of the traditional antibiotic growth promoters in the European Union. Butyrate may provoke its effects on metabolism via many different, yet not completely defined pathways. One of those pathways is that butyrate is known as a histone deacetylase inhibitor, inducing histone hyperacetylation *in vitro* and playing a predominant role in the epigenetic regulation of gene expression and cell function. It was hypothesized in this study that butyrate, applied as feed additive, might cause similar *in vivo* modifications in the chromatin structure of the hepatocytes of chickens in the early post-hatch period. Further, it could influence the expression of certain genes and therefore modify the activity of hepatic microsomal drug-metabolizing cytochrome P450 (CYP) enzymes, resulting in pharmacoepigenetic interactions with simultaneously applied xenobiotics. Most experiments of this PhD study were performed in broiler chickens, because chickens are not only target species of butyrate administration as feed additive, but they can also serve as model for the investigation of butyrate's actions.

Regarding the intestinal effects of butyrate, this study aimed to compare the influence of butyrate on small intestinal histomorphology in chicken and rat, the latter as a model animal for monogastric mammals. *In vivo* studies were carried out in chicken to investigate the molecular mechanisms of butyrate's epigenetic actions on the liver. Broiler chicks in the early post-hatch period received butyrate-supplemented diet (1.5 g/kg diet) or were treated once daily with orally administered bolus of butyrate following overnight fasting with two different doses (0.25 or 1.25 g/kg body weight per day) for five days. After slaughtering, cell organelles were separated by differential centrifugation from the livers and acetylation of hepatic core histones was screened from cell nuclei by western blotting. Effects of butyrate on CYP gene expression were tested at first *in vitro* on primary culture of chicken hepatocytes, followed by an *in vivo* trial with butyrate-fed chickens. The activity of the most important CYP enzymes was also monitored by aminopyrine N-demethylation, aniline hydroxylation and testosterone 6β-hydroxylation assays from the microsomal fractions of chickens. Furthermore, the interaction of butyrate and the macrolide antibiotic erythromycin was tested *in vitro* and finally also *in vivo* by studying the major pharmacokinetic parameters of erythromycin.

Growth promoting effects of butyrate might be based partially on the morphological changes of the small intestinal mucosa: butyrate tended to increase depth of crypts in both species, while butyrate had a positive trophic effect on the enterocytes in chicken, but not in rat. Plasma butyrate concentration significantly increased after receiving butyrate-supplemented diet in both species and also after bolus treatment of chicks, so alimentary added butyrate could reach the extrahepatic tissues and might act as a biologically active molecule.

Orally added butyrate, applied either as feed additive or in bolus, had a remarkable impact on nucleosome structure of hepatocytes *in vivo*: independently from the form of application or the dose, butyrate caused hyperacetylation of histone H2A, but no changes were monitored in the acetylation state of H2B. Intensive, approximately 18-fold hyperacetylation of H3 was caused by the higher administered dose in bolus, while the lower concentration did not alter the acetylation of H3 in bolus nor as feed additive. Acetylation ratio of H4 tended to be increased only by the lower dose of butyrate boli.

Butyrate had a pronounced effect on gene expression of certain CYP enzymes in primary cultures of chicken hepatocytes *in vitro* as well as *in vivo*, applied as feed additive. Expression of CYP2H1 gene increased in both cultured hepatocytes and the liver of butyrate-fed chicks, while CYP3A37 gene expression declined after *in vitro* butyrate treatment, but this down-regulation was ameliorated *in vivo*. Interestingly, CYP1A gene was observed to be suppressed by butyrate in primary hepatocyte cultures, but in contrast, it was overexpressed *in vivo* in butyrate-fed animals compared to the controls. In spite of the observed *in vivo* modifications in histone acetylation and CYP gene expression, no significant changes were observed in the activity of hepatic microsomal CYP2H and CYP3A37 enzymes in any cases of oral butyrate application, tested by specific enzyme assays. Interestingly, butyrate in bolus attenuated the stimulatory effect of the simultaneously administered enzyme-inducing xenobiotic, phenobarbital (PB) on CYP2H and CYP3A37.

Regarding the *in vitro* interaction of butyrate and erythromycin, this macrolide antibiotic showed an additive action with concomitant butyrate treatment on gene expression of CYP2H1, but antagonized butyrate's effect on CYP3A37 gene expression. Notwithstanding that butyrate concentration was found to be a potent effector of erythromycin metabolism, the drug-metabolizing activity of cultured hepatocytes was not affected significantly by butyrate. In the *in vivo* trial longer absorption half-life, T_{max} and shorter elimination half-life of erythromycin were observed in the butyrate treated group of chickens. Based on C_{max} values, the two groups were non-bioequivalent. It can be stated that although there were differences in certain pharmacokinetic parameters of erythromycin, the dietary administered butyrate did not alter relevantly the therapeutic activity nor the terminal elimination of erythromycin in chickens.

Based on our results, it can be concluded that orally added butyrate acted as an epigenetic factor by increasing the acetylation of core histones in the liver and had an impact on hepatic CYP gene expression of chicken *in vitro* and *in vivo* as well. Nevertheless these alterations did not affect the microsomal CYP activity of the liver, and the concomitant application of butyrate with veterinary pharmaceuticals possibly would not cause a major feed-drug metabolic interaction *in vivo* in chickens. So butyrate is suggested to be applied in safe as feed additive in poultry industry, from pharmacotherapeutical and food safety point of view as well, possibly not having any relevant pharmacoepigenetic interactions with simultaneously applied xenobiotics.

2. Introduction and literature overview

2.1. Butyrate as a short chain fatty acid

Short chain fatty acids (SCFA) are the major end products of the anaerobic microbial fermentation of carbohydrates and produced in high quantities in the forestomachs of ruminants and in the large intestine of monogastric mammals, birds and humans (Bergman, 1990).

The four-carbon, non-branched SCFA, the (n-)butyric acid, also mentioned as its ionized form (n-)butyrate (in the followings: butyrate) has a molar mass of 88.11 g/mol and is an oily colourless liquid at room temperature with a typical unpleasant odour and a density of 0.96 g/ml. Its melting and boiling points are -7.9°C and 163.5°C, respectively. Butyrate can be considered as a weak acid with a pK_a value of 4.82, it is easily soluble in water, ethanol and ether as well. Among the different SCFA, butyrate is of special interest due to its biological activity and its numerous positive effects on the health of gut and the extraintestinal tissues.

Butyrate is the most important energy source of the colonocytes (Roediger, 1982), regulating also the proliferation and differentiation of the gastrointestinal epithelium (Gálfi and Neogrády, 2001). It can induce apoptosis in genetically disordered cells (Medina et al., 1997; Leu et al., 2009), inhibit DNA synthesis and cell growth and reduce telomerase activity in certain tumor cell lines (Steliou et al., 2012). As a consequence, butyrate has a protective effect against cancer, which was reported in some *in vitro* (Young and Gibson, 1995) and also *in vivo* animal studies (McIntyre et al., 1993; Le Leu et al., 2007). Due to its selective antimicrobial action on most enteral pathogens (Ricke, 2003; Fernández-Rubio et al., 2008), butyrate improves the composition of the intestinal microflora, which can influence the health of the host animal (Candela et al., 2010).

Furthermore, as an epigenetic factor, butyrate regulates the transcription via influencing core histone acetylation, which is one of the most relevant epigenetic regulations of the cell function together with DNA methylation (Biancotto et al., 2010). In addition to its other biochemical effects, butyrate can increase insulin sensitivity of various tissues (Gao et al., 2009), induce absorption of water and sodium through the intestinal epithelium (Lu et al, 2008), stimulate neurogenesis in the ischemic brain, promote osteoblast formation and has a general anti-inflammatory effect (Steliou et al., 2012).

2.2. Butyrate as feed additive

Due to its numerous beneficial properties improving health and also the growth performance of chickens (Hu and Guo, 2007) and pigs (Gálfi and Bokori, 1990), butyrate is of special interest as feed additive, mainly applied as its sodium salt. Fiber-rich diet or uptake of resistant starch increases microbial butyrate production, but butyrate is also orally applicable in several forms. Butyrate's growth promoting effect was described already by Gálfi and Bokori (1990), who reported that dietary butyrate supplementation increased growth and had a positive influence on feed utilization in pigs. Hu and Guo (2007) described that the alimentary applied butyrate caused increased body weight (BW) and weekly BW gain in broiler chickens in the starter period. This effect of butyrate became more pronounced under suboptimal circumstances and health conditions, such as after *Escherichia coli* (*E. coli*) lipopolysaccharide (LPS)-challenge (Zhang et al., 2011).

The application of butyrate as feed additive is of special importance since the banning of the traditional antibiotic growth promoters in the European Union (EU) (Phillips, 2007). Until 2006 the EU permitted the use of sub-inhibitory concentrations of several antibacterial substances in animal diets. Licences for all sub-therapeutic levels of antibiotics for growth promotion have been withdrawn by the EU in 2006 due to the widespreading of bacterial resistencies against antibiotics. Notwithstanding in such countries, which are not affected by this ban, there is a demand as well for the application of alternatives instead of antibiotic growth promoters (Michard, 2008).

Nowadays some protected forms of butyrate, such as butyrate-coated micro-beads and its various esters, such as glycerides are applied as well with success in poultry nutrition, providing butyrate release only in the distal part of the gastrointestinal tract and reducing butyrate's odour (Antongiovanni et al., 2007).

Beneficial effects of the application of butyrate as feed additive are mostly based on the phenomenon that it can inhibit the growth of certain pathogenic bacteria, such as enterotoxic *E. 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 ability of the undissociated fatty acid molecule to pass across the cell membrane and to dissociate in the more alkaline interior milieu (Kashket, 1987). After dissociation, the ionized, anionic form cannot be transported out of the bacterial cell by passive transport any more and will be trapped in the intracellular space. At the same time, dissociating protons are involved in acidifying the cytoplasm. Most enteral pathogens are especially sensitive to the decreased intracellular pH and therefore the 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, increasing the turgor of the cell. Butyrate may also influence bacterial gene expression, which was already described 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), causing reduced invasiveness of microbes in intestinal epithelial cells *in vitro* (Van Immerseel et al., 2003).

However, it is now clear that many fermentative bacteria (such as *Lactobacillus spp.* and *Streptococcus bovis*), being part of the eubiotic enteral microflora, have the ability to let their intracellular pH decline when the extracellular pH becomes highly acidic. By letting the intracellular pH to be decreased, these bacteria have a much smaller pH gradient across their cell membranes and are protected against anion accumulation (Gálfi and Neogrády, 1996). Such bacteria can utilize butyrate as an energy source as well, acetyl~CoA, which is produced by its breakdown, can enter the citric acid cycle or can be used for replenishing intermediates of the citric acid cycle via the glyoxylate shunt.

Due to its bactericidal effect on most enteral pathogens, butyrate improves the composition of the intestinal microflora and can be also considered as a prebiotic. On the basis of these findings, butyrate can be a useful tool against the most common poultry-mediated zoonotic enteral pathogens as well, such as controlling *Salmonella enteritidis* and *Campylobacter jejuni* colonization in broiler flocks (Fernández-Rubio et al., 2008). In addition, butyrate is a potent anticoccidial agent by improving health of *Eimeria*-infected broiler chicks (Leeson et al., 2005).

Several other mechanisms are also involved in triggering butyrate's growth promoting action. First of all, butyrate improves the maturation of the gut associated lymphoid tissue (GALT) (Friedman and Bar-Shira, 2005), increases the expression of the tight junction proteins cingulin and occludin, enhancing the barrier function of the intestinal epithelium (Bordin et al., 2004) and stimulates the GALT mediated immune response in broiler chicken (Zhang et al., 2011). It is also known that butyrate can cause significant changes in the histomorphology of the intestines as well, increasing the surface area and hence the absorptive capacity of the gut (Antongiovanni et al., 2007; Hu and Guo, 2007). In addition, it was reported by Pászti-Gere et al. (2013) that oxidative stress induced bowel inflammation could be compensated by butyrate treatment, therefore the function of the intestinal epithelium as a mechanical barrier can be improved by butyrate.

2.3. Metabolism of butyrate

Butyrate, either of endogen or exogen origin, is presumed to be absorbed across the cell membrane of the enterocytes by simple diffusion in its undissociated butyric acid form, once inside the cell it is converted to its CoA thioester form, butyryl~CoA by the butyryl~CoA synthetase enzyme. This activated molecule enters the mitochondria via the carnitine shuttle and is broken down in the β-oxidation via acetoacetyl~CoA to two molecules of acetyl~CoA. The acetyl~CoA generated by butyric acid breakdown is mainly used for further oxidation in the citric acid cycle or can be released from the cell as acetate, deliberated by a thioesterase called acetyl~CoA hydrolase. A smaller amount of the produced acetyl~CoA can be involved in steroid metabolism as start molecule of the cholesterol synthesis. In addition, ketone bodies, such as acetoacetate and β-hydroxy-butyrate can be produced from the intermediates of butyrate oxidation as well. Most important steps and pathways of butyrate metabolism are presented in **Fig. 1**.

Although butyrate is greatly metabolized by the intestinal epithelium, a certain amount is also absorbed into the portal blood (Velázquez et al., 1997) and taken up by the liver (Demigné et al., 1986; Bloemen et al., 2009). The monocarboxylate transporter 1 (MCT-1) is mainly involved in the uptake of butyrate into liver cells in ruminants (Kirat et al., 2005), but this transport mechanism is not described yet in many species, such as chicken and rat. Butyrate is an important energy source for the liver as a substrate of the oxidative pathways, similarly as it was described previously regarding the intestinal epithelial cells. In addition, butyrate is also a potent effector of the hepatic metabolism, since it can decrease the mitochondrial oxidative phosphorylation yield and the ATP content of the liver due to its uncoupling-like effect (Beauvieux et al., 2001; Gallis et al., 2007) and can influence the mitochondrial ATP turnover linked to glycogen metabolism (Gallis et al., 2011).

The possibility of butyrate's clinical application is thwarted by its weak oral bioavailability due to its intensive metabolism in the colonocytes and its first-pass hepatic clearance. However, the non-metabolized, although small fraction of butyrate can act as an epigenetically active molecule in the hepatocytes or is being released from the liver to the blood stream and may reach the extrahepatic tissues as well.

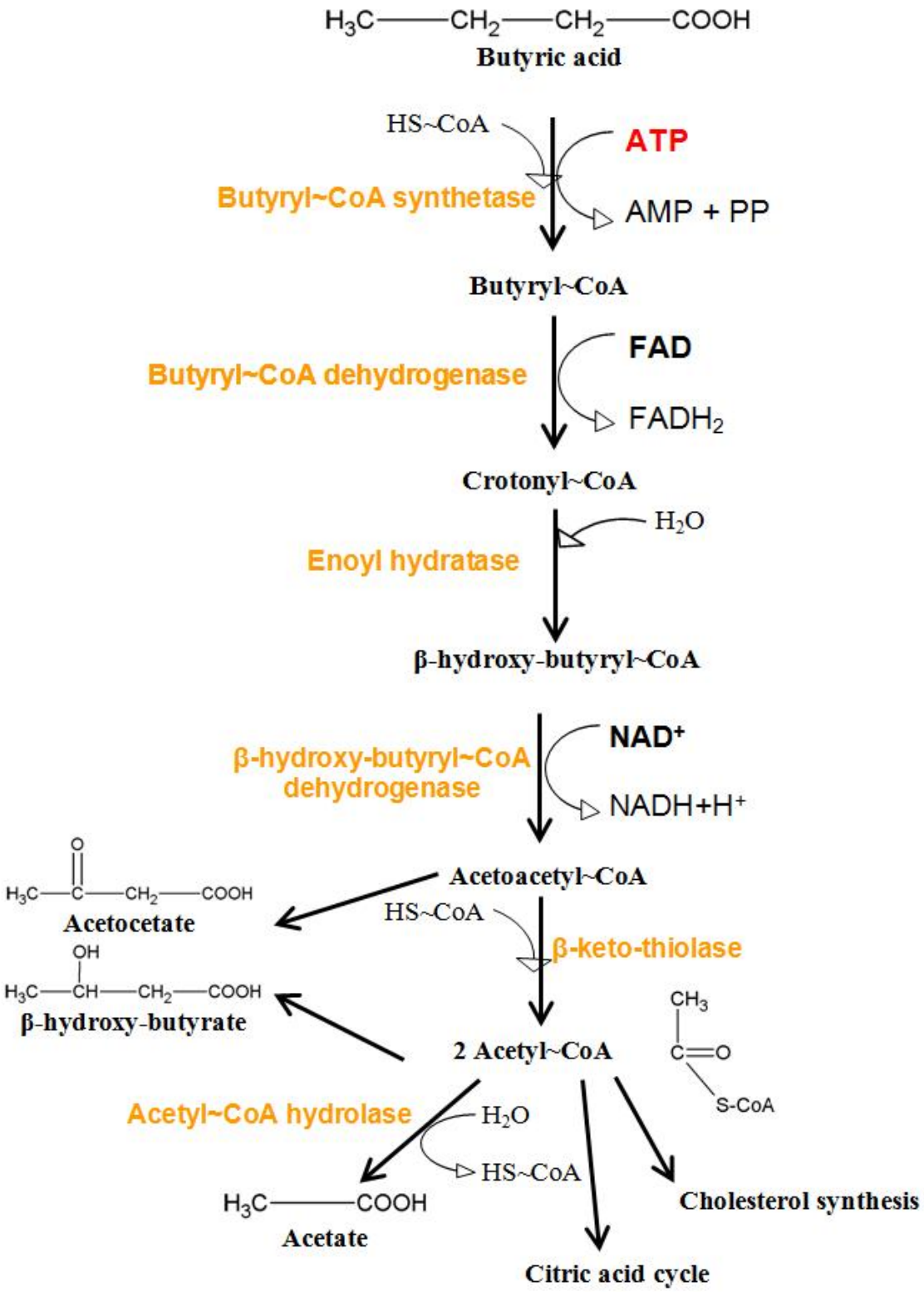


Fig. 1. Summary of the most important steps and pathways involved in butyrate metabolism. Explanation of details is included in the text.

2.4. Butyrate as an epigenetically active molecule

The term epigenetics covers heritable, functionally relevant changes in gene expression or cellular phenotype caused by mechanisms other than modifications in the DNA sequence. Butyrate is also well-known as an epigenetically active molecule by influencing histone acetylation (Davie, 2003) and DNA methylation (Cho et al., 2009).

Nucleosomes consist of DNA wrapped around a histone octamer comprised of dimers of core histones H2A, H2B, H3 and H4, also termed the nucleosome core particles (NCP), which are connected by linker DNA sections (Arents et al., 1991) (**Fig. 2**).

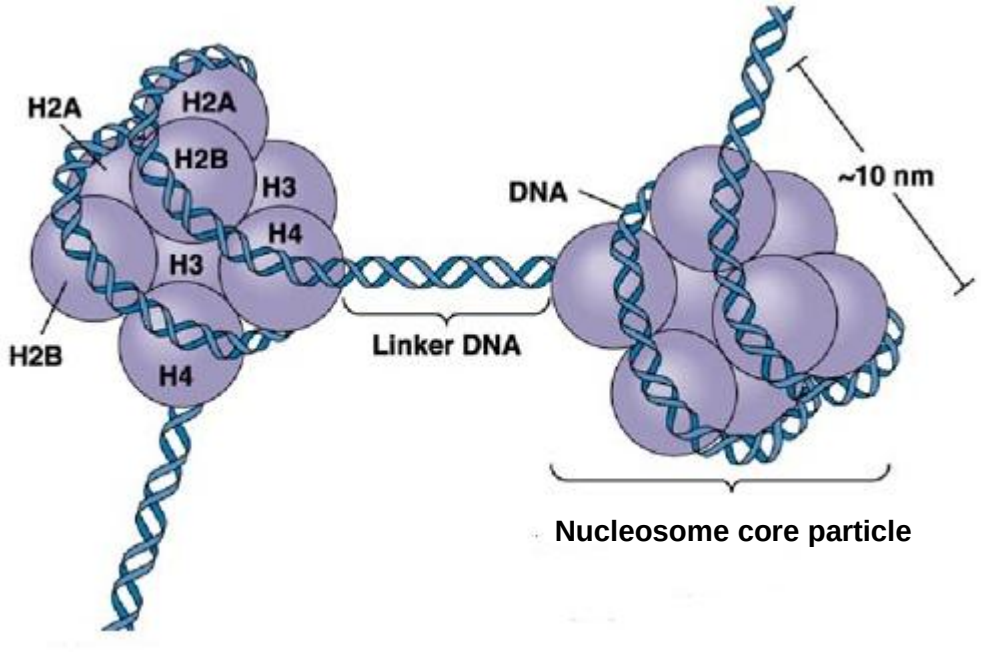


Fig. 2. Schematic structure of the nucleosome.
(Source: http://www.bhpress.org)

Posttranslational modifications of histones (**Fig. 3**) influence their interaction with DNA and nuclear proteins and are therefore highly involved in the alterations of chromatin structure and transcription pattern, regulating gene expression. Namely, acetylation, methylation, ubiquitinylation, sumoylation or phosphorylation of several amino acids provide a predominant epigenetic regulation of cell function. N terminal tails of histones, protruding from the histone core, are the most common target sections of covalent modifications, however some changes can occur in the core itself as well (Strahl and Allis, 2000; Jenuwein and Allis, 2001).

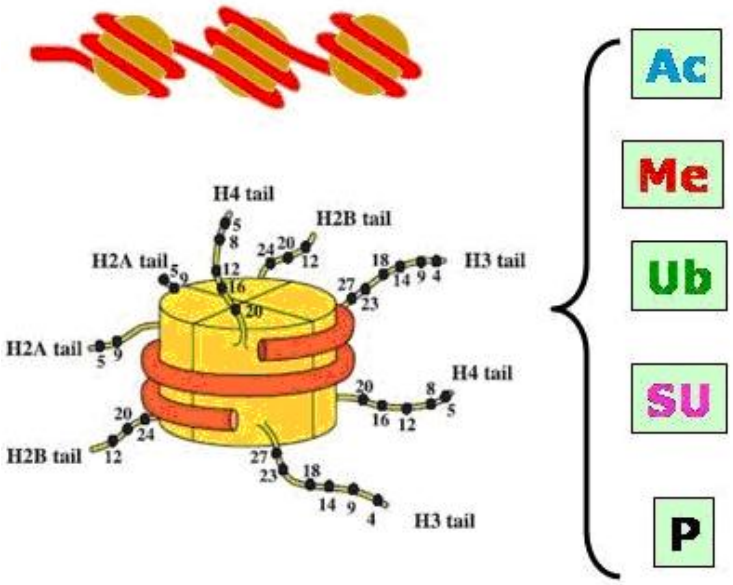


Fig. 3. The most important posttranslational modifications of histone proteins. Ac refers to acetylation, Me to methylation, Ub to ubiquitinylation, SU to sumoylation and P to phosphorylation. (Source: integratedhealthcare.eu)

The dynamic balance of acetylation and deacetylation of histone proteins at certain lysine residues is regulated by the opposing effects of histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Rada-Iglesias et al., 2007). HAT binds acetyl groups to the originally positively charged lysine residues, while HDAC removes them (**Fig. 4**). If HDAC is blocked by an inhibitor, it causes histone hyperacetylation in the N-terminal histone tails, which results in a modified, transcriptionally opened and active structure of the NCP, therefore it influences the transcriptional pattern of certain genes. In contrast, histone hypoacetylation can be considered as a conserved hallmark of heterochromatin (Davie, 2003; Gao et al., 2009). Therefore histone acetylation and deacetylation are highly involved in mitigating cellular function and chromatin-related processes (Wallace and Fan, 2010). It is suggested by Strahl and Allis (2000) that combinatorial sequences of histone modifications can be considered as a histone language or histone code, determining the transcription pattern and cellular proteomics as well.

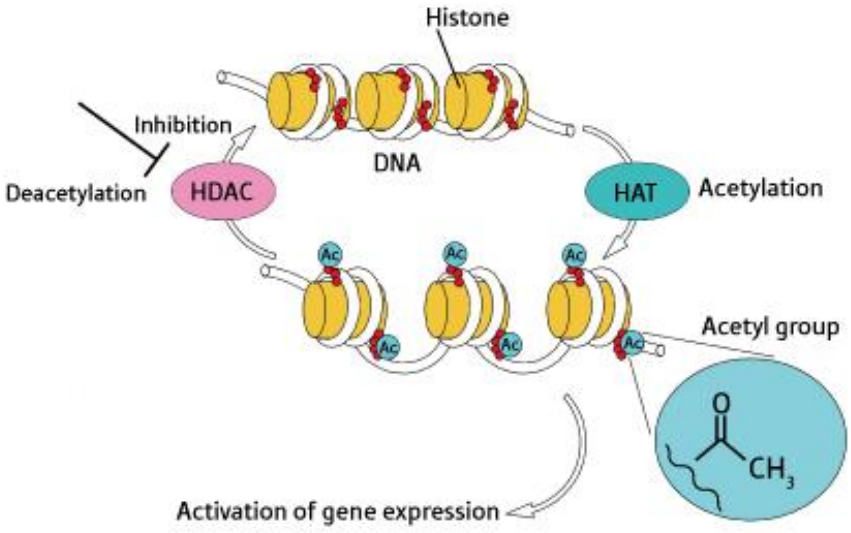


Fig. 4. The dynamic balance of histone acetylation (catalyzed by histone acetyltransferases, HATs) and deacetylation (catalyzed by histone deacetylases, HDACs), which might be influenced by HDAC inhibitors, such as butyrate. (Source: www.rikenresearch.riken.jp)

HDAC enzymes (of which at least 18 isoforms are described in mammals) are grouped into four principal classes according to their sequence homology to the yeast HDAC enzymes (Steliou et al., 2012; Minucci and Pelicci, 2006). Class I and most of the Class II HDAC isoenzymes are inhibited by butyrate, causing hyperacetylated core histones in cell cultures (Davie, 2003). It is already known for a long time that butyrate causes hyperacetylation of histones H3 and H4 in all examined vertebrate cell lines, while H2A and H2B are also affected in certain rat-derived cell types (Candido et al., 1978). In contrast to the wide range of *in vitro* results, only very little data are available in the literature regarding *in vivo* effects of butyrate on histone acetylation, and absolutely no data about such action in chicken. Significant increase in total histone acetylation was reported in case of porcine caecal tissue after dietary supplementation with the butyrate precursor lactulose by Kien et al. (2008).

It is known that butyrate-induced histone modifications are involved in butyrate's antitumor, antibacterial and metabolic effects (Guilloteau et al., 2010). Based on butyrate's epigenetic action on gene expression, activity of hepatic lactate dehydrogenase, alanine aminotransferase and γ-glutamyl transferase was also affected by butyrate (Engelmann et al., 1987). However, proliferation of hepatocytes could be inhibited by butyrate treatment on a hepatic cell line (Utesch et al., 1992). According to its epigenetic regulatory potential, butyrate has also several metabolic effects, such as influencing the plasma insulin level (Neogrády et al., 1989) and insulin sensitivity of extrahepatic tissues (Gao et al., 2009), which can be of interest in the therapy of diabetes type II by decreasing insulin resistance.

2.5. Microsomal cytochrome P450 (CYP) enzymes

Hepatic microsomal cytochrome P450 (CYP) enzymes, forming a superfamily of hemoproteins, are primarily involved in the oxidative metabolism of numerous endogenous and exogenous compounds (Yang et al., 2003). Regarding the endogenous substrates, CYPs play an essential role in steroid metabolism, such as synthesis of bile acids and steroid hormons. On the other hand, they are the key enzymes of the biotransformation of most xenobiotics. Importance of these biotransformation processes is to increase the polarity of apolar molecules, so that they can be excreted with the bile or with the urine, also avoiding renal tubular reabsorption. Xenobiotics are taken up by the hepatocytes with the action of organic anion transporters (OAT) or organic cation transporters (OCT), which cellular uptake can be considered as phase 0 of biotransformation (DeGorter and Kim, 2009). In phase I reactions, a polar functional group (such as hydroxyl, amino or thiol group) is introduced on the parent compound, which provides the possibility of conjugation in phase II, meaning covalent linkage formation with endogenously derived polar molecules (such as glucuronic acid, amino acids or sulphate group) (Singh, 2007).

CYP enzymes are involved mainly in the oxidative phase I reactions, the most common type of which is the hydroxylation of the substrate (Anzenbacher and Anzerbacherová, 2001). The mechanism of the CYP hydroxylation action will be summarized briefly in the followings (**Fig. 5**). As a reducing agent, $NADPH+H^+$ is required for the activity of CYP enzymes, hydrogens are transported from the coenzyme via riboflavin-derived prosthetic groups (FAD, FMN) of the NADP-CYP reductase to cytochrome b_5, where transportation of electrons and protons becomes detached. The central ferric ion of the CYP itself is being reduced by the transported electrons, while molecular oxygen and the appropriate substrate bind to the catalytic site of the enzyme. The substrate is being hydroxylated by one oxygen atom and the remaining oxygen will be utilized for water formation together with the transported electrons and protons (Anzenbacher and Anzerbacherová, 2001).

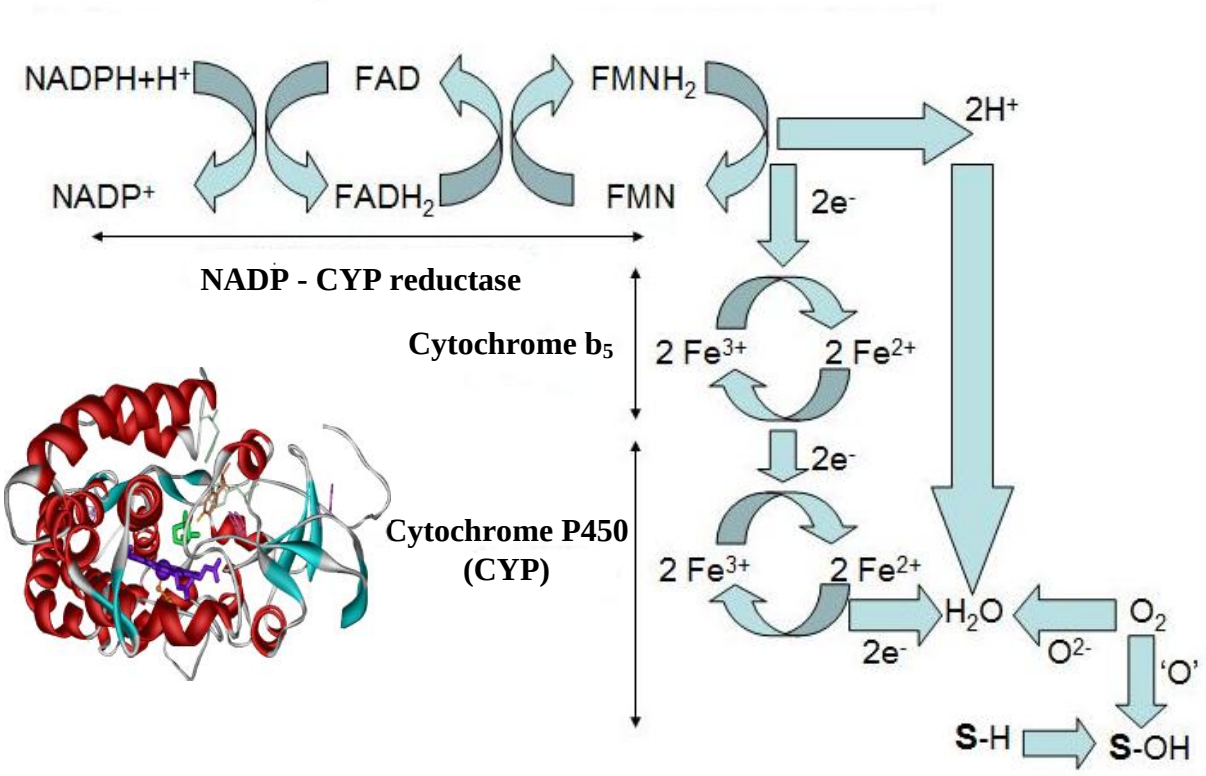


Fig. 5. Principles of the hydroxylation activity of the most common CYP enzymes. Explanation of details is included in the text. (Source of figure indicating the 3D structure of CYP enzymes: tifr.res.in)

CYP enzymes can be classified into families and subfamilies according to their genetic homology. In chicken, CYP1A (first of all CYP1A1), and CYP2H subfamilies, especially isoenzymes CYP2H1 and CYP2H2 (which are orthologs to the mammalian CYP2B and CYP2C proteins) and CYP3A37 (member of the CYP3A subfamily, ortholog to the mammalian CYP3A4) are the most important CYPs in drug metabolism (Ourlin et al., 2000). They are inducible by the well-known enzyme inductor phenobarbital (PB) (Hansen and May, 1989). In human liver microsomes, CYP3A4 plays a predominant role in the metabolism of therapeutic agents and it is responsible for the biotransformation of 40-60% of all clinically used drugs including the N-demethylation of erythromycin (Ourlin et al., 2000; Wang et al., 1997).

Most members of the CYP2 family are controlled by a nuclear receptor, namely the constitutive androstane receptor (CAR), which can bind the activator ligand together with the coactivator SRC-1. If this heterotrimer is associated with the transcription factor retinoid acid X-receptor (RXR), the formed complex can activate the responsive enhancer module of the appropriate CYP gene. Regarding CYP3A enzymes, inducer xenobiotics can join the ligand binding domain of the pregnane X-receptor (PXR) and then RXR, which complex again activates the responsible enhancer element of the promoter (Singh, 2007). It was recently

reported that HDAC inhibitors could influence CYP2 gene expression via the CAR pathway as well, through the dissociation of HDAC and recruitment of SRC-1 to receptor CAR (Takizawa et al., 2010). The stimulatory effect of PB on CYP2 enzymes was also found to be mediated mainly by the CAR system (Lempiäinen et al., 2011).

The nuclear-receptor-mediated pathway of the regulation of CYP gene expression is summarized briefly in **Fig. 6**.

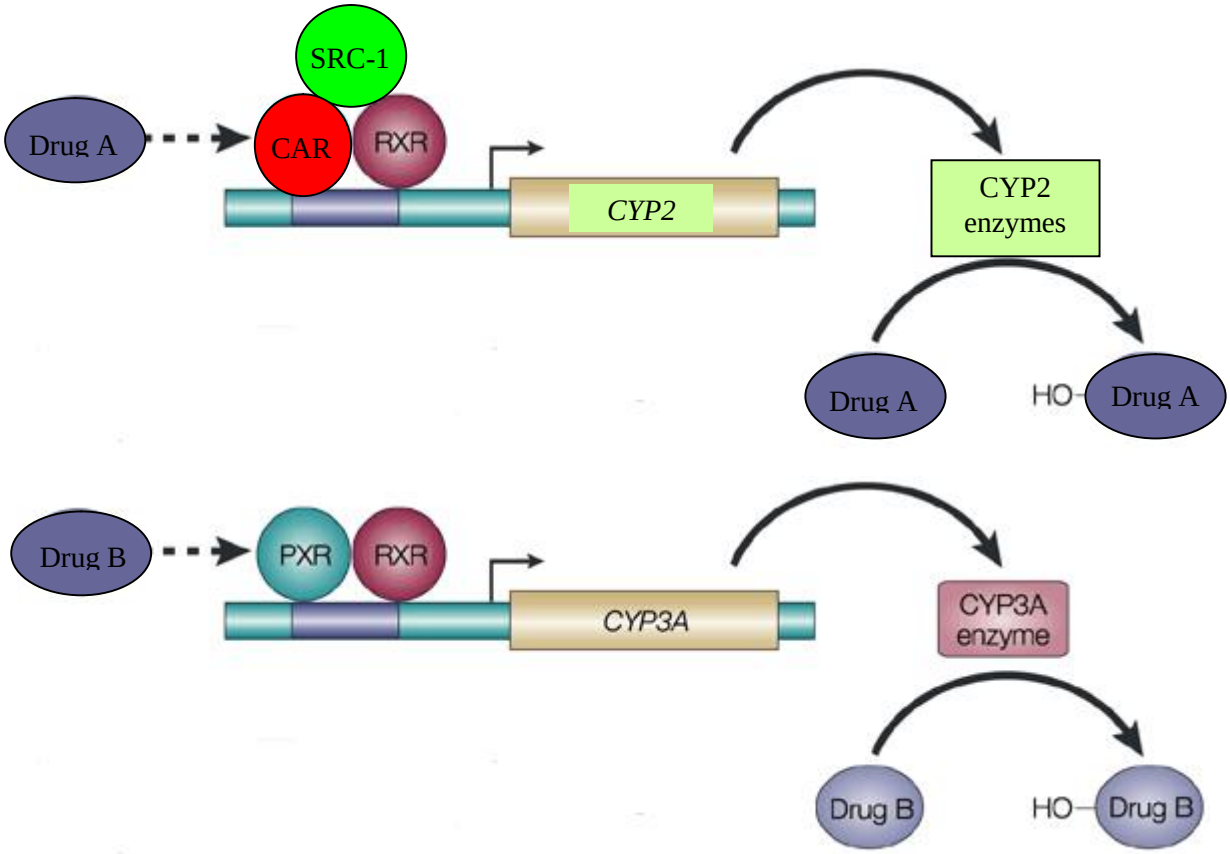


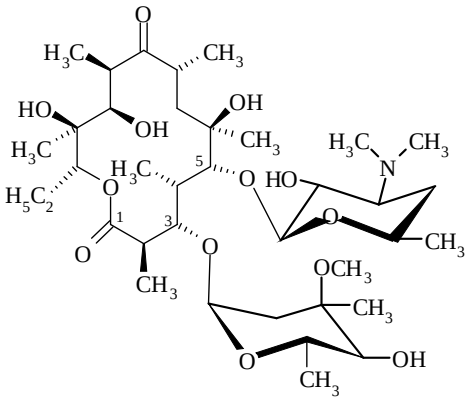
Fig. 6. The most important regulatory pathways of CYP2 and CYP3A gene expression. Full name of the abbreviated nuclear factors and explanation of the processes are included in the text. (Figure modified from the original source: Wilson and Kliewer, 2002)

Drugs or chemicals that cause CYP inhibition or induction are likely to have drug-drug interactions (Frassetto et al., 2007). This can lead to serious clinical consequences via reversible or irreversible means (McGinnity et al., 2006). Decrease in the activity of CYP enzymes can lead to an increase in hepatotoxicity (Hong and Yan, 2002) and it can cause a relevantly longer elimination half-life of xenobiotics, which is especially important in food-producing animals from food safety point of view. Increased CYP activity should be also monitored due to its possible negative therapeutical consequences.

It is known that the expression of certain drug-metabolizing microsomal CYP enzymes can be affected as well by histone modifications, altering the chromatin structure

and the affinity and binding possibility of the mentioned nuclear receptors to the promoter region of CYP genes (Baer-Dubowska et al., 2011). For instance, the HDAC inhibitor trichostatin A was shown to influence the *in vitro* expression of the CYP3A subfamily (Dannenberg and Edenberg, 2006). Alimentary added inulin, which is fermented by the colonic bacteria to SCFA, alleviates the reduction in the expression and activity of hepatic CYP1A1/2 and CYP2E1 enzymes in rats kept on a high-fat diet (Sugatani et al., 2012), possibly due to the epigenetic effects of the absorbed SCFA. On the basis of these findings, the enteral microbiome-produced or the orally added butyrate may also alter the activity of CYP enzymes, having an impact on hepatic detoxification capacity and drug metabolism, defined as possible pharmacoepigenetic influences. However, such possible effects of butyrate on hepatic microsomal CYP enzymes were not examined yet.

2.6. Erythromycin



Macrolide antibiotics, such as erythromycin are also potent effectors of the CYP enzymes (McGinnity et al., 2006). Erythromycin is widely applied in poultry medicine, so it may be of special interest regarding the possible metabolic interactions with butyrate.

It is effective *in vitro* against *Mycoplasma*, Gram positive *cocci*, *Neisseria*, some strains of *Haemophilus*, *Corynebacterium*, *Listeria*, *Brucella*, *Treponema spp.* and *Pasteurella multocida*. *Proteus*, *Pseudomonas* and *E. coli* are relatively resistant to the drug. The most important indications of its application in veterinary medicine are the followings: chronic mycoplasmosis in poultry, clinical and subclinical mastitis in lactating cows, infectious diseases caused by erythromycin sensitive bacteria (cattle, sheep, pig, poultry). Its most often recommended dose for broiler chickens and laying hens is 20-30 mg/kg BW/day (as erythromycin base) (EMEA, 2000).

Erythromycin is rather slowly absorbed from chicken intestine with some differences related to the mode of administration, the applied salt form (such as lactobionate, thiocyanate) and the coating of the applied compound. Only a very weak absorption of the orally applied erythromycin can be found in the stomach, the major site of absorption is the small intestine. Protein binding of the drug in blood plasma can be varied ranging from 90% in human to 38-45% in cattle. Due to its good properties for fast tissue penetration, tissue concentrations are usually much higher than that in serum and it persists longer. The most important way of excretion is the faeces through the bile. Rapid depletion of erythromycin was reported in tissues of chickens in 1 to 3 days after the treatment period. Even prolonged

treatment resulted in the same tissue residue concentration characteristics (Suárez and Ellis, 2006).

Its relatively fast elimination is based on the rapid metabolism of erythromycin by the N-demethylation activity of mainly the CYP3A subfamily of liver microsomes, producing N-desmethyl-erythromycin, which remains partly microbiologically active. However, other CYP subfamilies are involved as well in the biotransformation of erythromycin. Erythromycin may initially cause an induction of these CYP enzymes, which is mediated by certain nuclear receptors as it was mentioned previously (Sinh, 2007). Notwithstanding that erythromycin can act as an inductor on hepatic microsomal CYP enzymes, this action can be then followed in some cases by an inhibition through the formation of inactive enzyme/metabolite complexes (Al-Ghamdi et al., 2002). Biotransformation of erythromycin within CYPs generates nitroso compounds, produced by the oxidation of its tertiary amino group. These molecules can bind tightly to the heme prostethic group of CYPs to form a stable metabolic intermediate complex (McGinnity and Riley, 2001).

As a result, erythromycin may have multiple effects on various microsomal CYP enzymes, modifying the metabolism of other xenobiotics, which can give rise to serious drug-drug interactions (Bishop, 2005). Nevertheless the effects of erythromycin on CYP gene expression in chicken have not been investigated yet. Since erythromycin is a commonly applied antibiotic in poultry medicine, studying its effects on hepatic CYP enzymes of broilers would be of special importance.

In addition, simultaneous application of butyrate as feed additive with erythromycin may result in a relevant pharmacoepigenetic interaction by influencing the CYP gene expression. Induction of CYP enzymes would have therapeutical consequences by enhancing xenobiotic metabolism and shortening drug action, while an inhibitory effect might cause prolonged drug elimination, which is especially important from food safety point of view.

3. Significance and aims of the study

Several *in vitro* and *in vivo* actions of butyrate are already known, as it was mentioned above in the literature overview. However, numerous aspects of butyrate's epigenetic, trophic and metabolic effects still remained unclear, especially *in vivo*, after oral butyrate application. Since butyrate is widely applied in poultry nutrition, chickens are not only target species of butyrate administration as feed additive, but they can also serve as model for the investigation of butyrate's actions. Young chickens have a large capacity of growing, intensive hepatic metabolism and quite low rates of butyrate production in the large intestine (Snel et al., 2000), so they can be proper candidates to study the effects of the orally applied butyrate.

It is known that butyrate as feed additive can improve growth performance of broiler chickens in the early post-hatch period. This beneficial effect is partly maintained by butyrate-triggered histological changes of the intestinal mucosa and partly by the improvement of the composition of gut microflora, considered as the most important intestinal actions of butyrate. In this PhD study, after the assessment of the known butyrate-caused changes of the small intestinal micromorphology (height of villi, depth of crypts), the height of enterocytes was also measured to study butyrate's possible trophic effect on intestinal epithelial cells in both chickens and rats, the latter as monogastric mammalian model animals.

However, butyrate is greatly metabolized by the intestinal epithelium, it is also absorbed and transported to the liver by the hepatic portal system. To investigate butyrate's epigenetic and metabolic effects on the liver cells, hepatocellular butyrate uptake had to be examined at first. Further, butyrate's first-pass hepatic clearance and oral bioavailability were also aimed to be checked. The amount of butyrate released to the systemic circulation determines whether butyrate can act as a biologically active molecule in the extrahepatic tissues as well and may influence the metabolism, e.g. insulin sensitivity of certain cell types. This may be of special interest and one of the future perspectives of this study.

Butyrate is well-known as a HDAC inhibitor, causing *in vitro* histone hyperacetylation on all examined types of vertebrate-derived cell cultures. In contrast, very little data are available on butyrate's *in vivo* epigenetic action and absolutely no data has been published regarding butyrate-induced alterations of hepatic epigenetics. One of the most important goals of this study was to investigate the changes in hepatic histone acetylation caused by oral butyrate application in broiler chickens, providing novel data in this field of study.

It is described that certain HDAC inhibitors can alter the expression of hepatic microsomal drug-metabolizing CYP enzymes, but no such data is provided about butyrate. In this study it was hypothesized that based on its epigenetic action, butyrate might also alter

the expression and the activity of hepatic CYP enzymes. Therefore, CYP gene expression was aimed to be investigated after butyrate treatment *in vitro* and *in vivo* as well. In addition, activity of the most important CYP isoenzymes was also screened in the liver of butyrate-treated and control chickens.

The possible changes in CYP activities are of special importance due to their predominant role in xenobiotic biotransformation. To evaluate the possible pharmacoepigenetic interactions of butyrate with simultaneously applied drugs, the effects of concomitant butyrate and erythromycin treatment on hepatic CYP gene expression were aimed to be tested on primary cultures of chicken hepatocytes. Finally, pharmacokinetic properties of erythromycin were also determined in control and butyrate-fed chicks in order to evaluate the potential *in vivo* metabolic interactions, which could be highly relevant from food safety point as well.

Two different forms of oral butyrate application were compared in this study. Butyrate was added orally to broiler chickens either as feed additive or in daily bolus after overnight fasting. The latter treatment provided a fast, but short-term release of greater amount of butyrate to the portal vein and an intense butyrate exposure for the liver and therefore served as a model for investigating butyrate's possible *in vivo* epigenetic and metabolic action.

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

Ad 1

(1a) to investigate histomorphological changes in the small intestinal epithelium caused by oral butyrate application and to compare these data in chicken and rat. This would provide more information beyond the beneficial effects of butyrate on growth.

(1b) to study the butyrate uptake of primary cultures of hepatocytes as well as the first-pass hepatic clearance of butyrate in chicken and rat.

Ad 2

to evaluate the *in vivo* epigenetic effects of butyrate added orally to broiler chickens either as feed additive or in daily bolus. It was aimed to monitor the modifications in the acetylation state of hepatic core histones at the most frequent acetylation sites triggered by butyrate stimuli following both forms of application.

Ad 3

to detect the effects of butyrate on gene expression of hepatic microsomal drug-metabolizing CYP enzymes firstly *in vitro*, in primary cultures of chicken hepatocytes, then *in vivo*, after oral butyrate supplementation to broilers. Finally, the activity of CYP enzymes was aimed to be measured to screen, whether butyrate could influence the detoxification capacity and xenobiotic biotransformation of the liver.

Ad 4

to study the possible pharmacoepigenetic interaction between butyrate and erythromycin *in vitro* on CYP gene expression of cultured chicken hepatocytes. Additionally,

pharmacokinetic properties of erythromycin were measured following oral butyrate application in broiler chickens *in vivo* to study how butyrate could influence drug metabolism, having a huge impact from pharmacotherapeutical and food safety point of view.

An overview of the most important investigated topics related to the goals of this PhD study is presented in **Fig. 7**.

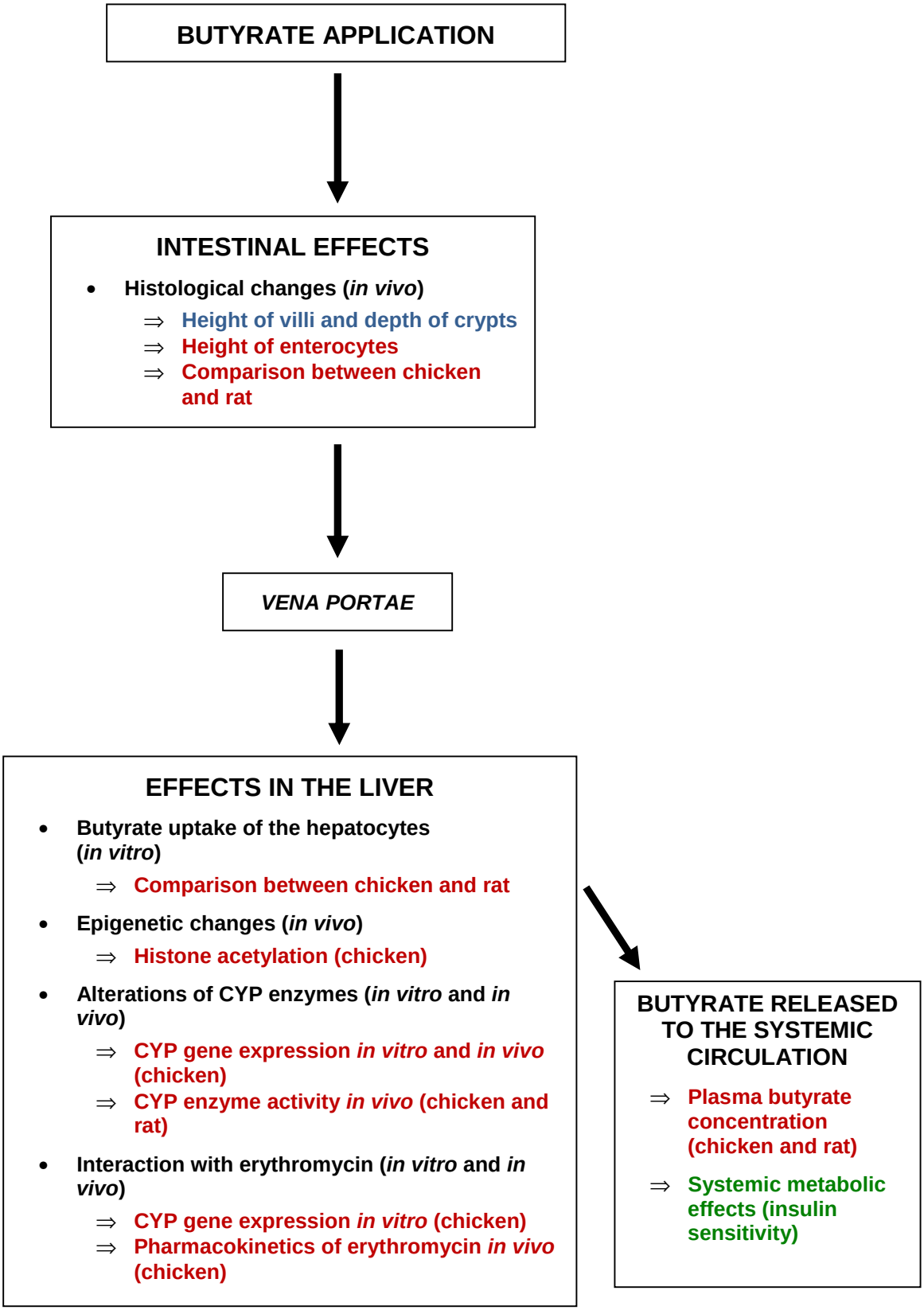


Fig. 7. Summary of the most important goals of the study. **Red** colour indicates aims providing novel data on butyrate's action, while **blue** colour represents effects to be confirmed and **green** colour refers to the future perspectives. The terms *in vitro* and *in vivo* reflect to the way of butyrate application.

4. Materials and methods

Chemicals

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

Animal welfare consideration

All experiments fully complied with legislation on research involving animal subjects according to the European and Hungarian law. Each procedure was approved by the Local Animal Test Committee of the Faculty of Veterinary Science, Szent István University, Budapest (Hungary), number of permission: 22.1/4719/003/2008.

4.1. In vitro studies on primary cultures of hepatocytes

4.1.1. Butyrate uptake of primary cultures of chicken and rat hepatocytes

4.1.1.1. Culturing of chicken hepatocytes

Three male, clinically healthy broiler chickens of the Ross 308 strain, obtained from a commercial hatchery (Uraiújfalu, Hungary), were housed and fed *ad libitum* according to the requirements of the Ross (2009) technology, as it is described later regarding our *in vivo* studies in section 4.2.1.1. At the age of six weeks, animals were anesthetized after 12 h fasting by intramuscular application (pectoral muscle) of the combination of xylazine (10 mg/kg BW), zolazepam (50 mg/kg BW) and tiletamine (50 mg/kg BW). After aseptic opening of the coelom, ribs were severed and the sternum was removed in order to permit free access to the liver. The hepatic portal system was cannulated via the pancreaticoduodenal vein and the liver was exsanguinated by 200 ml EGTA-free Hanks' Balanced Salt Solution (HBSS) (**Fig. 8**). The perfusion was continued by 200 ml 0.5 mM EGTA-containing HBSS buffer and then again by 200 ml of the same buffer solution as in the first step. Finally, 150 ml EGTA-free HBSS buffer, supplemented by 100 mg collagenase Type IV, 7 mM $MgCl_2$ and 7 mM $CaCl_2$ was applied and recirculated, in order to disintegrate the hepatocytes. All solutions, applied for perfusion were prewarmed at 40ºC and oxygenated previously with Carbogen (95% O_2, 5% CO_2, 1 l/min). Velocity of the perfusion was regulated by a peristaltic pump and set at 30 ml/min.

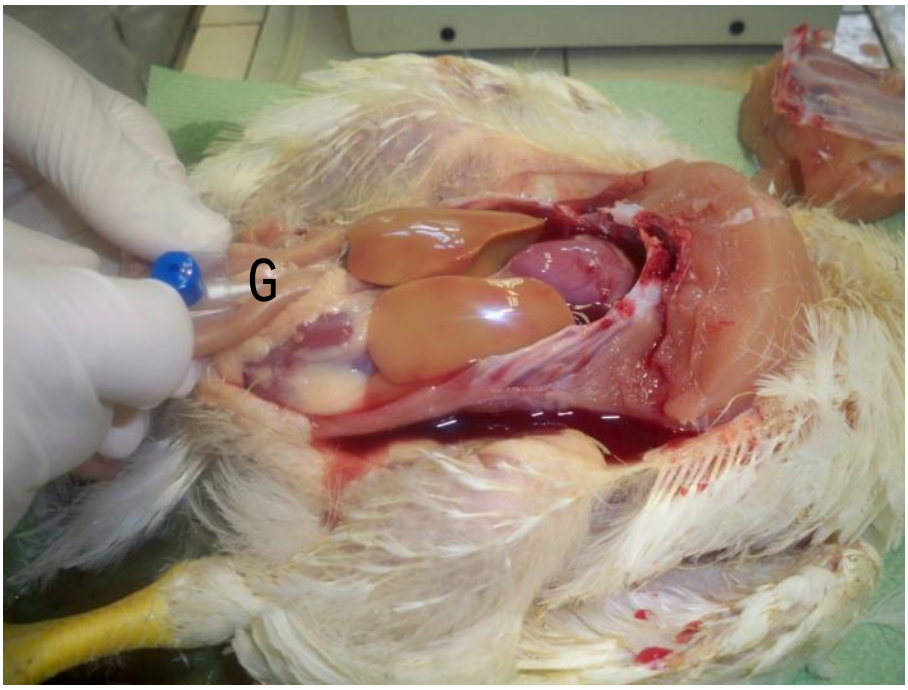


Fig. 8. Perfusion of the liver of chickens via the pancreaticoduodenal vein (in-flow, *).

After the collagenase digestion, the liver was excised, the capsule was disrupted and the digested parenchyma was filtered through a nylon mesh with 100 µm pore size (Millipore, Volketswil, Switzerland) to eliminate cell aggregates. Hepatocyte-enriched fractions were isolated and washed by low-speed centrifugation (50g, 3 min) firstly in BSA (2,5%) containing HBSS buffer and then twice in Williams' Medium E, supplemented previously with 50 mg/l gentamicin, 2 mM glutamine, 5% foetal bovine serum (FBS), 4 µg/l dexamethasone, 20 IU/l insulin and 0.22% sodium bicarbonate. Cell viability was assessed by the trypan blue exclusion test and it consistently exceeded 90% in all isolations. Yield of hepatocytes was determined by cell counting in Bürker chamber and cell concentration was adjusted to 10^6/ml. Hepatocytes (1.5 ml cell suspension/well) were seeded on 6-well Costar TC cell culture dishes (well diameter: 34.8 mm; Corning International, Corning, NY, USA), previously coated by collagen Type I (10 µg/cm^2) according to the manufacturer's instructions. Cell cultures were incubated at 37ºC in humid atmosphere with 10% CO_2, culture medium was changed 4 h after plating. A confluent monolayer of hepatocytes was gained after 24 h incubation (**Fig. 9**).

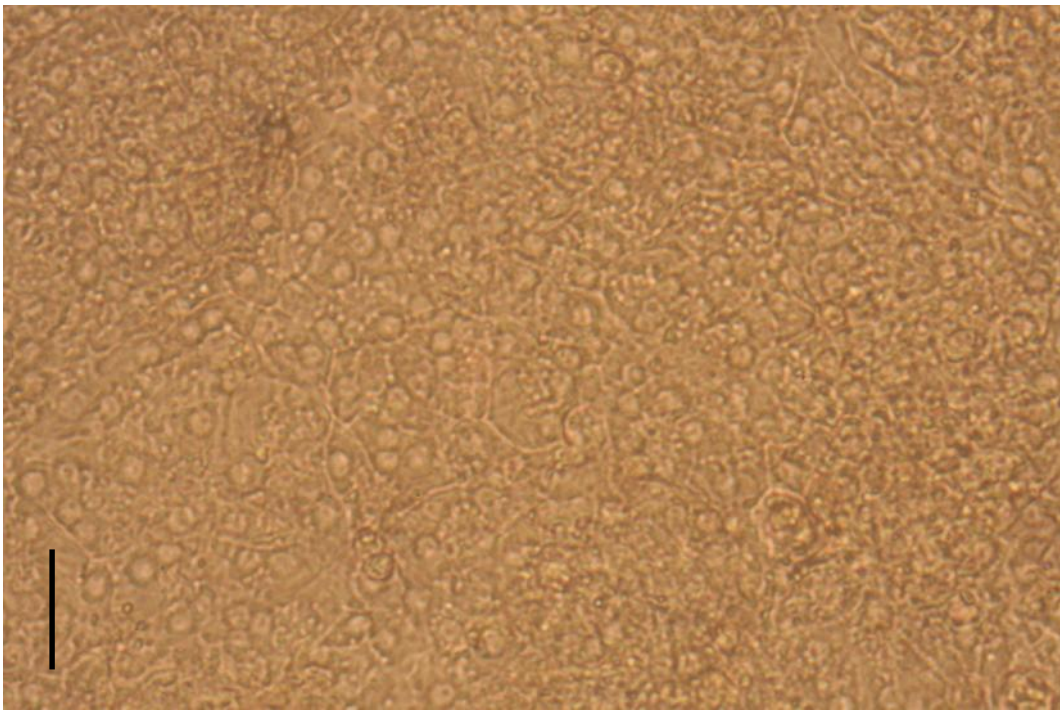


Fig. 9. Primary culture of chicken hepatocytes after 24 h incubation (phase contrast microscopy). Bar = 50 µm

Primary cultures of chicken hepatocytes were characterized by immunohistochemical detection of glutaminase. For this purpose, hepatocytes were cultured on glass inserts for 48 h, were frozen without fixation and stored at -80ºC until the examinations. After thawing on ice, cell cultures were blocked in a goat serum-containing blocking solution for 1 h and incubated with primary antibody specific for glutaminase (mouse, 1:250) overnight at 4ºC in a humidified chamber. Samples were gently washed three times with PBS and secondary anti-mouse antibody conjugated with cyanine 3 (Cy3) fluorescent dye was applied for 2 h at room temperature. After final triple PBS washing, inserts were examined by an Olympus IX70 microscope equipped with a Leica digital camera. Evaluation of the immunohistochemical staining was performed with the software Leica Application Suite 2.8.1. (Leica Microsystems, Switzerland).

High amount of the positively stained cells (**Fig. 10**) confirmed that the primary cultures consisted of hepatocytes and reached the quality required for the further examinations.

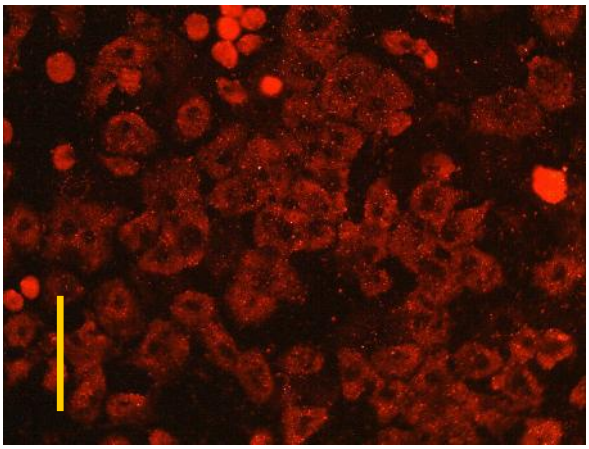


Fig. 10.A

Fig. 10.B

Fig. 10. A. Detection of glutaminase by immunohistochemical staining on primary culture of chicken hepatocytes after 48 h cultivation. **B.** A negative control performed without adding secondary antibody. Bar = 30 µm

4.1.1.2. Culturing of rat hepatocytes

Three male Wistar rats (Charles River, Wilmington, MA, USA) at the age of 8 weeks (200-250 g BW) were approved for the hepatocyte isolation. Animals were housed and fed *ad libitum* according to their requirements, as it is described later regarding our *in vivo* studies in section 4.2.1.8. Rats were fasted for 12 h prior to the cell isolation. After inducing general anesthesia by intraperitoneal administration of zolazepam (40 mg/kg BW) and tiletamine (40 mg/kg BW), inhalation narcosis was conducted by diethyl ether. The abdominal cavity was opened aseptically by a midline incision and the portal vein was cannulated (in-flow). The diaphragm and ribs were severed and the sternum removed in order to open the thoracic cavity. Thoracal section of the *vena cava caudalis* was cannulated via an incision on the right atrium (out-flow). The abdominal section of the *vena cava caudalis* was ligated over the kidneys to prevent the flow-out of the perfusion solutions. The liver was perfused through the performed closed perfusion system (**Fig 11-12**), regulated by a peristaltic pump.

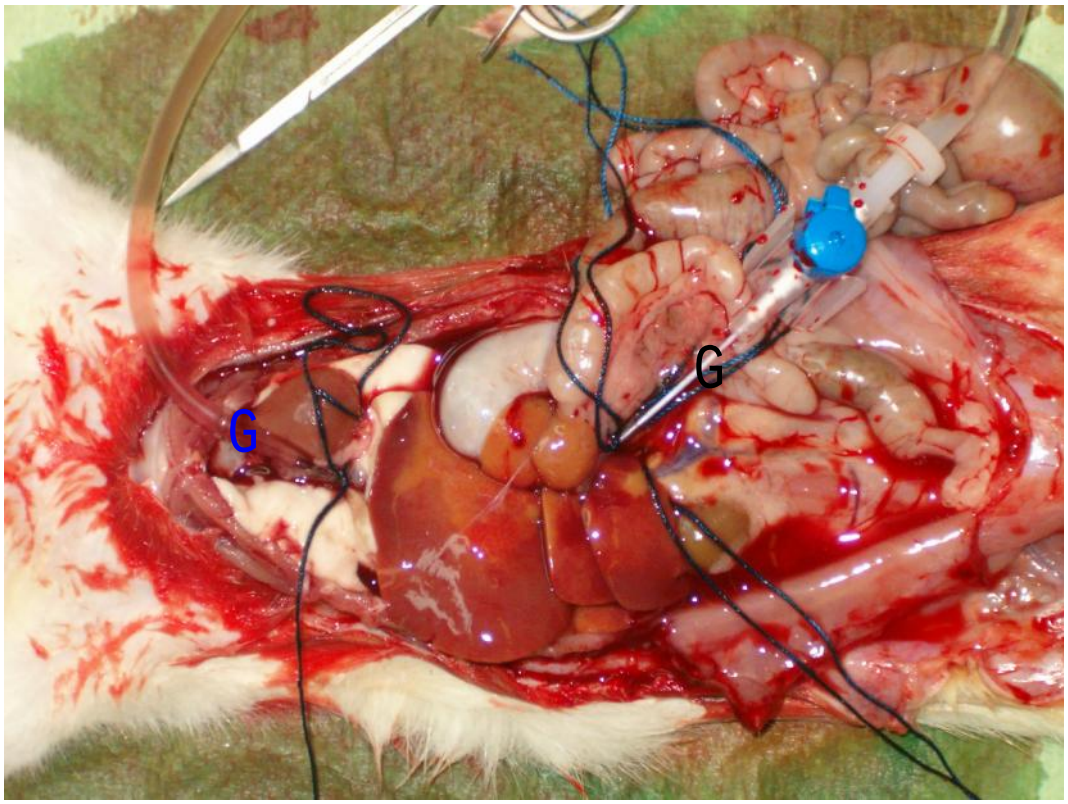


Fig. 11. Perfusion of the liver of rats. The perfusion solutions reach the liver through the *vena portae* (in-flow, *) and will be drained through the thoracal section of the *vena cava caudalis* (out-flow, G).

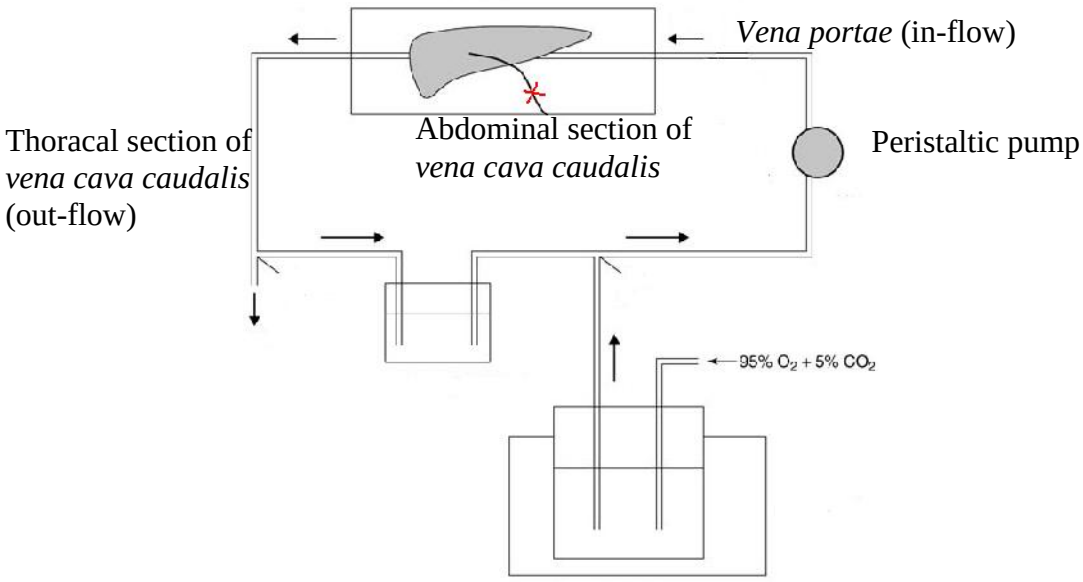


Fig. 12. Overview of the applied closed perfusion system.

The liver was firstly perfused by 300 ml EGTA-containing Earl's Balanced Salt Solution (EBSS) and thereafter by 200 ml EGTA-free EBSS solution. Finally, 25 mg collagenase Type IV was freshly dissolved in 130 ml of the same buffer, also supplemented

with $CaCl_2$ (end concentration 2.5 mM). This digesting solution was applied and recirculated in order to detach the hepatocytes. All solutions applied for perfusion were prewarmed at 40ºC and oxygenated previously with Carbogen (95% O_2, 5% CO_2, 1 l/min). Velocity of the perfusion was set at 30 ml/min.

After the collagenase digestion, the liver was ectomized, the capsule was disrupted and the digested parenchyma was filtered through a nylon mesh with 100 µm pore size (Millipore, Volketswil, Switzerland) to eliminate cell aggregates. Hepatocyte-enriched fractions were isolated and washed by low-speed centrifugation (100g, 2 min) firstly in a suspension solution and then the sediment was resuspended in the same solution supplemented with 15 ml diluted Percoll in order to purify the cell suspension disclosing the damaged cells. After spinning at 150g for 5 min, such injured cells were separated on the top of the tube, while intact hepatocytes could be found in the pellet. A final washing step (100g, 2 min) was performed in Williams' Medium E, supplemented previously by 50 mg/l gentamicin, 2.5 mg/l amphoterycin B, 2 mM glutamine, 5% FBS, 4 µg/l dexamethasone, 20 IU/l insulin and 0.22% sodium bicarbonate.

Trypan blue exclusion test and cell counting were conducted as with the isolated hepatocytes from chicken, described in section 4.1.1.1. Hepatocyte concentration was adjusted before seeding to 1.33 * 10^6/ml. Plating and cultivation were performed similarly to the chicken hepatocyte cultures. A confluent monolayer of hepatocytes was gained after 24 h incubation (**Fig. 13**).

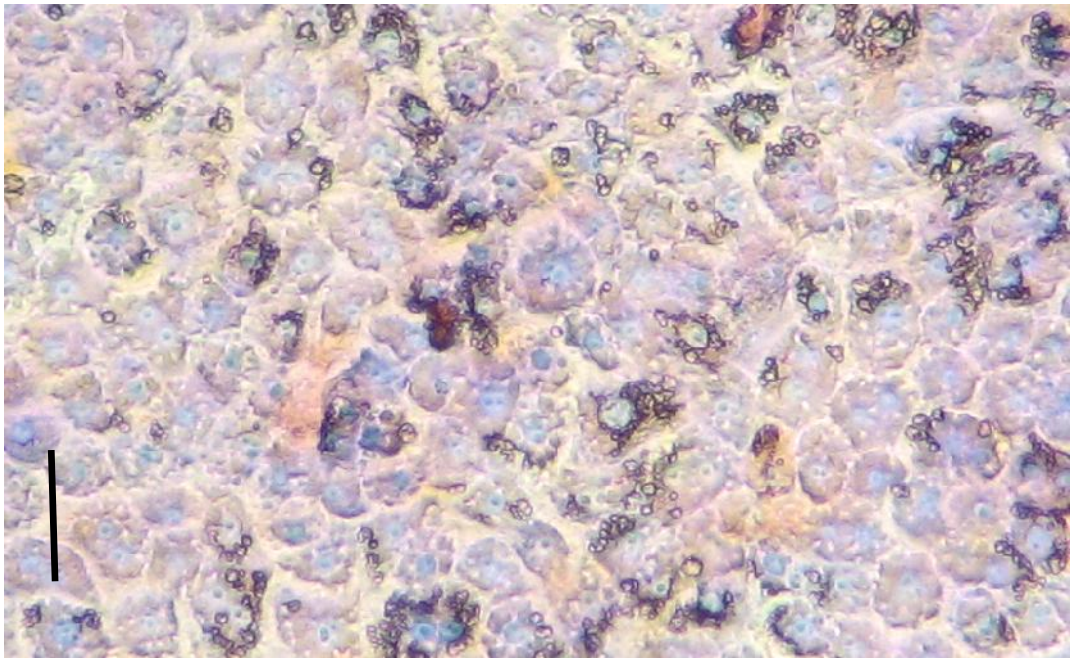


Fig. 13. Primary culture of rat hepatocytes after 24 h incubation (Giemsa staining). Bar = 30 µm

Primary cultures of rat hepatocytes were characterized by immunohistochemical detection of glutaminase according to the same protocol as that of the chicken hepatocyte cultures, mentioned in section 4.1.1.1.

High amount of the positively stained cells (**Fig. 14**) confirmed that the primary cultures consisted of hepatocytes and matched the requirements for the further examinations.

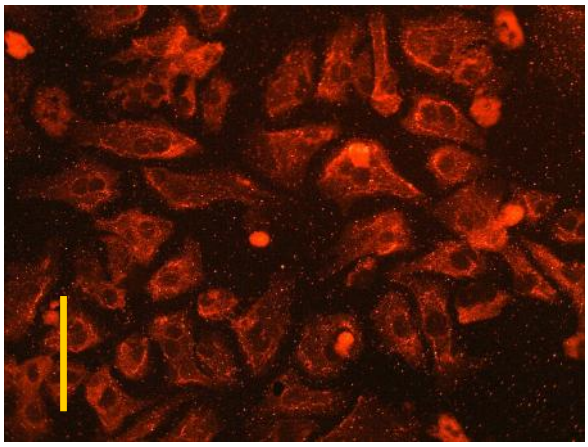


Fig. 14.A

Fig. 14.B

Fig. 14. A. Detection of glutaminase by immunohistochemical staining on primary culture of rat hepatocytes after 48 h cultivation. **B.** A negative control performed without adding secondary antibody. Bar = 30 µm

4.1.1.3. Experimental design

After 24 h cultivation, cultured hepatocytes from both chicken and rat were treated for 24 h with various concentrations of sodium butyrate (0, 1, 5 and 10 mM), dissolved in the appropriate cell culture medium (without FBS). Each treatment was conducted in triplicate.

4.1.1.4. Measurement of butyrate concentration in the culture medium of primary cultures of hepatocytes

Culture medium was removed from cell cultures at the end of the 24 h treatment period and after supplementation of the samples with 5% phosphoric acid and isovalerate inner standard (50 mg/50 ml) gas chromatographic analysis of SCFA was performed. Butyrate was separated and quantified by gas chromatography (Shimadzu GC 2010, Japan) using a 30 m (0.25 mm i.d.) fused silica column (Nukol column, Supelco Inc., Bellefonte, PA, USA).

4.1.2. CYP gene expression of primary cultures of chicken hepatocytes

4.1.2.1. Cell culturing and experimental design

Primary cultures of chicken hepatocytes were prepared as it was described previously in section 4.1.1.1. After 24 h cultivation, cells were treated for additional 24 h according to the following protocol: cell culture media (without FBS) contained six different concentrations of

sodium butyrate (0, 1, 2.5, 5, 7.5, 10 mM) and each sodium butyrate concentration was combined with the following concentrations of erythromycin: 0, 10, 50 and 100 µM, respectively. Each treatment was conducted in triplicate.

4.1.2.2. Isolation of CYP RNAs

RNA was isolated from control and treated cells (1.5 * 10^6 cells/culture dish) using the TRIzol reagent (Invitrogen, Paisley, UK), according to the manufacturer's instruction with slight modifications. Shortly, cells grown in mono-layer were lysed directly in the culture dish by adding the TRIzol reagent (1 ml / well) and passing the cell lysate several times through a pipette. The cell lysate was transferred immediately to microfuge tubes and was incubated at 4°C for 5 min in order to permit the complete dissociation of nucleoprotein complexes. Next, 200 µl ice-cold chloroform (Reanal, Budapest, Hungary) per 1 ml of TRIzol reagent was added to each sample, was shaken vigorously for 15 seconds and placed on ice at 4°C for 5 min. The homogenate was centrifuged at 12,000g (4°C) for 15 min. After spinning, the homogenate formed two phases: the lower phase was the organic phase, while the upper phase was the aqueous phase, containing the RNA. The aqueous phase was carefully transferred to a fresh microfuge tube, and then the chloroform extraction step had to be repeated once again. The aqueous phase was pipetted into a fresh tube again and an equal volume (400 µl) of ice-cold isopropanol (Merck, Darmstadt, Germany) was added. The sample was stored for 1 hour at -80°C and then centrifuged at 12,000g (4°C) for 10 min. After centrifugation, RNA became visible in form of a white pellet at the bottom of the tube. The supernatant was removed and RNA pellet was washed twice with 75% ice-cold ethanol (1 ml of 75% ethanol (Merck) / 1 ml initial solution used) by vortexing and subsequent centrifugation for 5 min at 7,500g (4°C). At the end of the procedure, the supernatant was removed and the pellet dried under laminar box with constant air flow for 10-15 min, then finally dissolved in 50 µl of molecular biology grade water (Eppendorf, Hamburg, Germany).

4.1.2.3. Quality and quantity control of the isolated RNA

Integrity of the isolated RNA was examined by electrophoresis. A 1% agarose gel containing 1 µg/ml ethidium bromide (Fluka, Buchs, Switzerland) was prepared with 1xTBE buffer. Two microliter of the RNA sample was mixed with 3 µl loading dye and was elecrophoresed at constant voltage of 80V for 25 min in 1xTBE buffer. The resulted bands were visualized and scanned by the InGenius LHR Gel Documentation and Analysis System (Syngene, Cambridge, UK). Quantity and A_{260}/A_{280} ratio of the isolated RNA were determined using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, USA). A_{260} and A_{280} values mean the absorbance of the sample at the wavelengths of 260 nm and 280 nm, respectively. A_{260} refers to the DNA/RNA concentration and A_{280} refers to the protein

concentration. A ratio of A_{260}/A_{280} > 1.8 suggests little protein contamination in a DNA/RNA sample.

4.1.2.4. Reverse transcription

Prior to the synthesis of the first strand of cDNA the RNA samples were treated with deoxyribonuclease (DNase) I according to the manufacturer's instruction in order to remove contaminating double and single stranded DNA. After DNase I treatment reverse transcription of RNA was achieved using RevertAid H Minus First Strand cDNA Synthesis kit (Fermentas, St. Leon-Roth, Germany) according to the manufacturer's recommendations. Shortly, 4 µl of RNA (approx. 500 ng) was mixed with 1 µl of random hexamer and 7 µl of molecular biology grade water, then incubated at 70°C for 5 min. Four microliter of 5x reaction buffer, 1 µl of RiboLock RNase Inhibitor and 2 µl of dNTP mix were mixed in a separate microfuge tube then added to the RNA and incubated at 25 °C for 5 min. Finally, 1 µl of reverse transcriptase was pipetted to the reaction. The thermal profile for reverse transcription was 25°C for 10 min, then 42°C for 1 hour and 70°C for 10 min.

4.1.2.5. Quantitative Real Time PCR

Quantitative real time PCR (qRT-PCR) was performed using the iQ SYBR Green Supermix kit (BioRad, Hercules, CA, USA) on the MiniOpticon System (BioRad). The cDNA was diluted 2-fold before equal amounts were added to duplicate qRT-PCR reactions. Tested genes of interest were CYP1A, CYP2H1 and CYP3A37, while the housekeeping gene β-actin was investigated as reference gene. Primer oligonucleotides were designed with Primer3 software (http://frodo.wi.mit.edu/). Primer sequences are listed in **Table 1**. For each PCR reaction, 2.5 µl cDNA was added directly to a PCR reaction mixture set to a final volume of 25 µl, containing 1x concentrated iQ SYBR Green Supermix and 0.2 µM of the appropriate primers. The thermal profile for all reactions was 2 min at 95°C, then 30 cycles of 10 sec 95°C, 20 sec at 56°C and 10 sec at 72°C. The fluorescence monitoring occured at the end of each cycle for 10 sec. Each reaction was completed with a melting curve analysis to ensure the specificity of the reaction. The PCR amplicons were separated by electrophoresis through a 1.5% agarose gel at constant voltage 60V for 35 min in 1xTBE buffer. The resulted bands were visualised and scanned by the InGenius LHR Gel Documentation and Analysis System and quantified by the GeneTools Software (Syngene). Relative expression ratio for CYP1A, CYP2H1 and CYP3A37 was determined by relative expression software tool (REST) (Pfaffl et al., 2002) available at http://www.gene-quantification.de/rest.html. Relative gene expression of target genes was calculated by REST using the formula $R=(E_{target})^{\Delta CPtarget(sample-control)}/(E_{ref})^{\Delta CPref(sample-control)}$, where R represents the relative gene expression ratio of the target gene, E stands for PCR efficiency, ΔCP is the crossing point difference of a sample versus

an untreated control, and ref represents the reference gene β-actin. PCR efficiency was calculated by the following formula: $E=10^{(-1/s)}-1$, where s is the slope of the standard curve.

Table 1. Sequences of the applied primers.

Gene	Accession number		Sequence (5' to 3')	Efficiency	Length (bp)
CYP1A*	NM_205146.1 (CYP1A1) NM_205147.1 (CYP1A4) X99454.1 (CYP1A5)	Forward Reverse	CCGTGACAACCGCCCTGTCC AGCCGTGGTCTCCTCTCCCG	0.912	115
CYP2H1	NM_001001616.1	Forward Reverse	ACAACCAGCACCACACTGAG GCATGTGGAACATTAAGGGG	0.921	206
CYP3A37	NM_001001751.1	Forward Reverse	TGGTTACCTGGCTTACCAGC ATAGAGCCGGAGGGTTTCAT	0.826	160
β-actin	NM_205518.1	Forward Reverse	GTCCACCTTCCAGCAGATGT ATAAAGCCATGCCAATCTCG	0.956	169

* CYP1A primers recognize gene sequences of CYP1A1, CYP1A4 and CYP1A5

4.1.3. Erythromycin elimination of primary cultures of chicken hepatocytes

4.1.3.1. Cell culturing and experimental design

Primary cultures of chicken hepatocytes were prepared as it was described previously in section 4.1.1.1. After 24 h cultivation, cells were treated with various sodium butyrate (0, 1, 2.5, 5, 7.5, 10 mM) and erythromycin (0, 10, 50 and 100 μM) concentrations for additional 24 h according to the protocol mentioned in section 4.1.2.1. Cell culture media without cells, containing the same concentration of sodium butyrate and erythromycin as those of the cultured hepatocytes were also incubated under the same conditions to serve as negative controls to screen the spontaneous degradation of erythromycin.

4.1.3.2. Measurement of erythromycin concentration in the culture medium of primary cultures of chicken hepatocytes

Concentration of erythromycin from culture media from cell cultures as well as from control wells without cells was determined with validated high performance liquid chromatography (HPLC) using sample derivatization method on a Merck-Hitachi LaCrom Elite HPLC system combined with Nucleosil C18 5 μm 25x0.46 column. Before extracting 200 μl of cell culture medium with 4 ml dichloromethane (Merck), first 600 μl 0.1 M Na_2HPO_4 (Spektrum3D, Budapest, Hungary) solution was added, pH was adjusted (8.8-9.3) and the mixture was homogenized using a vortex mixer two times one minute, respectively. Then dichloromethane phase was evaporated to dryness with the RotaDest apparatus at 40-45 °C. The sample residue was reconstituted with 200 μl acetonitrile by ultrasonication and vortex mixing. In derivatization procedure 125 μl 0.1M phosphate buffer (pH=7.5) and 125 μl 10 mg/ml (9 fluorenylmethyl)chloroformate (Merck) was added to each 200 μl sample and

gently mixed with vortex mixer. The reaction was performed at 50°C for 60 min, and then the samples were cooled down to room temperature. Finally, 150 µl of diluting solvent (50/50 acetonitrile / 0.03M phosphate buffer, pH=7.0) was added to each sample, which were then ready to be analyzed by the HPLC system. The injection volume was 50 µl. The mobile phase contained 70 (V/V)% acetonitrile (Merck) and 30 (V/V)% 0.03 M K_2HPO_4 (Merck) based phosphate buffer (pH=7.0) and pH was adjusted thereafter to 7.2. The flow rate was constantly set at 1 ml/min. UV detection method was applied with an excitation wavelength of 260 nm and an emission wavelength of 315 nm.The limit of quantification of the method was 0.002 µg/ml, and the linearity range was from 0.002 to 5 µg/ml. Intra-assay and interassay coefficients of variation were 3% and 3.5%, respectively, at a concentration of 0.004 µg/ml and 2.3% and 3.1%, respectively, at a concentration of 2.5 µg/ml.

This established validated HPLC method could be applied as well for the measurement of the erythromycin concentration from blood plasma samples as it was also used in our *in vivo* studies, mentioned later in section 4.2.4.2.

4.2. In vivo studies

4.2.1. Effects of butyrate applied as feed additive in chicken

4.2.1.1. Animals and treatments

Thirty one-day-old broiler chicks of the Ross 308 strain (mixed gender), obtained from a commercial hatchery (Bábolna Tetra Company, Uraiújfalu, Hungary), were included in the experiment. Broilers were housed together in metal pens (five animals per pen) with a floor area of 1.5 m^2, applying fresh sliwer as litter, under controlled light program (23 h/d from day 0 to 7; 18 h/d from day 8 to 14; 16 h/d from day 15 to 21). The temperature and other climatic circumstances were adjusted according to the requirements of the Ross technology (Ross, 2009). Daily BW gain and feed intake matched the requirements of the Ross technology during the whole examination period.

Experimental animals were randomized into three groups: ten chickens were fed with a control stock diet, free from any medication or chemical additives, formulated to the requirements of the starter period. Composition of the basal diet is shown in **Table 2**. Ten broilers were provided with the same feedstuff but supplemented with 1.5 g sodium butyrate/kg diet. Ten chickens received control diet, but were treated with PB injection intracoelomally (Phenobarbital sodium, Ph. Eur. 7.1, dissolved in sterile, pyrogen-free and endotoxin-free physiological saline solution) to induce CYP activity as a positive control. The applied dose was 80 mg/kg BW daily once over the last three days of the experiment.

Feed and water were provided *ad libitum* for all groups. Sodium butyrate content of the feedstuff of butyrate-fed animals was confirmed by gas chromatography (Shimadzu GC

2010, Japan) using a 30 m (0.25 mm i.d.) fused silica column (Nukol column, Supelco Inc., Bellefonte, PA, USA).

On day 21, 12 h post feeding all animals were slaughtered in anesthesia with carbon dioxide by decapitation. The duration of the experiment and the age of the applied chickens have been chosen for optimal observation of both the possible epigenetic influence of butyrate and the inducibility of CYP enzyme activity.

Table 2. Composition of the basal diet of chickens.

Item	Value
Ingredient, g/kg	
Corn	593.7
Soybean meal	310.0
Corn gluten meal	50.0
Limestone	15.0
Monocalcium phosphate	18.5
NaCl	4.0
Vitamin-mineral mixture[1]	6.0
L-Lysine HCl	1.8
DL-Methionine	1.0
Calculated nutrient composition	
Crude protein, g/kg	212.2
Ether extract, g/kg	29.4
Crude fiber, g/kg	25.3
Ash, g/kg	65.9
AME_n, MJ/kg	11.9
Lysine, g/kg	11.9
Methionine, g/kg	4.9
Methionine + Cysteine, g/kg	8.6
Calcium, g/kg	11.6
Available phosphorus, g/kg	4.5

[1]Provided (per kilogram of diet): Se, 0.24 mg; Fe, 135 mg; Mn, 136 mg; Cu, 22 mg; Zn, 110 mg; I, 1.2 mg; retinyl acetate, 4.14 mg; cholecalciferol 0.075 mg; α-tocopherol, 26.85 mg, choline chloride, 360 mg; menadione, 3.0 mg; riboflavin, 7.0 mg; cobalamine, 0.03 mg; niacin, 40 mg; pantothenic acid, 12 mg; folic acid, 1.0 mg; pyridoxine, 5.0 mg.

4.2.1.2. Determination of SCFA concentrations in blood plasma

Blood samples were taken from the brachial vein into heparinized tubes directly before decapitation. Plasma samples were separated from whole blood with centrifugation (1,300g, 10 min) and stored at -80°C until the further examinations. Concentrations of butyrate and acetate from plasma samples were measured by gas chromatography as described in the section 4.1.1.4.

4.2.1.3. Histometrical examinations on small intestinal micromorphology

Small intestine samples were taken from the proximal jejunum of control and butyrate-treated chickens, 100 mm distally from the *flexura duodenojejunalis* (**Fig. 15**, red

circle). The histological samples were fixed immediately in formaline-containing PBS (10%) and prepared according to the current laboratory procedure: dehydration with ethanol, inclusion into paraffin, rehydration and staining with hematoxylin-eosin. For the examination of the histological slides, the optical microscope Leitz “Dialux 20” was equipped with a digital camera JVC mod. TK-C 1380. Pannoramic Viewer 1.14.50 software was used for the histometric measurements: height of villi (20 villi in each animal), depth of crypts (20 crypts in each animal) and height of enterocytes (30 cells per animal) were measured.

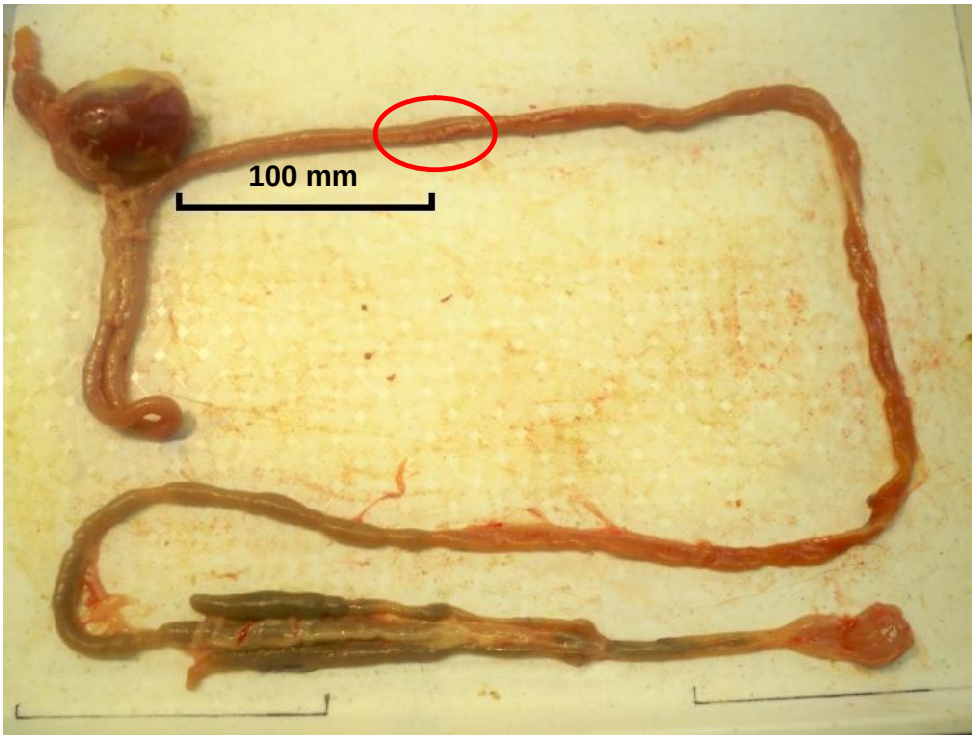


Fig. 15. Sampling from the proximal jejunum (100 mm distally from the *flexura duodenojejunalis*, red circle) of chickens for histometrical measurements

4.2.1.4. Liver sampling and isolation of subcellular organelles

After opening the coelom, the liver was exsanguinated with chilled physiological saline solution through the portal vein and was ectomized, weighed and shock-frozen immediately in liquid nitrogen. Before shock-freezing, 1 g piece of livers of butyrate-fed and control animals was cut and lysed in 1 ml TRIzol reagent (Invitrogen, Paisley, UK) for further PCR examinations on CYP gene expression.

Subcellular organelles were isolated by multi-step differential centrifugation according to the modified protocol of Van der Hoeven (1974). Briefly, liver samples were homogenized in adequate volume of a potassium chloride (1.15%)-based, EDTA (0.1 mM)-containing buffer solution (1 ml buffer per 1 g tissue) with a glass teflon dounce homogenizer on ice. After centrifugation at 2,000g for 10 min to gain the cell nucleus fraction, the pellet was resuspended in a lysis buffer supplemented with protease and phosphatase inhibitors (Halt Protease and Phosphatase Inhibitor Cocktail, Fisher Scientific, Rockford, IL, USA).

The upper phase was centrifuged at 10,000g for 25 min and microsomal fraction was isolated by a two-step ultracentrifugation at 100,000g for 75 min from the supernatant. Finally, the sediment was resuspended in a 0.05 M phosphate buffer, containing 20% glycerol and sonicated for 10 sec.

Microsomal total protein concentration was determined with a Bicinchoninic Acid Protein Assay Kit (Fisher Scientific, Rockford, IL, USA) on a microplate in triplicate, using bovine serum albumin (BSA) as a standard. Cell nuclei were isolated only from control and butyrate-fed chickens, while microsome fraction was prepared from PB-treated animals as well. All cell nucleus and microsomal fractions were shock-frozen in liquid nitrogen and were stored at -80ºC until further examinations.

4.2.1.5. Histone isolation and western blot analysis on histone acetylation

Histone isolation

Purified histone extracts were isolated by a Histone Purification Mini Kit (Active Motif, Carlsbad, CA, USA) from cell nucleus fractions according to the manufacturer's protocol. During the whole purification procedure kit reagents prevented further deacetylase activity to ensure acetylation status as *in vivo*.

Equal volume of ice-cold Extraction Buffer was added to the nucleus suspension. After homogenization, samples were incubated overnight at 4ºC on a rotating platform. Tubes were centrifuged at maximum speed (30,000g) for 5 min in a microfuge, and the supernatant, considered as the crude histone extract, was neutralized with one-fourth volume of 5x Neutralization Buffer (pH 8.0). Neutralized extract was loaded onto previously equilibrated histone isolation spin columns. After 3 washing steps with Wash Buffer, histones were eluted and precipitated overnight from the flow-through by 4% perchloric acid. Precipitate was sedimented by centrifugation at 30,000g for 60 min, the pellet was washed at first with 4% perchloric acid, later with acetone containing 0.2% HCl and finally with pure acetone. Histones were resuspended in sterile distilled water and the yield of total core histone proteins was quantified by measuring the absorbance at 230 nm.

Western blot analysis

Electrophoresis and western blotting were performed according to the instructions of the applied Acetyl Histone Antibody Sampler Kit (Cell Signaling, MA, USA). Histone preparates were diluted by SDS- and mercaptoethanol-containing loading buffer (supplemented with 50 mM dithiothreitol), sonicated for 15 sec in order to reduce viscosity and proteins were heat denatured at 95ºC for 5 min. Histones were separated by SDS-PAGE on polyacrylamide (4-20%) precast gradient gels (Biorad Laboratories, CA, USA), the amount of loaded protein was 3 µg per lane for the detection of histones H2A and H3, while 6 µg per lane for histones H2B and H4. After tank blotting of proteins onto nitrocellulose

membranes (0.2 µm pore size, Biorad Laboratories, CA, USA), histones were identified by immunodetection using antibodies of the Acetyl Histone Antibody Sampler Kit: after blocking with 5% fat-free milk-containing PBST for 2 h, the immunoblots were incubated overnight with primary antibodies against histones H2A (1:1000), H2B (1:500), H3 (1:1000), H4 (1:500) and their acetylated forms. Each acetyl histone antibody was specific for the target histone modified at the lysine residue of the most frequent acetylation site (H2A and H2B: Lys 5, H3: Lys 9, H4: Lys 8). Detection of the primary antibodies was performed using an anti-rabbit secondary antibody (1:2000) coupled with horseradish peroxidase. Primary antibodies were diluted in PBST, containing 5% BSA, while secondary antibodies in PBST, containing 5% fat-free milk. Bands were detected by the Chemidoc XRS enhanced chemiluminescence system (Biorad Laboratories, CA, USA). Membranes were finally stained by Indian Ink to detect all the separated proteins. Band intensities were quantified by the Quantity One 1-D Analysis software (Biorad Laboratories, CA, USA), trace quantities were standardized to the Indian Ink stained bands to ensure equal loading. Acetylation ratios were determined considering relative protein expression levels of each histone and its acetylated form.

All western blot examinations were carried out in duplicate. Regarding H3, two different bands were detected and due to their different molecular mass, trace quantities of bands representing H3.1 and H3.2 isoforms could be measured separately, but the acetylation state was calculated from the total amount of H3 and acetyl-H3.

4.2.1.6. Hepatic CYP gene expression

Expression of hepatic CYP1A, CYP2H1 and CYP3A37 genes was investigated by qRT-PCR. RNA isolation, reverse transcription and qRT-PCR examination were conducted as it is described in section 4.1.2.

4.2.1.7. Enzyme assays on hepatic microsomal CYP activity

Aminopyrine N-demethylation assay

Microsomal CYP2H/CYP3A37 activity (ortholog to the mammalian CYP2B/3A) was screened by the aminopyrine N-demethylation assay, in which formaldehyde production could be measured by the spectrophotometric method of Nash (1953). The enzyme assay was performed according to the modified protocol of García-Agúndez et al. (1990). The reaction mixture contained an $NADPH+H^+$-regenerating cofactor mixture, prepared from 0.5 mM $NADPH+H^+$ (Reanal, Budapest, Hungary), 50 mM glucose 6-phosphate, 4 IU/l glucose 6-phosphate dehydrogenase, 5 mM $MgCl_2$ and 50 mM semicarbazide.

After thawing on ice, 100 µl microsomal suspension was incubated with 200 µl cofactor mixture and 900 µl 0.05 M phosphate buffer (pH 7.4) in the presence of different concentrations (0, 1.25, 2.5, 5, 10 mM) of dimethylamino-antipyrine (aminopyrine substrate) for 10 min at 37ºC. The reaction was stopped by adding 200 µl 20% trichloroacetic acid. After

centrifugation at 4,500g for 10 min, 400 µl Nash reagent (0.16 ml acetyl acetone and 0.24 ml concentrated acetic acid in 20 ml of 4 M ammonium acetate solution) was added to 800 µl of the supernatant. The mixture was incubated at 60°C for 30 min, cooled down on ice and the absorbance was measured spectrophotometrically at 415 nm against reagent blank. Results were corrected by subtracting the absorbance of an inhibited blank per each substrate concentration (inhibited previously by adding 20% trichloroacetic acid). Formaldehyde standard curves were determined under the same conditions as used for microsomal activity measurements; each sample was examined in triplicate. Finally, the basic enzyme kinetic parameters, e.g. mean specific enzyme activity (reaction velocity), maximal reaction velocity (V_{max}) and the Michaelis-Menten's constant (K_M) were calculated and compared between groups. All results were standardized according to the total protein concentration of microsomal samples.

Aniline hydroxylation assay

CYP2H activity (ortholog to the mammalian CYP2B) was measured by aniline hydroxylation assay. The enzyme assay was carried out according to the modified protocol of Murray and Ryan (1982). The reaction mixture contained an $NADPH+H^+$-regenerating cofactor mixture with the same composition as for the aminopyrine N-demethylation assay. After thawing on ice, 100 µl microsomal suspension was incubated with 200 µl cofactor mixture and 900 µl 0.05 M phosphate buffer (pH 7.4) and different concentrations (0, 1.25, 2.5, 5, 10 mM) of aniline hydrochloride for 15 min at 37ºC. The reaction was terminated by adding 200 µl 20% trichloroacetic acid. Following centrifugation at 4,500g for 10 min, 400 µl 10% Na_2CO_3 solution and 400 µl alkaline phenol solution (0.8% phenol solution in 0.2 M NaOH) were added to 400 µl of the supernatant. The mixture was incubated at 37°C for 30 min, cooled down on ice and the absorbance was measured by spectrophotometer at 605 nm against reagent blank. An inhibited blank was approved for each substrate concentration similarly to the aminopyrine N-demethylation assay. To determine the amount of the produced 4-aminophenol, standard curves were prepared; each sample was examined in triplicate. Mean specific enzyme activity, V_{max} and K_M values were also determined and compared between groups. All results were standardized according to the total protein concentration of microsomal samples.

Testosterone 6β-hydroxylation assay

CYP3A37 activity (ortholog to the mammalian CYP3A) was determined by testosterone 6β-hydroxylation assay. All reactions were performed in total volumes of 200 µl. The incubation mixtures consisted of testosterone (0.5 mM), hepatic microsomal protein (1 mg/ml), $NADPH+H^+$ (1 mM) and a $NADPH+H^+$-generating system in 0.1 M Tris-HCl buffer (pH 7.4). The reactions were stopped after incubation for 15 min at 37°C by the addition of

200 µl ice-cold methanol. Samples were centrifuged at 13,000 rpm for 10 min at 4°C and the supernatants were analyzed by HPLC. The chromatographic method published by Lutz et al. (2002) was employed with some modifications. Potassium phosphate buffer (10 mM) at pH 3.2, containing methanol (57:43) was used as a mobile phase. Following isocratic separation of metabolites, the column was rinsed of remaining substrate as well as other hydrophobic components by employing gradients reaching 90% of organic solvent and then the column was re-equilibrated. The flow rate was 3 ml/min and the detector was set to 254 nm. Chromatography was performed on a Merck Hitachi HPLC system using an L-6200 A pump, an L-4250 UV detector and a D-6000 A interface (Darmstadt, Germany) with a 100×4.6 mm Chromolith Performance C18 (Merck) chromatographic column for analytical separation.

4.2.1.8. Comparative studies in rats

Thirty five-week-old male Wistar rats were included in the rat feeding experiment. Three animals were kept together in plastic pens with a floor area of 0.5 m^2 under controlled light (14 h/d). The temperature was set according to the ideal climatic circumstances for rodents (22-23^oC) as well as the relative air humidity (55%). Rats were randomly classified into three groups. Ten control animals were fed by commercially available pelleted feedstuff for rodents. Ten rats received the same feedstuff, previously reduced to powder, supplemented with sodium butyrate with the concentration of 1.5 g/kg and reformed to pellet. Finally, ten animals were kept on the control diet, but intraperitoneal PB injection (80 mg/kg BW) was administered daily once on days 19-21. Butyrate content of the diet was confirmed by gas chromatography as mentioned in section 4.2.1.1. Nutrient composition of the diet of rats can be seen in **Table 3**. Feed and water were provided *ad libitum* as well.

Table 3. Composition of the diet of rats.

Calculated nutrient composition	Value
Crude protein, g/kg	199.00
Ether extract, g/kg	3.61
Crude fiber, g/kg	4.60
Ash, g/kg	8.83
Digestable energy, MJ/kg	15.91
Lysine, g/kg	0.70
Methionine, g/kg	0.60
Calcium, g/kg	0.50
Available phosphorus, g/kg	0.40

Provided (per kilogram of diet): Se, 0.1 mg; Fe, 35 mg; Mn, 50 mg; Cu, 5.0 mg; Zn, 12 mg; I, 1.2 mg; retinyl acetate, 12 mg; cholecalciferol 0.25 mg; α-tocopherol, 50 mg, choline chloride, 1000 mg; menadione, 50 mg; riboflavin, 3.0 mg; cobalamine, 0.05 mg; niacin, 20 mg; pantothenic acid, 8 mg; folic acid, 1.0 mg; pyridoxine, 6.0 mg.

Similarly to the chickens, rats were decapitated in carbon dioxide anesthesia on day 21. Blood samples were taken from the retrobulbar venal plexus right before slaughtering. Plasma SCFA concentrations were investigated by gas chromatography as mentioned earlier (see section 4.1.1.4).

Small intestine samples of controls and animals fed with butyrate-supplemented diet were taken from the jejunum 300 mm distally from the pylorus (**Fig. 16**, red circle), fixed and examined as described in section 4.2.1.3.

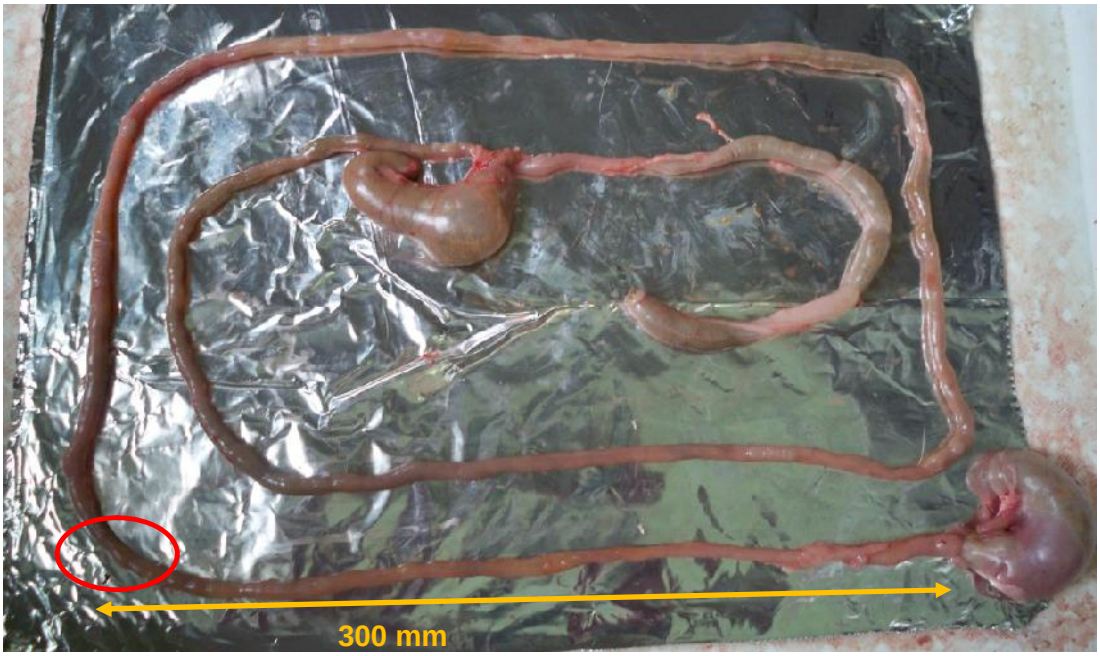


Fig. 16. Sampling from the proximal jejunum (300 mm distally from the pylorus, red circle) of rats for histometrical measurements

The portal vein was cannulated immediately after slaughtering and the exsanguinated liver was ectomized and was used for subcellular organelle isolation as mentioned in section 4.2.1.4. Microsomal CYP activities were studied by specific enzyme assays (CYP2B/3A by aminopyrine N-demethylation and CYP3A by testosterone 6β-hydroxylation) with the same protocols as applied in the chicken experiment (see section 4.2.1.7).

4.2.2. Effects of butyrate applied as daily bolus in chicken

4.2.2.1. Animals and treatments

Thirty-six day-of-hatch broiler chicks of the Ross 308 strain (mixed gender) were included in the experiment. Origin of the animals, housing conditions and ingredients of the applied diet were the same as those of the previous study described in section 4.2.1.1.

On days 20-24 experimental animals were fasted overnight for 12 h and thereafter treated once daily by a crop-tube with an intraingluvial bolus according to the following protocol: (i) ten chickens received 0.1 g/ml sodium butyrate solution (2.5 ml/kg BW, which equals 0.25 g sodium butyrate/kg BW daily); (ii) ten broilers were treated with 0.5 g/ml sodium butyrate solution (2.5 ml/kg BW, which equals 1.25 g sodium butyrate/kg BW daily); (iii) distilled water (2.5 ml/kg BW) was applied for ten chicks as a control group. In addition, (iv) six broilers were treated on days 20-24 by intracoelomal PB injection (Phenobarbital sodium, Ph. Eur. 7.1, dissolved in sterile, pyrogen-free and endotoxin-free physiological saline solution, applied dose: 80 mg/kg BW daily) to induce CYP activity as a positive control. The lower dose of butyrate boli provided approximately the same amount of sodium butyrate on each day of treatment, which was taken up daily as feed additive in the previous experiment (section 4.2.1.1). BW was measured individually on each day of treatment, boli and PB injections were adjusted to the measured BW per day. All animals were fasted for additional 2 h after each treatment in order to enhance the absorption of butyrate. Daily BW gain and feed intake matched the requirements of the Ross technology and no significant difference could be observed between the groups.

Animals were slaughtered in carbon dioxide anesthesia by decapitation on day 24. Last treatment was conducted 2 h prior to slaughtering.

4.2.2.2. Determination of SCFA concentrations in blood plasma

Blood samples were taken and concentrations of butyrate and acetate from plasma samples were measured by gas chromatography as described in section 4.2.1.2.

4.2.2.3. Liver sampling and isolation of subcellular organelles

Exsanguination and sampling of the liver, as well as isolation of subcellular organelles was performed as described in section 4.2.1.4. Cell nucleus fraction was isolated from the liver of the bolus-treated and control chickens (treatments i, ii, iii) in order to examine the acetylation state of the core histones, while microsomal fractions were prepared from all animals (treatments i, ii, iii, iv) to study the hepatic CYP activity.

4.2.2.4. Histone isolation and western blot analysis of histone acetylation

Preparation of hepatic core histones from cell nucleus fractions and western blot analysis of histone acetylation were performed as mentioned in section 4.2.1.5.

4.2.2.5. Enzyme assays on hepatic microsomal CYP activity

Microsomal CYP2H/CYP3A37 activity was detected by aminopyrine N-demethylation assay and CYP2H activity was investigated by aniline-hydroyxlation assay described in details in section 4.2.1.7.

4.2.3. Additional effects of butyrate and phenobarbital in chicken

Twenty-four day-of-hatch broiler chickens of the Ross 308 strain (mixed gender) were included in the experiment. Origin of the animals, housing and dietary conditions, as well as ingredients of the applied diet were the same as those of the experiment described in section 4.2.1.1. Broilers were randomized into the following groups (n=8/group) and received the following treatments after overnight fasting on days 19-24:

(i) Control group, distilled water *per os*

(ii) Distilled water *per os* + intracoelomal PB injection (80 mg/kg BW)

(iii) Sodium butyrate bolus, 0.25 g/kg BW *per os* + intracoelomal PB injection (80 mg/kg BW)

Butyrate boli were prepared and administered as it was written in section 4.2.2.1. All animals were slaughtered 2 h after the last received treatment as in the earlier experiments (see section 4.2.2.1). Liver sampling, microsome isolation and specific enzyme assays on CYP activity were carried out as it was mentioned in sections 4.2.1.4. and 4.2.1.7.

4.2.4. Effects of butyrate on pharmacokinetics of erythromycin in chicken

4.2.4.1. Animals, treatments and sampling

Twenty one-day-old broiler chicks of the Ross 308 strain (mixed gender) were included in the experiment. Origin of the animals, housing conditions and ingredients of the applied diet were the same as those of the experiment described in section 4.2.1.1. Animals were fed by a normal stock diet, with or without sodium butyrate supplementation (1.5 g/kg diet), n=10/group. At the end of the six-week long feeding period chickens were treated with a single intramuscular dose (30 mg erythromycin base/kg BW, pectoral muscle) of erythromycin (Gallimycin®, Ceva, Libourne, France) injection. Before the treatment control blood samples were collected. Further blood samples were drawn at 0.5, 1, 1.5, 2, 3, 4, 8 and 12 h after the injection. Blood sampling was conducted from the brachial vein into heparinized tubes.

4.2.4.2. Determination of plasma erythromycin concentration and pharmaco-kinetic properties of erythromicin

Plasma levels of erythromycin were determined with validated HPLC using sample derivatization method on a Merck-Hitachi LaCrom Elite HPLC system combined with

Nucleosil C18 5 µm 25x0.46 column. Details of the measurement are described in section 4.1.3.2,

The pharmacokinetic parameters of erythromycin were determined applying noncompartmental analysis with the software Kinetica 4.4.1 (Thermo Scientific, Schwerte, Germany). The bioequivalence between the results of butyrate-treated and control groups was established according to the method of European guideline (EMA/CVMP/016/00-Rev.2, 2011).

4.3. Statistical analysis

All values are expressed as mean ± SEM. Statistical analysis of data was performed with R 2.14.0 software (open access, downloaded from the following source: http://cran.r-project.org/bin/windows/base/old/2.14.0/ on 14 December 2011). Distribution of data was screened with Shapiro-Wilk's test of normality and by plotting out data as histograms. One-way ANOVA or ANCOVA and Student's t-test were used for the comparison of results of the treated groups with those of controls at normal distributions, while non-parametric Mann-Whitney's test was approved at not normal distributions. Person's test of correlation was applied in order to analyze the correlation between datasets. Level of significance was set at $P<0.05$.

5. Results

5.1. In vitro studies on primary cultures of hepatocytes

5.1.1. Butyrate uptake of primary cultures of chicken and rat hepatocytes

Butyrate concentration was measured from cell culture media after 24 h incubation with different initial concentrations of butyrate by gas chromatography. Relative butyrate uptake values (%) were calculated regarding the measured and initial concentrations. Results of cultured chicken and rat hepatocytes are presented in **Table 4**.

Table 4. Relative butyrate uptake of cultured rat and chicken hepatocytes after 24 h incubation with different concentrations of butyrate.

Initial butyrate concentration of medium (mM)	Relative butyrate uptake (%)	
	Rat	Chicken
1	100	100
5	100	19.67 ± 3.86***
10	75.71 ± 6.26	15.12 ± 1.31***

Results are expressed as mean ± SEM, n=3 animals. Each experiment was conducted in triplicate.
***Significant difference between species, P<0.001.

Regarding our results, it could be stated that hepatocytes of both chicken and rat took up the whole amount of butyrate at 1 mM concentration, there was no difference between species. However, after incubation with 5 mM butyrate, chicken hepatocytes could take up only a relatively small amount (approx. one-fifth) of the applied butyrate, while the whole amount of butyrate was taken up by cultured rat liver cells. Similarly to these, at the initial concentration of 10 mM butyrate, hepatocytes of rats showed significantly (P<0.05) higher butyrate consumption than cell cultures from chickens. It means that butyrate uptake of chicken hepatocytes reached a plateau after elevation of butyrate concentration, which phenomenon could not be observed in rats. This finding is clearly visible also in **Fig. 17**, comparing the absolute amounts of butyrate taken up by the hepatocytes.

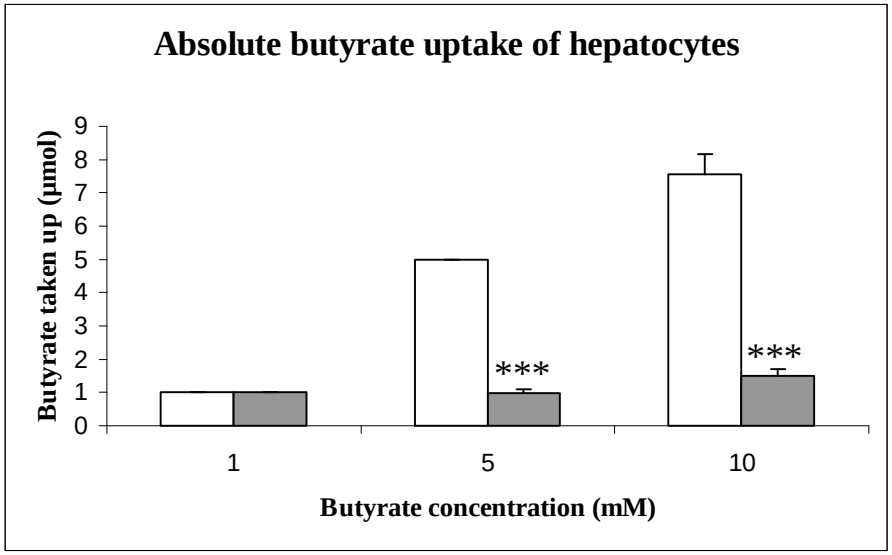


Fig. 17. Absolute amount of butyrate taken up by cultured hepatocytes of rat and chicken. Open bars refer to the primary cultures of hepatocytes from rat and closed ones to those from chicken. Results are expressed as mean ± SEM, n=3 animals. Each experiment was conducted in triplicate.
***Significant difference between species, P<0.001.

5.1.2. CYP gene expression of primary cultures of chicken hepatocytes

The PCR amplicons, separated by agarose gel electrophoresis were visualized to confirm the presence of the required products. It was clearly visible that only one product was amplified in each case and the molecular mass of the amplicons matched the literature data (CYP1A: 115 bp, CYP2H1: 206 bp, CYP3A37: 160 bp and β-actin: 169 bp).

In vitro applied butyrate and erythromycin caused notable, significant changes in the expression of the examined CYP genes on primary cultures of chicken hepatocytes. To evaluate their effects on gene expression, both butyrate and erythromycin were included in the applied ANCOVA model as continuous variables. The expression of CYP2H1 genes significantly increased with butyrate concentration (P<0.001) (**Fig. 18.B**). In contrast to this finding, both CYP1A and CYP3A37 genes were significantly suppressed after butyrate treatment (P<0.001), but this suppression was even more pronounced on CYP1A (**Fig. 18.A**) than on CYP3A37 (**Fig. 18.C**). Increasing erythromycin concentrations caused elevated relative gene expression levels of both CYP2H1 and CYP3A37 genes (P<0.001). Interestingly, CYP1A was not affected by erythromycin treatment (P=0.615).

The concomitant application of butyrate and erythromycin resulted in a significantly higher CYP2H1 gene expression than that of butyrate or erythromycin alone (**Fig. 18.B**). The inhibitory action of butyrate on CYP3A37 was alleviated by erythromycin because the decline of CYP3A37 gene expression was less pronounced in the presence of erythromycin, especially at the highest (100 µM) erythromycin concentration (**Fig. 18.C**). Erythromycin did

not affect the gene suppression action of butyrate on CYP1A gene relevantly, except for 1 mM butyrate simultaneously with 50 µM erythromycin, where significant increase in gene expression could be detected (**Fig. 18.A**). Relative CYP gene expression levels can be seen in **Table 5**.

Table 5. Relative CYP gene expression levels of primary cultures of chicken hepatocytes after incubation with different concentrations of butyrate and erythromycin. Gene expression of untreated cells (no butyrate, no erythromycin) was considered as 1 in each case. Results are expressed as mean ± SEM, n=3 animals. Each experiment was conducted in triplicate.

CYP1A

Concentration of butyrate (mM)	Concentration of erythromycin (µM)			
	0	**10**	**50**	**100**
0.0	1	0.66 ± 0.20	1.53 ± 0.49	0.94 ± 0.24
1.0	0.55 ± 0.13	0.36 ± 0.05	1.71 ± 0.09	0.43 ± 0.05
2.5	0.23 ± 0.04	0.24 ± 0.08	0.25 ± 0.13	0.28 ± 0.06
5.0	0.10 ± 0.03	0.09 ± 0.01	0.10 ± 0.03	0.24 ± 0.01
7.5	0.14 ± 0.01	0.16 ± 0.02	0.12 ± 0.02	0.12 ± 0.01
10.0	0.24 ± 0.03	0.11 ± 0.03	0.18 ± 0.01	0.10 ± 0.02

CYP2H1

Concentration of butyrate (mM)	Concentration of erythromycin (µM)			
	0	**10**	**50**	**100**
0.0	1	2.06 ± 0.03	3.41 ± 0.32	11.72 ± 0.66
1.0	0.87 ± 0.06	1.99 ± 0.27	1.85 ± 0.18	7.27 ± 0.27
2.5	3.24 ± 0.28	6.43 ± 0.61	5.68 ± 0.95	11.26 ± 0.50
5.0	8.13 ± 0.09	12.97 ± 1.00	15.02 ± 0.96	22.85 ± 1.71
7.5	9.90 ± 1.56	12.69 ± 0.83	11.07 ± 1.14	21.83 ± 0.80
10.0	9.10 ± 1.04	11.02 ± 1.46	12.35 ± 1.07	20.32 ± 2.16

CYP3A37

Concentration of butyrate (mM)	Concentration of erythromycin (µM)			
	0	**10**	**50**	**100**
0.0	1	1.55 ± 0.20	1.21 ± 0.15	1.59 ± 0.33
1.0	0.63 ± 0.03	1.05 ± 0.04	1.00 ± 0.03	1.16 ± 0.05
2.5	0.61 ± 0.01	1.26 ± 0.09	0.87 ± 0.08	1.69 ± 0.14
5.0	0.53 ± 0.03	0.82 ± 0.02	0.90 ± 0.03	1.60 ± 0.03
7.5	0.48 ± 0.01	0.74 ± 0.06	0.77 ± 0.03	1.43 ± 0.16
10.0	0.39 ± 0.06	0.60 ± 0.02	0.76 ± 0.06	1.17 ± 0.20

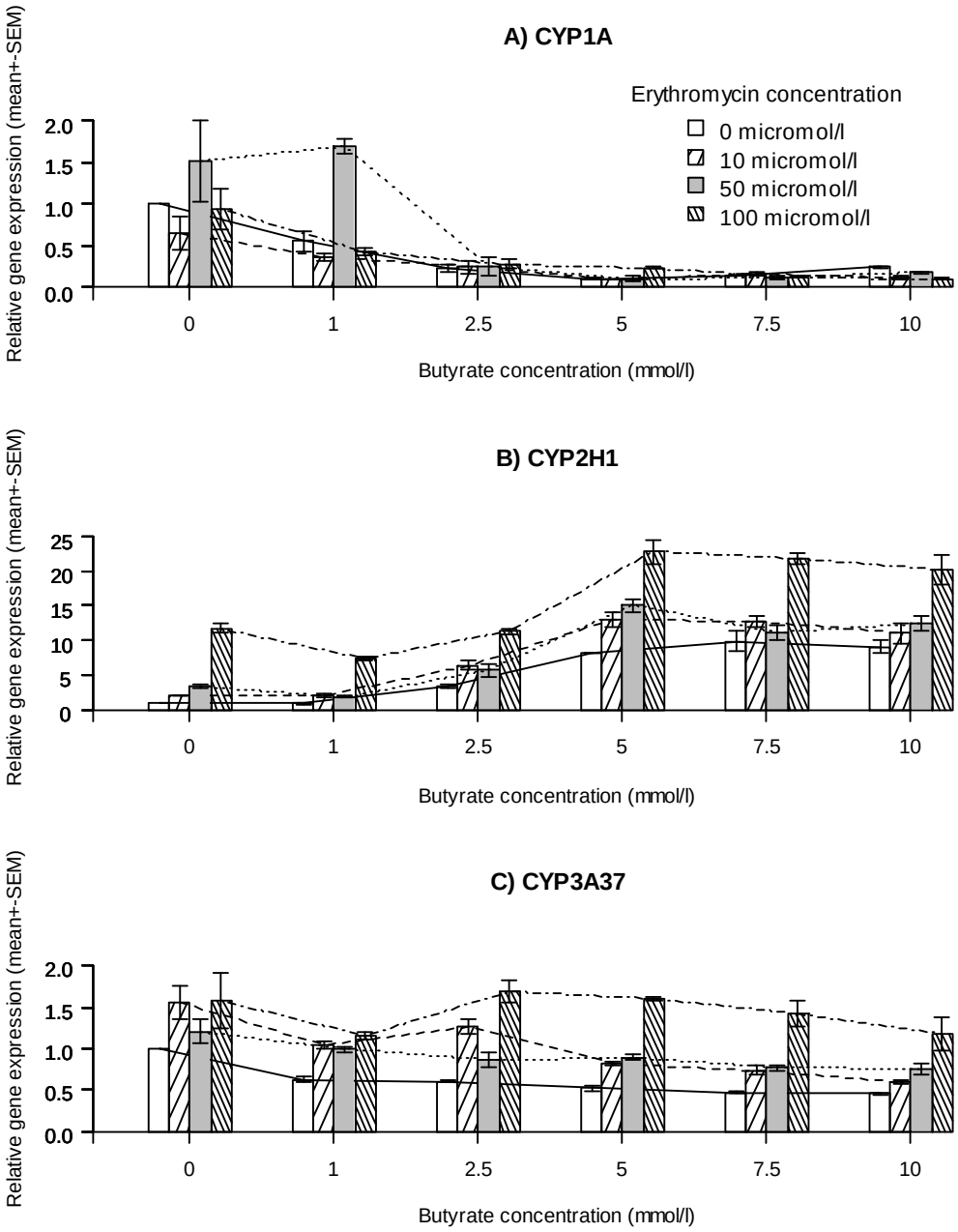


Fig. 18. The relative gene expression or suppression effect (y axis) of the different concentrations of butyrate (x axis) and erythromycin (columns with different shade) on CYP1A (A), CYP2H1 (B) and CYP3A37 (C) genes in cell culture of chicken hepatocytes. Both butyrate and erythromycin were included in the model as a continuous variable. The baseline value - neither butyrate nor erythromycin was added to the medium - is determined as 1, and is represented in each chart with the first empty column. Results are expressed as mean ± SEM, n=3 animals. Each experiment was conducted in triplicate.

5.1.3. Erythromycin elimination of primary cultures of chicken hepatocytes

Erythromycin concentration in cell culture media of primary cultures of chicken hepatocytes and control wells without cells after 24 h incubation with different initial concentrations of butyrate and erythromycin was measured by HPLC. Percentages of eliminated erythromycin, compared to the sample that contained no cells and no butyrate, were determined from the measured and the initial erythromycin concentrations. Results are shown in **Fig. 19**.

Based on the calculated percentageous erythromycin elimination values, the effects of various factors influencing erythromycin degradation, such as cellular uptake, butyrate concentration and erythromycin concentration could be evaluated. It was clearly visible that some spontaneous erythromycin degradation occured even without cellular metabolism. However, the presence of hepatocytes obviously increased the dissappearence of erythromycin highly significantly ($P<0.001$) in each case, indicating the erythromycin uptake of the cultured liver cells. Butyrate concentration seemed to be a potent, increasing effector of erythromycin consumption as well ($P<0.001$). Interestingly, erythromycin elimination in hepatocyte cultures and controls without cells increased more or less parallelly at elevating butyrate concentrations.

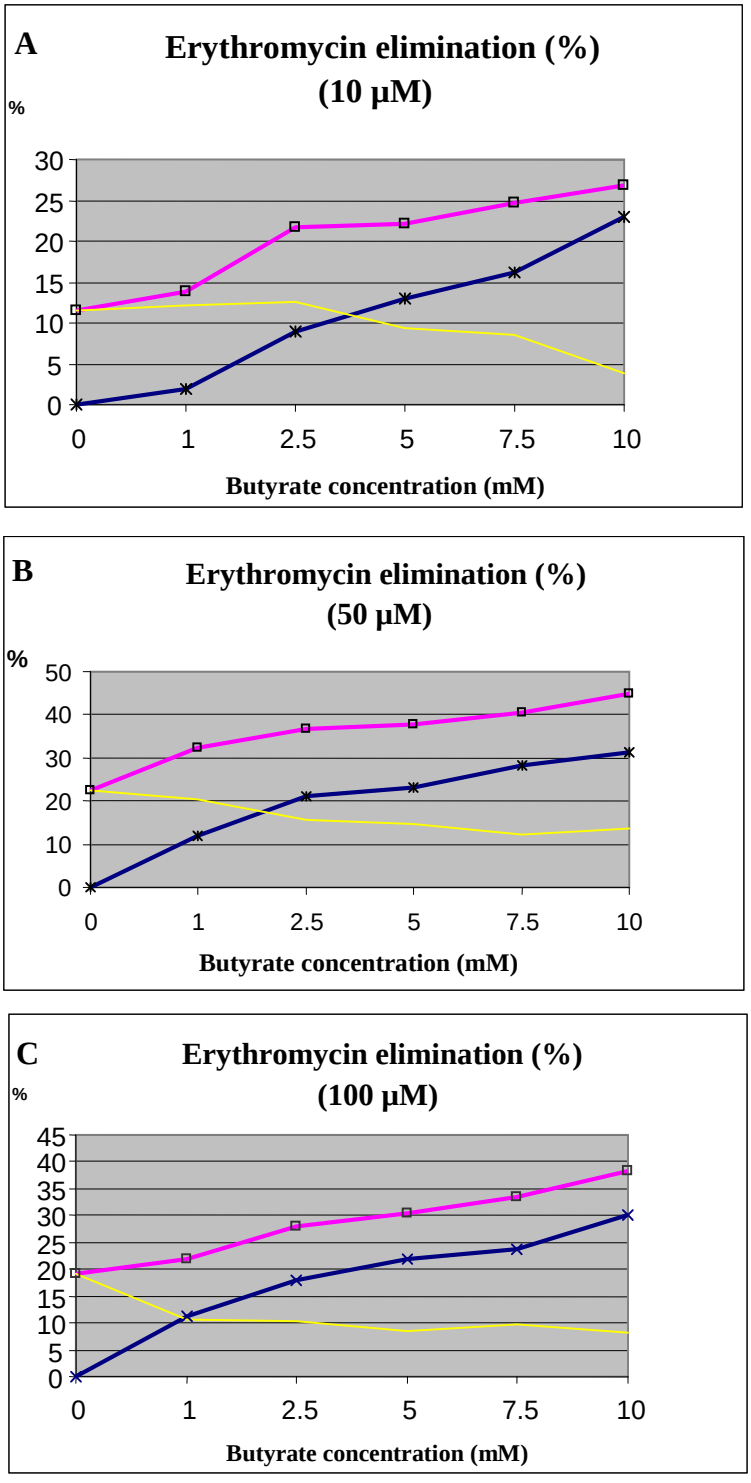


Fig. 19. Percentage of erythromycin elimination in the samples spiked with an initial concentration of **A.** 10, **B.** 50 and **C.** 100 µM erythromycin, respectively, observed at different butyrate concentrations. Purple lines indicate erythromycin degradation values of primary cultures of chicken hepatocytes, blue ones refer to those of the controls with no cells, while yellow lines show the netto erythromycin degradation rate of the hepatocytes (results of controls subtracted from those of cell cultures). n=3 animals.

5.2. In vivo studies

5.2.1. Effects of butyrate applied as feed additive in chicken

5.2.1.1. Concentrations of SCFA in blood plasma

Significantly elevated (P<0.01) plasma butyrate concentration was measured in chickens kept on butyrate-supplemented diet, compared to the level of the control animals (**Table 6**). However, no changes in plasma acetate level could be detected (**Table 6**).

Table 6. Effects of dietary butyrate supplementation on SCFA concentrations of the blood plasma in broiler chickens.

	Control	Butyrate[1]
Butyrate (µM)	24.65 ± 6.21	53.75 ± 6.25**
Acetate (mM)	1.15 ± 0.06	1.30 ± 0.08

[1]Butyrate = supplemented with 1.5 g/kg diet sodium butyrate for 21 days.
Results are expressed as mean ± SEM, n=10/group.
**Significant difference compared to control, P<0.01.

5.2.1.2. Small intestinal micromorphology

The following histological changes could be observed in the proximal jejunum of chickens: depth of crypts tended to be increased in the proximal jejunum after butyrate supplementation, but this tendency did not reach statistical significance (P=0.089). No significant difference was found in the height of villi (P=0.280) (**Table 7**). In spite of this, height of enterocytes was increased significantly (P<0.001) in butyrate-treated chickens compared to the control.

Table 7. Effects of dietary butyrate supplementation on small intestinal micromorphology in broiler chickens.

	Control	Butyrate[1]
Height of villi (µm)	1082.52 ± 61.15	1165.61 ± 72.14
Depth of crypts (µm)	147.17 ± 10.41	158.43 ± 10.94
Height of enterocytes (µm)	25.77 ± 0.68	30.33 ± 0.47***

[1]Butyrate = supplemented with 1.5 g/kg diet sodium butyrate for 21 days.
Results are expressed as mean ± SEM, n=10/group.
***Significant difference compared to control, P<0.001.

5.2.1.3. Acetylation of hepatic core histones

Screening of important acetylation sites at core histones revealed that butyrate caused a significantly increased ratio in H2A acetylation at Lys 5 (P=0.038) in broiler chickens fed with a butyrate-supplemented diet during the early post-hatch period. In contrast, dietary butyrate had no significant influence on the acetylation state of core histones H2B, H3 and H4 at the examined acetylation sites (H2B: P=0.397, H3: P=0.181, H4: P=0.382). Quantitative results and representative bands obtained by Western blotting are shown in **Fig. 20**.

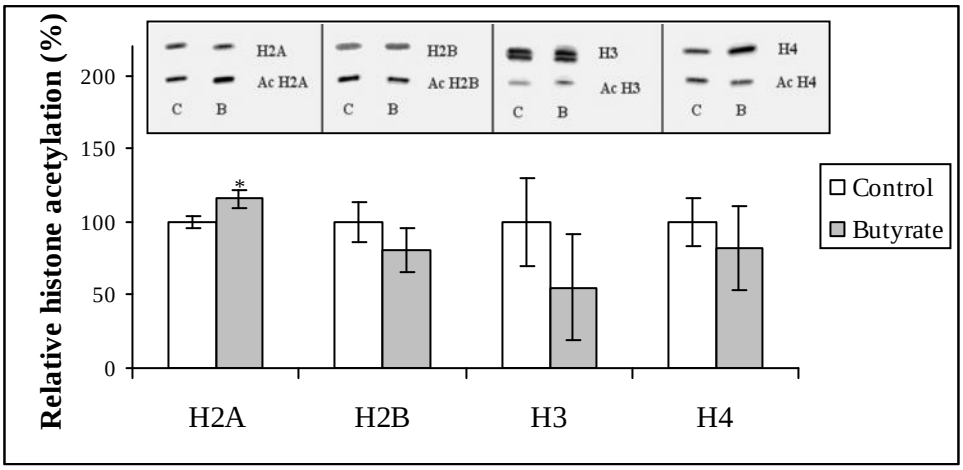


Fig. 20. Relative acetylation of hepatic core histones in broiler chickens with dietary butyrate supplementation (1.5 g/kg diet) for 21 days, compared to those of controls (considered as 100%). Results are expressed as mean ± SEM, n=10/group. Representative bands are also shown as obtained by western blotting, in each case open bars refer to the control (C) and the closed ones to the butyrate-fed group (B). The upper row indicates the relative protein expression levels of H2A, H2B, H3 and H4, respectively, while the bands specific for acetylated histones can be seen below. *Significant difference compared to control, P<0.05.

5.2.1.4. Hepatic CYP gene expression

According to our results, expression of hepatic CYP1A and CYP2H1 genes was influenced by butyrate supplementation of the diet in chicken. Both CYP1A and CYP2H1 were significantly (CYP1A: P<0.001, CYP2H1: P=0.005), more than twofold overexpressed in butyrate-fed animals compared to the control group (**Fig. 21**). However, no alterations could be detected by screening the relative gene expression of CYP3A37 (P=0.557).

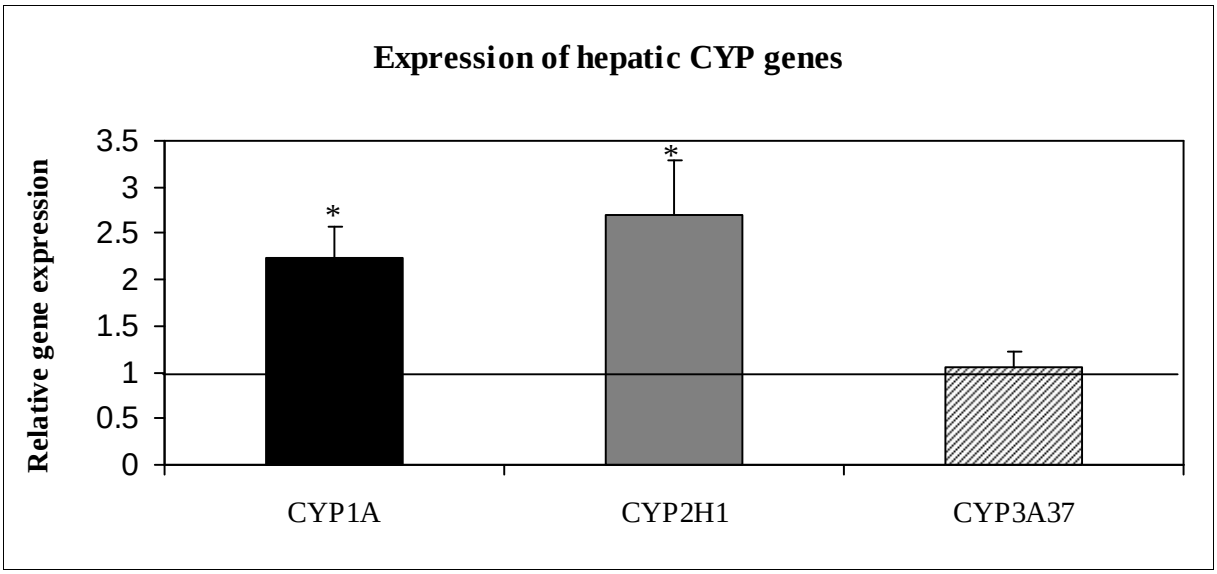


Fig. 21. Relative gene expression of hepatic CYP genes in chickens after butyrate supplementation of the diet (1.5 g/kg) for 21 days. Gene expression of control animals was considered as 1, indicated by a horizontal line. Results are expressed as mean ± SEM, n=10/group.
*Significant difference compared to control, P<0.05.

5.2.1.5. Hepatic microsomal CYP activity

Aminopyrine N-demethylation assay

No significant difference was observed between the average aminopyrine N-demethylation activity (P=0.541), nor between V_{max} (P=0.436) and K_M (P=0.114) values of hepatic microsomal CYP2H/CYP3A37 enzymes of butyrate-fed and control broiler chickens. PB treatment caused notable enzyme induction with significantly increased mean specific activity (P=0.006), V_{max} (P=0.003) and K_M values (P=0.011) (**Fig. 22.A and Table 8**).

Aniline hydroxylation assay

Butyrate supplementation did not alter the aniline hydroxylation activity of hepatic microsomal enzymes, which indicated the activity of the CYP2H subfamily. There was no significant difference in the average enzyme activity (P=0.321), V_{max} (P=0.370) and K_M (P=0.673) values between control and butyrate-fed chickens. PB treatment significantly induced the mean enzyme activity (P<0.001) and increased V_{max} (P= 0.028) values, while K_M tended to decrease (P= 0.050) (**Fig. 22.B and Table 8**).

Testosterone 6β-hydroxylation assay

Similarly, butyrate did not influence the hepatic microsomal CYP3A37 activity in broiler chicken, tested by the testosterone 6β-hydroxylation assay (P=0.436), while PB treatment resulted in a highly significant, approximately seven times higher mean enzyme activity (P<0.001) (**Fig. 22.C**).

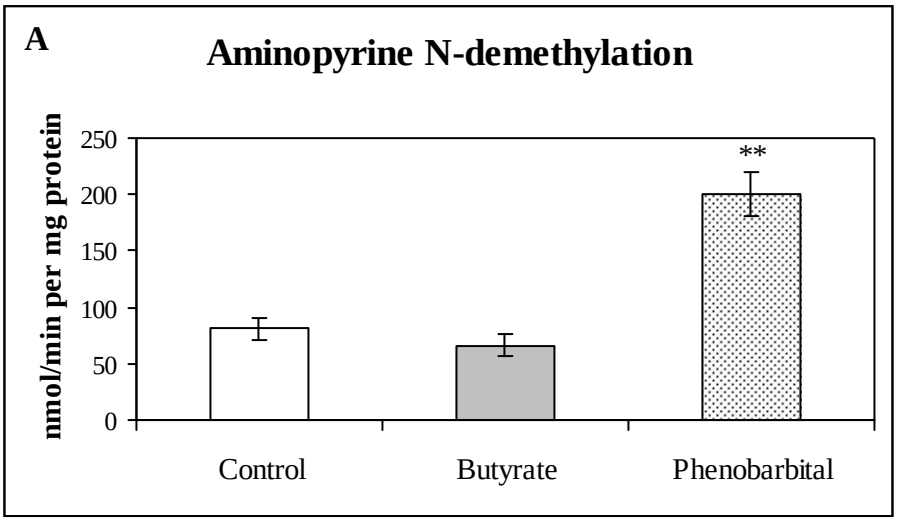


Fig. 22. A. Average amount of formaldehyde produced in aminopyrine N-demethylation assay (nmol/minute per mg microsomal protein), indicating the mean specific activity of hepatic microsomal CYP2H/CYP3A37 enzymes.

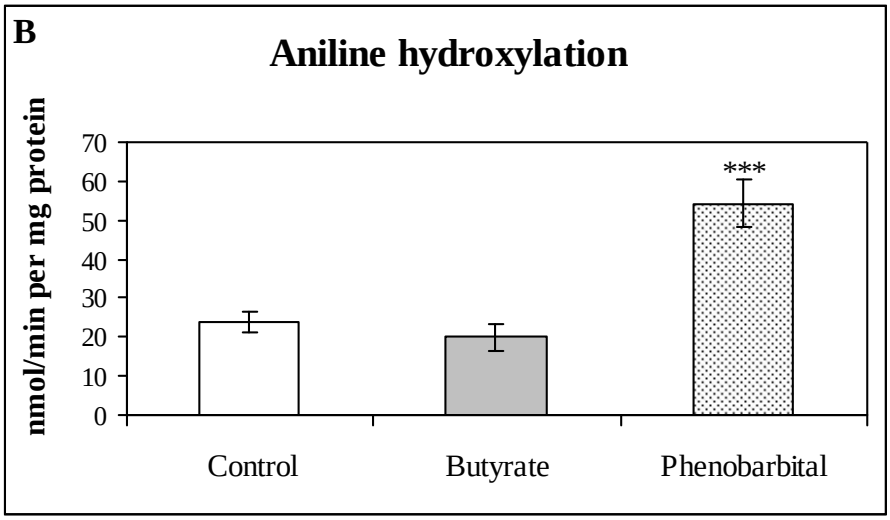


Fig. 22. B. Average amount of 4-aminophenol produced in aniline hydroxylation assay (nmol/minute per mg microsomal protein), indicating the mean specific activity of hepatic microsomal CYP2H subfamily.

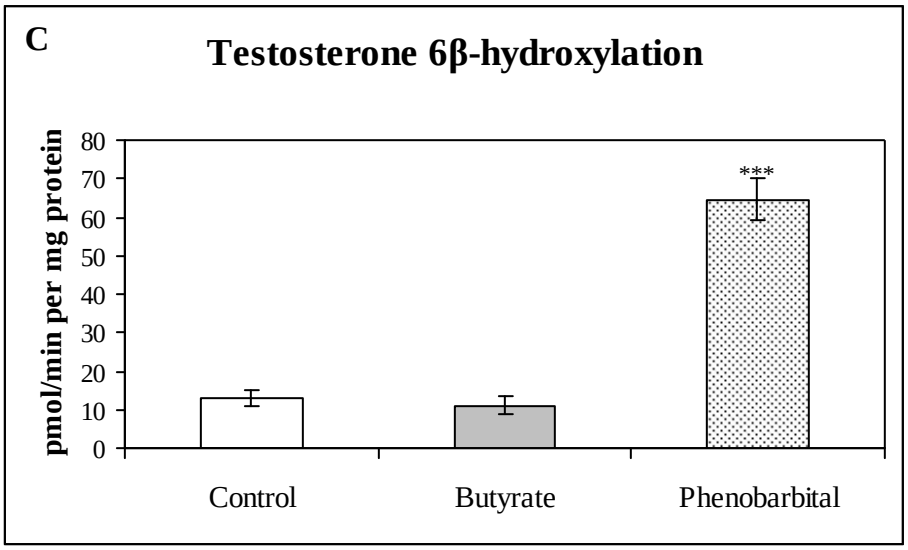


Fig. 22. C. Average amount of 6β-hydroxy-testosterone produced in testosterone 6β-hydroxylation assay (pmol/minute per mg microsomal protein), indicating the mean specific activity of hepatic microsomal CYP3A37 isoenzyme.

Fig. 22. A-C. All tests were carried out with hepatic microsome fractions of broiler chickens fed with control or butyrate-supplemented diet (1.5 g/kg diet) for 21 days or treated with intracoelomal phenobarbital injection (80 mg/kg BW) on days 19-21 of the experiment. Results are expressed as mean ± SEM, n=10/group.
Significant difference compared to control, **P<0.01, ***P<0.001.

Table 8. Effects of dietary butyrate supplementation on kinetic properties of cytochrome P450 (CYP) enzymes in broiler chickens.

Enzyme action	Responsible CYP	Kinetic parameter	Control	Butyrate[1]	PB[2]
Aminopyrine N-demethylation	CYP2H/3A37	V_{max} (µmol/mg/min)	0.116 ± 0.018	0.144 ± 0.049	0.380 ± 0.057**
		K_M (mM)	0.574 ± 0.184	0.869 ± 0.210	1.734 ± 0.117*
Aniline hydroxylation	CYP2H	V_{max} (µmol/mg/min)	0.090 ± 0.013	0.072 ± 0.016	0.144 ± 0.016*
		K_M (mM)	8.282 ± 2.246	6.531 ± 1.288	4.165 ± 0.391

[1]Butyrate = diet supplemented with 1.5 g/kg diet sodium butyrate.
[2]PB = chickens received 80 mg/kg BW intracoelomal phenobarbital injection on days 18-21.
Results are expressed as mean ± SEM, n = 10/group.
Significant difference compared to control, *P<0.05, **P<0.01.

Significant positive correlation was found between aniline hydroxylation (CYP2H) and testosterone 6β-hydroxylation (CYP3A37) activity of the chicken liver microsomes (P<0.001). Similarly, aminopyrine N-demethylation and testosterone 6β-hydroxylation also positively correlated (P=0.025). Interestingly, a significant positive correlation could also be found between the acetylation state of H3 histone and microsomal CYP3A37 activity (P=0.045). However, all of these correlations could be observed both in butyrate-fed and control chickens.

5.2.1.6. Comparative studies in rats

Concentrations of SCFA in blood plasma

Butyrate concentration of the blood plasma increased significantly in rats after butyrate supplementation of the feedstuff compared to the control group (P<0.01), but no significant difference could be observed in the plasma acetate concentration. Results are presented in **Table 9**.

Table 9. Effects of dietary butyrate supplementation on SCFA concentrations of the blood plasma in rats.

	Control	**Butyrate[1]**
Butyrate (µM)	25.00 ± 7.61	31.36 ± 4.88**
Acetate (mM)	25.68 ± 6.15	25.39 ± 5.82

[1]Butyrate = supplemented with 1.5 g/kg diet sodium butyrate for 21 days.
Results are expressed as mean ± SEM, n=10/group.
**Significant difference compared to control, P<0.01.

Small intestinal micromorphology

In the histological studies on small intestinal morphology, height of villi tended to be increased in the butyrate-supplemented group compared to the control animals, but the difference was not significant (P<0.10). A low, not significant increase of crypt depth could be also observed after butyrate supplementation of the diet (P<0.10). According to our results, height of enterocytes was not affected by butyrate. Results are presented in **Table 10**.

Table 10. Effects of dietary butyrate supplementation on small intestinal micromorphology in rats.

	Control	**Butyrate[1]**
Height of villi (µm)	444.49 ± 25.20	537.77 ± 26.60
Depth of crypts (µm)	123.40 ± 10.55	159.93 ± 11.64
Height of enterocytes (µm)	18.60 ± 0.67	18.52 ± 0.72

[1]Butyrate = supplemented with 1.5 g/kg diet sodium butyrate for 21 days.
Results are expressed as mean ± SEM, n=10/group.

Hepatic microsomal CYP activity

According to our results, the hepatic microsomal aminopyrine N-demethylation activity of rats, specific for CYP2B/3A subfamilies, was not affected by butyrate suplementation of the diet. No significant difference could be observed by comparing mean reaction velocities (P=0,841), K_M (P=0.421) and V_{max} (P=0.548) values of butyrate-fed and control animals. PB administration caused notable induction of CYP2B/3A activity, mean specific activity (P=0.016) and V_{max} (P=0.008) increased significantly, while K_M value was not influenced (P=0.151) by the applied butyrate supplementation. Results are shown in **Fig. 23.A**, K_M and V_{max} values are included in **Table 11**.

Similarly, butyrate supplementation of the diet did not cause any significant alterations in the activity of microsomal CYP3A subfamily in the hepatocytes of rats, monitored by the testosteron-6β-hydroxylation assay (P=0.310). However, intraperitoneal PB treatment resulted in a remarkable, approximately three-fold induction of CYP3A (P=0.036) (**Fig. 23.B**).

Table 11. Effects of dietary butyrate supplementation on kinetic properties of CYP2B/3A enzymes in rats.

Kinetic parameter	Control	Butyrate[1]	PB[2]
V_{max} (µmol/min/mg)	0.159 ± 0.018	0.190 ± 0.027	0.389 ± 0.066*
K_M (mM)	1.656 ± 0.544	1.963 ± 0.445	3.683 ± 0.350

[1]The diet of butyrate-treated rats was previously supplemented with sodium butyrate at the dose of 1.5 g/kg diet for 21 days.
[2]Phenobarbital-treated animals received an intraperitoneal injection (80 mg/kg BW) on days 19-21 of the experiment.
Results are expressed as mean ± SEM, n=10/group.
*Significant difference compared to control, P<0.05.

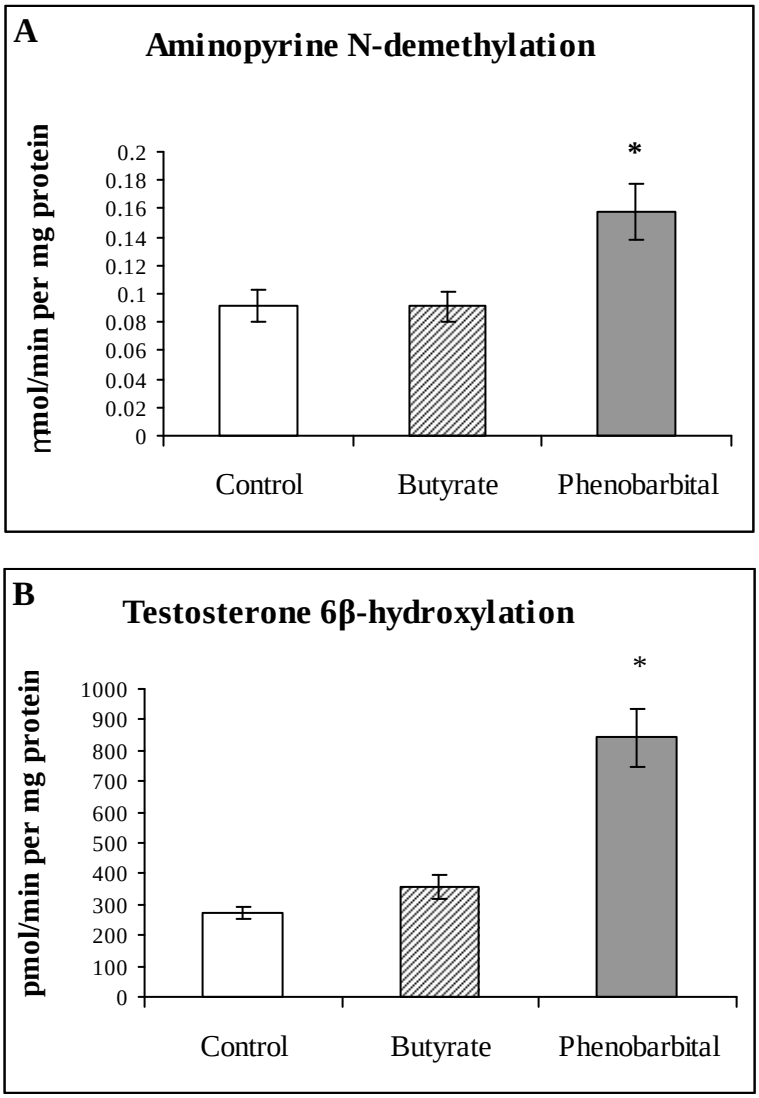


Fig. 23. Specific activity of hepatic microsomal CYP enzymes of rats.

A. Columns indicate the amount of formaldehyde (μmol/minute per mg microsomal protein) produced in the aminopyrine N-demethylation assay, specific for the CYP2B/3A subfamilies.

B. Columns indicate the amount of 6β-hydroxy-testosterone (pmol/minute per mg microsomal protein) produced in the testosterone 6β-hydroxylation assay, specific for the CYP3A subfamily.

The diet of the butyrate-treated group was previously supplemented with sodium butyrate at the dose of 1.5 g/kg diet for 21 days, while phenobarbital-treated animals received an intraperitoneal injection (80 mg/kg BW) on days 19-21 of the experiment. Results are expressed as mean ± SEM, n=10/group.

*Significant difference compared to control, $P<0.05$.

5.2.2. Effects of butyrate applied as daily bolus in chicken

5.2.2.1. Concentrations of SCFA in blood plasma

A highly significant (P<0.001) and notable increase in plasma butyrate concentration was measured in broilers treated by a daily bolus of butyrate, compared to that of the control animals (**Table 12**). The lower applied dose (0.25 g/kg BW) caused approx. 7-fold, while the higher dose more than 11-fold elevation of butyrate level in the blood plasma. In contrast, acetate concentration was not affected by the oral butyrate application.

Table 12. Effects of oral application of butyrate bolus on SCFA concentrations of the blood plasma in broiler chickens.

	Control	**Butyrate 0.25 g/kg BW[1]**	**Butyrate 1.25 g/kg BW[1]**
Butyrate (µM)	16.67 ± 3.35	38.38 ± 3.81***	64.36 ± 6.60***
Acetate (mM)	1.45 ± 0.07	1.52 ± 0.10	1.52 ± 0.11

[1]Butyrate = chickens treated with oral application of butyrate bolus in a lower (0.25 g/kg BW) and a higher (1.25 g/kg BW) dose.
Results are expressed as mean ± SEM, n=10/group.
***Significant difference compared to control, P<0.001.

5.2.2.2. Acetylation of hepatic core histones

Screening of the important acetylation sites of core histones showed that butyrate treatment in bolus at the lower dose (0.25 g/kg BW) tended to increase acetylation of H2A at lysine 5 (P=0.063), and the higher applied dose (1.25 g/kg BW) caused significant, approximately twofold increase in acetylation (P=0.048) at the same acetylation site of H2A compared to the control group (**Fig. 24 and Fig. 25.A**). In contrast, butyrate bolus did not influence the acetylation of H2B at lysine 5 with the lower (P=0.274) nor the higher dose of butyrate (P=0.714) (**Fig. 24 and Fig. 25.B**). There was no significant difference in the acetylation ratio of total H3 at lysine 9 after the application of butyrate in the lower dose (P=0.146). However, higher dose of butyrate caused relevant, approximately 18-fold increased H3 acetylation ratio (P=0.009) (**Fig. 24 and Fig. 25.C**). The H3 isoforms H3.1 and H3.2 could be also separated on the immunoblots and it was found that butyrate increased the relative protein expression level of the H3.1 isoform, which was poorly expressed in control animals, but was detected in high amount in both butyrate-treated groups. Regarding the acetylation of H4 at lysine 8, butyrate tended to induce hyperacetylation at the lower administered dose (P=0.063) (**Fig. 24 and Fig. 25.D**).

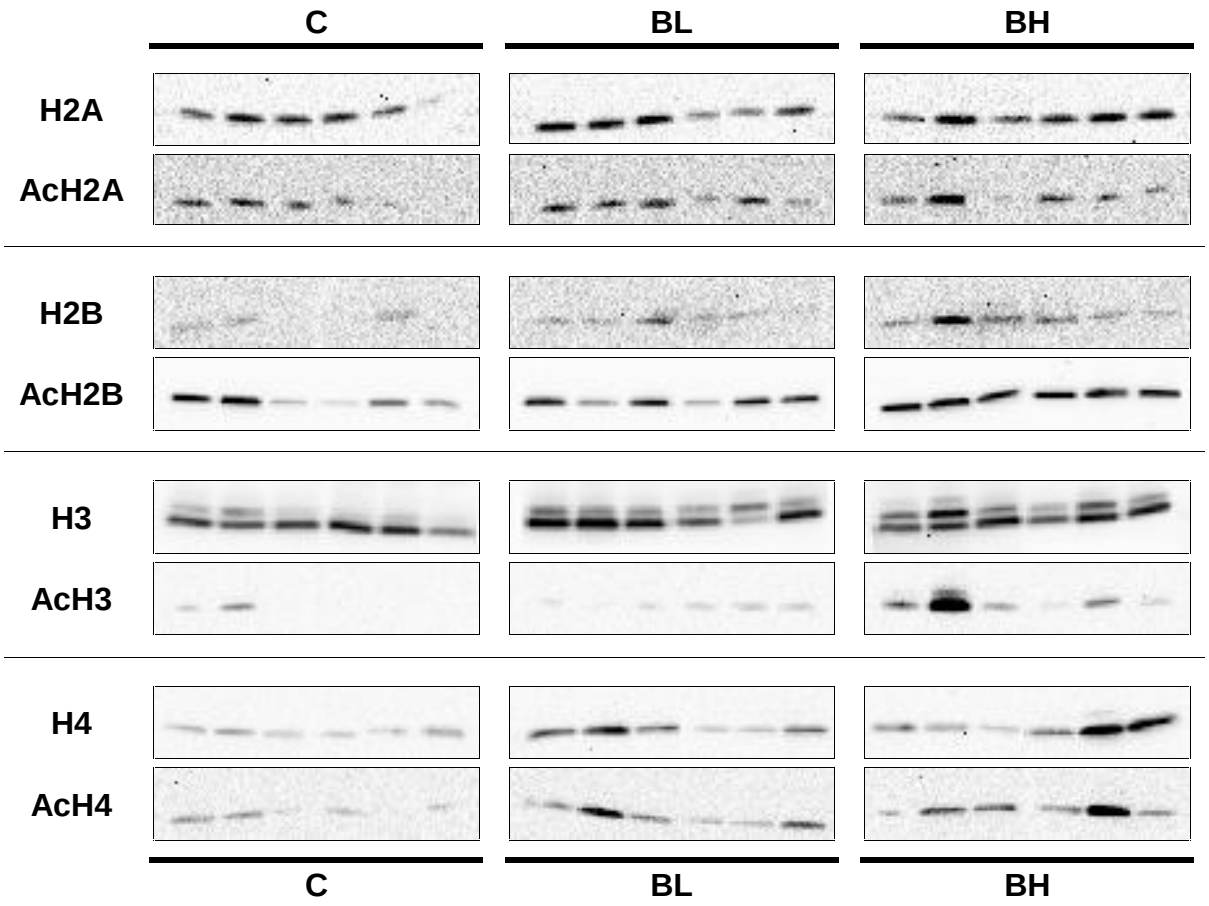


Fig. 24. Representative bands as obtained by western blotting from isolated hepatocyte histones of chickens. Columns show the bands of control animals (C) and chicks after oral application of butyrate bolus on days 20-24 at the dose of 0.25 g/kg BW (BL) and the dose of 1.25 g/kg BW (BH). The upper rows show the relative protein expression levels of total H2A, H2B, H3 and H4, respectively, while the bands specific for acetylated histones of the same animals can be seen below (AcH2A-AcH4). At H3, the upper band can be identified as the H3.1 isoform and the lower as H3.2. Western blots were done in duplicate for all histones.

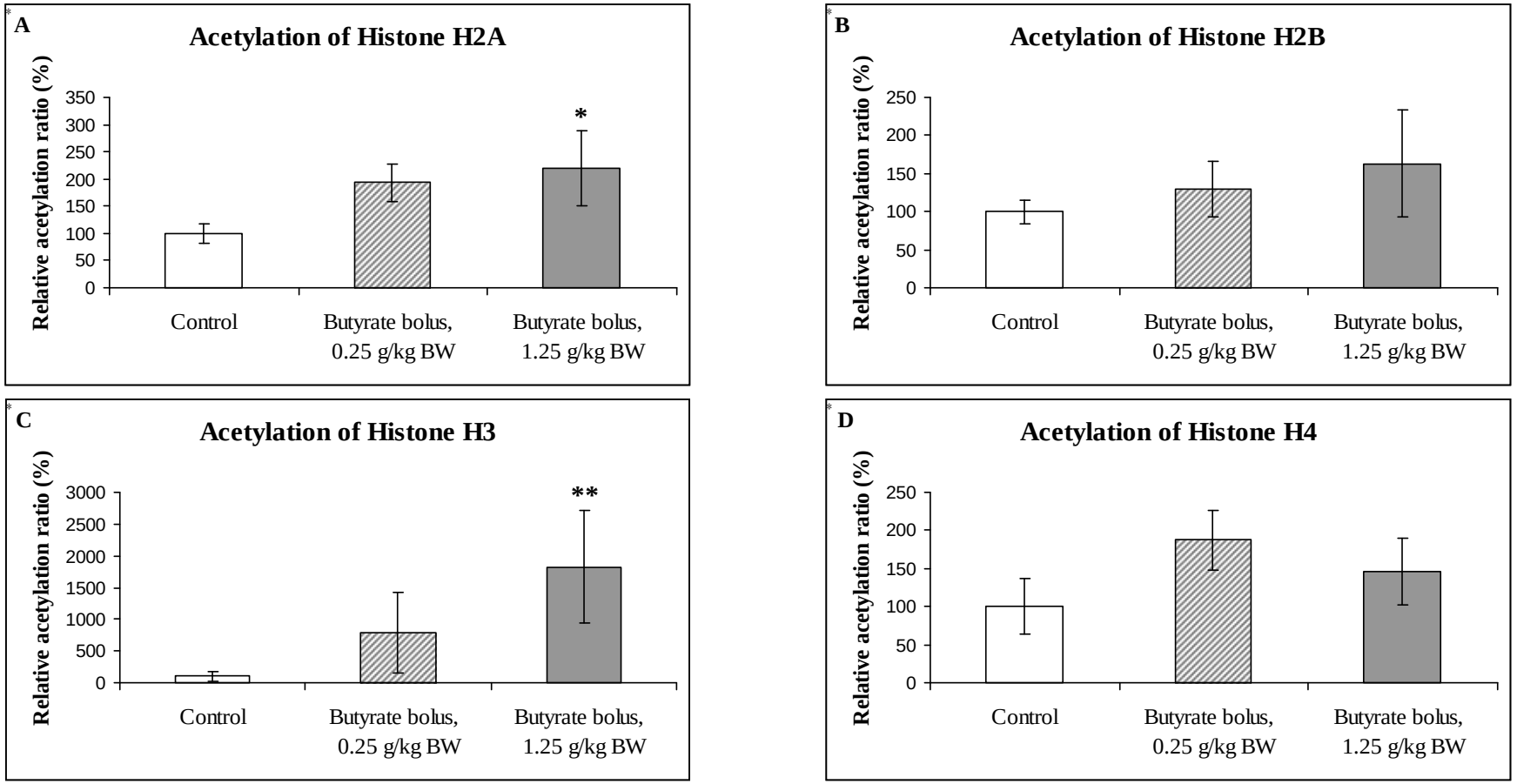


Fig. 25. A-D. Relative acetylation of hepatic core histones in chicken after oral application of butyrate bolus. Butyrate was applied at a lower (0.25 g/kg BW) and a higher (1.25 g/kg BW) dose on days 20-24, relative acetylation ratios were compared to those of controls (considered as 100%). Acetylation ratios were determined considering relative protein expression levels of each histone and its acetylated form. Results are expressed as mean ± SEM, n=6/group. Significant difference compared to control, *P<0.05, **P<0.01.

5.2.2.3. Hepatic microsomal CYP activity

Screening the aminopyrine N-demethylation activity of hepatic microsomal fractions, catalyzed by CYP2H and CYP3A37 enzymes, no significant difference was found between the mean specific enzyme activity (reaction velocity) of the butyrate-treated animals and those of controls (lower dose: P=0.196, higher dose: P=0.523) (**Fig. 26.A**). Similarly, administration of butyrate bolus caused no significant differences in the V_{max} values, independently of the applied dose (lower dose: P=0.368, higher dose: P=0.911). The lower concentration of butyrate did not affect the K_M value (P=0.713), but the higher dose tended to decrease it (P=0.095), however, due to high standard error of mean it can be considered only as a near-significant trend (**Table 13**). As an enzyme inductor, PB treatment caused notable enzyme induction with significantly increased mean specific activity (P=0.003) (**Fig. 26.A**) and V_{max} values (P=0.009), but did not influence the K_M (P=0.878) of the reaction (**Table 13**).

In agreement with these results, butyrate treatment in bolus did not alter the aniline hydroxylation activity of the liver, specific for the microsomal CYP2H subfamily. No significant difference was found in the mean specific enzyme activity (lower dose: P=0.211, higher dose: P=0.848) (**Fig. 26.B**), V_{max} (lower dose: P=0.700, higher dose: P=0.640) and K_M (lower dose: P= 0.354, higher dose: P=0.542) values (**Table 13**) between control and butyrate-stimulated chickens, independently of the applied dose. PB treatment enhanced significantly the CYP2H activity: increased mean specific activity (P=0.002) was measured (**Fig. 26.B**), but V_{max} (P=0.267) and K_M values were not affected (P= 0.760) (**Table 13**).

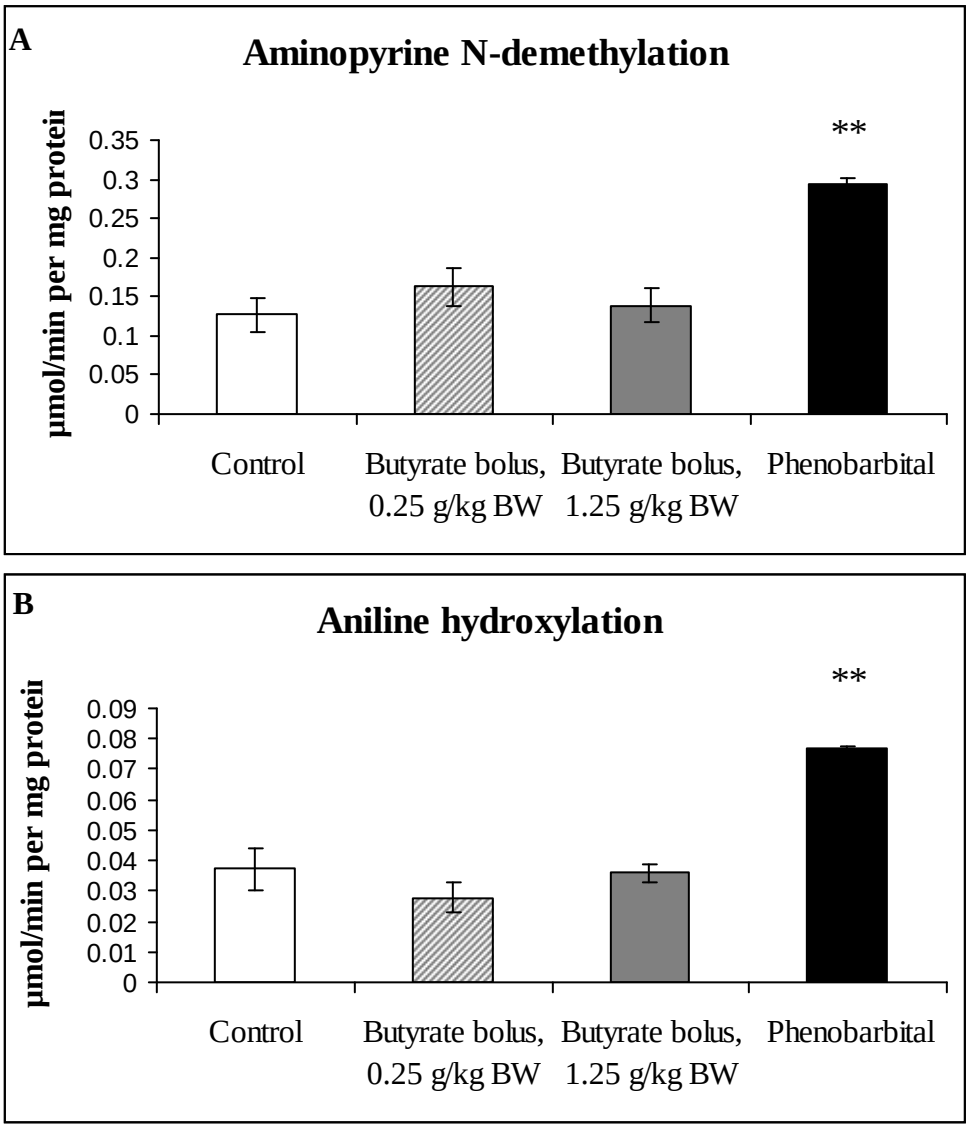


Fig. 26. Mean specific activity of hepatic microsomal CYP enzymes of chickens.

A. Average amount of formaldehyde produced in aminopyrine N-demethylation assay (µmol/minute per mg microsomal protein), indicating the mean specific activity of hepatic microsomal CYP2H/CYP3A37 enzymes.

B. Average amount of 4-aminophenol produced in aniline hydroxylation assay (µmol/minute per mg microsomal protein), indicating the mean specific activity of hepatic microsomal CYP2H subfamily.

Results of enzyme assays, carried out with the hepatic microsomal fraction of chickens after oral application of butyrate bolus at a lower (0.25 g/kg BW, n=10) and a higher (1.25 g/kg BW, n=9) dose or treated with intracoelomal phenobarbital (PB) injection (80 mg/kg BW, n=6) on days 20-24, are compared to those of controls (n=9). Results are expressed as mean ± SEM. **Significant difference compared to control, $P<0.01$.

Table 13. Effects of oral application of butyrate on kinetic properties of cytochrome P450 (CYP) enzymes in chickens.

Enzyme action	Responsible CYP	Kinetic parameter	Control	Butyrate 0.25 g/kg BW[1]	Butyrate 1.25 g/kg BW[1]	PB[2]
Aminopyrine N-demethylation	CYP2H/ 3A37	V_{max} (µmol/mg/min)	0.22 ± 0.06	0.21 ± 0.04	0.17 ± 0.03	0.38 ± 0.01**
		K_M (mM)	1.07 ± 0.24	1.20 ± 0.33	0.57 ± 0.15	1.02 ± 0.14
Aniline hydroxylation	CYP2H	V_{max} (µmol/mg/min)	0.11 ± 0.02	0.15 ± 0.04	0.13 ± 0.03	0.19 ± 0.01
		K_M (mM)	5.27 ± 0.48	12.25 ± 4.36	7.86 ± 2.55	3.27 ± 0.45

[1]Butyrate = chickens treated with oral application of butyrate bolus in a lower (0.25 g/kg BW) and a higher (1.25 g/kg BW) dose on days 20-24.
[2]PB = chickens received intracoelomal phenobarbital injection (80 mg/kg BW) on days 20-24.
Results are expressed as mean ± SEM (control: n=9; butyrate, lower dose: n=10; butyrate, higher dose: n=9; PB: n=6)
**Significant difference compared to control, P<0.01.

5.2.3. Additional effects of butyrate and phenobarbital in chicken

Simultaneous application of butyrate bolus and PB significantly increased the mean specific activity of hepatic microsomal CYP2H/3A37 enzymes, screened by both aminopyrine N-demethylation and aniline hydroxylation assays (P=0.029 and P=0.022, respectively). However, the stimulatory effect of the butyrate-PB combination was significantly lower than that of PB alone (P=0.040 and P=0.029, respectively), so the enzyme inducing action of PB was partly alleviated by butyrate (**Fig. 27**). Similar results of V_{max} values could be observed in both enzyme assays: butyrate ameliorated the PB-triggered elevation of V_{max} as well (P=0.054 and P=0.008, respectively), while K_M was not affected significantly by the applied treatments (**Table 14**).

Table 14. Additional effects of butyrate and phenobarbital (PB) on kinetic properties of cytochrome P450 (CYP) enzymes in chickens.

Enzyme action	Responsible CYP	Kinetic parameter	Control	PB[1]	Butyrate bolus + PB[2]
Aminopyrine N-demethylation	CYP2H/ 3A37	V_{max} (µmol/mg/ min)	0.08 ± 0.03	0.32 ± 0.10***	0.16 ± 0.03
		K_M (mM)	1.42 ± 0.43	3.82 ± 2.83	1.68 ± 0.49
Aniline hydroxylation	CYP2H	V_{max} (µmol/mg/ min)	0.10 ± 0.02	0.19 ± 0.06**	0.11 ± 0.02*
		K_M (mM)	7.47 ± 1.79	4.34 ± 0.44	3.67 ± 0.51

[1]PB = chickens received intracoelomal PB injection (80 mg/kg BW) on days 20-24.
[2]Butyrate bolus + PB = chickens treated simultaneously with oral application of butyrate bolus (0.25 g/kg BW) and intracoelomal PB injection (80 mg/kg BW) on days 20-24.
Results are expressed as mean ± SEM, n=8/group.
Significant difference compared to control, *P<0.05, **P<0.01, ***P<0.001.

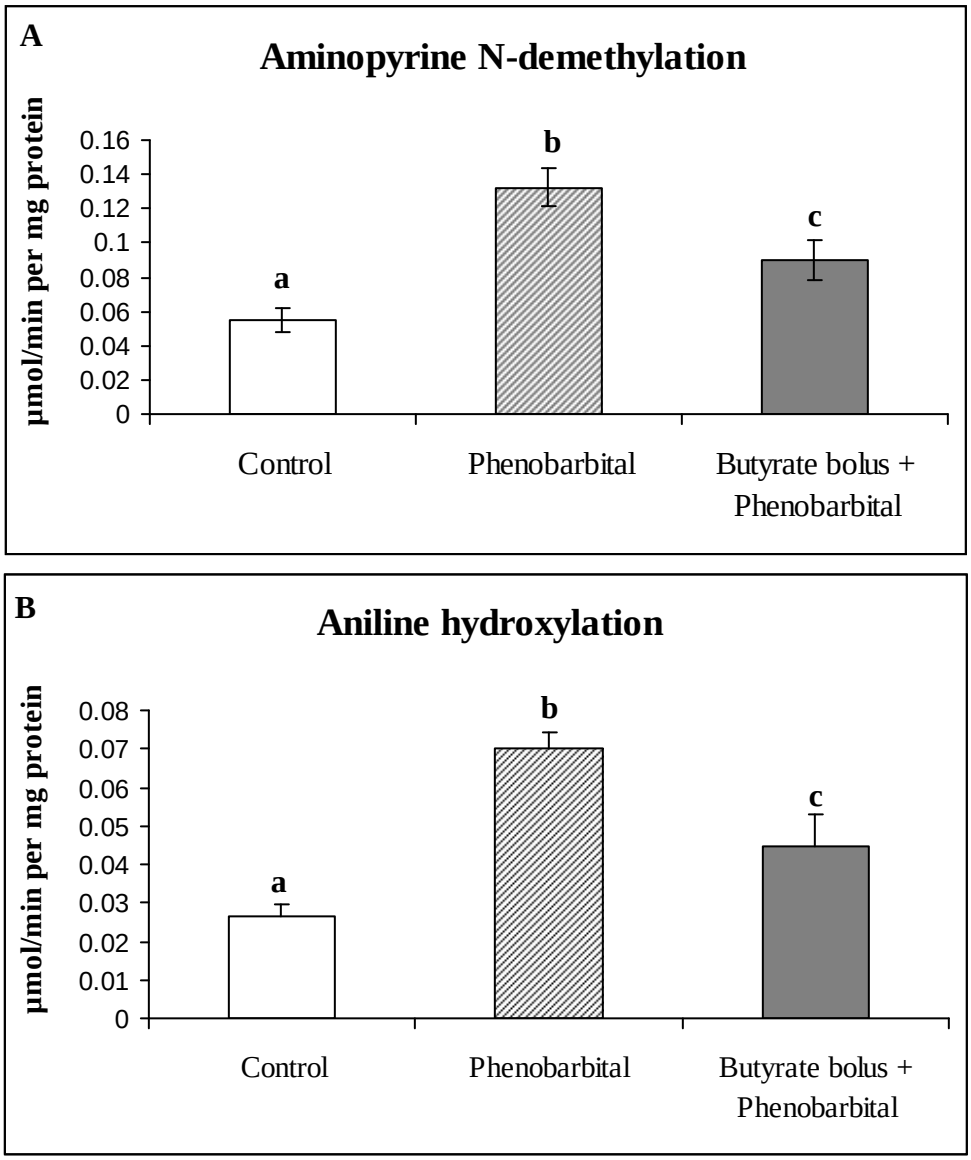


Fig. 27. Mean specific activity of hepatic microsomal CYP enzymes of chickens.

A. Average amount of formaldehyde produced in aminopyrine N-demethylation assay (µmol/minute per mg microsomal protein), indicating the mean specific activity of hepatic microsomal CYP2H/CYP3A37 enzymes.

B. Average amount of 4-aminophenol produced in aniline hydroxylation assay (µmol/minute per mg microsomal protein), indicating the mean specific activity of hepatic microsomal CYP2H subfamily.

Results of enzyme assays, carried out with the hepatic microsomal fraction of chickens treated by intracoelomal phenobarbital (PB) injection (80 mg/kg BW) and by the same treatment in combination with oral application of butyrate bolus (0.25 g/kg BW), compared to those of controls. Results are expressed as mean ± SEM, n=8/group. Bars marked with different letters mean significant difference, $P<0.05$.

5.2.4. Effects of butyrate on pharmacokinetics of erythromycin in chicken

The absorption half-life ($T_{half-abs}$) and the time to maximum plasma concentration (T_{max}) values were significantly elevated in butyrate-fed animals compared to the control ($T_{half-abs}$: P=0.005; T_{max}: P=0.012). However, the plasma elimination half-life ($T_{half-el}$) was significantly decreased by butyrate supplementation of the diet (P=0.018). Interestingly, the mean maximum plasma concentration (C_{max}: P=0.062) and the area under the plasma concentration–time curve (AUC: P=0.135) tended to be higher in butyrate treated chicks. However, the mean residence time (MRT: P=0.057) showed a tendency to be reduced by the applied butyrate, but these changes did not reach the level of statistical significance (P<0.10). Based on the AUC values the two groups revealed to be bioequivalent, but not according to C_{max} values. The plot of plasma concentrations versus time after intramuscular erythromycin application can be found in **Fig. 28**. The determined pharmacokinetic parameters are included in **Table 15.**

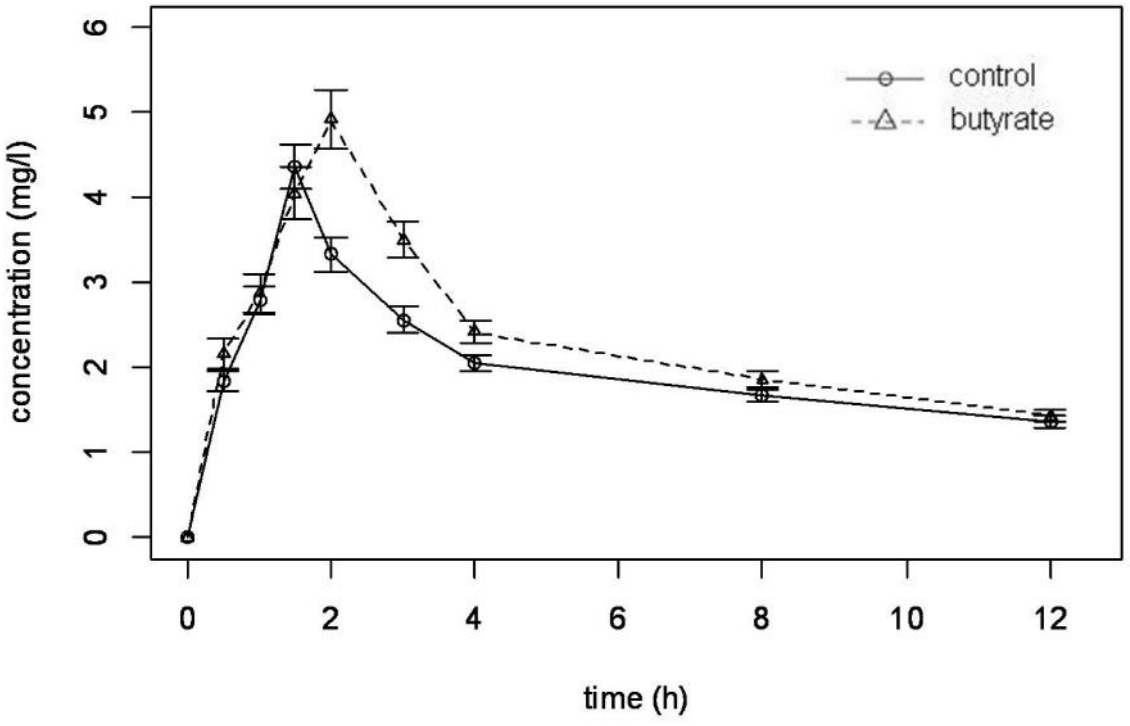


Fig. 28. The plot of plasma erythromycin concentrations versus time after erythromycin application (30 mg/kg BW, single intramuscular injection) in chickens fed with butyrate-supplemented (1.5 g sodium butyrate/kg diet for 6 weeks) or control diet. Results are expressed as mean ± SEM, n=10/group.

Table 15. The main calculated pharmacokinetic parameters of erythromycin following intramuscular injection (30 mg/kg BW) in chickens fed with butyrate-supplemented (1.5 g sodium butyrate/kg diet for 6 weeks) or control diet, determined by applying noncompartmental analysis with the software Kinetica 4.4.1.

Group	$T_{half-abs}$ (hour)	C_{max} (mg/l)	T_{max} (hour)	$T_{half-el}$ (hour)	AUC (mg x l/hour)	MRT (hour)
Control	0.39 ± 0.07	3.30 ± 0.50	1.62 ± 0.21	13.82 ± 2.40	33.53 ± 4.67	8.48 ± 1.19
Butyrate	0.56 ± 0.15*	3.93 ± 0.83#	1.98 ± 0.32*	11.15 ± 2.06*	37.18 ± 5.41	7.35 ± 1.23

Results are expressed as mean ± SEM, n=10/group.
*Significant difference compared to control, P<0.05.
non-bioequivalent within 90% confidence interval.

6. Discussion

6.1. Effects of oral butyrate application on the small intestinal histomorphology

The well-known beneficial effects of butyrate on growth parameters might be based partially on the morphological changes of the small intestinal mucosa. Butyrate tended to increase depth of crypts and had a significant positive trophic effect on the height of enterocytes in chicken (**Table 7**). These results are mainly in agreement with those of Antongiovanni et al. (2007), who reported increased depth of crypts in the jejunum; while in contrast, Hu and Guo (2007) found increased small intestinal villi height, caused by dietary butyrate. Multiple effects of butyrate on gastrointestinal epithelial cell proliferation and differentiation were described by Gálfi and Neogrády (2001), depending on the age and the status of gut flora as well. Increased height of enterocytes after dietary butyrate supplementation, observed in our study, makes butyrate not only a potent regulator of cell division, but a molecule that can cause hypertrophic changes in the gastrointestinal epithelial cells due to its direct trophic effect. All these morphological alterations can lead to a larger surface of small intestine and consequently more efficient nutrient absorption, which may enhance the growth rate of these animals. However, it is clear that such improvements in growth performance are based on many other different effects as well, such as balancing the gut flora or increasing the immune response.

Partly similarly to broilers, height of villi and depth of crypts tended to be higher in butyrate-fed rats than those in controls, but the difference did not match the requirements of statistical significance. In contrast to the study in chickens, no hypertrophic effect of butyrate on height of enterocytes was detected in rats (**Table 10**). Among mammals, histometrical data on jejunal micromorphology found in literature were mainly published about pigs, only very little data can be found about such studies in rats. Sakata (1986) reported increased crypt cell production rate in rats after oral application of butyrate. Kotunia et al. (2004) observed increased heigth of villi, but decreased mucosal thickness in the duodenum, while increased height of villi, depth of crypts and improved mucosal thickness in the distal jejunum and ileum of 3 to 10 days old piglets. In an other experiment, butyrate supplementation of pig diets after weaning led to decreased villi height and mucosal thickness of the jejunum (Le Gall et al., 2009). Data found in literature could be so widespreading because of the multiple effects of butyrate on epithelial cell proliferation and differentiation (Gálfi and Neogrády, 2001), depending on the age, state of the enteral microbiome and other local circumstances in the intestinal lumen.

6.2. Butyrate uptake of cultured hepatocytes and fate of orally applied butyrate beyond the gut

After studying some intestinal aspects beyond butyrate's growth promoting action, its epigenetic and metabolic effects were studied. To investigate butyrate-induced processes in the liver, its uptake and metabolism had to be monitored firstly.

No data can be found in literature regarding the mechanisms of butyrate uptake into the hepatocytes in chicken and rat, however, the role of the MCT-1 was described by Kirat et al. (2005) in ruminants. Regarding our *in vitro* examinations, butyrate was greatly taken up from cell culture media by cultured hepatocytes, but this uptake was limited at higher butyrate concentrations in chicken by reaching a maximal amount of butyrate to be able to be taken up (**Fig. 17**). In contrast, no such limitation could be found in primary cultures of rat hepatocytes, where higher amount of butyrate could be taken up even at 10 mM initial butyrate concentration (**Fig. 17**). According to this difference, we can suggest that different transport mechanisms may exist in the two examined species, possibly being in connection with altered expression of certain transporters, such as MCT-1, highly involved in butyrate uptake. However, it should be pointed out that further studies are needed to confirm these hypotheses.

It was reported by Steliou et al. (2012) that the first-pass hepatic clearence and poor bioavailability of butyrate could highly restrict its biological action. We found that however butyrate is partly metabolized in the liver, a certain amount is passed through to the systemic circulation, causing elevation of its plasma concentration in chickens and rats receiving oral butyrate treatment either as feed additive or in case of chickens as daily bolus (**Table 6, 9, 12**). In this later form of administration, significant dose-dependency of plasma butyrate concentration was also detected.

Similar increase of plasma butyrate concentration was found in mice by Gao et al. (2009), but at higher dose of butyrate, and by Knudsen et al. (2005) in pigs after rye bread consumption. According to our results, butyrate fed at 1.5 g/kg diet increases plasma butyrate concentration, so it can act as a biologically active molecule not only in the intestine and the liver, but also in different peripheral tissues. However, there was no significant difference in plasma acetate concentrations either in chicken or rat. It means that although the liver can produce acetate from the absorbed butyrate, it mainly enters in its active form of acetyl~CoA into certain pathways of the intermediary metabolism, such as the citric acid cycle or the ketogenesis.

6.3. Epigenetic effect of orally applied butyrate on hepatic histone acetylation

The non-metabolized fraction of butyrate may act as an epigenetic factor in the liver, influencing chromatin structure with its numerous consequences. Regarding acetylation state of hepatic core histones, butyrate had a remarkable impact on nucleosome structure of hepatocytes in both *in vivo* trials with chickens in the early post-hatch period, when butyrate was applied either as feed additive or in daily bolus. It is well-known that the epigenetically active butyrate caused hyperacetylation of histones H3 and H4 in all examined vertebrate cell lines, while H2A and H2B were affected only in certain rat-derived cell cultures (Candido et al., 1978). In contrast to the large amount of *in vitro* results, very few reports are found about *in vivo* trials on HDAC inhibitors. Significant increase in total histone acetylation was reported in case of porcine caecal tissue after dietary supplementation with lactulose, precursor of butyrate production by anaerobic gut bacteria in a recent study (Kien et al., 2008). However, absolutely no data can be found in the literature regarding the *in vivo* effects of butyrate on histone acetylation in chicken, so our present studies provide novel data on this field of butyrate's epigenetic action.

To study the results of our experiments involving butyrate application as feed additive or in daily bolus, the most important differences have to be pointed out regarding the different forms of butyrate administration. In our first study with butyrate evenly mixed in the feedstuff of the chicken, butyrate could be taken up together with the diet continuously during the whole examination period, but this uptake might be followed by a prolonged absorption and a long-acting butyrate exposure of the liver. In contrast, butyrate administered in bolus after overnight fasting provides a fast, but short-term release of greater amount of butyrate to the portal vein and an intensive stimulus for the liver. Dosage of butyrate as a supplement (1.5 g/kg diet), chosen regarding the usual applied concentration of butyrate as feed additive was approximately equivalent with the lower dose bolus (0.25 g/kg BW). With the higher administered concentration, 1.25 g/kg BW, we aimed to provide high amount of butyrate for the hepatocytes to study also the dose-dependency of its action.

In both experiments, significant hyperacetylation of H2A at lysine 5 was observed, providing modifications in the epigenetic regulation of cell function, and no dose-dependency could be detected after bolus treatment (**Fig. 20, 25**). H2A has the largest number of subtypes among all core histones, 13 variants have been identified by Bonenfant et al. (2006). Butyrate-induced hyperacetylation of H2A was also described by Tobisawa et al. (2010) on a colonic epithelial cell line *in vitro*. Acetylation of H2A is of special importance since its acetylation state is highly involved in conformational changes of the nucleosome, working synergistically with acetylation of the N-terminal histone tails (Ishibashi et al., 2009).

Brower-Toland et al. (2005) underlined that H2A acetylation played an important role in decreasing the histone-DNA interactions in the acetylated NCP, making the chromatin transcriptionally active.

In spite of these results, it can be stated that butyrate did not affect the acetylation state of H2B at lysine 5 after butyrate administration in bolus, nor in the feed additive study (**Fig. 20, 25**). However, there are still some other lysine residues in H2B, which may be potential targets of HDAC inhibitors (Zhang et al., 2002), and possible effects of butyrate on these other acetylation sites cannot be excluded.

Similarly to the first experiment, where butyrate was applied as feed additive, the lower dose of butyrate bolus did not cause any changes in the acetylation of H3 at lysine, but the higher dose induced a highly relevant, approx. 18-fold hyperacetylation of H3 at lysine 9 (**Fig. 20, 25**). Due to the key-role of H3 modifications in gene expression (Shin et al., 2012; Mathew et al., 2010), this action seems to be a very important change in the epigenetic regulation of transcription. Hyperacetylation of H3 after butyrate exposure was reported by several *in vitro* studies in a variety of cultured mammalian cells, but not yet described *in vivo*. It was found already in 1973 that butyrate in millimolar concentrations caused hyperacetylation of H3 and H4 in all examined cultured cell types from vertebrates (Candido et al., 1978). Butyrate-induced dynamic histone acetylation was compared between mammalian and avian cells *in vitro* (Davie, 2003), where huge amount of highly acetylated H3 isoforms was found after butyrate treatment in human breast cancer cells, in contrast of terminally differentiated avian immature erythrocytes, 2% of which participated in the acetylation process. Among the many acetylation sites, in agreement with our results, it was recently described that butyrate induced H3 hyperacetylation first of all at lysine 9, an acetylation site that plays a critical role in the epigenetic regulation of cell function (Shin et al., 2012). Since this acetylation site is linked to histone phosphorylation and methylation processes, these site-specific modifications together can cause distinct chromatin alterations and cell cycle modifications (Mathew et al., 2010).

The H3 isoforms H3.1 and H3.2 could be also separated on the immunoblots and it was found that butyrate increased the relative protein expression level of the H3.1 isoform, which was poorly expressed in control animals, but was detected in high amount in both butyrate boli treated groups. It is known that three H3 variants (H3.1, H3.2, H3.3) do exist in mammals, specifically, H3.1 is involved in both chromatin activation and repression, while H3.2 plays an important role in gene repression and H3.3 is especially enriched in active marks (Hake et al., 2006). Unlike in the case of mammals, only H3.1 and H3.2 could be separated from chicken cells (Zhang et al., 2002b). Due to the pleiotropic effect of H3.1 on transcription, increased protein expression level of H3.1 after butyrate treatment, detected in our present study, may be also of special importance.

Lower dose of butyrate tended to increase acetylation of H4 at lysine 8, differing from the feed additive experiment, underlining the critical role of the application (**Fig. 20, 25**). Similarly to H3, H4 is also a highly involved target of butyrate-induced hyperacetylation in cell cultures (Candido et al., 1978). It is known that acetylation and deacetylation of H4 is a well-coordinated process, and butyrate-induced tetra- and tri-acetylated forms of H4 are always acetylated at lysine 8 (Zhang et al., 2002a). Therefore, the lysine residue examined in this study is considered as one of the most important acetylation sites of H4. It was recently also stated that H3 at lysine 9 and H4 at lysine 8 are critical targets of butyrate-induced histone hyperacetylation, which process is associated with the G-protein-coupled receptor-41, also activated by butyrate (Wu et al., 2012).

The lacking hyperacetylation effect of butyrate on H2B in all examined groups and on H3 and H4 in certain cases of butyrate application might be explained by several causes. The applied primary antibodies recognised the most frequent individual acetylation possibility of each histone (H2A and H2B: Lys 5, H3: Lys 9, H4: Lys 8) close to the N-terminal end of the molecule. However, there are still some other lysine residues in all core histones, which may be potential targets of HDAC inhibitors (Zhang et al., 2002a). The study of Davie (2003) also underlines the importance of the acetylation site, as well as the actual phase of the cell cycle in the histone hyperacetylating effect of butyrate. The critical role of HAT was reported by Rada-Iglesias et al. (2007), who found decreased histone acetylation on cell lines (HepG2 and HT-29) caused by butyrate treatment, according to its pleiotropic effect on HAT and HDAC. In association with these reports, lack of histone hyperacetylation at the prior mentioned acetylation sites in certain cases may be also in connection with the modified activity of HATs.

We can summarize that orally applied butyrate as feed additive or in bolus influenced hepatic histone acetylation *in vivo*; core histones H2A, H3 and H4 were involved in this partly application- and dose-dependent action. Since butyrate modified the chromatin structure, it can be considered as an important epigenetic effector of gene expression of hepatocytes. Therefore, hepatic drug-metabolizing microsomal CYP enzymes were investigated as genes potentially affected by butyrate-induced histone acetylation.

6.4. Effects of butyrate on hepatic CYP gene expression and enzyme activity in vitro and in vivo

Based on our results, butyrate had a pronounced effect on gene expression of certain CYP enzymes in primary cultures of chicken hepatocytes *in vitro* as well as *in vivo*, applied as feed additive mixed into the feedstuff of broiler chickens (**Table 5; Fig. 18, 21**). Expression of CYP2H1 gene was significantly increased in both cultured hepatocytes and in the liver of butyrate-fed chicks. CYP3A37 gene expression significantly declined after *in vitro* butyrate treatment, but this down-regulation was ameliorated *in vivo*. Interestingly, CYP1A gene expression was suppressed by butyrate in primary culture of hepatocytes, but in contrast, it was overexpressed *in vivo* in butyrate-fed animals compared to the control.

Very little data are presented in the literature on the effect of drugs or other xenobiotics on CYP enzymes in chicken. Zhang et al. (2011) reported that enrofloxacin decreased the expression of CYP1A and CYP3A subfamilies, while marbofloxacin decreased the expression of only CYP1A in broilers. It was reported that histone acetylation had a huge impact on gene expression of several members of the CYP2 family (Baer-Dubowska et al., 2011). It is also known that alterations in H3 acetylation are involved in the expression of CYP3A subfamily in the adult mouse (Li et al., 2009). Confirmingly, a significant positive correlation was also found in our study between the acetylation state of H3 histone and microsomal CYP3A37 activity.

Butyrate treatment of cultured hepatocytes provides direct butyrate exposure for the cells, unlike the *in vivo* model where countless factors may influence the fate and the action of butyrate on the liver. However, butyrate caused hyperacetylation of histones H3 and H4 in all examined vertebrate cell types, only H2A was affected by oral butyrate application as feed additive, while acetylation state of H2A, H3 and H4 could be altered by butyrate administration in bolus in chicken. Thus, it is clearly visible that butyrate's histone hyperacetylating activity can be highly influenced by certain circumstances, such as the examination model itself or the dose and the form of application. Different histone modifications can cause various alterations in the transcription pattern of genes, so it can be considered as a possible explanation for the partly contradictory data regarding butyrate's *in vitro* and *in vivo* effects on CYP gene expression.

The activity of hepatic microsomal CYP2H/3A enzymes was also screened by specific enzyme assays either after butyrate supplementation of the diet or butyrate application in bolus in chicken. In contrast to the results of CYP gene expression, no changes of enzyme activity were found after any forms of butyrate application (**Fig. 22, 26**). In case of CYP3A37, the butyrate-triggered *in vitro* suppression was already alleviated *in vivo* on the level of gene expression and obviously did not cause any changes in enzymatic action.

As a known enzyme inductor, intracoelomally administered PB caused a notable increase in CYP2H and CYP3A37 enzyme activity in all of the applied enzyme assays (**Fig. 22, 26**), also confirming the efficacy of our methodological approach. Induction of CYP3A and CYP2H1 gene expression by PB was described by Paolini et al. (1997) and Davidson et al. (2001). Goriya et al. (2005) compared PB-induced CYP3A37 and CYP2H1 gene expression in chickens, where PB induced the expression of CYP3A37 and CYP2H1 genes in a similar manner. One possible explanation of this is that all the currently known drug-inducible CYPs in chickens share a conserved arrangement of DNA elements that mediate induction by PB and other xenobiotics (Handschin and Meyer, 2003). In agreement with these results, we also found CYP2H and CYP3A37 isoenzymes well-inducible by PB.

Independently from the butyrate treatments, testosterone 6β-hydroxylation (CYP3A37) activity showed a significant positive correlation with aniline hydroxylation (CYP2H) and aminopyrine N-demethylation (CYP2H/3A37) activity of the chicken liver microsomes. As it was also reported in this study that acetylation of hepatic histone H3 directly correlates with microsomal CYP3A37 activity, the observed correlations are also of special interest because of the recognized direct connection between changes at the level of histone proteins and those of the CYP enzyme activity, in addition to the cooperativity among the various CYP isoenzymes.

A feeding trial was also conducted with rats in order to compare the possible effects of the alimentary added butyrate between chicken and a monogastric mammalian model animal. Similarly to the results obtained in broilers, butyrate as feed additive did not cause any alterations in the activity of hepatic drug-metabolizing CYP2B and CYP3A enzymes in rats, tested by any of the applied enzyme assays (**Fig. 23**). In agreement with our expectations, intraperitoneal PB treatment as a positive control increased the activity of both examined subfamilies (**Fig. 23**).

Now, on the basis of these results, it can be stated that independently of the form of application and the applied dose, alimentary butyrate did not modify the activity of the examined CYP enzymes under physiological circumstances. However, butyrate's potential effects on other CYP subfamilies cannot be excluded. It is also not clear, whether under special dietary conditions butyrate may modify the liver enzymes of biotransformation. For example, dietary supplementation of inulin, a precursor of colonic butyrate production, in rats, suffering from high-fat-diet-induced hyperlipidaemia and hepatic steatosis, counteracted the decrease in the expression and activity of hepatic CYP1A1/2 and CYP2E1 enzymes (Sugatani et al., 2012).

The PB-caused elevation of hepatic microsomal CYP2H/3A37 activity was revealed to be decreased by the concomitant oral butyrate application (**Fig. 27**). PB is known as a potent effector of the CAR pathway as well, mediating an epigenetic switch and regulating

the gene expression of the CYP2 family (Lempiäinen et al., 2011). Stimulatory effect of HDAC inhibitors on CYP2B (CYP2H in birds) subfamily is also conducted partly via the activation of the CAR nuclear receptor (Takizawa et al., 2010). HDAC inhibitors, such as valproic acid and phenylbutyrate diminish the complex of CAR and HDAC, therefore augment the binding of SRC-1 to CAR (Takizawa et al., 2010). Based on these data, the PB-triggered CAR-mediated CYP enzyme activation might be affected by the epigenetic action of butyrate, resulting in a declined rate of enzyme induction. Similarly, other xenobiotics, administered for therapeutical purpose could also interact with butyrate applied as feed additive, modifying the expression and activity of the microsomal phase I drug-metabolizing enzyme system, which can be of special importance. However, additional studies on the signal transduction and epigenetic mechanisms of butyrate- and PB-induced transcriptional alterations are required to understand the interaction of butyrate and PB.

6.5. Interaction of butyrate and erythromycin in vitro and in vivo

Since orally applied butyrate could cause certain posttranslational modifications of histones and influenced CYP gene expression, but not the activity of these enzymes, finally the effects of butyrate on the metabolism of erythromycin, a xenobiotic used in poultry veterinary practice and metabolized mainly in the liver, was investigated *in vitro* and *in vivo*.

To fully evaluate and understand the pharmacoepigenetic interaction of butyrate and erythromycin, the effects of erythromycin itself on chicken hepatic CYP gene expression should have been investigated first. Erythromycin is known to have multiple effects on CYP gene expression, inductive processes are mediated by the nuclear receptors CAR, PXR and RXR, influencing the transcription from the appropriate gene (Sinh, 2007), while inhibitory action can be explained by inactive metabolite/enzyme complex formation (Al-Ghamdi et al., 2002).

Erythromycin alone caused an increased expression of both CYP2H1 and CYP3A37 genes, while CYP1A was not affected *in vitro* (**Table 5, Fig. 18**). Since both butyrate and erythromycin had an inducing effect on CYP2H1, these actions seemed to be summarized (additive action) after the concomitant application of butyrate and erythromycin. The suppressive action of butyrate on CYP3A37 was counteracted by the simultaneously applied erythromycin (contraversal action). Based on our *in vitro* results, simultaneously applied butyrate and erythromycin might cause notable alterations in the biotransformation of such xenobiotics, which are primarily metabolized by the hepatic CYP2H1 isoenzyme.

As erythromycin elimination was evaluated on primary cultures of chicken hepatocytes, decreased erythromycin concentration was measured in culture medium paralelly with increasing butyrate concentrations (**Fig. 19**). Notwithstanding that butyrate concentration was found to be a potent effector of erythromycin disappearance from the

medium, rate of erythromycin consumption in wells with cultured hepatocytes and in controls without cells increased approximately parallelly. Therefore it can be stated that the drug-eliminating activity of liver cells was not affected significantly by butyrate. It is known that erythromycin, similarly to other drugs can be accumulated in the hepatocytes even without immediate intracellular metabolism (Yabe et al., 2011). Based on this data, the observed decreased erythromycin concentration of the culture medium of hepatocytes could mainly indicate the intensive hepatocellular erythromycin uptake and not directly its metabolism, which was not in connection with the presence of butyrate.

In our *in vivo* pharmacokinetic examination, the plasma concentrations of erythromycin following one single IM administration in butyrate-supplemented and control groups were comparable to those, published by Goudah et al. (2004). Although some differences among the two groups were demonstrated, in conclusion, the significant longer $T_{half\text{-}abs}$, T_{max} and the shorter $T_{half\text{-}el}$ observed for butyrate treated group seems to be of negligible clinical significance (**Table 15**). Furthermore, based on the means of C_{max} values of erythromycin, the butyrate treated and control groups were non-bioequivalent, but according to AUC values the two groups were found to be bioequivalent (**Table 15**). Based on these results, the obtained differences of pharmacokinetic parameters may not alter significantly either the therapeutic activity or the terminal elimination of erythromycin from the body.

The gene expression of CYP1A and CYP2H1 were increased *in vivo* by oral butyrate application, while CYP3A37, by which erythromycin is mainly metabolized, was not affected by butyrate. As a consequence of these findings, some pharmacokinetic parameters of erythromycin were only slightly influenced by butyrate. Since other CYP subfamilies than that of CYP3A enzymes are also involved in erythromycin metabolism, altered expression of CYP1A and CYP2H1 could even cause the observed pharmacokinetic differences. However, we would like to point out that – in spite of the modifications in gene expression – no butyrate-induced changes were observed in the CYP2H/3A37 activity of liver microsomes, so the observed alterations on the level of mRNA were not realized in modified enzyme activity.

Based on our results, the concomitant application of butyrate with erythromycin did not cause a major feed-drug interaction *in vivo* in chickens. However, possible interactions provided by long-term simultaneous erythromycin and butyrate administration cannot be excluded by taking the gene expression modulatory activity of erythromycin into consideration as well.

It can be concluded from the results of the present study that orally added butyrate could epigenetically modify the chromatin structure of the hepatocytes in chicken *in vivo* and alter the gene expression of the hepatic microsomal drug-metabolizing CYP enzymes *in vitro* and *in vivo* as well, but these changes did not result finally in modified *in vivo* CYP enzyme activity. However, butyrate attenuated the stimulatory effect of PB on CYP activity, but did

not cause any major pharmacoepigenetic interactions with simultaneously applied erythromycin. So butyrate is suggested to be applied in safe as feed additive in poultry industry, from pharmacotherapeutical and food safety point of view as well.

7. New scientific results

Ad 1

Butyrate applied as feed additive (1.5 g/kg diet) increased the height of enterocytes in the jejunum of broiler chickens in the early post-hatch period, having a positive trophic effect on the gastrointestinal epithelium.

Ad 2

Orally applied butyrate had a remarkable *in vivo* epigenetic effect on nucleosome structure of hepatocytes of chickens. Butyrate supplementation of the diet with the dose of 1.5 g/kg caused hyperacetylation of hepatic histone H2A, while it did not alter the acetylation state of the other core histones. Butyrate, administered in daily bolus for five days with the dose of 1.25 g/kg body weight increased the acetylation state of H2A and H3, but no significant changes could be monitored in the acetylation state of H2B and H4.

Ad 3

Butyrate had a pronounced effect on gene expression of certain hepatic cytochrome P450 (CYP) enzymes of chicken *in vitro* on primary hepatocyte cultures (in the concentration of 1 to 10 mM) as well as *in vivo*, applied as feed additive (1.5 g/kg diet). Expression of CYP2H1 gene was increased by butyrate in both cultured hepatocytes and the liver of butyrate-fed chicks, while CYP3A37 gene expression declined after *in vitro* butyrate treatment, but this down-regulation was ameliorated *in vivo*. CYP1A gene was observed to be suppressed by butyrate in primary hepatocyte cultures, but in contrast, it was overexpressed *in vivo* in butyrate-fed animals compared to the controls. Orally applied butyrate, either as feed additive (1.5 g/kg diet) or in bolus (0.25 or 1.25 g/kg body weight), did not affect the activity of hepatic microsomal CYP2H and CYP3A37 enzymes in the liver of chickens, tested by aminopyrine N-demethylation (CYP2H/CYP3A37), aniline hydroxylation (CYP2H) and testosterone 6β-hydroxylation (CYP3A37) assays. However, butyrate in bolus attenuated the stimulatory effect of simultaneously administered phenobarbital on CYP2H and CYP3A37.

Ad 4

The macrolide antibiotic erythromycin (10 to 100 μM) increased the expression of CYP2H1 gene of cultured chicken hepatocytes, which was additive with the effect of simultaneous butyrate (1 to 10 mM) treatment. Erythromycin ameliorated butyrate's suppressing action on CYP3A37 gene in the same primary cell culture model. Alimentary supplemented butyrate (1.5 g/kg diet) modified the pharmacokinetic parameters of erythromycin followed by its single intramuscular injection (30 mg/kg body weight) in chicken: increased absorption half-life, T_{max} and shorter elimination half-life could be observed in chickens receiving such butyrate application. However, these alterations modified relevantly neither the therapeutic activity nor the terminal elimination of erythromycin.

8. References

Al-Ghamdi, M. S., Jain S. (2002). **Clinically reported interactions with Erythromycin - An update**, Pakistan J. Med. Res., 41. 4, 2002.

Antongiovanni, M., Buccioni, A., Petacchi, F., Leeson, S., Minieri, S., Martini, A. and Cecchi, R.: **Butyric acid glycerides in the diet of broiler chickens: effects on gut histology and carcass composition**, Ital. J. Anim. Sci., 6. 19-25, 2007.

Anzenbacher, P. and Anzenbacherová, E. (2001): **Cytochromes P450 and metabolism of xenobiotics, cellular and molecular**, Cell. Mol. Life Sci., 58. 737-747, 2001.

Arents, G., Burlingame, R. W., Wang, B. C. and Love, W. E.: **The nucleosomal core histone octamer at 3.1 A resolution: a tripartite protein assembly and a left-handed superhelix**, P. Natl. Acad. Sci. USA, 88. 10148-10152, 1991.

Baer-Dubowska, W., Majchrzak-Celinska, A. and Cichocki, M.: **Pharmacoepigenetics: a new approach to predicting individual drug responses and targeting new drugs**, Pharmacol. Rep., 63. 293-304, 2011.

Beauvieux, M. C., Tissier, P., Gin, H., Canioni, P. ad Gallis, J. L.: **Butyrate impairs energy metabolism in isolated perfused liver of fed rats**, J. Nutr., 131. 1986-1992, 2001.

Bergman, F. N.: **Energy contributions of volatile fatty acids from the gastrointestinal tract in various species**, Physiol. Rev., 70. 567-590, 1990.

Biancotto, C., Frigè, G. and Minucci, S.: **Histone modification therapy of cancer**, Adv. Genet., 70. 341-86, 2010.

Bishop, Y. (Ed.): **The Veterinary Formulary** (6th edition), Pharmaceutical Press, London, UK, 2005.

Bloemen, J. G., Venema, K., van de Poll, M. C., Olde Damink, S. W., Buurman, W. A. and Dejong, C. H.: **Short chain fatty acids exchange across the gut and liver in humans measured at surgery**, Clin. Nutr., 28. 657-61, 2009.

Bonenfant, D., Coulot, M., Towbin, H., Schindler, P. and Van Oostrum, J.: **Characterization of histone H2A and H2B variants and their post-translational modifications by mass spectrometry**, Mol. Cell. Proteomics, 5. 541-552, 2006.

Bordin, M., D'Atri, F., Guillemot, L. and Citi, S.: **Histone deacetylase inhibitors up-regulate the expression of tight junction proteins**, Mol. Canc. Res., 2. 692-701, 2004.

Brower-Toland, B., Wacker, D. A., Fulbright, R. M., Lis, J. T., Kraus, W. L., Wang, M. D.: **Specific contributions of histone tails and their acetylation to the mechanical stability of nucleosomes**, J. Mol. Biol. 346. 135-146, 2005.

Candela, M., Maccaferri, S., Turroni, S., Carnevali, P. and Brigidi, P.: **Functional intestinal microbiome, new frontiers in prebiotic design**, Int. J. Food Microbiol., 140. 93-101, 2010.

Candido, E. P. M., Reeves, R. and Davie, J. R.: **Sodium butyrate inhibits histone deacetylation in cultured cells**, Cell, 14. 105-113, 1978.

Cho, H. J., Kim, S. Y., Kim, K. W., Kang, W. K., Kim, J. I., Oh, S. T., Kim, J. S. and An, C. H.: **The combination effect of sodium butyrate and 5-Aza-2'-deoxycytidine on radiosensitivity in RKO colorectal cancer and MCF-7 breast cancer cell lines**, World J. Surg. Oncol., 7. 49, 2009.

Dannenberg, L. O. and Edenberg, H. J.: **Epigenetics of gene expression in human hepatoma cells: expression profiling the response to inhibition of DNA methylation and histone deacetylation**, BMC Genomics, 7. 181, 2006.

Davidson, B. P., Dogra, S. C. and May, B. K.: **The antiglucocorticoid RU486 inhibits phenobarbital induction of the chicken CYP2H1 gene in primary hepatocytes**, Mol. Pharmacol., 60. 274-281, 2001.

Davie, J. R.: **Inhibition of histone deacetylase activity by butyrate**, J. Nutr., 133. 2485S-2493S, 2003.

DeGorter, M. K. and Kim, R. B.: **Hepatic drug transporters, old and new: Pharmacogenomics, drug response, and clinical relevance**, Hepatology, 50. 1014-1016, 2009.

Demigné, C., Yacoub, C. and Rémésy, C.: **Effects of absorption of large amounts of volatile fatty acids on rat liver metabolism**, J. Nutr., 116. 77-86, 1986.

European Agency for the Evaluation of Medicinal Products, Committee for Veterinary Medicinal Products: **Erythromycin**, Summary Report (1), EMEA/720/99-FINAL [URL: http://www.ema.europa.eu/docs/en_GB/document_library/Maximum_Residue_Limits_-_Report/2009/11/WC500014184.pdf]

Engelmann, J. L., Staecker, G. L. and Richardson, A. G.: **Effect of sodium butyrate on primary cultures of rat hepatocytes**, In Vitro Cell. Dev. B., 25. 1504-1510, 1987.

Fernández-Rubio, C., Ordónez, C., Abad-González, J., Garcia-Gallego, A., Pilar Honrubia, M., Jose Mallo, J. and Balana-Fouce, R.: **Butyric acid based feed additives help**

protect broiler chickens from *Salmonella enteritidis* infection, Poultry Sci., 88. 943-948, 2008.

Frassetto, L. A., Poon, S., Tsourounis, C., Valera, C. and Benet, L. Z.: **Effects of uptake and efflux transporter inhibition on erythromycin breath test results**, Clin. Pharmacol. Ther., 81. 828-832, 2007.

Friedman, A. and Bar-Shira, E.: **Effect of nutrition on development of immune competence in chickens gut associated lymphoid system**. WPSA, Proc. 15th Eur. Symp. on Poultry Nutrition, Balatonfüred, Hungary, 234-242, 2005.

Gallis, J. L., Tissier, P., Gin, H. and Beauvieux, M. C.: **Decrease in oxidative phosphorylation yield in presence of butyrate in perfused liver isolated from fed rats**, BMC Physiol., 7. 8, 2007.

Gallis, J. L., Gin, H., Roumes, H. and Beauvieux, M. C.: **A metabolic link between mitochondrial ATP synthesis and liver glycogen metabolism: NMR study in rats re-fed with butyrate and/or glucose**, Nutr. Metab. 8. 38, 2011.

Gantois, I., Ducatelle, R., Pasmans, F., Hautefort, I., Thompson, A., Hinton, J. C. and Van Immerseel, F.: **Butyrate specifically down-regulates Salmonella pathogenicity island 1 gene expression**, Appl. Env. Microbiol., 72. 946-949, 2006.

Gao, Z., Yin, J., Zhang, J., Ward, R. E., Martin, R. J., Lefevre, M., Cefalu, W. T. and Ye, J.: **Butyrate improves insulin sensitivity and increases energy expenditure in mice**, Diabetes, 58. 1509-1517, 2009.

García-Agúndez, J. A,. Luengo, A. and Benítez, J.: **Aminopyrine N-demethylase activity in human liver microsome**, Clin. Pharmacol. Ther., 48. 490-495, 1990

Gálfi, P. and Bokori, J.: **Feeding trial in pigs with a diet containing sodium n-butyrate**, Acta Vet. Hung., 38. 3-17, 1990.

Gálfi, P. and Neogrády, S.: **Short shain fatty acids (acidifiers) as probiotics in diets for piglets**. In: Piva, G.: Proceedings of the 4th International Feed Production Conference. Piacenza, Italy, 1996.

Gálfi, P. and Neogrády. S.: **The pH-dependent inhibitory action of n-butyrate on gastrointestinal epithelial cell division**, Food Res. Int., 34. 581-586, 2001.

Goriya, H. V., Kalia, A., Bhavsar, S. K., Joshi, C. G., Rank, D. N. and Thaker, A. M.: **Comparative evaluation of phenobarbital-induced CYP3A and CYP2H1 gene expression by quantitative RT-PCR in Bantam, Bantamized White Leghorn and White Leghorn chicks**, J. Vet. Sci., 6. 279–285, 2005.

Goudah, A., El-Sooud, K. A. and El-Aty, A. M. A.: **Pharmacokinetics and tissue residue profiles of erythromycin in broiler chickens after different routes of administration**, Deut. Tierarztl. Woch., 111. 162–165, 2004.

Guilloteau, P., Martin, L., Eeckhaut, V., Ducatelle, R., Zabielski, R. and Van Immerseel, F.: **From gut to the peripheral tissues: the multiple effects of butyrate**, Nutr. Res., 23. 366-384, 2010.

Hake, S. B., Garcia, B. A., Duncan, E. M., Kauer, M., Dellaire, G., Shabanowitz, J., Bazett-Jones, D. P., Allis, C. D. and Hunt, D. F.: **Expression patterns and post-translational modifications associated with mammalian histone H3 variants**, J. Biol. Chem., 281. 559–568, 2006.

Handschin, C. and Meyer, U. A.: **Induction of Drug Metabolism: The role of nuclear receptors**, Pharmacol. Rev., 55, 649–673, 2003.

Hansen, A. J. and May, B. K.: **Sequence of a chicken Phenobarbital-inducible cytochrome P450 cDNA: regulation of two P450 mRNAs transcribed from different genes**, DNA, 8. 179-191, 1989.

Hong, L. U. and Yan, L. I.: **Effects of bicyclol on aflatoxin B and hepatotoxicity in rats**, Acta Phamac. Sin., 23. 942-945, 2002.

Hu, Z. and Guo, Y.: **Effects of dietary sodium butyrate supplementation on the intestinal morphological structure, absorptive function and gut flora in chickens**, Anim. Feed Sci. Tech., 132. 240-249, 2007.

Ishibashi, T., Dryhurst, D., Rose, K. L., Shabanowitz, J., Hunt, D. F. and Ausio, J.: **Acetylation of vertebrate H2A.Z and its effect on the structure of the nucleosome**, Biochemistry-US, 48. 5007–5017, 2009.

Jenuwein, T. and Allis, C. D.: **Translating the histone code**, Science 293. 1074–1080, 2001.

Kashket, E. R.: **Bioenergetics of lactic acid bacteria: Cytoplasmic pH and osmotolerance**, FEMS Microbiol. Rev., 46. 233–244, 1987.

Kien, C. L., Peltier, C. P., Mandal, S., Davie, J. R. and Blauwiekel, R.: **Effects of the in vivo supply of butyrate on histone acetylation of cecum in piglets**, JPEN-Parenter. Enter., 32. 51-56, 2008.

Kirat, D., Inoue, H., Iwano. H., Hirayama. K., Yokota, H., Taniyama, H. and Kato, S.: **Expression and distribution of monocarboxylate transporter 1 (MCT1) in the gastrointestinal tract of calves**, Res. Vet. Sci., 79. 45–50, 2005.

Knudsen, K. E., Serena, A., Kjaer, A. K., Jorgensen, H. and Engberg, R.: **Rye bread enhances the production and plasma concentration of butyrate but not the plasma concentrations of glucose and insulin**, J. Nutr., 135. 1696-1704, 2005.

Kotunia, A., Woliński, J., Laubitz, D., Jurkowska, M., Romé, V., Guilloteau, P. and Zabielski, R.: **Effect of sodium butyrate on the small intestine development in neonatal piglets feed by artificial sow**, J. Physiol. Pharmacol., 55. 59-68, 2004.

Le Gall, M., Gallois, M., Sève, B., Louveau, I., Holst, J. J., Oswald, I. P., Lallès, J. P. and Guilloteau, P.: **Comparative effect of orally administered sodium butyrate before or after weaning on growth and several indices of gastrointestinal biology of piglets**, Br. J. Nutr., 102. 1285-1296, 2009.

Le Leu, R. K., Brown, I. L., Hu, Y., Morita, T., Esterman, A. and Young, G. P.: **Effect of dietary resistant starch and protein on colonic fermentation and intestinal tumourigenesis in rats**, Carcinogenesis, 28. 240-245, 2007.

Leeson, S., Namkung, H., Antongiovanni, M. and Lee, E. H.: **Effect of butyric acid on the performance and carcass yield of broiler chickens**, Poult. Sci., 84. 1418-22, 2005.

Lempiäinen, H., Müller, A., Brasa, S., Teo, S. S., Roloff, T. C., Morawiec, L., Zamurovic, N., Vicart, A., Funhoff, E., Couttet, P., Schübeler, D., Grenet, O., Marlowe, J., Moggs, J. and Terranova, R.: **Phenobarbital mediates an epigenetic switch at the constitutive androstane receptor (CAR) target gene Cyp2b10 in the liver of B6C3F1 mice**, PLoS ONE, 6. e18216, 2011.

Leu, R. K. L., Hu, Y., Brown, I. L. and Young, G. P.: **Effect of high amylose maize starches on colonic fermentation and apoptotic response to DNA-damage in the colon of rats**, Nutr. Metab., 6. 11, 2009.

Li, Y., Cui, Y., Hart, S. N., Klassen, C. D. and Zhong, X. B.: **Dynamic patterns of histone methylation are associated with oncogenic expression of the CYP3A4 genes during mouse liver maturation**, Mol. Pharmacol., 75. 1171–1179, 2009.

Lu, J. J., Zou, X. T., and Wang, Y. M.: **Effects of sodium butyrate on the growth of weanling pigs**, J. Anim. Feed Sci., 17. 568-578, 2008.

Lutz, E. S. M., Markling, M. E. and Masimirembwa, C. M.: **Monolithic silica rod liquid chromatography with ultraviolet or fluorescence detection for metabolite analysis of cytochrome P450 marker reactions**, J. Chromatogr. B, 780. 205-215, 2002.

Mathew, O. P., Ranganna, K., Yatsu, F. M.: **Butyrate, an HDAC inhibitor, stimulates interplay between different posttranslational modifications of histone H3 and**

differently alters G1-specific cell cycle proteins in vascular smooth muscle cells, Biomed Pharmacother., 64. 773-740, 2010.

McGinnity, D. F. and Riley R. J.: **Predicting drug pharmacokinetics in humans from in vitro metabolism studies**, Biochem. Soc. Trans., 29. 135-139, 2001.

McGinnity, D. F., Berry, A. J., Kenny, J. R., Grime, K., and Riley R. J.: **Evaluation of time-dependent cytochrome P450 inhibition using cultured human hepatocytes**, Drug Metab. Dispos., 34. 1291-1300, 2006.

McIntyre, A., Gibson, P. R. and Young, G. P.: **Butyrate production from dietary fibre and protection against large bowel cancer in a rat model**, Gut, 34. 386-391, 1993.

Medina, V., Edmonds, B., Young, G. P,. James, R., Appleton, S. and Zalewski, P. D.: **Induction of caspase-3 protease activity and apoptosis by butyrate and trichostatin A (inhibitors of histone deacetylase): Dependence on protein Synthesis and Synergy with a mitochondrial/cytochrome c-dependent pathway**, Cancer Res., 57. 3697-3707, 1997.

Michard, J.: **Seeking new broiler growth promoters**, Poult. Int., 47. 28-30, 2008.

Minucci, S. and Pelicci, P. G.: **Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments of cancer**, Nat. Rev. Cancer, 6. 38-51, 2006.

Murray, M. and Ryan, A. J.: **Inhibition and enhancement of mixed-function oxidases by nitrogen heterocycles**, Biochem. Pharmacol., 31. 3002–3005, 1982.

Nash, T.: **The colorimetric estimation of formaldehyde by means of the Hantzsch reaction**, Biochem. J., 55. 416-421, 1953.

Neogrády, Z., Gálfi, P. and Kutas, F.: **Effect of intraruminal butyrate infusion on the plasma insulin level in sheep**, Acta Vet. Hung., 37. 247-253, 1989.

Ourlin, J. C., Baader, M., Fraser, D., Halpert, J. R. and Meyer, U. A.: **Cloning and functional expression of a first inducible avian cytochrome P450 of the CYP3A subfamily (CYP3A37)**, Arch. Biochem. Biophys., 373. 375-384, 2000.

Paolini, M., Pozzetti, L., Sapone, A., Biagi, G. L. and Cantelli-Forti, G.: **Development of basal and induced testosterone hydroxylase activity in the chicken embryo in ovo**, Brit. J. Pharmacol., 122. 344-350, 1997.

Pászti-Gere, E., Palócz, O., Fébel, H., Jakab, Cs. and Gálfi, P.: **A nátrium-n-butirát in vivo és in vitro hatásának vizsgálata oxidatív stressz okozta bélgyulladásokban (In vivo and in vitro examination of the effect of sodium-n-butyrate on oxidative stress-induced bowel inflammations)**, Magy. Állatorv. Lapja, 134. 726-732, 2012.

Pfaffl, M. W., Horgan, G. W. and Dempfle, L.: **Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR**, Nucleic Acids Res., 30., e36, 2002.

Phillips, I.: **Withdrawal of growth-promoting antibiotics in Europe and its effects in relation to human health**, Int. J. Antimicrob. Ag., 30. 101-107, 2007.

Rada-Iglesias, A., Enroth, S., Ameur, A., Koch, C. M., Clelland, G. K., Respuela-Alonso, P., Wilcox, S., Dovey, O. M., Ellis, P. D., Langford, C. F., Dunham, I,, Komorowski, J, and Wadelius, C.: **Butyrate mediates decrease of histone acetylation centered on transcription start sites and down-regulation of associated genes**, Genome Res., 17. 708-719, 2007.

Ricke, S. C.: **Perspectives on the use of organic acids and short chain fatty acids as antimicrobiols**, Poultry Sci., 82. 632-639, 2003.

Roediger, W. E.: **Utilization of nutrients by isolated epithelial cells of the rat colon**, Gastroenterology, 83. 424-429, 1982.

Ross Broiler Management Manual. Ross, Newbridge, Midlothian, UK, 2009

Sakata, T.: **Stimulatory effect of short-chain fatty acids on epithelial cell proliferation in the rat intestine: a possible explanation for trophic effects of fermentable fibre, gut microbes and luminal trophic factors**, Br. J. Nutr., 58. 95-103, 1987.

Shin, J., Li, R., Gao, Y., Baldwin, R. and Li, C.: **Genome-wide ChIP-seq mapping and analysis reveal butyrate-induced acetylation of H3K9 and H3K27 correlated with transcription activity in bovine cells**, Funct. Integr. Genomics, 12. 119-130, 2012.

Singh, K. B.: **Cytochrome P450 enzyme isoforms and their therapeutic implications: an update**, Indian J. Med. Sci., 61. 102-116, 2007.

Snel, J., Harmsen, H. J. M., Van der Wielen, P. W. J. J. and Williams, B. A.: **Dietary strategies to influence thegastrointestinal microflora of young animals and its potential to improve intestinal health**. In: Blok, M. C., Vahl, H. A., De Lange, L., Van de Braak, A. E., Homko, G. and Hessing, M. Wageningen: Nutrition and health of the gastrointestinal tract. Wageningen Academic Publishers, 37-60, 2000.

Steliou, K., Boosalis, M. S., Perrine, S. P., Sangerman, J and Faller, D. V.: **Butyrate histone deacetylase inhibitors**, BioResearch, 1. 4, 2012.

Strahl, B. D. and Allis, C. D.: **The language of covalent histone modifications**, Nature, 403. 41–45, 2000.

Suárez, A. F. and Ellis, R.: **Erythromycin**. In: Residue evaluation of certain veterinary drugs, Food and Agriculture Organization of the United Nations, Rome, Italy, 2006.

Sugatani, J., Sadamitsu, S., Wada, T., Yamazaki, Y., Ikari, A. and Miwa, M.: **Effects of dietary inulin, statin, and their co-treatment on hyperlipidemia, hepatic steatosis and changes in drug-metabolizing enzymes in rats fed a high-fat and high-sucrose diet**, Nutr. Metab. 9. 23, 2012.

Takizawa, D., Kakizaki, S., Horiguchi, N., Tojima, H., Yamazaki, Y., Ichikawa, T., Sato, K. and Mori, M.: **Histone deacetylase inhibitors induce cytochrome P450 2B by activating nuclear receptor CAR**, Drug Metab. Dispos., 38. 1493-1498, 2010.

Tobisawa, Y., Imai, Y., Fukuda, M. and Kawashima, H.: **Sulfation of colonic mucins by N-acetylglucosamine 6-O-sulfotransferase-2 and its protective function in experimental colitis in mice**, J. Biol. Chem., 285. 6750-6760, 2010.

Utesch, D., Diener, B., Molitor, E., Oesch, F. and Platt, K. L.: **Characterization of cryopreserved rat liver parenchymal cells by metabolism of diagnostic substrates and activities of related enzymes**, Biochem. Pharmacol., 44. 309–315, 1992.

Van der Hoeven, T. A. and Coon, M. J.: **Preparation and properties of partially purified cytochrome P-450 and reduced nicotinamide adenine dinucleotide phosphate-cytochrome P-450 reductase from rabbit liver microsomes**, J. Biol. Chem., 249. 6302-6310, 1974.

Van Immerseel, F., De Buck, J., Meulemans, G., Pasmans, F., Velge, P., Bottreau, E., Haesebrouck, F. and Ducatelle, R.: **Invasion of Salmonella Enteritidis in avian intestinal epithelial cells in vitro is influenced by short-chain fatty acids**, Int. J. Food Microbiol., 85. 237-248, 2003.

Velázquez, O. C., Lederer, H. M. and Rombeau, J. L.: **Butyrate and the colonocyte. Production, absorption, metabolism, and therapeutic implications**, Adv. Exp. Med. Biol., 427. 123-134, 1997.

Wallace, D. C. and Fan, W**.: Energetics, epigenetics, mitochondrial genetics**, Mitochondrion, 10. 12-31, 2010.

Wang, R. W., Newton, D. J., Scheri, T. D. and Lu, A. Y. H.: **Human cytochrome P450 3A4-catalysed testosterone 6ß-hydroxylation and erythromycin N-demethylation**. In: The American Society for Pharmacology and Experimental Therapeutics, 25. 502-507, 1997.

Wilson, T. M. and Kliewer, S. A.: **Pxr, car and drug metabolism**, Nat. Rev. Drug Disc., 1. 259-266, 2002.

Wu, J., Zhou, Z., Hu, Y. and Dong, S.: **Butyrate-induced GPR41 Activation Inhibits Histone Acetylation and Cell Growth**, J. Genet. Genomics, 39. 375-384, 2012.

Yabe, Y., Galetin, A. and Houtson, J. B.: **Kinetic characterization of rat hepatic uptake of 16 actively transported drugs**, Drug Metab. Dispos., 39. 1808-1814, 2011.

Yang, L. Q., Yu, W. F., Cao, Y. F., Gong, B., Chang, Q. and Yang, G. S.: **Potential inhibition of cytochrome P450 3A4 by propofol in human primary hepatocytes**, World J. Gastro., 9. 1959-1962, 2003.

Young, G. P., Gibson PR, eds: **Butyrate and the human cancer cell**. Cambridge: Cambridge University Press; 1995.

Zhang, K., Williams, K. E., Hunag, L., Yau, P., Siino, J. S., Bradbury, E. M., Jones, P. R., Minch, M. J. and Burlingame, A. L.: **Histone acetylation and deacetylation**, Mol. Cell. Proteomics, 1. 500-508, 2002a.

Zhang, K., Tang, H., Huang, L., Blankenship, J. W., Jones, P. R., Xiang, F., Yau, P. M. and Burlingame, A. L.: **Identification of acetylation and methylation sites of histone H3 from chicken erythrocytes by high-accuracy matrix-assisted laser desorption ionization-time-of-flight, matrix-assisted laser desorption ionization-postsource decay, and nanoelectrospray ionization tandem mass spectrometry**, Anal. Biochem., 306. 259-269, 2002b.

Zhang, W. H., Jiang, Y., Zhu, Q. F., Gao, F., Dai, S. F., Chen, J. and Zhou, G. H**.: Sodium butyrate maintains groth performance by regulating the immune response in broiler chickens**, Brit. Poultry Sci., 52. 292-301, 2011.

9. Own scientific publications

9.1. Publications related to the topic of the present dissertation

Full text papers in peer-reviewed journals:

Mátis, G., Neogrády, Zs., Csikó, Gy., Kulcsár, A., Kenéz, Á. and Huber, K.: **Effects of orally applied butyrate bolus on histone acetylation and cytochrome P450 enzyme activity in the liver of chicken – a randomized controlled trial**, Nutr. Metab., 10. 12, 2013. (**IF:** 2.89)

Mátis, G., Neogrády, Zs., Csikó, Gy., Gálfi, P., Fébel, H., Jemnitz, K., Veres, Zs., Kulcsár, A., Kenéz, Á. and Huber, K.: **Epigenetic effects of dietary butyrate on hepatic histone acetylation and enzymes of biotransformation in chicken**, Acta Vet. Hung., 61. (4), 2013. (DOI: 10.1556/AVet.2013.033) (last known **IF:** 0.642)

Csikó, Gy., Nagy, G., **Mátis, G.**, Neogrády, Zs., Kulcsár, A., Jerzsele, Á., Szekér, K. and Gálfi, P.: **Possible effects of dietary n-butyrate on hepatic biotransformation and pharmacokinetic behaviour of drugs in chicken**, J. Vet. Pharmacol. Ther., [submitted] (last known **IF:** 1.181)

Mátis, G., Csikó, Gy., Jemnitz, K., Veres, Zs., Fébel, H., Kulcsár, A., Petrilla, J. and Neogrády, Zs.: **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)**, Magy. Állatorv. Lapja, 135. 109-116, 2013. (last known **IF:** 0.3)

Oral presentations on international conferences:

Csikó, Gy., Nagy, G., **Mátis, G.**, Neogrády, Zs. and Gálfi, P.: **The effect of dietary sodium butyrate on the pharmacokinetics of erythromycin in broiler chickens**, 12th International Conference of the European Association for Veterinary Pharmacology and Toxicology, Utrecht, The Netherlands, 2012.

Mátis, G., Kulcsár, A., Petrilla, J., Kenéz, Á., Csikó, Gy., Neogrády, Zs. and Huber, K.: **Epigenetic consequences of oral butyrate application in chicken**, GfE Conference, Göttingen, Germany, 2013.

Poster presentations on international conferences:

Mátis, G., Kenéz, Á., Kulcsár, A., Csikó, Gy., Neogrády, Zs. and Huber, K.: **Trophic and epigenetic effects of dietary butyrate supplementation in chicken**, GfE Conference, Göttingen, Germany, 2012.

Mátis, G., Csikó, Gy., Jemnitz, K., Veres, Zs., Szabó, M., Kulcsár, A., Kenéz, Á., Gálfi, P. and Neogrády, Zs.: **Effects of dietary butyrate supplementation on hepatic microsomal cytochrome P450 activity in chicken and rat**, FEBS3+ Conference, Opatija, Croatia, 2012.

Oral presentations on Hungarian national conferences:

Mátis, G., Csikó, Gy., Annus, K., Neogrády, Zs. and Gálfi, P.: **A nátrium-n-butirát metabolizmusa patkány és csirke primer májsejttenyészetben**, MTA Akadémiai Beszámolók, Budapest, Hungary, 2010.

Mátis, G., Neogrády, Zs., Csikó, Gy. and Annus, K.: **A butirát hatásának vizsgálata májsejttenyészetben: a primer tenyészet jellemzése immunhisztokémia és western blot segítségével**, MTA Akadémiai Beszámolók, Budapest, Hungary, 2011.

Mátis, G., Csikó, Gy., Fébel, H. and Neogrády, Zs.: **A takarmánykiegészítőként adott butirát növekedési teljesítményre és egyes vérparaméterekre gyakorolt hatásának vizsgálata brojlercsirkében és patkányban**, MTA Akadémiai Beszámolók, Budapest, Hungary, 2012.

Mátis, G., Kenéz, Á., Kulcsár, A., Csikó, Gy., Neogrády, Zs. and Huber, K.: **A takarmánykiegészítőként adott butirát epigenetikus hatásának vizsgálata brojlercsirkében**, MTA Akadémiai Beszámolók, Budapest, Hungary, 2012.

Mátis, G., Csikó, Gy., Kulcsár, A., Kenéz, Á., Jemnitz, K., Veres, Zs., Szabó, M. and Neogrády, Zs.: **A takarmánykiegészítőként adott butirát máj citokróm P450 izonezimekre gyakorolt hatásának vizsgálata brojlercsirkében és patkányban**, MTA Akadémiai Beszámolók, Budapest, Hungary, 2012.

Mátis, G., Kenéz, Á., Kulcsár, A., Csikó, Gy., Neogrády, Zs. and Huber, K.: **A bolusban adott butirát májsejtek hiszton-acetilációjára gyakorolt hatásának vizsgálata brojlercsirkében**, MTA Akadémiai Beszámolók, Budapest, Hungary, 2013.

Mátis, G., Csikó, Gy., Kulcsár, A., Petrilla, J., Pleva, D., Neogrády, Zs. and Gálfi, P.: **Máj citokróm P450 enzimek aktivitásának vizsgálata bolusban adott butirátkezelést követően brojlercsirkében**, MTA Akadémiai Beszámolók, Budapest, Hungary, 2013.

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

Full text papers in peer-reviewed journals:

Domokos, M., Ígyártó, B., Glávits, R., Pécsi, A., **Mátis, G.**, Földi, J., Kulcsár, M., Huszenicza, Gy., Neogrády, Zs. and Gálfi, P.: **Az apoptotikus sejt arányának csökkenése hyperketonaemiás tehenek endometriumában az involúció korai időszakában**

(Decreasing the proportion of apoptotic cells in the endometrium in early period of involution in hyperketonaemic cows), Magy. Állatorv. Lapja, 132. 641-646, 2010. (**IF:** 0.3)

Pászti-Gere, E., **Mátis, G.**, Farkas, O., Kulcsár, A., Palócz, O., Csikó, Gy., Neogrády, Zs. and Gálfi, P.: **The effects of intestinal LPS exposure on inflammatory responses in porcine enterohepatic co-culture system**, Inflammation, [submitted] (last known **IF:** 1.747)

Oral presentations on Hungarian national conferences:

Mátis, G., Mitze, S., Neogrády, Zs. and Gálfi, P**.: Bakteriális lipopoliszacharidok által kiváltott interleukin-6 termelés bendőhámsejteken**, MTA Akadémiai Beszámolók, Budapest, Hungary, 2009.

Mátis, G., Mitze, S., Vonza, É., Neogrády, Zs. and Gálfi, P.: **Bendőnyálkahártya hám- és kötőszöveti eredetű sejtjeinek bakteriális lipopoliszacharidok által kiváltott interleukin-6 termelése**, MTA Akadémiai Beszámolók, Budapest, Hungary, 2010.

Mátis, G., Csikó, Gy., Kulcsár, A., Petrilla, J., Farkas, O., Palócz, O., Neogrády, Zs. and Gálfi, P.: **Bakteriális lipopoliszacharidok által kiváltott oxidatív stressz hatása sertés primer májsejttenyészetre**, MTA Akadémiai Beszámolók, Budapest, Hungary, 2013.

Farkas, O., Palócz, O., Csikó, Gy., Pászti-Gere, E., **Mátis, G.**, Kulcsár, A., Petrilla, J., Neogrády, Zs. and Gálfi, P.: **Oxidatív stressz és gyulladás hatásának vizsgálata in vitro bélhám és májsejt ko-kultúra modellen**, MTA Akadémiai Beszámolók, Budapest, Hungary, 2013.

Kulcsár, A., Molnár, A., **Mátis, G.**, Petrilla, J., Pál, L., Jerzsele, Á., Neogrády, Zs. and Dublecz, K.: **A különböző koncentrációban adott butirát gátló hatásának *in vitro* vizsgálata *Campylobacter jejuni* törzsekre**, MTA Akadémiai Beszámolók, Budapest, Hungary, 2013.

9.3. Supervising of DVM theses

Annus, K.: **A nátrium-n-butirát felvételének és metabolizmusának vizsgálata patkány primer májsejttenyészetben**, TDK dolgozat, 2010. Supervisor: **Mátis, G.**

Nyáry, D.: **A takarmánykiegészítőként adott butirát növekedésre és vékonybél-morfológiára gyakorolt hatásainak vizsgálata csirkében és patkányban**, TDK dolgozat, 2011. Supervisors: **Mátis, G.** and Kenéz, Á.

Pleva, D.: **A *per os* felvett butirát máj citokróm P450 enzimek aktivitására gyakorolt hatásának vizsgálata brojlercsirkében**, TDK dolgozat, 2012. Supervisor: **Mátis, G.**

10. Acknowledgement

I would like to express my honest gratitude to my supervisor **Zsuzsanna Neogrády** for her continous encouragement and intensive efforts in supervising my work. It was never too late at night for her to discuss a problem, she was always behind me if I needed some help in research or in any field of life. Throughout the years, she became much more for me than just a supervisor, I am grateful for her kind support, and not only in my scientific work. A special thank has to be granted to **György Csikó**, my co-supervisor for his generous help, advices, for all his patience and for introducing me into the amazing world of pharmacological research. I am especially grateful to Prof. **Korinna Huber**, my external supervisor for providing the opportunity to work together on this project in her laboratory in Hannover, establishing an outstanding cooperation. Special thanks have to be given for her kind support and useful advices during the last three years.

I would like to thank to Prof. **László Frenyó** for the possibility that I could start to work in the motivating milieu of the Department of Physiology and Biochemistry. I am especially grateful to Prof. **Péter Gálfi** for his useful advices and support from the Department of Pharmacology and Toxicology.

I am very grateful and would like to express many warm thanks to my former TDK supervisor **Mónika Domokos**, who kindly introduced me into the laboratory the first time and showed me, what scientific work is. This PhD work should be dedicated to her memory.

It is my honor to acknowledge the work of my colleagues in the Department of Physiology and Biochemistry, which was essential for the success of this PhD project. It was a pleasure for me to work in this fantastic team, where lab work was conducted not only preciously, but it was also full of fun. Among them, first of all I would like to thank to **Ákos Kenéz**, with whom we worked together from the beginning of my PhD studies, as well in Hannover. I am also grateful to **Anna Kulcsár**, as well for her excellent contribution in the statistical evaluations, and also to **Janka Petrilla** for her special patience and kindness even during the hard moments. I would like to thank for the help of **Tünde Benedek** in the maintenance of the animals and also for her outstanding laboratory work. I am very grateful to **Tamás Veresegyházy**, who opened new horizonts for me by introducing me into the fascinating world of Biochemistry on his lectures. Special thanks have to be granted to Prof. **Ferenc Kutas** for his wise advices and support throughout the years.

I am very grateful for the contribution of **Katalin Jemnitz** and **Zsuzsanna Veres** to introduce me into the amazing method of hepatocyte isolation and for the team-work together through my PhD studies regarding the microsomal CYP enzyme studies. Special thanks to **Hedvig Fébel** for the excellent cooperation regarding the animal feeding trials and for her

work in the gas chromatographic measurements. The precise work of **Gábor Nagy** in the HPLC assays of erythromycin determinations is also gratefully acknowledged. Many thanks should be granted as well as to **Krisztina Szekér** for her continous help, being also my consultant.

Many thanks to **Győző Kaján**, **Éva Fejes** and **Zsolt Wagner** for their essential help in the ultracentrifugation procedures. I would like to thank for the excellent assistance of **Erika Lajtai**, **Kathrin Hansen** and **Susanne Hoppe** in the laboratory work. I want to express special thanks to my graduate students **Kata Annus**, **Daniella Nyáry**, **Dániel Pleva** and **Katalin Hermándy-Berencz**, who wrote or currently write their TDK theses under my supervising. Their help and excellent work was necessary for the success of this project.

At last, but not least I am especially grateful for **my family** and **my friends** for their warm support. Special thanks to my parents and grandparents for all their love and for being behind me all the time when I needed their support. As teachers, they showed me a true way to follow, and they always did all their best to help me to reach my dreams. In addition, I would like to gratefully acknowledge the professional assistance of my father in editing the figures and formatting the dissertation. The help of my brother, Zsolti is also really appreciated, he was not only my brother and my friend at the same time, but if it was necessary, he was there to help me during the long animal sampling days and in the laboratory work as well.