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Evaluation of feed additives and their potential in antimicrobial replacement in farm animals

PhD thesis

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Content

List of abbreviations

AGP: antimicrobial growth promoter
ANF: alpha-naphthoflavone
ANOVA: analysis of variance
bp: base pair
BCA: bicinchoninic acid
Blich*, B. licheniformis: Bacillus licheniformis*
BSA: bovine serum albumin
bw: body weight
CAR: constitutive androstane receptor
cDNA: copy deoxyribonucleic acid
CycA: cyclophilin A
CYP: cytochrome P450
CXR: chicken xenobiotic-sensing orphan nuclear receptor
DMEM: Dulbecco's Modified Eagle's Medium and Ham's F-12 Nutrient Mixture
DWA: drinking water acidifier
ECF, *E. faecium: Enterococcus faecium*
E. coli: Escherichia coli
EDTA: ethylene diamine tetraacetic acid
EGF: epidermal growth factor
EGTA: ethylene glycol tetraacetic acid
ELISA: enzyme-linked immunosorbent assay
FA: fulvic acid
FBS: foetal bovine serum
GAPDH: glyceraldehyde-3-phophate dehydrogenase
GIT: gastrointestinal tract
HPRT: hypoxanthine phosphoribosyl transferase
Hsp70: heat shock protein 70
icoe: intracoelomal
IL: interleukin
IPEC-J2: intestinal porcine epithelial cell line
KTZ: ketoconazole
LGG: *Lactobacillus rhamnosus*
L. plantarum: Lactobacillus plantarum
LPS: lipopolysaccharide

MRS: DeMan, Rogosa, Sharpe broth

NADPH: nicotinamide adenine dinucleotide phosphate

PB: phenobarbital

PBS: phosphate buffered saline

PCR: polymerase chain reaction

P. multocida: Pasteurella multocida

qPCR: quantitative/real-time polymerase chain reaction

REST: Relative Expression Software Tool

RLU: relative luminescence unit

RNA: ribonucleic acid

mRNA: messenger ribonucleic acid

SCS: spent culture supernatant

SD: standard deviation

SE: standard error of mean

SN: sanguinarine containing product

TEER: transepithelial electric resistance

TNF-α: tumor necrosis factor alpha

TSB: tryptic soy broth

ttkg: testtömeg kilogramm

UDP: uridine 5'-diphospho

Summary

Improving food animal production is a major aspect because of the constantly increasing demand in human nutrition. A wide range of non-drug substances have health and growth promoting abilities and with their use it is possible to reduce the antimicrobial drug consumption. These feed additives may have immunomodulatory effect or beneficial effect on the composition of the host microbiota. The favourable impact of these supplements are observed and described, but the detailed mechanism of action and potential side effects of them is mostly unknown. Bearing this knowledge would lead to more targeted administration and proper utilization of them. Several substances available to use as feed additives or supplementation of drinking water in livestock farming, in our study we are focusing on the effect of fulvic acid (FA), a sanguinarine containing product (SN), a drinking water acidifier (DWA), the metabolites of probiotics, and beta-glucan among them.

Our aim was to describe the effect of these feed additives in porcine intestinal cells and in broiler chickens on immunomodulatory responses and xenobiotic metabolizing enzymes. Furthermore, their influence on the rabbit hepatic drug metabolizing enzymes was investigated *ex vivo*. For this purpose a recently developed luminescence method for predicting the activity of human drug metabolizing enzymes was tested *a priori* for use in rabbit hepatic cytochrome P450 enzymes.

Both *in vitro* and *in vivo* experiments were carried out during our studies. As a model of intestinal epithelium IPEC-J2 cells were grown on 0.4 µm pore-size membrane insert. Primary rabbit hepatocytes and liver microsomes obtained from rabbits and chickens were used to measure the activity of CYP enzymes. The interleukin, cytokine and CYP mRNA levels were monitored by qPCR method, the inflammatory markers and antibody levels were measured with ELISA, and the CYP450 activity levels were determined by luminescence method.

The drinking water supplements prevented the upregulation of the pro-inflammatory cytokine TNF-alpha in LPS-treated enterocytes. Together with LPS the β-glucan and fulvic acid treatment further increased the level of IL-8 mRNA. The level of CYP3A29 mRNA was not affected by any of the treatments, but the acidic supplements at higher doses enhanced the intestinal gene expression of CYP1A genes. The simultaneous treatment of LPS and the supernatant of *Enterococcus faecium* or the *Lactobacillus rhamnosus* restrained the LPS-evoked production of IL-6 and IL-8 protein in porcine enterocytes.

The *in vitro* application of fulvic acid or the sanguinarine-containing supplement did not alter the activity of rabbit hepatic CYP2C and CYP3A6 enzymes. Based on this outcome, it can be presumed, that the use of the two examined additives together with drug substances in rabbits is safe. This is supported by the result of the concomitant use of tylosin

and SN or FA. Tylosin inhibited the activity of both CYP enzymes; CYP2C9 and CYP3A6, by adding sanguinarine containing or fulvic acid drinking water additive to the mixture do not expose any impact on the activity of these xenobiotic metabolizing enzymes.

The orally applied drinking water supplements for five consecutive days did not change the activity of chicken hepatic CYP2C and CYP3A enzymes. The administration of 50 mg/kg bw. beta-glucan, 5 mg/kg bw. SN, 50 mg/kg bw. SN, 250 mg/kg bw. fulvic acid and 1 ml/kg bw. DWA to the chickens significantly increased the hepatic CYP1A activity. Nevertheless, the four applied drinking water supplements did not cause clinically important modifications in the gene expression level and the activity of the avian CYP2C, which considered the most crucial in avian drug metabolism. Consequently, the risk of cytochrome mediated interactions between the examined supplements and pharmaceuticals used in the chicken industry are unlikely. The concurrent application of SN or FA or *Bacillus licheniformis* supernatant with tiamulin did not influence the CYP activity reducing effect of tiamulin.

Three of the orally applied drinking water supplements strengthened the impact of *Pasteurella multocida* vaccination in the chicken flock. Compared to controls the SN and the 10 day long DWA treatments doubled, while the fulvic acid treatments tripled the amount of produced *Pasteurella* antibody in chickens after immunization. Among the tested feed additives; fulvic acid, SN and DWA would be powerul tools to optimize the effect of *P. multocida* vaccination in the flock.

Összefoglalás

Az élelmiszer célú állattenyésztés javítása a folyamatosan növekvő kereslet miatt fontos. Számos nem gyógyszer hatóanyagnak van egészségmegőrző és növekedést elősegítő hatása, így felhasználásukkal csökkenthető lenne az antimikrobás gyógyszer fogyasztás. Ezek a takarmány-adalékanyagok immunmoduláló hatással rendelkeznek vagy kedvező hatással lehetnek a gazdaszervezet mikrobiota összetételére. Ezen kiegészítő szerek kedvező hatásait korábban megfigyelték és leírták, azonban részletes hatásmechanizmusuk és lehetséges mellékhatásaik kevéssé ismertek. Részletesebb ismeretek célzottabb alkalmazást és hatékonyabb felhasználást tennének lehetővé. Számos takarmánykiegészítő hatóanyag elérhető, melyek közül kutatásaink során a fulvosavak, szangvinarin tartalmú készítmény, ivóvíz savanyító, probiotikumok metabolitjainak, valamint béta-glükán hatásait vizsgáltuk.

Célunk volt, hogy leírjuk a takarmánykiegészítők hatással vannak-e a sertés bélhámsejtek és brojlercsirkék immunválaszára, valamint xenobiotikum lebontó enzimjeire. Továbbá, hogy befolyásolják-e a nyúl hepatikus gyógyszer lebontó enzimjeinek szintjét. Emiatt egy a humán gyógyszermetabolizáló enzimek vizsgálatára fejlesztett lumineszcens módszer állati eredetű metabolikus enzimek vizsgálatára való alkalmasságát teszteltük.

In vitro valamint *in vivo* vizsgálatokat is végeztünk tanulmányunk során. A bél epitélium modellezésére IPEC-J2 sejteket tenyésztettünk 0,4 µm pórusátmérőjű membrán inzerteken. Primer nyúl májsejteket, valamint nyúl és csirke máj mikroszómát izoláltunk a CYP enzim aktivitás méréséhez. Az interleukin, citokin és CYP mRNS szinteket qPCR módszerrel, a gyulladásos marker és az antitest szinteket ELISA-val mértük, és a CYP450 aktivitási szinteket lumineszcencia módszerrel határoztuk meg.

Az itatóvízben alkalmazott adalékanyagok megakadályozták a proinflammatorikus citokin, TNF-alfa termelődését LPS-kezelt enterocitákban. Az LPS és a béta-glükán vagy fulvosav együttes kezelés tovább növelte az IL-8 mRNS szintjét. A CYP3A29 mRNS szintjét egyik kezelés sem befolyásolta, de a savas karakterű kiegészítők serkentették a CYP1A gének kifejeződését. Az LPS és az *Enterococcus faecium* vagy a *Lactobacillus rhamnosus* felülúszója egyidejűleg alkalmazva megakadályozta az LPS által előidézett IL-6 és IL-8 többlet fehérjetermelést a sertés bélhámsejtekben.

A fulvosav vagy a szangvinarin tartalmú szer nem befolyásolta a nyúl máj CYP2C és CYP3A6 enzimjeinek aktivitását. Valószínűleg ezek a kiegészítő szerek nyúlban biztonságosan alkalmazhatók gyógyszerekkel együtt adva. Ezt támasztja alá, hogy tilozin antibiotikummal együtt alkalmazva nem befolyásolták a tilozin CYP enzimgátló hatását.

A fulvosavak, szangvinarin tartalmú készítmény, ivóvíz savanyító, valamint béta-glükán itatóvízben öt napon át alkalmazva nem volt hatással a csirke hepatikus CYP2C és

CYP3A enzimjeinek aktivitására. Az 50 mg/ttkg béta-glükán, 5 és 50 mg/kg Sangrovit, a 250 mg/ttkg fulvosav és az 1 ml/ttkg ivóvíz savanyító adása megemelte a csirkék máj CYP1A enzimjeinek aktivitását. Azonban, a négy vizsgált készítmény nem okozott jelentős különbséget a madár gyógyszermetabolizmusban fontos CYP2C izoenzimek génexpressziójában valamint aktivitásában, tehát a gyógyszerhatóanyagok és az itatóvízben adott kiegészítők között valószínűleg nem áll fenn citokróm-közvetítette kölcsönhatás csirkékben. A tiamulinnal együttesen alkalmazott Sangrovit, fulvosav vagy *Bacillus licheniformis* felülúszó nem befolyásolta a tiamulin CYP gátló hatását.

A szájon át alkalmazott takarmány-kiegészítők közül három megerősítette a *Pasteurella multocida* vakcinázás hatását a csirkeállományban. A kontroll csoporttal összehasonlítva a Sangrovit és a 10 napos ivóvíz savanyító kezelések megduplázták, míg a fulvosav kezelések megháromszorozták a csirkékben képződött *Pasteurella*-antitest mennyiségét az immunizálás után. A vizsgált takarmány-adalékanyagok közül; a fulvosav, a Sangrovit és az itatóvíz savanyító hatásos eszköz lehetne a *P. multocida* vakcinázás optimalizálására csirkeállományokban.

1. Introduction

Economical livestock production without antimicrobial consumption is one of the main aspect of European food production. Since the antimicrobial drugs are prohibited as growth promoters and their prophylactic use should be kept in a minimum, other solutions are necessary. One of them is the usage of feed additives, hence large amount of supplements are available on the market to improve production, either via feed or via drinking water use. The efficacy of these products are somewhat proven, but the mechanism of their action and their side effects are unknown.

Swine and chicken are the two most important protein sources for humans, therefore the food safety of feed and drinking water additives should be evaluated in these species. The porcine intestinal epithelial cell line (IPEC-J2) originated from the jejunum is a good *in vitro* alternative for preliminary investigations. The IPEC-J2 as a non-cancerous cell line is a suitable model of the swine intestine (Langerholc et al., 2011).

Rabbits are livestock animals in increasing demand for meat production, widely used laboratory models and popular pet species. Despite this, only a very few medicines have been registered for rabbits. Veterinary practitioners are forced to use medicines registered for other pet animals, and the high incidence of resulting iatrogenic illness reflects the differences in drug metabolizing capacity between species.

The cytochrome P450 (CYP450) system plays a key role in xenobiotic metabolism, where the CYP enzymes catalyse Phase I reactions. In humans, for instance CYP1A1 is involved in the biotransformation of polycyclic aromatic hydrocarbons, CYP1A2 metabolizes phenacetin, theophylline, caffeine, imipramine and propranolol, and CYP3A is the most abundant CYP isoenzyme, metabolising over half of current drugs (Boek-Dohalská et al., 2001). Despite the highly conserved functions of cytochrome P450 enzymes, significant inter-species variance exists in the activity and substrate-specificity of individual enzymes.
Valuable information about the safety of drugs, feed additives etc. can be obtained by measuring their impact on CYP enzyme activities. Animal models are the gold standard but ethical and cost considerations favour *in vitro* systems such as primary hepatocyte cell cultures or liver microsome fractions, which closely approximate to parent cells and provide high initial CYP450 activity (Guillouzo, 1998).

1.1 Swine production

Nowadays more than 109 million tons of pork produced per year in the world (Jha and Berrocoso, 2015). During the growing period, the pigs are exposed to numerous adverse factors, including inflammation or bacterial, viral, and parasitic pathogens, which will lead to decreased weight gain and impaired general condition. Antimicrobial growth promoters (AGP) have widely been used in pig diets, especially in nursery diets, to control incidences of post-weaning diarrhoea and to improve growth performance (Omonijo et al., 2018). The AGPs are prohibited to use in food animal production since 2006 in the European Union, 2016 in the United States and 2017 in Canada. Successful replacement of them is necessary in mass production to improve the health of the animals and to enhance growth performance. Feed supplements are universally used for these reasons. Although various additives can be used in swine husbandry, none of them has the comprehensive beneficial effect as AGPs. It is essential to determine the mechanism of action and side effects of the available feed additives. In possession of these information more tailored usage of them would be possible.

It is substantial in pig production to maintain a healthy gastrointestinal tract (GIT) to achieve maximal efficiency of converting feed mass into pig body mass (Liao and Nyachoti, 2017). The GIT is the first barrier between orally introduced materials and the internal body environment; it is the entering point of nutrients, vitamins, xenobiotics, and it has an expansive immune system, furthermore it is in close cooperation with the gut microbiota. Studying its complex function requires myriads of parameters to deal with. Intestinal cell lines provide a good *in vitro* alternative to evaluate the effect of a substance on the bowel epithelium. IPEC-J2 cells are porcine intestinal columnar epithelial cells that were isolated from neonatal piglet mid-jejunum. The IPEC-J2 cell line provides a model of the intestinal epithelium for swine based infection studies, and also for comparative investigations between porcine and human bowel mechanisms (Brosnahan and Brown, 2012).

1.2 Poultry production

It is incredibly important to produce good quality meat with high food safety in large-scale production. Poultry production is a main source of meat and eggs for human consumption. Administration of supplements to the animals during the growth period may lead to decreased antibiotic consumption in the chicken industry.

Determining the xenobiotic metabolizing capacity of the liver in each species is important to evaluate the species or individual sensitivity to foreign chemical compounds. It is substantial

to define the mechanism of each feed additives particularly their effect on the drug metabolizing enzymes to predict any possible medicine-supplement interaction.

Commercial poultry production is raising primarily chickens, ducks, turkeys and geese for the production of meat, eggs and feathers. To prevent economical loss and to ensure the health of animals, it is very important to reduce the chance of infectious diseases and respond promptly and properly in case of an infection.

Examples of infectious diseases comprising major risk in the poultry industry are; fowl cholera (avian *pasteurellosis*), chronic respiratory disease caused by *Mycoplasma gallisepticum* (Xiao et al., 2016), avian intestinal *spirochaetosis* caused by *Brachyspira spp.* (Le Roy et al., 2015), and coccidiosis caused by different *Eimeria* species.

To reduce the risk of an infection, the birds are treated almost their entire life with drugs like antibiotics and anticoccidials. The coccidiostats are automatically incorporated to the flock's feed, therefore in case of a disease outbreak the choice of the antimicrobial treatment is severely constrained. Especially, because ionophor antibiotics are causing serious metabolic interactions with other antimicrobials; such as pleuromutilines, lincosamides, macrolides. Feed additives may prevent the diseases' outbreak in the poultry flock and they also enhance their quality of meat, eggs or feathers correlating to their purpose of breeding. Since these substances can be metabolized in the liver, the evaluation of possible metabolic interactions between supplements and antimicrobials in chicken is crucial.

1.3 Rabbit production

Rabbit (*Oryctolagus cuniculus var. domesticus*) production has increasing significance recently which leads to the growing importance of health promotion in rabbit herds. There are several infectious diseases in rabbits that can occur commonly amongst wild, pet, laboratory or meat rabbits, e.g. *myxomatosis*, rabbit haemorrhagic disease, *encephalitozoonosis* caused by *Encephalitozoon cuniculi* and diseases caused by *Pasteurella multocida*. The use of natural additives in the rabbit industry has increased during the last years, not only due to the ban on the subtherapeutic use of antibiotics as growth promoters in Europe, but also due to the demand for natural products by consumers, and therefore synthetic active compounds should be replaced by natural ones (Zotte et al., 2016). There are plenty of supplementing products available to use in livestock farming, although their detailed mechanism of action is absent from the scientific knowledge. Their beneficial effect is known and observed, but the secondary effects or side effects of them remain unknown.

Pasteurellosis is a disease which frequently occurs and has a high probability in rabbit herds. It can be treated with antibiotics sufficiently, but for prevention of the outbreak of the

disease feed additives can be used due to their positive effect on the functioning immune system. It has been proven that feed additives can possess disease-preventing abilities in rabbits, for instance β-glucans, which are cell wall constituents of fungi, are an effective protection against physiological *P. multocida* infections in rabbits, preventing any histological damage (Palocz et al., 2014).

1.4 Feed additives

Improving livestock production is a central concern because of the constantly increasing demand in human nutrition. Infectious diseases represent greater risk in livestock than in individual animals. Antimicrobial therapy is often the only efficient response to these infections, nonetheless the use of antimicrobial agents in large quantity entails high risk due to the rapidly expanding resistance to these agents. Antibacterials should be used less frequently and with great responsibility, their impact should be supported with various feed supplements or vaccination or both. There are numerous commercially available vaccines for bacterial infections, however, immunization alone does not provide complete protection at population level. In addition to the inoculation with specific vaccines, administration of other feed additives is recommended, because of supposed positive effect of them on the immune system.

One of the major purposes of using feed additives in animals is to improve the quality of the feed and thereby improving the animals' performance and health. The nutritional additives are normally composed of vitamins, amino acids, fatty acids and minerals. Feed additives such as probiotics, acidifiers or plant extracts can be additionally added to the feed. The direct effects of nutritional deficiencies on the gastrointestinal tract are a very frequent cause of enteritis and other serious gastrointestinal diseases (Zotte et al., 2016).

Administration of feed additives and other safe compounds to the animal stocks and poultry flocks is essential in reducing the therapeutic drug usage, especially the antibiotic consumption in the food processing industry. It is substantial to define the mechanism of action of each substance, in particular their effect on the drug metabolizing enzymes to estimate any possible drug-supplement interaction.

Among the available feed additives we investigated the effect of *Lactobacillus rhamnosus, Enterococcus faecium*, fulvic acid, a sanguinarine containing product, a drinking water acidifier and β-glucan.

1.4.1 Beta-glucan

Beta-glucans derived from fungi and yeast, which consist of a (1,3)-β-linked backbone with small numbers of (1,6)-β-linked side chains, are essentially known for their immune-modulating effects, oral delivery of them impact mucosal immunity, as shown by an increase of intraepithelial lymphocytes in the intestine (Stier et al., 2014). β-glucan–mediated immunomodulatory effects on dendritic cell activation, macrophage phagocytosis, T-helper cell, and cytotoxic T-lymphocyte priming and differentiation, and *in vivo* antitumor immune responses, are dependent of the dectin-1 pathway (Qi et al., 2011).

The (1-3), (1-6) β-glucans, cell wall constituents of fungi, are recognized by mammalian cells as pathogen-associated molecular patterns and thus act as biological response modifiers. This recognition plays an important role in host defense and presents specific opportunities for clinical modulation of the host innate immune response. Experimental and clinical results show that the (1-3), (1-6) β-glucans act as broad-spectrum enhancers of host defense mechanisms, positively influencing the immunological response of mammals including humans to bacterial, viral, and fungal infections (Hofer and Pospisil, 2011). Furthermore, there is evidence that β-glucans have influence on the adaptive immune system; dietary β-glucan supplementation improved the T-lymphoproliferative response and the growth of primary and secondary lymphoid organs (Guo et al., 2003). These properties make the β-glucans good candidate enhancer of vaccination.

1.4.2 Fulvic acid

Humic acids are a principal component of humic substances, which are the major organic constituents of soil, peat and coal. The substances consist of a complex mixture of various acids containing carboxyl and phenolate groups. Fulvic acid is one fraction of humic substances and is soluble in water under any pH conditions (MacCarthy, 2001).

Fulvic acid is one of the most active fractions of humic substances which are commonly found in soil. Humic substances have been widely used in animal nutrition to improve the profitability of animal production and the health status of animals (Szabo et al., 2017). Fulvic acid contains carboxyls, hydroxyls, carbonyls, phenols, quinones, and semiquinones, which are responsible for its metal chelating and antioxidant activity (Plaza et al., 2005). Previous studies indicated that fulvic acid formed a film on the mucus epithelium of the gastrointestinal tract, protected against infections and toxins, and improved utilization of nutrients in animal feed (Kunavue, 2012). Dietary supplementation with fulvic acid improved feed efficiency and immunity as well (Chang et al., 2014).

As a nutritional supplement, fulvic acid can be found as a component of mineral colloids. Fulvic acids are poly-electrolytes and they are unique colloids that diffuse easily through membranes, whereas all other colloids do not (Yamauchi et al., 1984). Fulvic acid has natural antibacterial, antiviral and fungicidal effects which help the animals in the battle against pathogens and stress factors appearing during intensive breeding. It is also known to help the uptake of microelements and deplete mycotoxins.

The feed supplement fulvic acid belongs to the humates, which summarizes substances originated by the deterioration of organic plant particles caused by bacteria. This group of substances consist of humus, humic acid, fulvic acid, ulmic acid and trace minerals and has the ability to decrease the usage of antibiotics in poultry production. While the concrete effects of humates are not fully known, their usage enhances the feed conversion ratio, feed digestibility and growth performance (Nagaraju et al., 2014). According to other studies, these natural substances can inhibit the growth of bacteria and fungi, which produce toxins such as aflatoxin by binding to them and thus diminishing their absorption in the intestines in broiler chickens. Furthermore it can reduce hormonal stress, and act as an anti-inflammatory and antiviral mediator and stabilizes the intestinal microbiota (Arafat et al., 2017).

1.4.3 Sanguinarine

Sanguinarine belongs to the family of benzoquinolines, which are organic compounds containing a benzene fused to a quinoline ring system. Sanguinarine, also known as sanguinarine chloride is a quaternary ammonium salt of benzylisoquinoline alkaloids and can be extracted from several plants, which are members of the *Papaveracea* family such as Mexican poppy (*Argemone mexicana*) and five-seeded plume-poppy (*Macleaya cordata*). Sanguinarine has been shown to exhibit antibiotic, anti-apoptotic, anti-fungal, anti-inflammatory and anti-angiogenic functions. It is used as a naturopathic therapy for treating infections and managing pain, and also as an expectorant, sedative and emetic (Mitscher et al., 1987). Sanguinarine was originally shown to possess anti-inflammatory properties and it has been used to prevent various inflammatory diseases. In a study about the anti-inflammatory effects of sanguinarine and its modulation of inflammatory mediators from peritoneal macrophages, sanguinarine displayed significant anti-inflammatory effects both *in vitro* and *in vivo*. This study also demonstrated that sanguinarine effectively inhibited the expression of inflammatory mediators and inflammation in general (Niu et al., 2012). Sanguinarine has also shown to increase feed intake and therefore to promote animal growth, meat quality and yield. The administration of Sangrovit® has significant beneficial effects on growth and health in weaning pigs; increased body weight and average daily gain, as well as the reduction of the feed conversion ratio was observed (Kantas et al., 2015).

Furthermore, it has inhibitory properties on cell proliferation, it can stop the growth of some cancer cell lines and enhance their apoptosis.

In a study different groups of poultry were fed with diets either containing two different concentrations of sanguinarine, 20 and 50 ppm. The groups that were fed with the supplement had a higher weight gain and feed conversion ratio, more caecal lactic acid bacteria were present in the gut, the total cholesterol level of the chicken decreased and the amount of malondialdehyde was lower than in the control group. This represents less lipid peroxidation and a positive influence on the meat quality (Lee et al., 2015).

Sanguinarine is used not only in swine and poultry, but in bovine diets as well (Vieira et al., 2008), quaternary benzophenanthridine alkaloids have been shown to have antimicrobial, anti-plaque, antioxidant, anti-inflammatory and immunomodulatory effects (Aguilar-Hernandez et al., 2016), and have selective effects on microbial growth along the digestive tract (Estrada-Angulo et al., 2016). Dietary supplementation with Sangrovit® significantly improved body weight and feed conversion ratio in broiler chickens (Vieira et al., 2008).

1.4.4 Probiotic bacteria

Probiotics have been proven to be suitable feed additives as they can promote health and performance parameters of livestock animals. *Enterococcus faecium* (*E. faecium*) is used as a probiotic supplement in farm animals. It has shown positive effects on diarrhoea incidence effects and on immunological parameters in the gastrointestinal tract of pigs (Lodemann et al., 2015).

Lactobacilli are natural inhabitants of the gastrointestinal tract and are able to ferment various sugars (hexoses, pentoses, and disaccharides) to lactic acid. They can influence anti-pathogen properties of the protective surface to be more efficient and ultimately may prevent the proliferation of undesirable bacteria and fungi, and are maintaining the microbiological balance in the intestines. *Lactobacilli* exhibit competitive exclusion abilities, attachment to epithelial cells of the intestine, fermentation of a broad spectrum of sugars including complex sugars, enhancement of the immune system and resistance to inner digestive track conditions (Aleksandrzak-Piekarczyk et al., 2018).

The probiotic *Lactobacillus rhamnosus* GG (LGG) is a lactic acid bacterium known to have beneficial effect on human and animal gut biota. LGG is inhibiting the pathogen biofilm formation, as well as modulating the gut microbiota, help maintenance of intestinal barrier function and regulating the host immune response (Zhang et al., 2017a).

Bacillus licheniformis is a Gram-positive, spore forming bacterium belongs to the family *Bacillaceae*. The species can be located in the soil as a non-pathogenic, saprophytic microorganism. It is used for different purposes such as the prevention of intestinal diseases

and industrially for the production of enzymes like amylases and peptide antibiotics (Wiegand et al., 2013).

In the chicken industry, *Bacillus licheniformis* is commonly used to prevent necrotic enteritis, which is a gastrointestinal disorder caused by the *Clostridium perfringens*, a Gram-positive, obligate pathogenic and anaerobic bacterium (Knap et al., 2010). This disease causes enhanced mortality and a decrease in growth performance. The probiotic *B. licheniformis* increases the growth performance of the chickens and their feed conversion ratio by preventing their intestines from enteric diseases, stabilizing their microbiota and also increasing their nutrient digestion with the ability to produce digestive enzymes such as protease, lipase and amylase (Zhou et al., 2016).

1.4.5 Drinking water acidifiers

Organic acids have been shown to have beneficial effects on growth performance and have long been used in farm animals. A wide range of organic acids with variable physical and chemical properties exists, of which many are used as drinking water supplements or as feed additives (acidifiers) (Huyghebaert et al., 2011). The ability of organic acids to change from undissociated to dissociated form, depending on the environmental pH, makes them effective antimicrobial agents (Partanen and Mroz, 1999).

Immunofort® is a solution for use in drinking water; it contains volatile fatty acids, amino acids, phosphoric acid, zinc, and copper salt complexes. The aqueous solution of the product contains undissociated organic acids, which inhibit the growth of pathogen microorganisms. The acidification of poultry and swine diets with various weak organic acids such as formic, fumaric, propionic, lactic and sorbic acid can reduce colonization by pathogens and the formation of toxic metabolites (Rodjan et al., 2018). Other organic acids such as sodium-n-butyrate inhibit the growth of enteric pathogenic bacteria, such as *E. coli, Salmonella* and *Clostridium* species, and are therefore suitable tools against certain intestinal inflammatory microorganisms (Ricke, 2003).

Zinc is one of the essential trace elements in poultry as it is an important cofactor of carbonic anhydrase that plays a vital role in eggshell formation (Zhang et al., 2017b). In laying hens, the addition of zinc, manganese, and copper to the diet improved the eggshell quality and ultrastructure (Stefanello et al., 2014).

1.5 Antimicrobial treatment

Antimicrobial therapy is often the only efficient response to the diseases, therefore their use in case of a disease outbreak is inevitable. During our studies a macrolide and a pleuromutilin antibiotic were used as model agents, because of their known inhibitory effect on drug metabolizing enzymes. The macrolide antibiotics include natural agents, prodrugs and semisynthetic substances. Tylosin is effective against Gram-positive and fastidious Gram-negative organisms, like *Pasteurella, Mannheimia, Actinobacillus* and *Haemophilus* species, but also against *Mycoplasma* species and *Clostridium perfringens*. Its absorption is good after *per os* administration. Tylosin phosphate is used to treat and prevent proliferative enteropathy in swine and tylosin tartrate in mycoplasmosis and other respiratory infections caused by fastidious organisms. Macrolides inhibit protein synthesis by inhibiting aminoacyl-tRNA and peptidyl-tRNA binding to the ribosomes.

Macrolides may inhibit the drug metabolism in the liver by complex formation and inactivation of microsomal drug oxidizing enzymes. They are frequently given in combination with other drugs, so possible pharmacokinetic interactions might occur (Periti, 1993).

Tiamulin is a pleuromutilin derivative and is a frequently used drug in the poultry, pig and rabbit industry. It has bacteriostatic mode of action against Gram-positive, fastidious Gram-negative and intracellular pathogens such as *Mycoplasma* and *Brachyspira* species by inhibiting their protein synthesis. Tiamulin is given orally to the animals for the treatment of respiratory or intestinal diseases. The tiamulin metabolism in the liver is not well evaluated in chickens.

1.6 Drug metabolism

Cytochrome P450 enzymes (CYP450) are a group of heme-thiolate monooxygenase enzymes. The majority of drug metabolism is carried out in the liver by these redox enzymes. A large number of families of CYP450 enzymes exists, each member of which catalyses the biotransformation of a unique spectrum of drugs, with some overlap in the substrate specificities. The primary location of CYP450 is the liver, which has the greatest specific enzymatic activity and the highest total activity, but in addition to that, there are also various forms of CYP450 expressed in other organs and tissues such as the kidney, breast, prostate, skin and nasal epithelium, gonads, placenta, brain, lung, spleen, pancreas and gastro-intestinal tract. The intestinal epithelium is allowing the absorption of water and nutrients and serves as a first barrier to microbes, and also inducing and modulating immune responses (Negroni et al., 2015). The large monolayer surface comprised of the intestinal epithelial cells

is also the first point of entry to the different orally applied drugs and other chemicals. The intestinal cytochrome enzymes play key role in the initial step of xenobiotic metabolism.
The CYP enzyme's subcellular location is the smooth endoplasmic reticular membrane (Lewis, 2001). The most important CYP isoenzymes belong to the families CYP1-3. The CYPs' mRNA expression levels are in correlation with their enzymatic activity, however the intensity of the correlation depends on the CYP isoform (Temesvari et al., 2012).
In the overall reaction of the phase I drug metabolism, the drug is oxidized and oxygen is reduced to water. Reducing equivalents are provided by NADPH, and the formation of this cofactor is coupled to cytochrome P450 reductase. CYP450 enzymes has the ability to bind and activate two atoms of oxygen, in most cases this is the dioxygen molecule, and thereby activating the dioxygen for chemical reactions (Anzenbacher and Anzenbacherova, 2001), which leads to the activation or inactivation of these chemical compounds. During the phase II of drug metabolism, a group of conjugation enzymes, which include e.g. UDP-glucuronyl-transferases or glutathione S-transferases, conjugate the metabolites with water-soluble groups to prepare them further for the excretion with the bile or urine, and thereby completing the drug metabolism process.

There are specific aspects about the drug metabolism in rabbits, because their drug metabolism is faster due to their higher initial enzyme activity and abundance compared to other species. While horses and pigs show significantly lower levels than rabbits, chickens have the lowest enzyme activity among these species (Nebbia et al., 2003). Veterinary medicines are often used off-label and also human preparations being used for certain indications in companion animals or maybe in farm animals, individually. Comparing different animal species, the catalytic activity was described to be rather consistent in mammals for CYP2E1, CYP1A2, CYP4A, CYP2D, and CYP3A. Major inconsistencies were reported in the extrapolation of substrate specificity regarding CYP2A, CYP2B and CYP2C (Fink-Gremmels, 2008).

1.6.1 CYP1A subfamily

The CYP1 can be categorised into three subgroups; CYP1A, CYP1B and CYP1C. This group binds polycyclic, often halogenated, aromatic hydrocarbons, as well as aromatic amines, and some endogenous substrates. Birds have generally two isoforms of the CYP1A; CYP1A4 and CYP1A5, which can be grouped and compared to the CYP1A1 and CYP1A2 in mammals (Goldstone and Stegeman, 2006). By comparing CYP1A4 and CYP1A5 of the birds, both can be distinguished by their affinity to different substances. CYP1A4 enhances the activity of several enzymes like the 7-ethoxyresorufin O-deethylase (EROD) and aryl-hydrocarbon-hydroxylase (AHH) as well as the metabolism of 3,3´4,4- tetrachlorobiphenyl

(TCB). The CYP1A5 on the other hand mediates the action of arachidonic acid epoxygenase, which is more specific for the liver of birds than of mammals (Sigel and Pyle, 2007).

1.6.2 CYP2C subfamily

The CYP2 enzyme family is of broad interest because they play a major role in the biotransformation of a variety of endogenous substances such as lipids, steroids and vitamins, as well as several xenobiotics (Nelson et al., 1993). Typical substrates of CYP2C9 enzyme are nonsteroidal anti-inflammatory drugs and hypoglycaemics. CYP2C9 substrates are weakly anionic and fairly lipophilic (Anzenbacher and Anzenbacherova, 2001). The CYP2C8 is an enzyme which is highly active during drug metabolism and present in a great quantity in the human liver (Backman et al., 2016; Daily and Aquilante, 2009).
CYP2C45 is the first detected avian CYP gene belonging to CYP2C subfamily (Baader et al., 2002). Other members are the CYP2C23b, which can be induced by phenobarbital, and the CYP2C23a (Watanabe et al., 2013). Both were formerly called CYP2H in chickens but due to their close relation to the human and mouse CYP2C members they were reclassified and renamed. The high abundance of CYP2C subfamily in chickens indicates the importance of these enzymes in avian drug metabolism (Watanabe et al., 2013). The CYP2Cs also can be used as biological markers to detect accumulation and contamination of the environmental pollution in wild animals (Kubota et al., 2011).

1.6.3 CYP3A subfamily

The CYP3A subfamily in mammals is responsible for up to half of the total cytochrome P450 in the liver and accounts for approximately 50% of the metabolism of clinically important drugs. The principle reaction of the CYP3A enzymes is the testosterone-6β-hydroxylation in various animal species, except for dogs, which have a very low activity of this enzyme. This mode of action is covered by the CYP3A6 in rabbits, which is also the predominantly expressed isoform of its family in the rabbit liver. The rabbit CYP3A6 isoenzyme seems to have high similarity with the human CYP3A4 isoenzyme (Palocz et al., 2017), their substrate spectrum presumably overlaps greatly.

1.6.4 Drug interactions

Any given drug preferentially induces one form of cytochrome P450 or a particular group of P450s. Drug-provoked induction is the most important source of drug interactions from a pharmacological point of view. A drug may induce its own metabolism and also other drugs can be further catalysed by the initially induced P450.

Competitive or non-competitive inhibition of CYP450 enzyme activity can result in the reduced metabolism of other drugs or endogenous substrates. Competitive inhibition refers to the direct competition of two substances for the catalytic site of the respective CYP. It is called non-competitive inhibition when substrates bind to other parts of the CYP proteins resulting in conformational changes. Inhibition can be caused by a number of commonly used drugs.

The highest risk of drug toxicity following co-medications is associated with inhibitors causing the generation of reactive metabolites that can bind irreversibly to individual CYPs and terminate their function (suicide inhibition).

The ability of macrolide antibiotics to interact with the biotransformation of some other drugs has been widely recognized. Macrolides can induce their own hepatic biotransformation into nitrosoalkanes. Nitrosoalkanes subsequently form inactive complexes with the iron of CYP enzymes and this then leads to the inhibition of the CYP3A-mediated catalytic activity (Pessayre et al., 1985). This mechanism accounts for most of the drug interactions produced by macrolides (Westphal, 2000). Macrolide antibiotics can interact adversely with commonly used drugs, by altering their metabolism due to the inhibition of CYP3A (von Rosensteil and Adam, 1995). This results in a decreased biotransformation rate of simultaneously administered drugs. Drug interactions with carbamazepine, cyclosporin, terfenadine and theophylline represent the most common encountered interactions with macrolide antibiotics.

In previous studies about the relative CYP3A inhibiting potency of a series of macrolides and pleuromutilin in microsomal fractions of goat and cattle, tiamulin and triacetyloleandomycin were proven to be effective inhibitors of CYP450 activity. Tylosin tartrate showed a typical complex formation and was found to be a weak inhibitor of the CYP3-catalysed hydroxylation of testosterone (Zweers-Zeilmaker et al., 1999). Tiamulin is mainly metabolized in the liver, and causes CYP450-mediated toxic interactions with other substances, e.g. coccidiostats such as ionophore antibiotics, mainly monensin or narasin, which leads to the accumulation of ionophores consequently lethal intoxication that causes damage of the liver and other tissues and will result in the death of the birds. Other drug-drug interactions was observed with tetracyclines, tiamulin has been shown to have an enhanced activity with them (Islam et al., 2009).

Despite the highly conserved functions of cytochrome P450 enzymes, significant inter-species variance exists in the activity and substrate-specificity of individual enzymes.
In general, the beneficial effect of feed additives as e.g. probiotics, plant extracts or fungal polysaccharides have been described and proven, but the specific mechanism of action usually remains unknown. Understanding their participation in metabolic processes brings us closer to clarifying their mechanism of action.

2 Aims of the study

The protective effect of two probiotics and four commercially available feed additives on endotoxin-induced inflammatory responses was observed in porcine intestinal cells. Furthermore, the impact of the test supplements on the expression of cytochrome P450 genes was investigated.

A highly sensitive luminescence assay has been developed for assaying the human cytochrome system (Cali et al., 2006), and is specific for CYP isoenzyme activity. Our goal is to test the luminescent assay's applicability for rabbit CYP detection in rabbit hepatocytes, assuming functional similarity between rabbit CYP3A6 and human CYP3A4 as the two enzymes are highly similar (Franklin, 1995). We also aim to compare the changes of CYP1A1, CYP1A2 and CYP3A6 gene expression levels, *in vivo* and *in vitro*.

The aim of this study is to examine the effect of four substances; β-glucan, a drinking water acidifier (DWA), a sanguinarine containing product (SN) and fulvic acid to the hepatic CYP mRNA expression level and CYP enzyme activity of chickens (*Gallus gallus domesticus*).

In order to measure expression levels accurately, normalization by multiple housekeeping genes instead of one is required (Vandesompele et al., 2002). However regarding to the gene expression levels in the chicken species the scientific data contains calculations with only one housekeeping gene (Feng et al., 2012; Ghareeb et al., 2013; Kim et al., 2014; Watanabe et al., 2013; Zhao et al., 2014). The CYP mRNA expression levels during our examinations are calculated using both one and two reference genes to demonstrate the potential differences between the two calculation methods.

Another goal of our examinations is to evaluate the effect of the aforementioned additives and *Bacillus licheniformis*, which are commonly used supplements in the chicken industry, on the chicken hepatic CYP enzymes and detect whether there are interactions between feed additives and the antibiotic tiamulin. Their effect on the liver enzymes CYP1A and CYP2C are measured. Tiamulin is used as a model CYP inhibitor compound and its action in different concentrations on the CYP enzymes is also determined.

Further aim of this study is to examine the effect of two commercially available feed supplements in rabbit drug metabolism: fulvic acid and a sanguinarine-containing product. Their influence on CYP2C and CYP3A6 of the CYP450 enzyme system is investigated *ex vivo*. The antibiotic tylosin is used as model agent that acts as CYP enzyme inhibitor to monitor the possible supplement-antibiotic interaction on the CYP enzymes. Tylosin's effect on the CYP450 enzyme system, as well as the supplements' effect is examined separately, and also the effect of fulvic acid and sanguinarine combined with tylosin is measured.

The other goal of our study is to investigate the effect of four authorized drinking water supplementers; (1-3), (1-6) β-glucans, fulvic acid, sanguinarine containing product and a water acidifier on the efficacy of *Pasteurella multocida* vaccination of chickens.

3 Materials and methods

3.1 *In vitro* studies

3.1.1 Bacterial culturing

Lactobacillus rhamnosus DSM7133 (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures), *Enterococcus faecium* CECT 4515 (Spanish Type Culture Collection) were grown in DeMan, Rogosa, Sharpe broth (MRS). *Bacillus licheniformis* DSM5749 (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures) was grown in tryptone soya broth (TSB). Inoculation of bacteria was accomplished with a stationary culture of a bacterial strain (1% inoculum). The bacteria were grown for 24 h at 37 °C and subcultured at least twice prior to the experiments.
Before the experiments, the bacterial cultures were centrifuged for 15 minutes at 1500 g, and then the cell culture supernatant was filtered through a 0.22 µm filter. Prior to the treatment the spent culture supernatant (SCS) of each bacterium were diluted in culture medium to achieve the desired concentration, and the pH value of them were adjusted to 7.2.

3.1.2 Cell line and culture conditions

The non-transformed porcine intestinal epithelial cell line IPEC-J2, originally isolated from jejunal epithelia of a neonatal unsuckled piglet (Schierack et al., 2006), was a kind gift of Dr. Jody Gookin, Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, NC, USA. IPEC-J2 cells were grown and maintained in complete medium, which consisted of a 1:1 mixture of Dulbecco's Modified Eagle's Medium and Ham's F-12 Nutrient Mixture (DMEM/F12) (plain medium) supplemented with 5% foetal bovine serum (FBS), 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenium, 5 ng/ml epidermal growth factor and 1% penicillin-streptomycin (all from Lonza Group Ltd, Belgium). Cells were grown at 37 °C in a humidified atmosphere of 5% CO_2.
IPEC-J2 cells were seeded onto six-well transwell polyester membrane inserts (Corning Inc., Corning, NY, USA), the latter coated with 8 µg/cm^2 rat tail collagen type I (Sigma-Aldrich, Steinheim, Germany), at a density of 10^6 cells/ml (the volume of complete medium was 1.5 ml on the apical side and 2.5 ml on the basolateral side per well according to the manufacturer's instructions). Cells could adhere for 24 h before being washed and re-fed every other day. Transepithelial electrical resistance (TEER) measurement of monolayers was performed on alternate days after seeding, from day 5 of culture, using an EVOM Epithelial Tissue Volt/Ohmmeter (World Precision Instruments, Berlin, Germany).

3.1.3 Neutral Red uptake assay for cell viability

Influence of lipopolysaccharide (LPS), SCS of *E. faecium* and *L. rhamnosus*, sanguinarine containing product (SN) (5 and 50 µg/ml.), drinking water acidifier (DWA), β-glucan and fulvic acid on the viability of enterocytes was tested. The substances were dissolved and diluted in cell culture medium. IPEC-J2 cells were seeded onto a 96-well plate and incubated with the test substances for 1, 4 and 24 h. Viability of IPEC-J2 cells was measured 24 hours after treatment by Neutral Red uptake assay as described by Repetto et al (Repetto et al., 2008).

3.1.4 Treatment of cell cultures

Before treatment, confluent monolayers of the IPEC-J2 cells were washed with plain medium. LPS and the other solutions were prepared freshly prior to each experiment. LPS (Lipopolysaccharides from *Escherichia coli* O26:B6, Sigma-Aldrich, Steinheim, Germany) was added in plain medium at 10 µg/ml on the apical side of the IPEC-J2 layer. The other substances were added alone or simultaneously with LPS in the following concentrations: *E. faecium* SCS and *L. rhamnosus* SCS (1 and 10%), sanguinarine containing product (SN) (5 and 50 µg/ml.), drinking water acidifier (DWA) (0.1 and 1 µl/ml), β-glucan (5 and 50 µg/ml) and fulvic acid (25 and 250 µg/ml). After 1 h incubation with LPS and the other substances, cells were washed with plain medium and cultured for additional 1 h for PCR studies and additional culture plates for another 24 h for ELISA measurements. TEER measurements were performed both before and after the LPS treatment.

Applied supplements:

Bakers' yeast β-glucan (Wellmune WGP®, Biothera Company, USA)

Sanguinarine containing product (Sangrovit® WS, Phytobiotics Gmbh., Germany)

Drinking water acidifier (Immunofort®, Europharmavet Ltd., Hungary)

Fulvic acid (Fulvix pulvis®, Alpha-Vet Ltd., Hungary) (Palócz et al., 2019a)

3.1.5 Quantitative PCR

One hour after the treatment, culture medium was removed and 1 ml of ice-cold RNAzol RT reagent (Sigma-Aldrich, Steinheim, Germany) was added to the IPEC-J2 samples. Samples were collected and kept at -80°C until further processing. Total RNA was isolated from the cells according to the manufacturer's instructions. Quantity, A260/A280 and A260/A230 ratios of the extracted RNA were determined using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, USA).

Synthesis of the first strand of cDNA from 1000 ng of total RNA was achieved using RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Roth, Germany) according to the manufacturer's recommendations, using the random hexamer as a priming method. Quantitative real-time PCR (qPCR) was performed according to (Palocz et al., 2016) on the MiniOpticon System (BioRad). The cDNA was diluted 5-fold, before equal amounts were added to duplicate qPCR reactions. Tested genes of interest were IL-6, IL-8, TNF-α, CYP1A1, CYP1A2, CYP3A29 and Hsp70. Hypoxanthine phosphoribosyl transferase (HPRT) and Cyclophilin-A (CycA) were used as reference genes. 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 (BioRad, Hercules, CA, USA) and 0.2 µM of the appropriate primers. The thermal profile for all reactions was 3 min at 95 °C, then 40 cycles of 20 sec at 95 °C, 30 sec at 60 °C, and 30 sec at 72 °C. At the end of each cycle, the fluorescence monitoring was set for 10 seconds.

To determine the efficiencies of the PCR reactions, standard curves were obtained for each target and reference gene, using serial dilutions of a reference cDNA. Real-time PCR efficiencies (E) were calculated according to the equation: E=10(-1/slope).

Table 1. Sequence of primer sets for porcine genes, used for quantitative PCR

Gene symbol	Accession number	Primer sequences (5'-3')	Product size (bp)	Efficiency	Reference
IL-8	NM_213867	F: AGAGGTCTGCCTGGACCCCA R: GGGAGCCACGGAGAATGGGT	126	1.972	(Paszti-Gere et al., 2012)
IL-6	NM_214399	F: TTCACCTCTCCGGACAAAAC R: TCTGCCAGTACCTCCTTGCT	122	1.970	(Sakumoto et al., 2006)
TNF-α	NM_214022	F: TTCCAGCTGGCCCCTTGAGC R: GAGGGCATTGGCATACCCAC	146	1.873	(Hyland et al., 2006)
CYP1A1	NM_214412.1	F: CAGAGCTGCTTAGCCTTATCAACC R: CTGGATGCTGGGATTTGTCACCAG	386	2.00	(Kojima et al., 2010)
CYP1A2	NM_001159614.1	F: GTGAGGAGATGTTCAGCATCGTGAAG R: CTTCTGTATCTCAGGATATGTCACA	386	1.750	(Kojima et al., 2008)
CYP3A29	NM_214423.1	F: TTCGTGCTTCACAGAGAGACCC R: TACTAGGTGGGGGTGGATGG	576	1.975	(Farkas et al., 2014)
Hsp70	NM_001123127	F: GCCCTGAATCCGCAGAATA R: TCCCCACGGTAGGAAACG	152	2.0	(Zhong et al., 2010)
CycA	NM_214353	F: GCGTCTCCTTCGAGCTGTT R: CCATTATGGCGTGTGAAGTC	160	1.907	(Hyland et al., 2006)
HPRT	NM_001032376	F: GGACTTGAATCATGTTTGTG R: CAGATGTTTCCAAACTCAAC	91	1.963	(Nygard et al., 2007)

CycA: cyclophilin A, CYP: cytochrome P450, HPRT1: hypoxanthine phosphoribosyl transferase-1, Hsp70: heat shock protein 70, IL: interleukin, TNF-α: tumor necrosis factor alpha, F: forward, R: reverse

3.1.6 ELISA measurement

After the treatments with 10 µg/ml LPS, 25 µg/ml fulvic acid, 1 and 10% SCS of *E. faecium* and *L. rhamnosus* (1 h), IPEC-J2 cells, were washed and incubated in culture medium for another 24 h. Culture media were collected after 6, 12 and 24 h after treatment to measure the IL-6 and IL-8 protein concentrations. Level of IL-6 and IL-8 secretion was determined by IL-6 and IL-8 porcine-specific ELISA kits (ThermoFisher Scientific, Waltham, USA) according to the manufacturer's instructions.

3.1.7 Rabbit hepatocyte isolation and plating

Livers were obtained from female, six-week-old New Zealand white rabbits weighing approximately 1.5 kg. The animals were housed in stainless steel cages, two rabbits per cage, provided food and water *ad libitum* for one week before use. Rabbits were anesthetized by the combination of xylazine (5 mg/kg bw., 2% solution) and ketamine (60 mg/kg bw., 10% solution) intramuscularly. After aseptic opening of the abdominal cavity the portal vein of the liver was cannulated, and rabbit liver was perfused with calcium-free Hank's buffer containing 0.5 mM EGTA for 10 min at a rate of 30 ml/min. The liver was then removed from the abdominal cavity and perfused with Hank's buffer containing 2.5 mM calcium-chloride and 2.5 mM magnesium-chloride and collagenase type IV (0.5 mg/ml) for 25 min at a flow rate of 30 ml/min. All perfusion buffers were preheated to 37 °C and were equilibrated with 95% O_2/5% CO_2 before use.

The next steps were performed under laminar flow. The liver was removed from the Glisson capsule and gently shaken in BSA buffer (25 mg/ml) to release the hepatocytes. The obtained suspension was filtered through a 125 µm pore size Nylon mesh and then centrifuged at 105 g for 4 min., after which the cell pellet was re-suspended in Williams E medium (Sigma-Aldrich, St. Louis, USA) containing 2 mM glutamine and 7.5% $NaHCO_3$. The washing procedure was repeated twice. The plating medium was Williams E supplemented with 5% FBS, 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenium, 5 ng/ml EGF, 50 IU/ml penicillin, 50 µg/ml streptomycin, 1 µM dexamethasone. The viability of the hepatocytes as assessed by the trypan blue exclusion test was 88%. Hepatocyte cells (10^6/0.5 ml/well) were added to 24-well polystyrene plates (Corning, New York, USA) coated with collagen type I (Sigma-Aldrich, St. Louis, USA). After 4 hours of incubation at 37 °C in 5% CO_2 atmosphere the cells were attached to the surface (Figure 1).

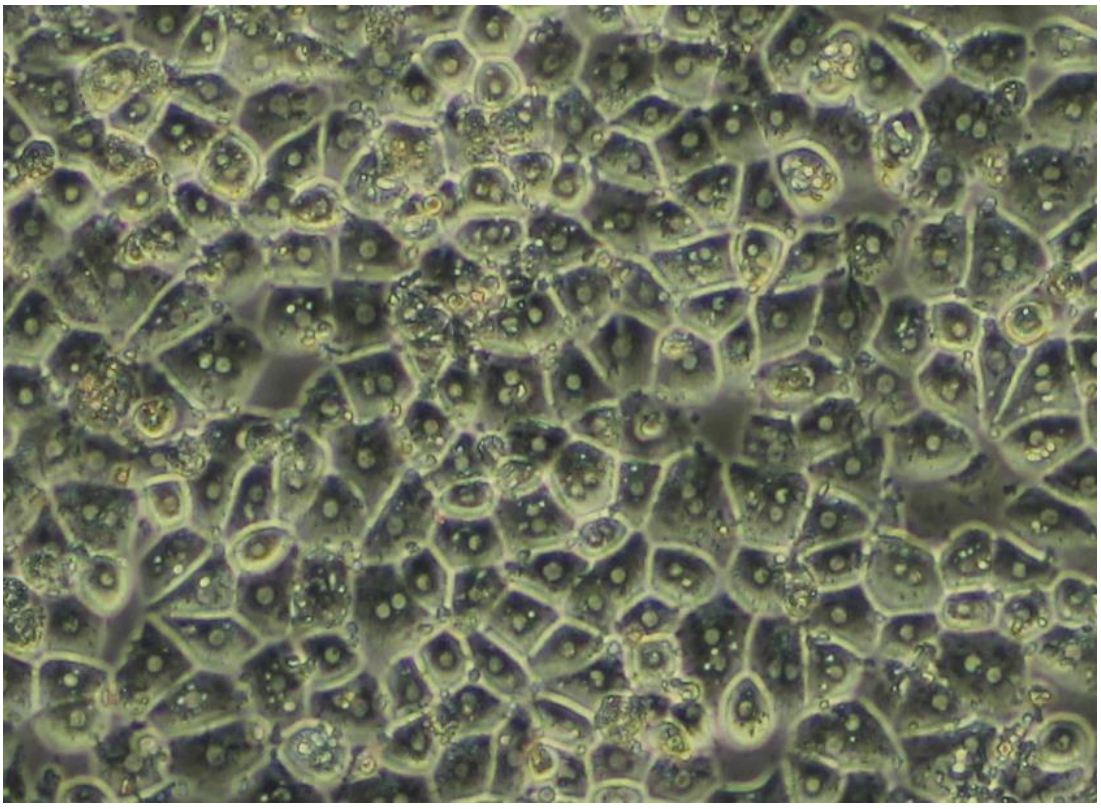


Figure 1. Rabbit primary hepatocyte cell culture after 4 h incubation (Palocz et al., 2017). Phase-contrast micrograph, magnification: 200×. Primary hepatocytes display the typical cubic cell shape and often contain two nuclei.

3.1.8 Rabbit hepatocyte treatment

Four hours after isolation the cells were washed twice with serum-free medium and treated either with inducer or with inhibitors for 2 hours. Phenobarbital, ketoconazole and alpha-naphthoflavone were used as model compounds to induce stimulation or inhibition of the CYP enzymes. For general CYP450 enzyme inhibition we used 500 µM phenobarbital, for CYP1A2 inhibition 50 µM alpha-naphthoflavone and for CYP1A1 and CYP3A6 inhibition 25 µM ketoconazole. After the treatment the cells were washed twice in PBS, and then the P450-Glo™ assays (Luciferin-CEE, Luciferin-1A2, Luciferin-IPA, Luciferin-PFBE; Promega, Madison, USA) were performed according to the manufacturer's recommendation, the luminogenic substrates were added to each well respectively. After the required time the Luciferin Detection Reagents (Promega, Madison, USA) were added to the cells and the luminescence was detected by a luminometer (Victor X2, PerkinElmer, Massachusetts, USA). The hepatocyte cells were collected in tergitol buffer for protein determination (Bicinchoninic Acid Protein Assay Kit, Thermo Scientific, Rockford, USA).

Xenobiotics: Our study utilised phenobarbital and ketoconazole, which are characteristic inducer and inhibitor agents of CYP450 enzymes.

3.1.9 Quantitative PCR

After treatment, culture medium was removed. Five hours after treatment, 1 ml of ice-cold RNAzol RT reagent (Sigma-Aldrich) was added to the hepatocyte culture and the samples collected and kept at -80 °C until further processing. Total RNA was isolated from the cells as it was described at 2.1.4 chapter. The tested genes were CYP1A1, CYP1A2 and CYP3A6. Hypoxanthine phosphoribosyl transferase-1 (HPRT1), glyceraldehyde-3-phophate dehydrogenase (GAPDH) and beta-actin were used as reference genes. Primer sequences are listed in Table 2. 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 3 min at 95 °C, then 40 cycles of 20 sec at 95 °C, 30 sec at 60 °C, and 30 sec at 72 °C. At the end of each cycle, fluorescence monitoring was set for 10 seconds.

Table 2. Sequence of primer sets for rabbit genes, used for quantitative PCR

Gene symbol	Accession number	Primer sequences*	Product size (bp)	Efficiency	R^2
GAPDH	NM_001082253	F 5'-GGCAAAGTGGATGTTGTCGC-3' R 5'-GCCGTGGGTGGAATCATACT-3'	87	0.887	0,988
Beta-actin	NM_001101683	F 5'-GTGCTTCTAGGCGGACTGTT-3' R 5'-CGGCCACATTGCAGAACTTT-3'	240	0.766	0,986
HPRT1	NM_001105671	F 5'-AGCCCCAGCGTTGTGATTAG-3' R 5'-TCGAGCAAGCCTTTCAGTCC-3'	141	0.905	0,982
CYP1A1	NM_001171072	F 5'-CTTCGGAGCTGGGTTTGACA-3' R 5'-AGAGGTGTGTCGGAAGGTCT-3'	199	0.960	0,982
CYP1A2	NM_001171121	F 5'-GTGGCAAATCAACCACGACC-3' R 5'-CGTGACCTTCTCACTCAGGG-3'	115	0.813	0,983
CYP3A6	NM_001171268	F 5'-CAAAAGCGCCGAGTGGATTT-3' R 5'-AAGTCTGCCAGCAATTGGGT-3'	327	0.731	0,990

*All primers were designed using the Primer-BLAST tool (Jian et al., 2012).

HPRT1: hypoxanthine phosphoribosyl transferase-1, GAPDH: glyceraldehyde-3-phophate dehydrogenase, CYP: cytochrome P450, F: forward, R: reverse

3.2 *In vivo* experiments

3.2.1 Rabbits housing and treatments

Twelve, clinically healthy, six-week-old New Zealand White rabbits (S & K Lap Nyúltenyésztő Kft., Kartal, Hungary) were used in this study. The animals were housed in stainless steel cages (40x60x80 cm), two rabbits per cage in the animal housing facility of the Department of Pharmacology and Toxicology, acclimated at 20 ± 2 °C on a 12 h light 12 h dark schedule. One hundred grams of commercial pellet feed was provided daily, water was available *ad libitum*. The rabbits (mean weight 1350 ± 96 g) were randomly divided into three groups comprising a control group, a phenobarbital group (80 mg/kg bw.) and a ketoconazole group (40 mg/kg bw.). The xenobiotics were administered to the rabbits for three consecutive days, *per os*. On the third day the rabbits were anesthetized by a combination of Zoletil® injection (0.3 ml/kg bw.) and Xylavet® injection (0.25 ml/kg bw.) intramuscularly. After aseptic opening of the abdominal cavity the portal vein of the liver was cannulated, the liver was perfused with neutral buffered saline and then removed, flash-frozen in liquid nitrogen and stored at -80 °C till further processing.

3.2.2 Chickens housing and treatments

Forty-five clinically healthy five-week-old broiler chickens (Herbro Kft., Hernád, Hungary) were used in this study. The animals were housed in stainless steel cages four birds per cages, acclimated at 20 ± 2 °C on a 12 h light 12 h dark schedule. Feed and water were provided *ad libitum*.

The chickens (mean weight 749 ± 94 g) were randomly divided into 9 groups (5 chickens/group) comprising 1 control group and 8 experimental groups. Four of the 8 experimental groups received the supplement at the recommended dose, while the other 4 were given tenfold dose of the additive. The groups were as follows. Sanguinarine containing product receiving groups (SN) (5 and 50 mg/kg bw.), drinking water acidifier receiving groups (DWA) (0.1 and 1 ml/kg bw.), beta-glucan receiving groups (5 and 50 mg/kg bw.) and fulvic acid receiving groups (25 and 250 mg/kg bw.). The test substances were freshly dissolved in water daily and were administered via probe (2 ml/kg bw.) to the birds individually for five consecutive days. The controls received the same amount of water via probe (placebo).

A day after the last treatment day the chickens were euthanized (Euthasol® 1 ml/kg bw. icoe.) and their livers were perfused *in situ* with physiological saline solution until the blood was drained. Then the livers were collected and shock frozen in liquid nitrogen and stored at -80 °C until further processing.

3.2.3 Microsome separation

The livers were homogenised with two volumes of ice-cold buffer (1.15% KCl, 0.1 mM EDTA, pH 7.4) by a Potter-Elvehjem homogenizer (Schuett Biotec GmbH, Göttingen, Germany). Microsomes were isolated by a two-step differential ultracentrifugation (Beckman L7-65 Ultracentrifuge, Beckman-Coulter) according to (Nebbia et al., 2001).

For the isolation, KCl (1.15%)/EDTA (0.1 mM) solution was used, as well as a phosphate buffer with the pH of 7.4 (0.1 M) with EDTA (0.1 mM) and glycerol. All steps were carried out at 4 °C. The liver was put in a beaker and cold KCl-solution was added – approximately twice as much as the liver's weight. Then the liver was cut into small pieces and homogenized with a Potter apparatus. The suspension was then centrifuged for 20 minutes at 10 000 g. Afterwards the supernatant was carefully pipetted into a clean ultra-centrifuge, without pipetting the fat layer on top of the pellet. This was centrifuged for 1 hour at 105 000 g. The supernatant was decanted and KCl/EDTA solution was added to it. The pellets (subcellular fractions) were homogenized with the ultra-turrax. This mixture was then centrifuged again for 1 hour at 105 000 g. The gained microsome fractions were resuspended with 0.1 M phosphate buffer, pH 7.4, containing 0.1 mM EDTA and 20% glycerol (w/v) to get a protein concentration of about 20 mg/ml. The suspension was pipetted into microcentrifuge tubes and shock-frozen in liquid nitrogen. At the end, the microsome suspension was stored at -80 °C until further processing.

Protein concentrations were measured by Pierce™ BCA protein assay kit (ThermoFisher Scientific, Waltham, MA, USA).

3.2.4 Quantitative PCR

The liver samples (100 mg) were homogenised in 1 ml of ice-cold RNAzol RT reagent (Sigma-Aldrich, Steinheim, Germany) by a Potter-Elvehjem homogenizer (Schuett Biotec GmbH, Göttingen, Germany). Total RNA was isolated from the cells as it was described at 2.1.4 chapter. Quantitative real-time PCR (qPCR) was performed according to (Palócz et al., 2019b). The tested genes of interest were the avian cytochrome P450 1A4 (CYP1A4), cytochrome P450 2C23a (formerly known as CYP2H1), cytochrome P450 2C45 (CYP2C45) and cytochrome P450 3A37 (CYP3A37). As reference (housekeeping) genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and beta-actin were selected. Primer sequences are listed in Table 3. The thermal profile for all reactions was 2 min at 95 °C, then 30 cycles of 10 sec at 95 °C, 20 sec at 56 °C, and 10 sec at 72 °C.

Table 3. Primer sequences specific for chicken genes used for qPCR

Gene	Accession number	Primer sequence (5'-3')	Efficiency	Length (bp)	Reference
B-actin	NM_205518.1	F: GTCCACCTTCCAGCAGATGT	0.956	169	(Csiko et al., 2014)
		R: ATAAAGCCATGCCAATCTCG			
GAPDH	NM_204305.1	F: GGTGGTGCTAAGCGTGTTAT	0.957	264	(Hong et al., 2006)
		R: ACCTCTGTCATCTCTCCACA			
CYP1A4	NM_205147.1	F: CCGTGACAACCGCCCTGTCC	0.912	115	(Csiko et al., 2014)
		R: AGCCGTGGTCTCCTCTCCCG			
CYP2C23a	NM_001001616.1	F: ACAACCAGCACCACACTGAG	0.921	206	(Csiko et al., 2014)
		R: GCATGTGGAACATTAAGGGG			
CYP2C45	NM_001001752.1	F: TGGTTACCTGGCTTACCAGC	1.00	151	(Watanabe et al., 2013)
		R: ATAGAGCCGGAGGGTTTCAT			
CYP3A37	NM_001001751.2	F: TGGTTACCTGGCTTACCAGC	0.826	160	(Csiko et al., 2014)
		R: ATAGAGCCGGAGGGTTTCAT			

F: forward, R: reverse, GAPDH: Glyceraldehyde 3-phosphate dehydrogenase, CYP: cytochrome P450

3.2.5 Cytochrome activity determination

The CYP1A, CYP3A and CYP2C activity of liver microsome samples were measured using the P450-Glo™ substrates (Luciferin-CEE, Luciferin-IPA, Luciferin-ME; Promega, Madison, USA) (Palocz et al., 2017). The assays were performed according to the manufacturer's recommendation; the luminogenic substrates and the NADPH regeneration system (Promega, Madison, USA) were added to each 5-fold diluted microsome sample respectively. After the required time, the Luciferin Detection Reagents (Promega, Madison, USA) were added to the mixture and the formed luminescence signal was detected by a luminometer (Victor X2, PerkinElmer, Massachusetts, USA).

The test substances were sanguinarine-containing product (Sangrovit®, Phytobiotics Gmbh., Germany), fulvic acid (Fulvix pulvis®, Alpha-Vet Ltd., Hungary), tiamulin fumarate and tylosin tartrate (Alfa Aesar, Massachusetts, USA). A stock solution of tiamulin was made with luciferin free water and then diluted to 0.5 µM, 5 µM, 50 µM, 500 µM, 2.5 mM and 5 mM final concentrations. A stock solution of tylosin 40 mM was prepared with luciferin free water and then diluted to 0.5 mM, 1 mM, 2.5 mM, and 5 mM final concentrations. A stock solution of SN was prepared and diluted to 5 µg/ml. The stock solution of fulvic acid was diluted to 25 µg/ml. 5% SCS of *B. licheniformis* were used.

Bioluminescence assay: The luminogenic compounds are beetle luciferin derivatives that are converted by CYP enzymes to luciferin. D-luciferin is formed and detected in a second reaction with the Luciferin Detection Reagent, generating luminescence proportional to CYP activity.

3.3 Semi-field trial

3.3.1 Chickens housing and treatments

Fifty plus ninety, clinically healthy, three week old Broiler chickens (Herbro Kft., Hernád, Hungary) were used (mean weight: 601 ± 92 g). The animals were housed in stainless steel cages (30x90x50 cm) five individuals per cages, acclimated at 20 ± 2 °C on a 12 h light 12 h dark schedule. The chickens for the first experiment were randomly divided into 5 groups (10 chickens/group) comprising 1 control group and 4 experimental groups: two pre-treated, and two pre- and post-treated groups, treated individually with 5 or 50 mg/kg bw. β-glucan (Immivet® 3-6 dispersible powder; Biothera Company, USA) for 5 or 10 days, respectively. Each animal was inoculated subcutaneously via *Pasteurella multocida* vaccine (Poulvac Pabac IV vaccine A.U.V., Zoetis Hungary Kft., Budapest, Hungary) once at age of one month.

The chickens for the second experiment were randomly divided into 9 groups (10 chickens/group) comprising 1 control group and 8 experimental groups: four pre-treated, and four pre- and post-treated groups. The pre-treated groups received either water soluble β-glucan (5 mg/kg bw.) (Wellmune WGP®, Biothera Company, USA) or fulvic acid (25 mg/kg bw.) (Fulvix pulvis®, Alpha-Vet Ltd., Hungary) or sanguinarine containing product (5 mg/kg bw.) (SN) (Sangrovit®, Phytobiotics Gmbh., Germany) or water acidifier (0.1 ml/kg bw.) (DWA) (Immunofort®, Europharmavet Ltd., Hungary) prior to vaccination for 5 days, the pre- and post-treated groups received the same treatment prior to and following vaccination for 5 days. Each supplements were mixed into the animal's drinking water. Each animal was inoculated subcutaneously via *Pasteurella multocida* vaccine (Poulvac Pabac IV vaccine A.U.V., Zoetis Hungary Kft., Budapest, Hungary) twice; for the first time at the age of one month and for the second time three weeks later.

3.3.2 Detection of *Pasteurella multocida* antibody

For the determination of *Pasteurella multocida* antibody titers from the first study; blood samples were collected from each chicken before vaccination and two weeks after the inoculation of *P. multocida* vaccine. For the determination of *Pasteurella multocida* antibody titers from the second study, blood samples were collected from each chicken before both inoculations and one week after the second vaccination. The blood samples were centrifuged at 1000 g for 10 min, the obtained serum samples were collected in clear tubes. ProFLOK® *Pasteurella multocida* ELISA kit (Synbiotics, MO, USA) were used for antibody detection.

The average sample per positive (S/P) ratio of each group was calculated according to the manufacturer's recommendation. The S/P ratios were calculated according to the following equation: S/P ratio (%) = [corrected optical density of a sample/corrected optical density of a positive reference serum].

3.3.3 Statistical analyses

Relative gene expression levels of the genes of interest were calculated by the Relative Expression Software Tool (REST) 2009 Software. Statistical analyses were performed by Statistica 13 software (Dell Inc., Round Rock, USA). Differences between means were evaluated by one-way analysis of variance (ANOVA) followed by a post hoc comparison using Dunnett's test or Fisher's least significant difference (LSD) test or Tukey's 'Honest Significant Difference' method. Differences were considered significant if the p-value was < 0.05.

3.3.4 Ethical approval

The animal trials were conducted according to approved laboratory animal experimentation ethics regarding to the national and European law, compatible with the conditions set up by the Guidelines for Ethical Conduct in the Care and Use of Nonhuman Animals in Research. The study was authorised by the Local Institutional Animal Care Committee of Faculty of Veterinary Science Szent István University (permit numbers: 3/2015, 27/2015).

4 Results

4.1 Results of the *in vitro* experiments

4.1.1 Cell viability test

Viable IPEC-J2 cells incorporated and bind the supravital neutral red dye in their lysosomes (Figure 2). Viability of the cells was monitored after LPS (10 µg/ml), SN (5 and 50 µg/ml.), DWA (0.1 and 1 µl/ml), β-glucan (5 and 50 µg/ml) and fulvic acid (25 and 250 µg/ml) treatment. Viability of the IPEC-J2 cells was not altered after 1, 4 and 24 h of the treatment with each test substance (Figure 3).

According to the measured TEER of the cell cultures before and after the treatments, the integrity of the monolayer IPEC-J2 cell cultures was not damaged due to the 1-hour treatment with the test substances.

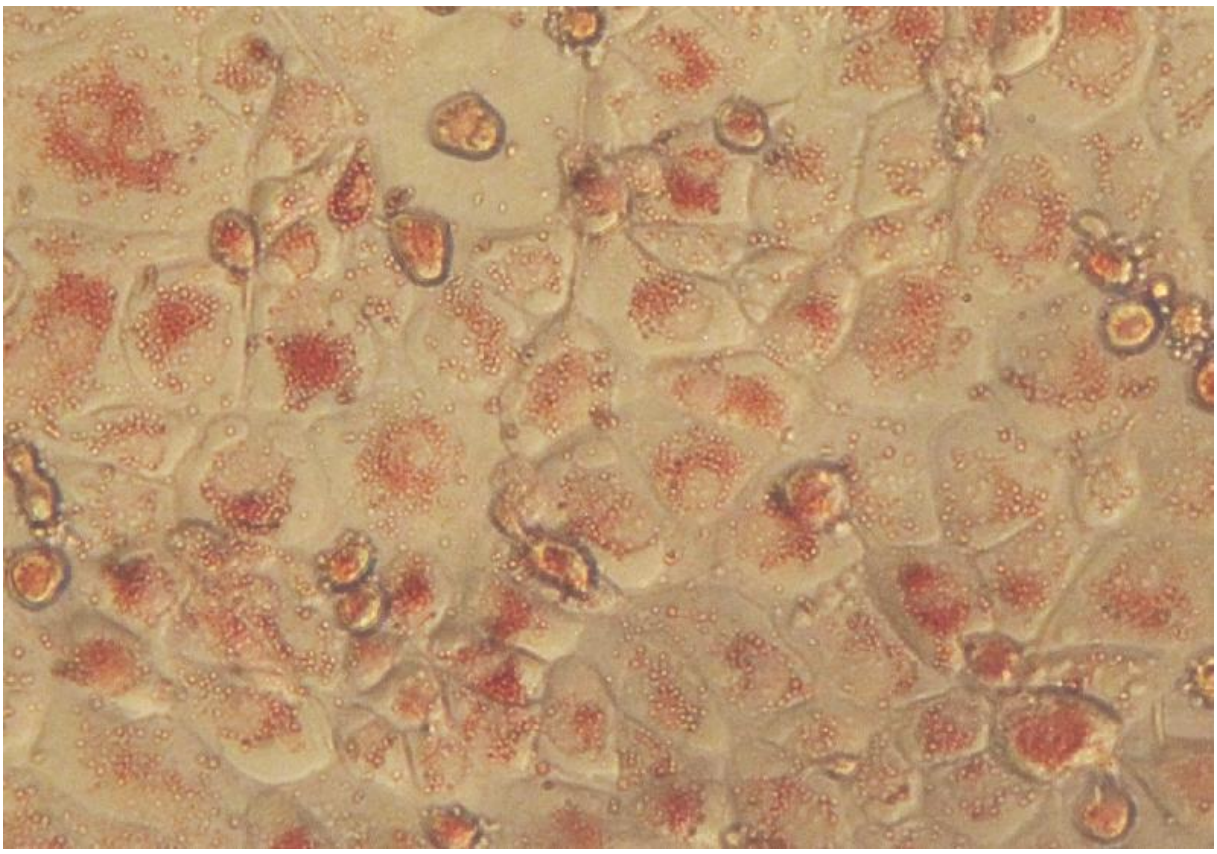


Figure 2. Phase contrast micrograph of IPEC-J2 cells, neutral red dye incorporated in their lysosomes. Magnification 200×.

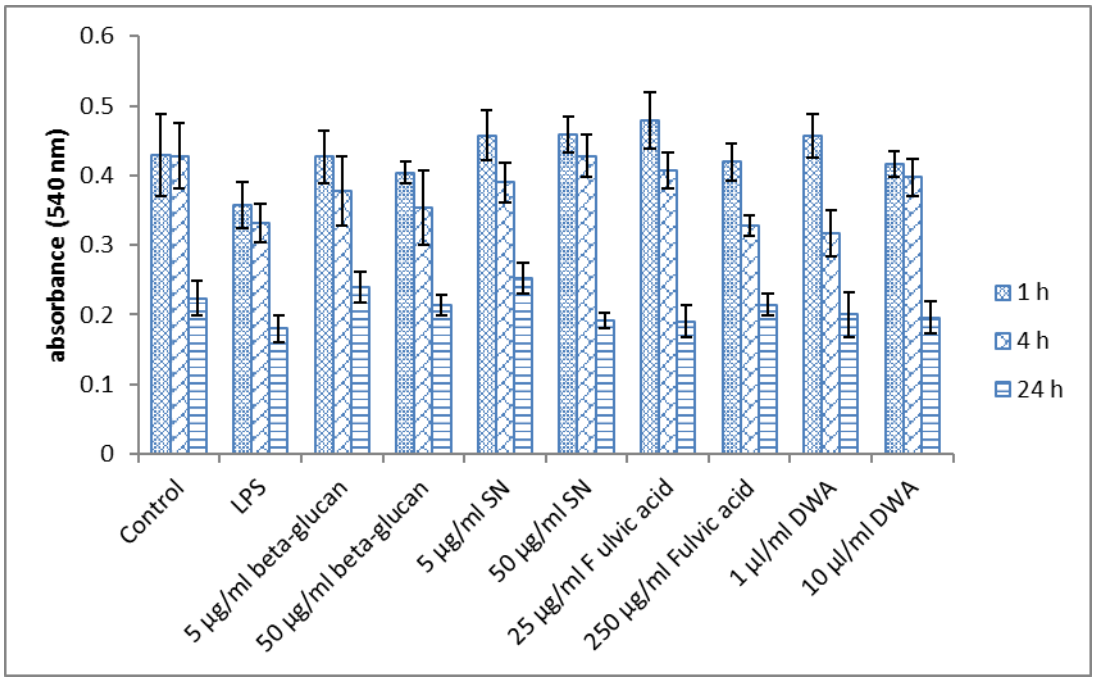


Figure 3. IPEC-J2 cell viability after 1, 4, and 24 h treatment. LPS concentration is 10 µg/ml; n=8/group; Data are shown as mean+SD. SN - Sanguinarine containing product, DWA - Drinking water acidifier

4.1.2 Effect of feed additives on inflammatory markers after LPS-treatment

The one-hour treatment with 10 µg/ml LPS upregulated the gene expression of IL-8 and TNF-alpha. The relative gene expression of IL-6 remained unchanged and TNF-alpha and Hsp70 were downregulated after the treatment with each supplement compared to the LPS-treated group. The simultaneous treatment of both β-glucan and fulvic acid with LPS resulted in increased IL-8 mRNA level (Figure 4).

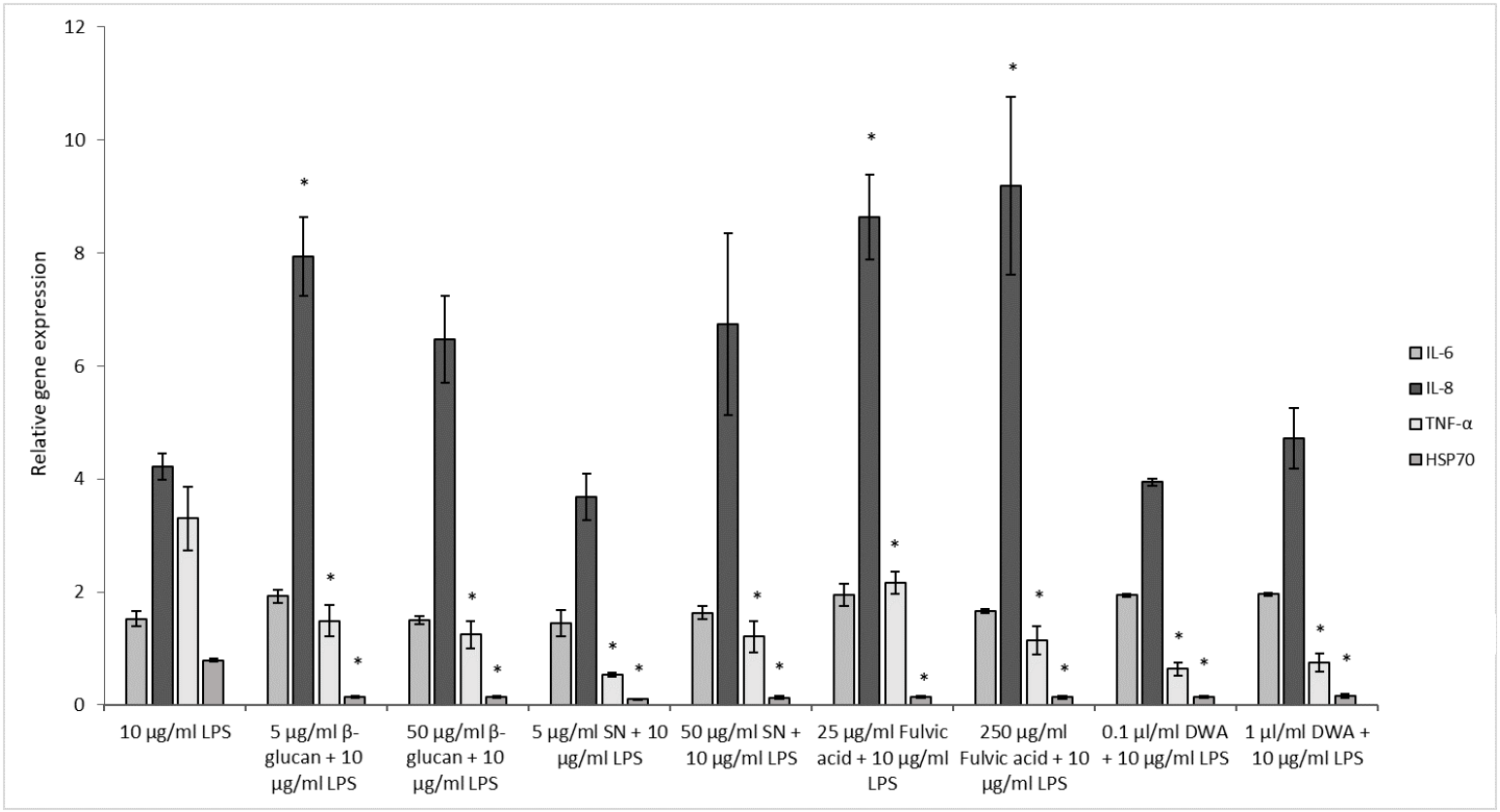


Figure 4. Gene expression (mRNA) of IL-6, IL-8, TNF-α, Hsp70 of IPECJ2 cells exposed to the treatments for 1 h relative to untreated controls (n=6/group) Significant differences are displayed compared to the LPS-treated group, *p<0.05. Data are shown as mean+SE. SN - Sanguinarine containing product, DWA - Drinking water acidifier

The measured IL-6 protein concentrations after LPS, LGG, ECF and fulvic acid treatment are shown in Figure 5. The 10 µg/ml LPS treatment increased the IL-6 protein level, but all other treatment layout prevented the extra IL-6 production. The produced IL-6 protein was found in the apical compartment 6 h after treatment, in the basolateral compartment no detectable IL-6 protein was measured.

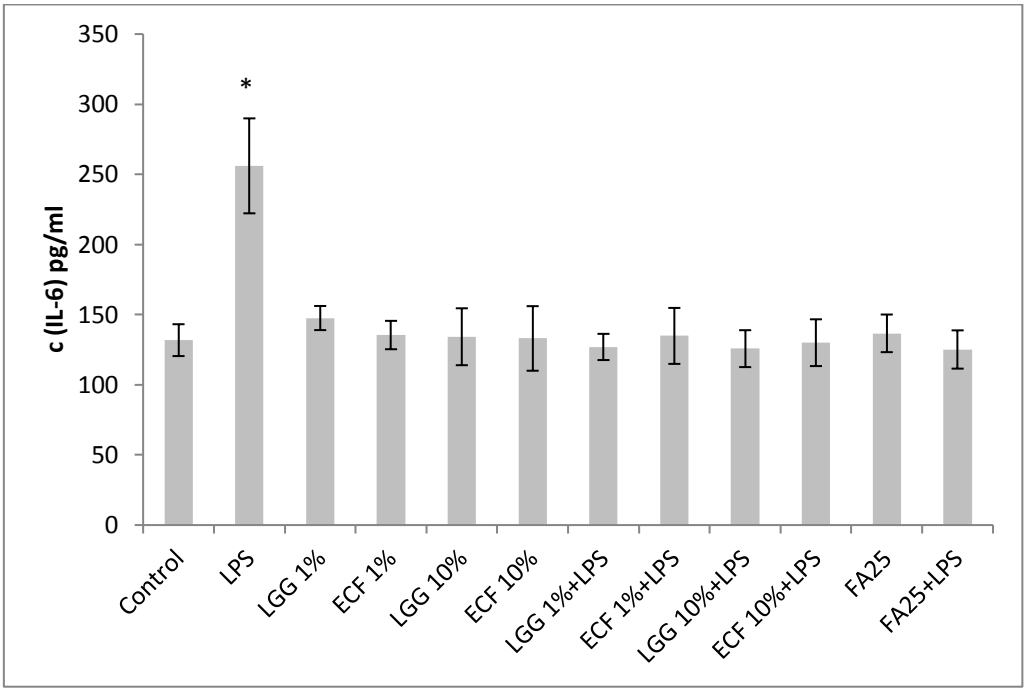


Figure 5. IL-6 protein level of IPEC-J2 cells after treatment with 10 µg/ml LPS, 1 and 10% of the supernatant of *Lactobacillus rhamnosus* (LGG) or *Enterococcus faecium* (ECF) or 25 µg/ml fulvic acid (FA). n = 3/group, *p<0.05 Data are shown as mean+SD.

The measured IL-8 protein concentrations after LPS, LGG, ECF and fulvic acid treatment are shown in Figure 6. The 10 µg/ml LPS treatment increased the IL-8 protein level, but all the other treatments prevented the IL-8 production. The produced IL-8 protein was found in the apical compartment 24 h after the cells were treated, in the basolateral compartment the IL-8 protein level was undetectable.

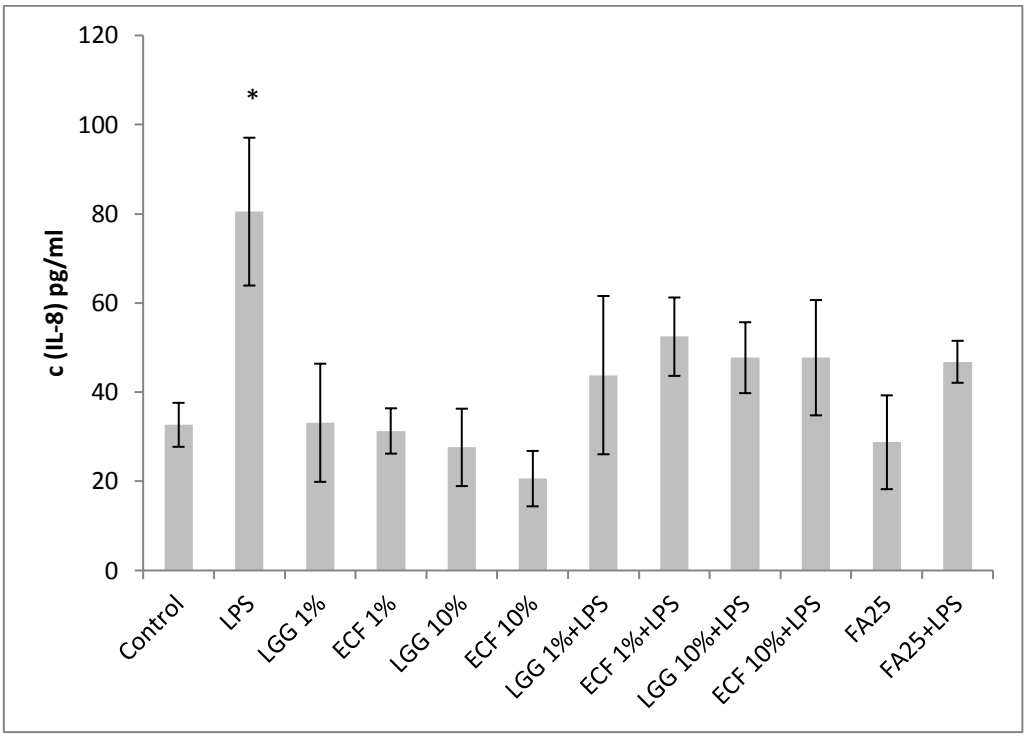


Figure 6. IL-8 protein level of IPEC-J2 cells after treatment with 10 µg/ml LPS, 1 and 10% of the supernatant of *Lactobacillus rhamnosus* (LGG) or *Enterococcus faecium* (ECF) or 25 µg/ml fulvic acid (FA). n = 3/group *p<0.05 Data are shown as mean+SD.

4.1.3 Effect of feed additives on the relative expression of CYP450 genes

CYP1A1 expression increased after one-hour SN, fulvic acid and high dose DWA treatment. CYP1A2 mRNA level enhanced following the treatment with both doses of SN, high dose fulvic acid and high dose DWA (Figure 7). None of the treatments changed CYP3A29 gene expression. The β-glucan treatment did not alter any of the CYP450 mRNA levels.

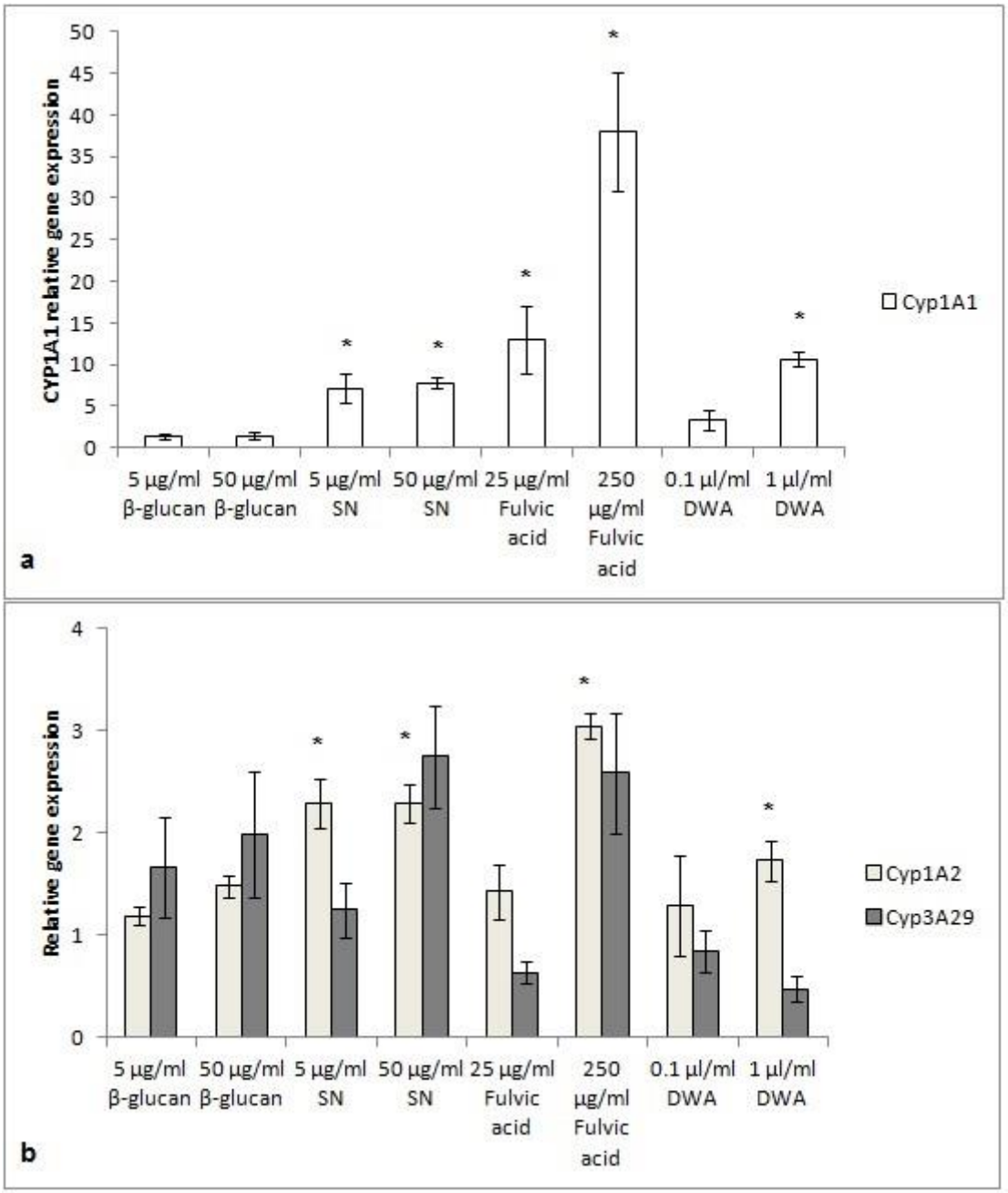
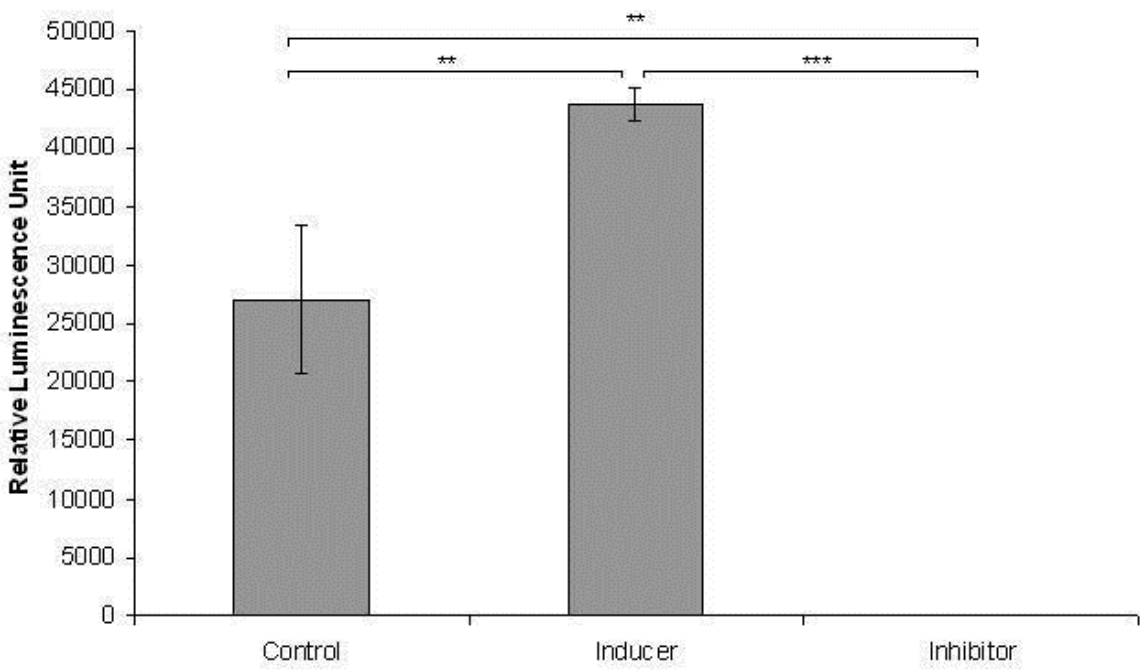


Figure 7. Relative expression of CYP1A1 (a), CYP1A2 and CYP3A29 (b) genes in feed additive treated IPEC-J2 cells compared to untreated controls (n=6/group, *p<0.05). Data are shown as mean+SE. SN - Sanguinarine containing product, DWA - Drinking water acidifier

4.1.4 CYP450 activity of rabbit primary hepatocytes

The rabbit primary hepatocyte cell culture protein concentration was effectively standardised (800 ± 30 µg/ml) in each well. By exclusion, any differences in CYP activity were therefore caused by induction or inhibition. Phenobarbital at 500 µM was an effective inducer of all three tested isoenzymes. Alpha-naphthoflavone at 50 µM had no effect on any of the three examined isoenzymes. Ketoconazole at 25 µM significantly inhibited CYP1A1 and CYP3A6.
In respect to CYP1A1, ketoconazole-evoked inhibition was so powerful that CYP1A1 activity was almost totally suppressed (Figure 8A). CYP1A2 was also inhibited, albeit not quite so effectively (Figure 8B). Regarding CYP3A6, both induction and ketoconazole-mediated inhibition were highly effective (Figure 8C).

A


CYP1A1
Relative Luminescence Unit
50000
45000
40000
35000
30000
25000
20000
15000
10000
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0
**
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Control
Inducer
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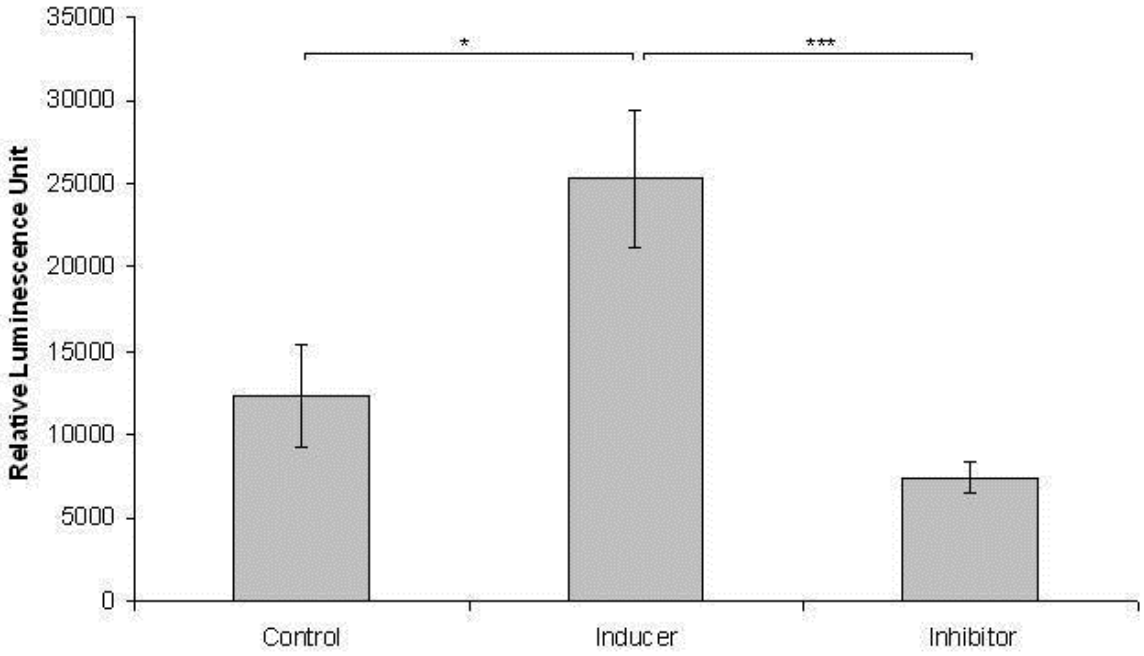
B


CYP1A2
Relative Luminescence Unit
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30000
25000
20000
15000
10000
5000
0
*
***
Control
Inducer
Inhibitor

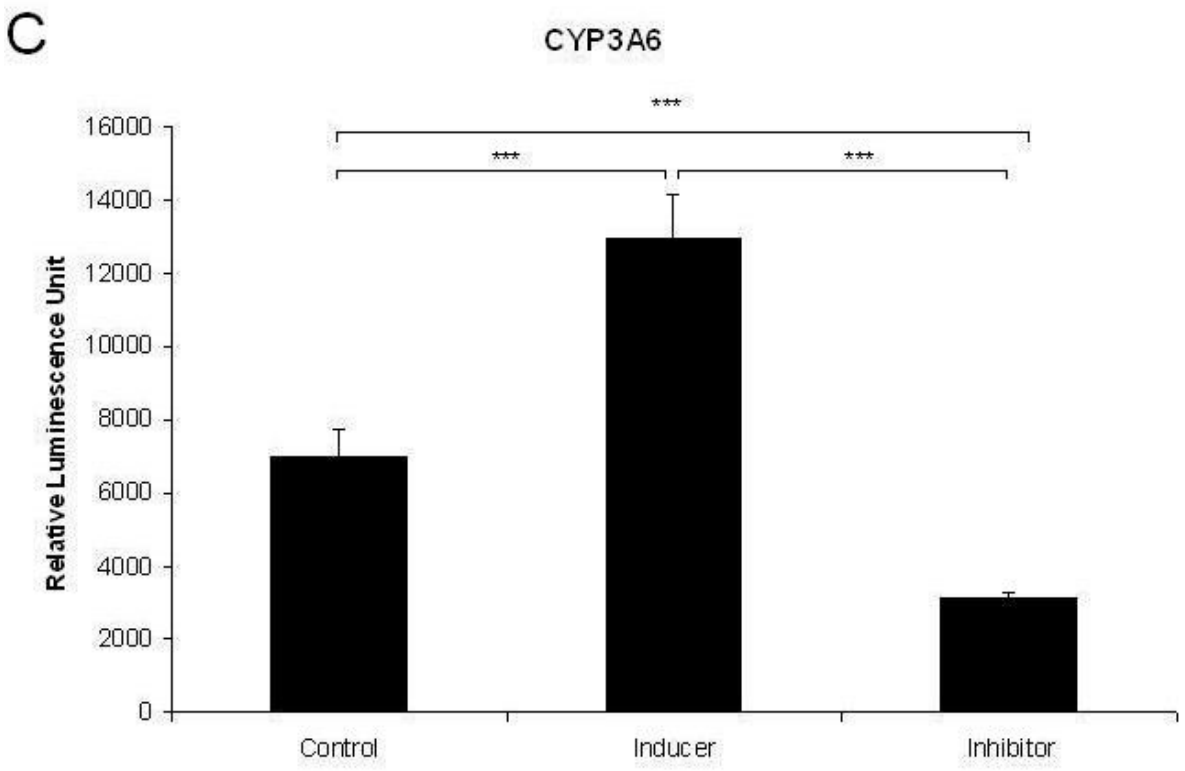


Figure 8. Changes in cytochrome enzyme activity in rabbit primary cell cultures. Control – non-treated, Inducer – treated with 500 μM phenobarbital for 2 h, Inhibitor – treated with 50 μM alpha-naphthoflavone and 25 μM ketoconazole for 2 h. (n = 4/group; *p < 0.05; **p < 0.01; ***p < 0.001) Data are shown as mean ± SE. (A) CYP1A1 activity. (B) CYP1A2 activity. (C) CYP3A6 activity.

Nonetheless only the Luciferin-IPA substrate was suitable for the rabbit CYP3A6 activity measurement, as the detected luminescent signal was not high enough when the Luciferin-PFBE substrate was applied. The obtained RLU (Relative luminescence unit) was 261±26 for the control cells, 312±77 for the induced cells and 212±15 for the inhibited cells. This substrate did not work well with the rabbit CYP3A6 enzyme.

4.1.5 Gene expression of rabbit primary hepatocytes

PCR results are shown in Figure 9. Phenobarbital treatment upregulated the CYP3A6 gene, while expression of CYP1A1 and CYP1A2 remained unchanged. CYP1A1 and CYP3A6 genes were upregulated after treatment with ketoconazole.

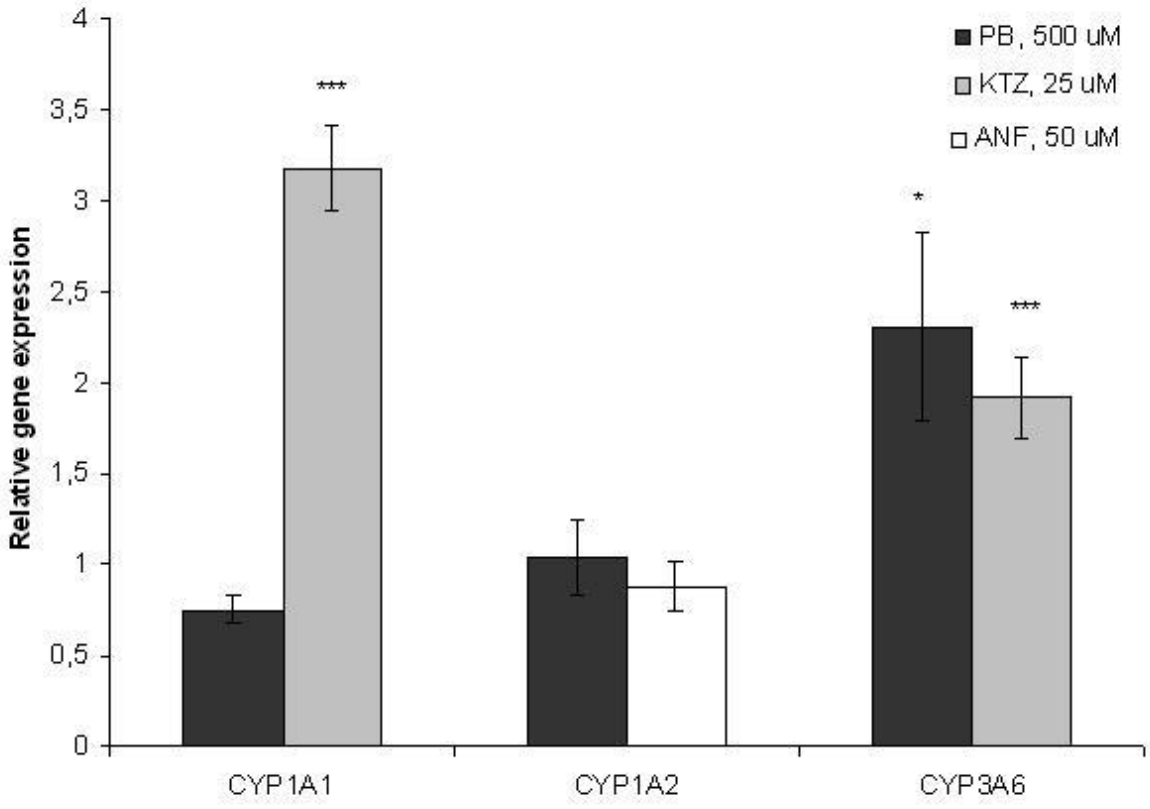


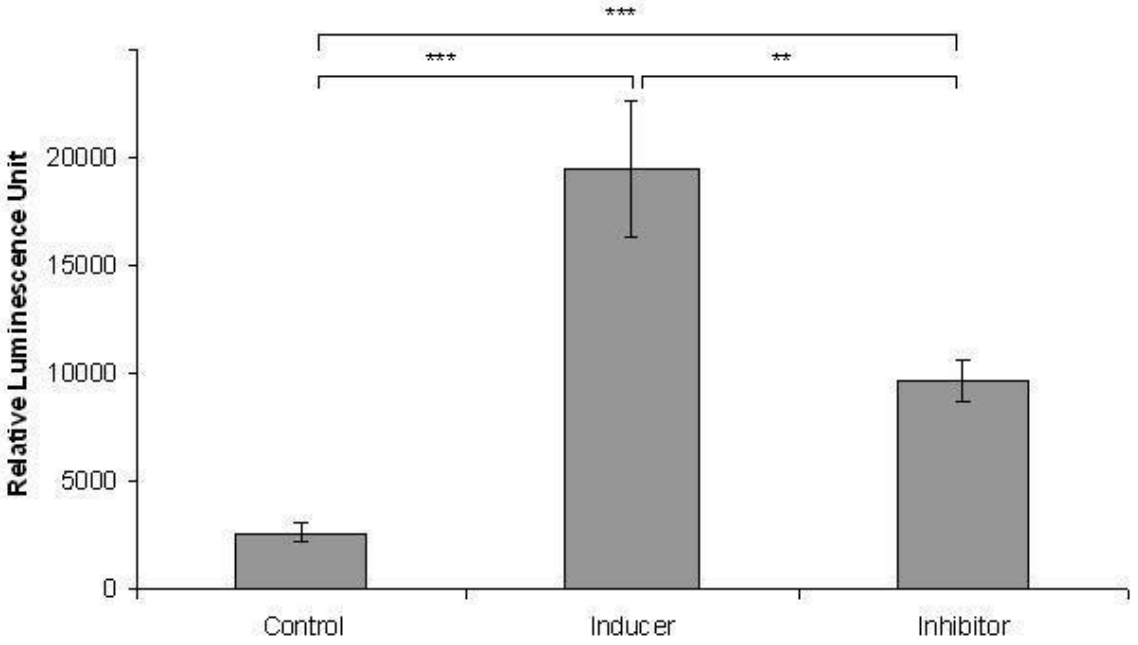
Figure 9. Relative gene expression of the CYP genes in rabbit primary cell cultures, compared to untreated controls. PB – phenobarbital, KTZ – ketoconazole, ANF – alpha-naphthoflavone. The cell cultures were treated for 2 h. (n = 4/group; *p < 0.01, ***p < 0.001) Data are shown as mean ± SE.

4.2 Results of the *in vivo* experiments

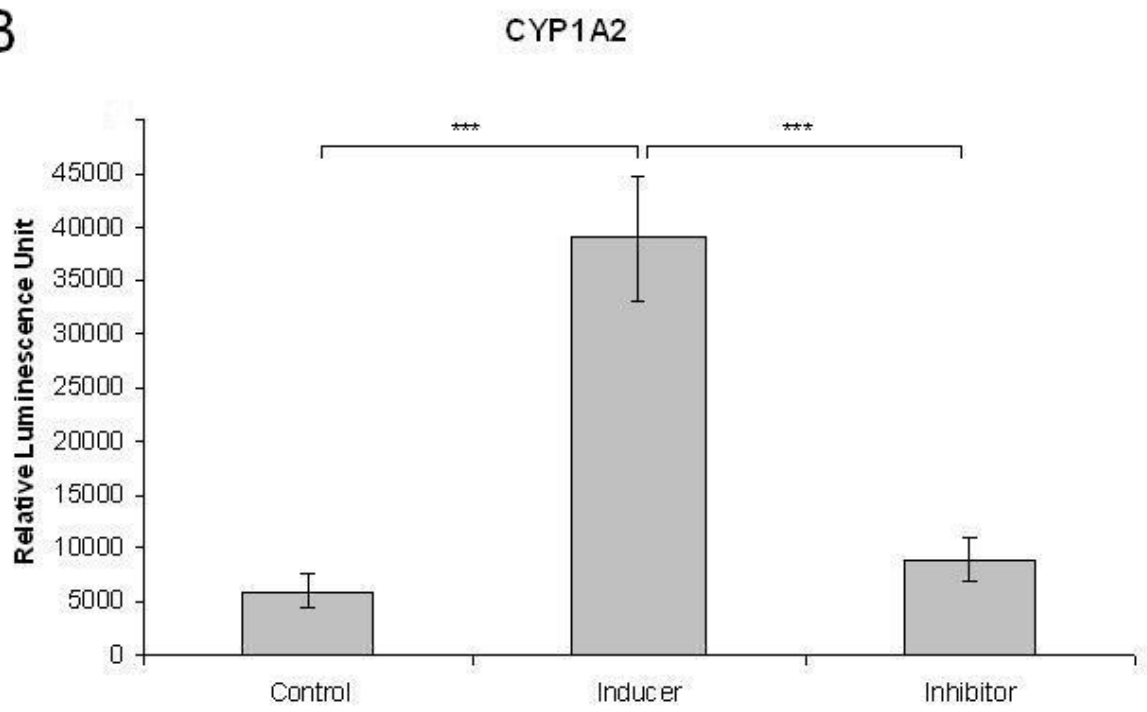
4.2.1 Hepatic CYP450 activity of rabbit

Protein concentrations of the microsomes from the different groups of animals were uniform. Microsomes from the phenobarbital treated rabbits showed increased activity of all three isoenzymes (CYP1A1, CYP1A2, CYP3A6) (Figure 10). Ketoconazole treatment increased the activity of CYP1A1, decreased the activity of CYP3A6, and had no effect on CYP1A2 (Figure 10).

A


CYP1A1
***
***
**
Relative Luminescence Unit
20000
15000
10000
5000
0
Control
Inducer
Inhibitor

B


CYP1A2
***
***
Relative Luminescence Unit
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40000
35000
30000
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20000
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10000
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Control
Inducer
Inhibitor

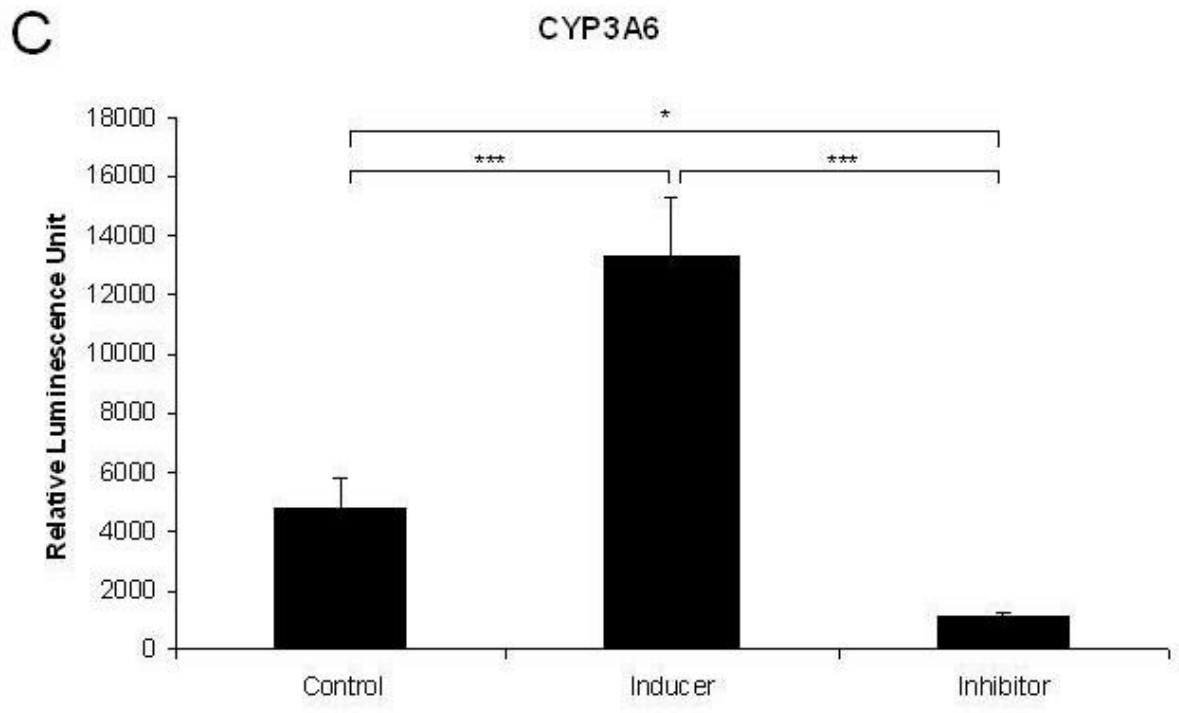


Figure 10. Changes in cytochrome enzyme activity in rabbit microsomes. Control – non-treated, Inducer – treated with 80 mg/kg bw. phenobarbital for 3 days, Inhibitor – treated with 40 mg/kg bw. ketoconazole for 3 days. (n = 4/group; *p < 0.05; **p < 0.01; ***p < 0.001) Data are shown as mean ± SE. (A) CYP1A1 activity. (B) CYP1A2 activity. (C) CYP3A6 activity.

4.2.2 Gene expression of CYP450 in rabbit liver

PCR results are shown in Figure 11. After three days of phenobarbital treatment, gene expression levels were increased for all three isoenzymes. Ketoconazole treatment did not alter the gene expression of CYP1A1 and CYP1A2, but increased expression of the CYP3A6 gene.

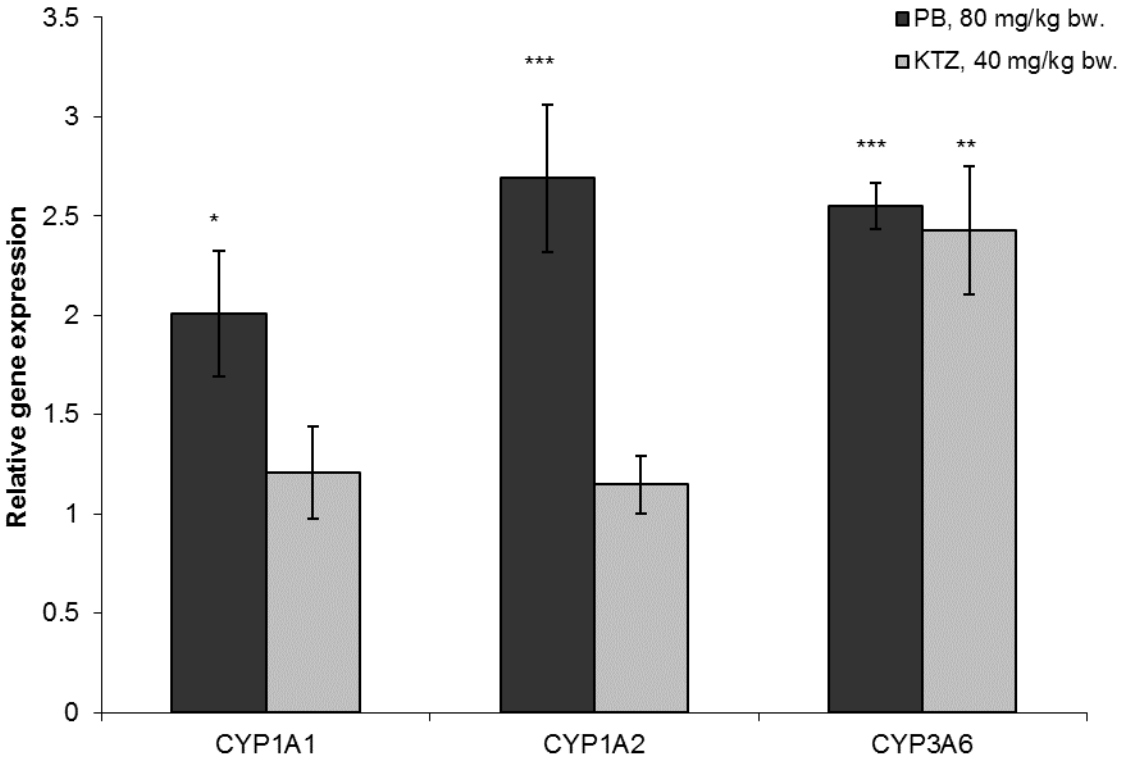


Figure 11. Relative gene expression of the CYP genes in rabbits, compared to untreated controls. PB – phenobarbital, KTZ – ketoconazole. The rabbits were treated orally for 3 days. (n = 4/group; *p < 0.05; **p < 0.01; ***p < 0.001) Data are shown as mean ± SE.

4.2.3 Hepatic CYP450 activity of chicken

The microsome protein concentration was practically equal (23.17 ± 1.52 mg/ml) in each sample.

The activity of CYP1A, CYP2C and CYP3A are shown in Figure 12, 13 and 14, respectively. The administration of 50 mg/kg bw. beta-glucan, 5 mg/kg bw. SN, 50 mg/kg bw. SN, 250 mg/kg bw. fulvic acid and 1 ml/kg bw. DWA to the chickens significantly increased the hepatic CYP1A activity ($p=0.002$, $p=0.010$, $p=0.015$, $p<0.001$ and $p<0.001$ respectively). The orally applied drinking water supplements for five consecutive days did not alter the activity of chicken hepatic CYP2C and CYP3A enzymes.

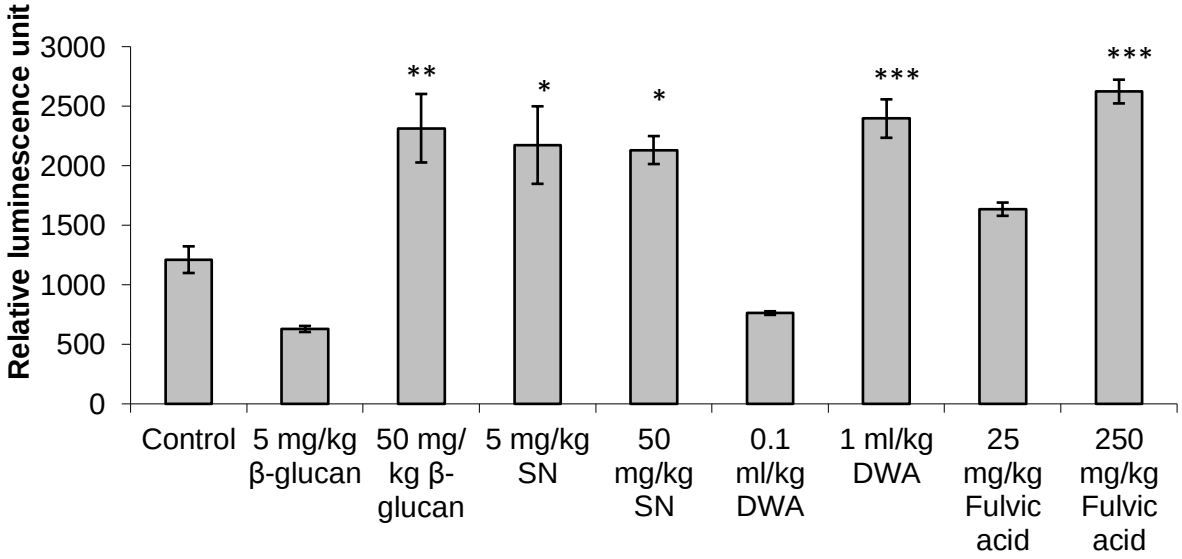


Figure 12. Changes in CYP1A enzyme activity in chicken liver microsomes. (n = 5/group; *p < 0.05; **p < 0.01, ***p < 0.001; each treatment group compared to control group) Data are shown as mean ± SE. SN - Sanguinarine containing product, DWA - drinking water acidifier. The chickens were treated orally for 5 days.

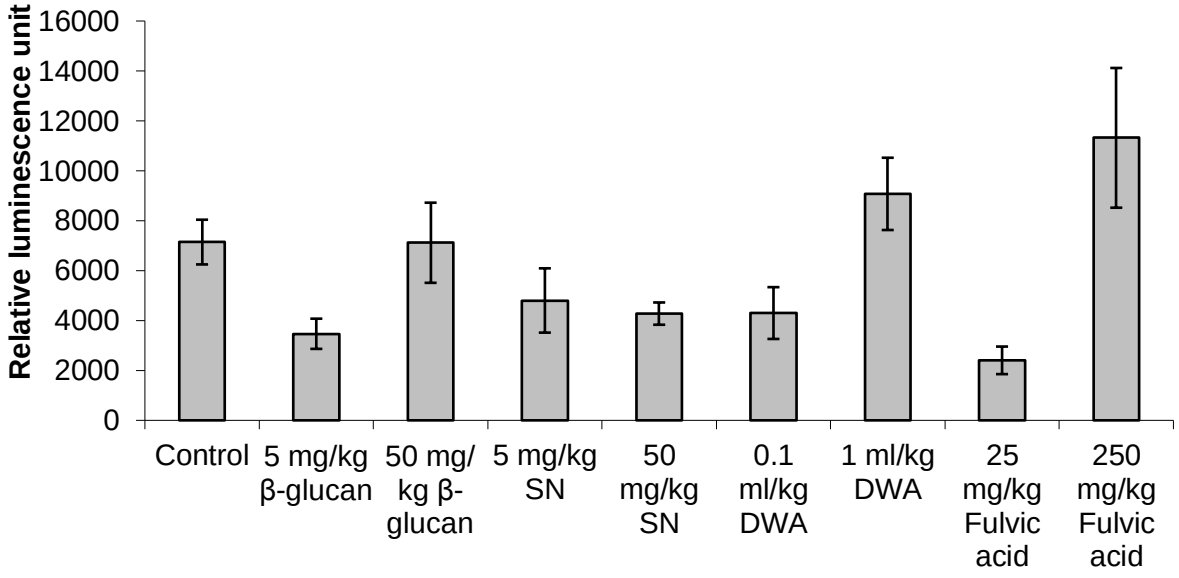


Figure 13. Changes in CYP2C enzyme activity in chicken liver microsomes. (n = 5/group; The level of significance was set at $p < 0.05$; each treatment group compared to control group.) Data are shown as mean ± SE. SN - Sanguinarine containing product, DWA - drinking water acidifier. The chickens were treated orally for 5 days.

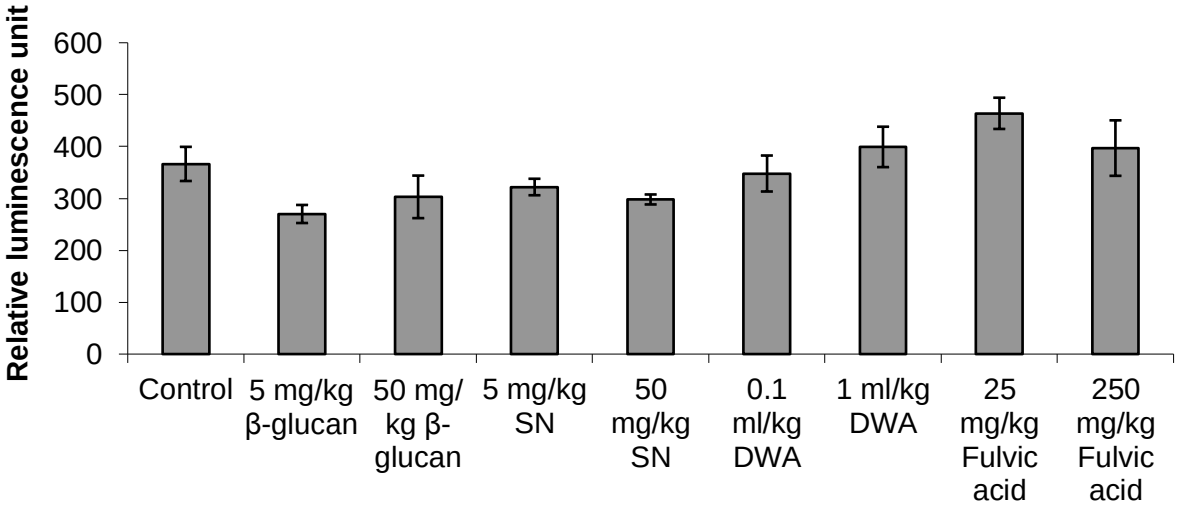


Figure 14. Changes in CYP3A enzyme activity in chicken liver microsomes. (n = 5/group; The level of significance was set at $p < 0.05$; each treatment group compared to controls.) Data are shown as mean ± SE. SN - Sanguinarine containing product, DWA - drinking water acidifier. The chickens were treated orally for 5 days.

4.2.4 Gene expression of CYP450 in chicken liver

Regarding the results of qPCR, the CYP1A4, CYP2C23a, CYP2C45 and CYP3A37 mRNA expression levels normalized to a single reference gene (beta-actin or GAPDH) are compared to the mRNA expression levels using two housekeeping genes (beta-actin and GAPDH) for normalization (Table 4). Overall, differences occurred seven times between the two calculation methods; three differences occurred compared to beta-actin and five discrepancies occurred compared to GAPDH reference gene.

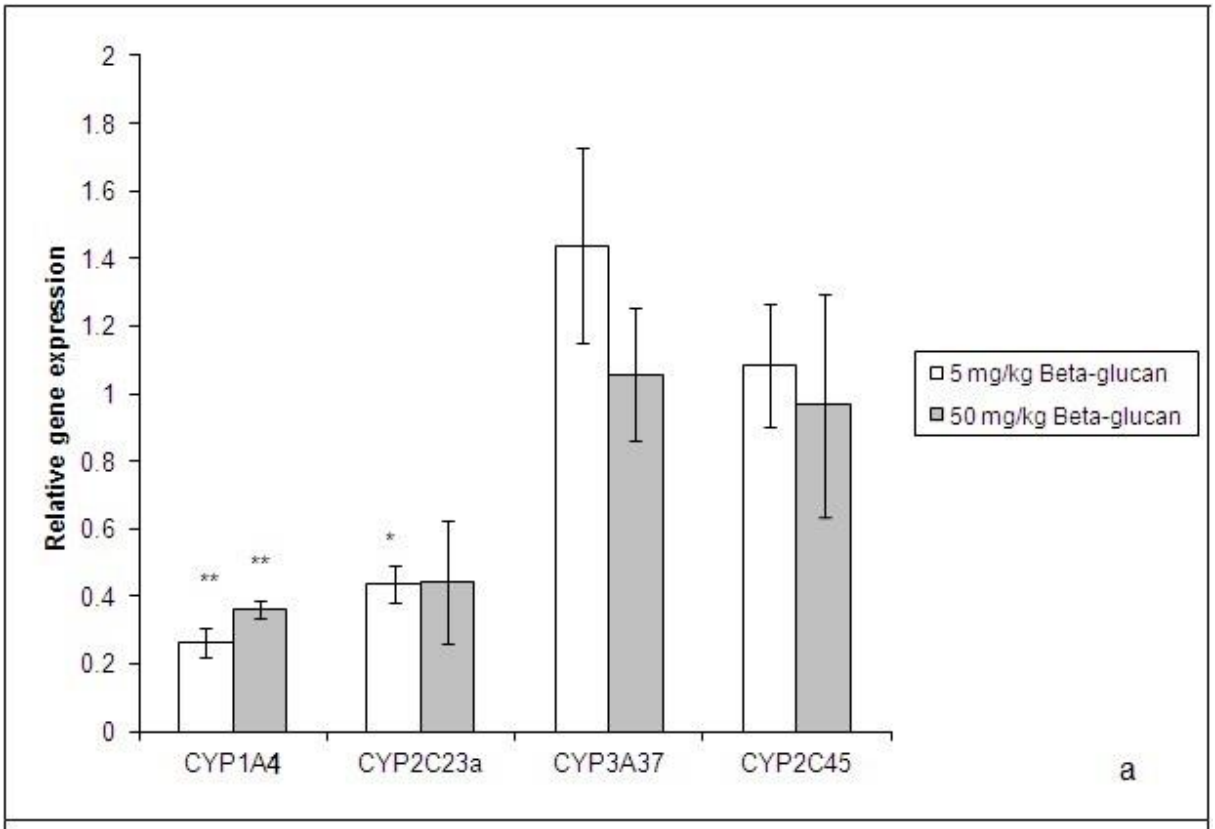
Table 4. Comparison of cytochrome gene expression levels normalized to one (either beta-actin or GAPDH) or two (beta-actin and GAPDH) reference genes

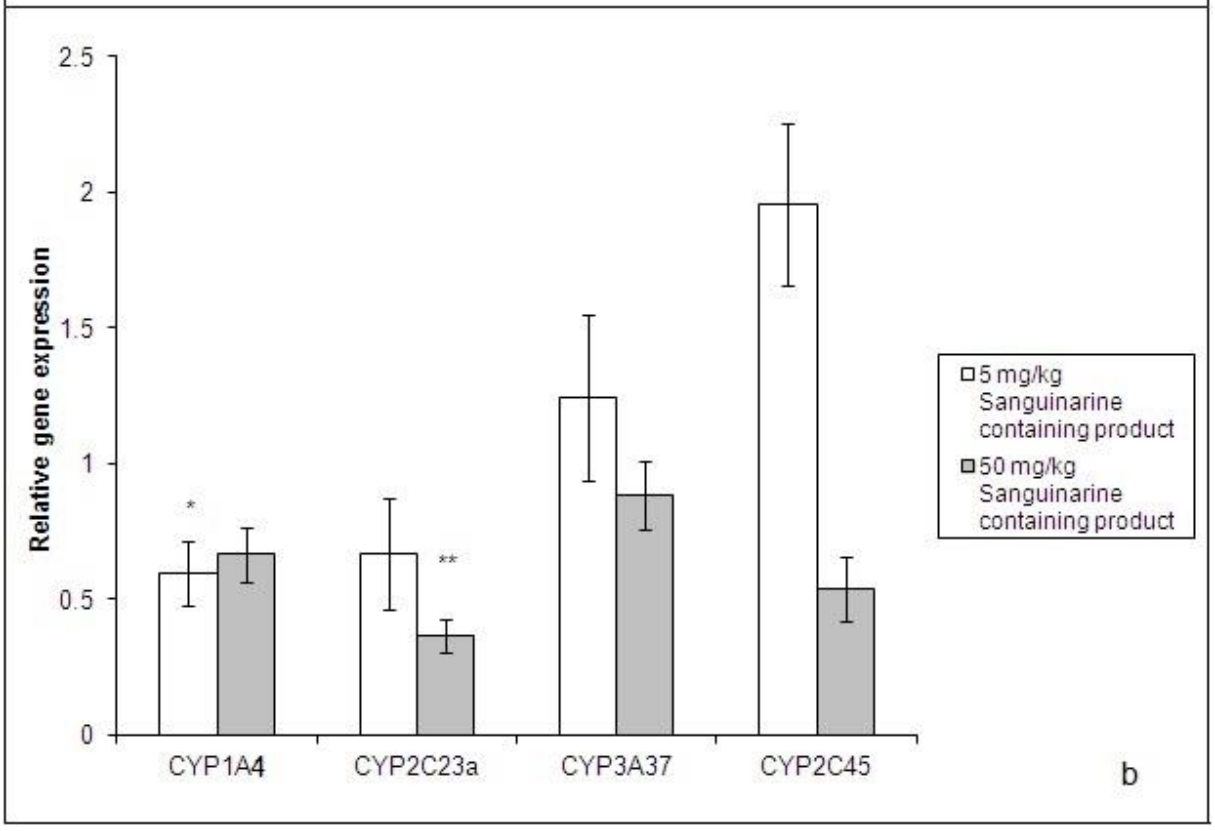
	one reference gene: beta-actin				two reference genes: beta-actin and GAPDH				one reference gene: GAPDH			
Treatment	CYP1A4	CYP2C23a	CYP3A37	CYP2C45	CYP1A4	CYP2C23a	CYP3A37	CYP2C45	CYP1A4	CYP2C23a	CYP3A37	CYP2C45
5 mg/kg beta-glucan	Down	Down	–	–	Down	***Down***	–	–	Down	**–**	–	–
50 mg/kg beta-glucan	Down	–	–	–	Down	–	–	–	Down	–	–	–
5 mg/kg SN	Down	***Down***	–	–	***Down***	**–**	–	–	**–**	–	–	–
50 mg/kg SN	–	**–**	–	–	–	***Down***	–	**–**	–	Down	–	***Down***
0.1 ml/kg DWA	Down	Down	–	–	***Down***	Down	–	–	**–**	Down	–	–
1 ml/kg DWA	**–**	–	–	–	***Down***	–	–	–	**–**	–	–	–
25 mg/kg Fulvic acid	–	–	–	–	–	–	–	–	–	–	–	–
250 mg/kg Fulvic acid	–	–	–	–	–	–	–	–	–	–	–	–

Down: downregulation, UP: upregulation, –: unchanged, GAPDH: Glyceraldehyde 3-phosphate dehydrogenase, CYP: cytochrome P450, DWA: drinking water acidifier, SN: Sanguinarine containing product

Differences between the two calculations are marked in bold and italic.

The results of the chicken CYP450 gene expression levels normalized to two housekeeping genes are shown in Figure 15. The 5 mg/kg bw. β-glucan and the 0.1 ml/kg bw. DWA treatment downregulated the relative gene expression of CYP1A4 and CYP2C23a. The level of CYP1A4 was decreased after 50 mg/kg bw. β-glucan, 5 mg/kg bw. SN and 1 ml/kg bw. DWA administration. Furthermore, the administration of 50 mg/kg bw. SN caused downregulation of the CYP2C23a gene.


Relative gene expression
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
**
*
CYP1A4
CYP2C23a
CYP3A37
CYP2C45
5 mg/kg Beta-glucan
50 mg/kg Beta-glucan
a


Relative gene expression
2.5
2
1.5
1
0.5
0
*
**
CYP1A4
CYP2C23a
CYP3A37
CYP2C45
5 mg/kg Sanguinarine containing product
50 mg/kg Sanguinarine containing product
b

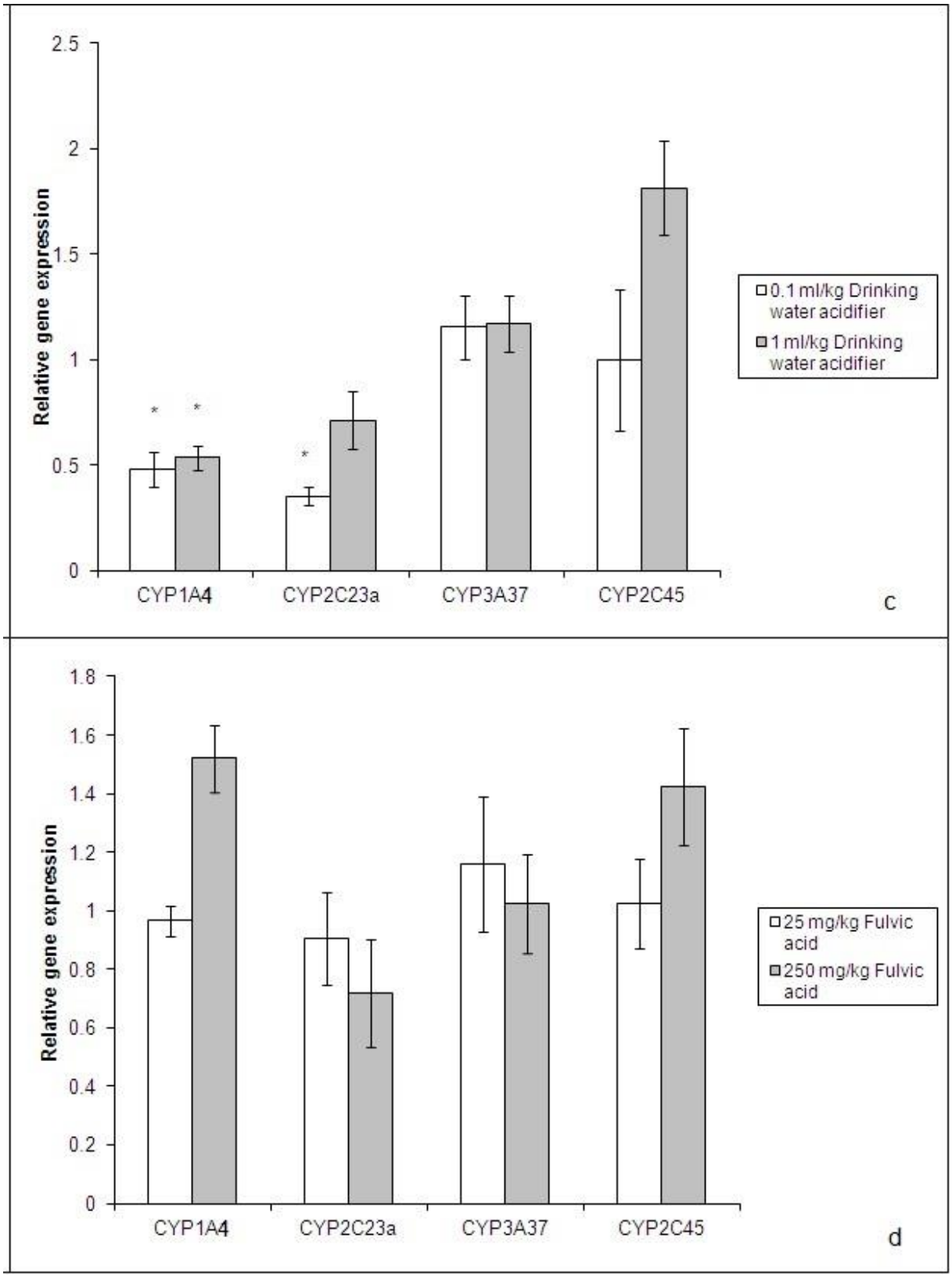


Figure 15. Relative gene expression of the CYP genes in chicken liver, compared to untreated controls. (n = 5/group; *p < 0.05; **p < 0.01) Data are shown as mean ± SE. (a) beta-glucan, (b) sanguinarine containing product, (c) drinking water acidifier, (d) fulvic acid treatment. The chickens were treated orally for 5 days.

4.2.5 Tiamulin's effect on chicken CYP450 activity *ex vivo*

Figure 16 shows the effect of tiamulin on the activity of CYP2C enzymes. The applied dosages of tiamulin fumarate decreased the activity of the CYP2C enzymes; 5 mM tiamulin decreased the activity by 90%.

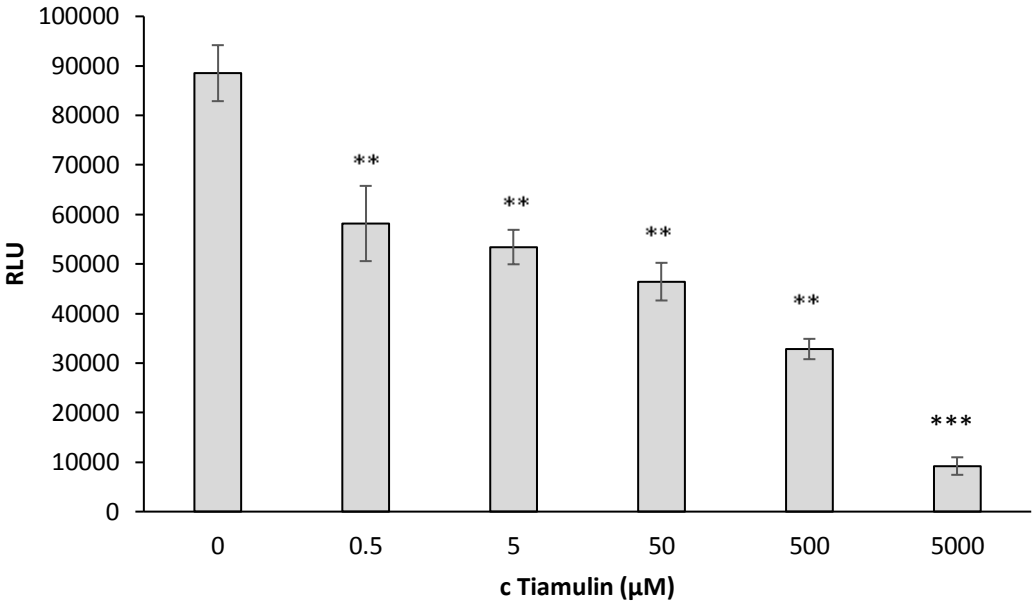


Figure 16: The effect of tiamulin fumarate on the chicken hepatic CYP2C activity. Data are shown as mean±SD. RLU – Relative luminescence unit, **p<0.01, ***p<0.001

The following Figure 17 illustrates the simultaneous effect of the different feed supplements and tiamulin on the CYP2C enzymes. The enzyme activity decreased as a result of 2.5 and 5 mM tiamulin with the same tendency as without the additives ($p<0.01$), although at 2.5 mM tiamulin concentration, the CYP2C activity was slightly ($p<0.07$) higher in the control sample than in the treated samples.

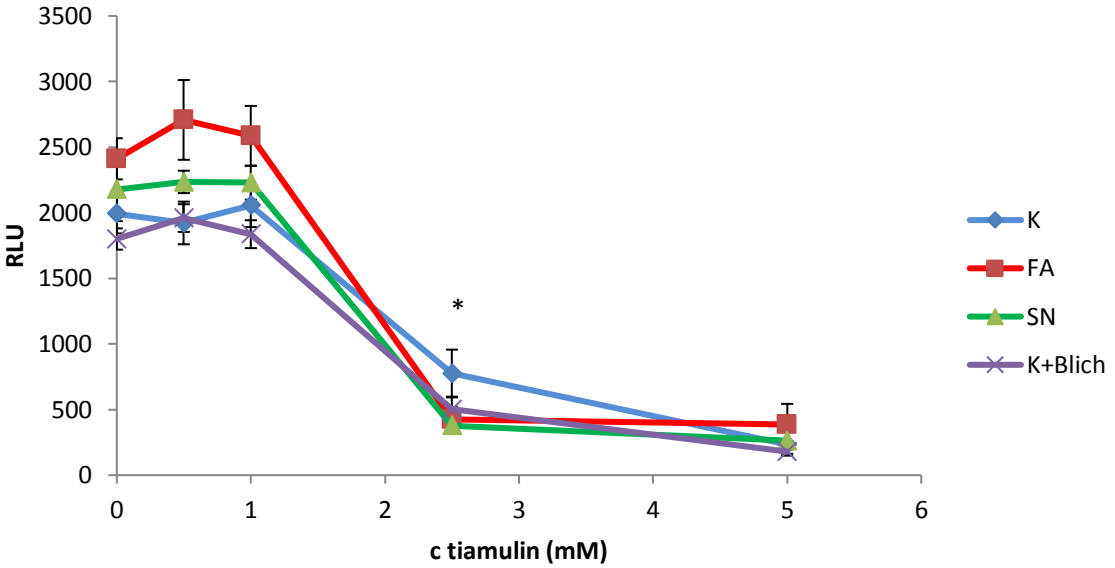


Figure 17: The effect of tiamulin fumarate and supplements on the chicken hepatic CYP2C enzymes. FA= 250 mg/kg bw. fulvic acid, SN= 50 mg/kg bw. sanguinarine, Blich= 5% supernatant of *Bacillus licheniformis*, K= control group, tiamulin alone; Data are shown as mean±SE. RLU – Relative luminescence unit; *$p<0.07$

The activity of CYP2C is generally higher compared to the activity of CYP1A enzymes. The following diagram (Figure 18) shows the effect of tiamulin on the activity of CYP1A enzymes. The 0.5, 1, 2.5 and 5 mM tiamulin fumarate reduced the activity of CYP1A enzymes to 70, 40, 12 and 8%, respectively.

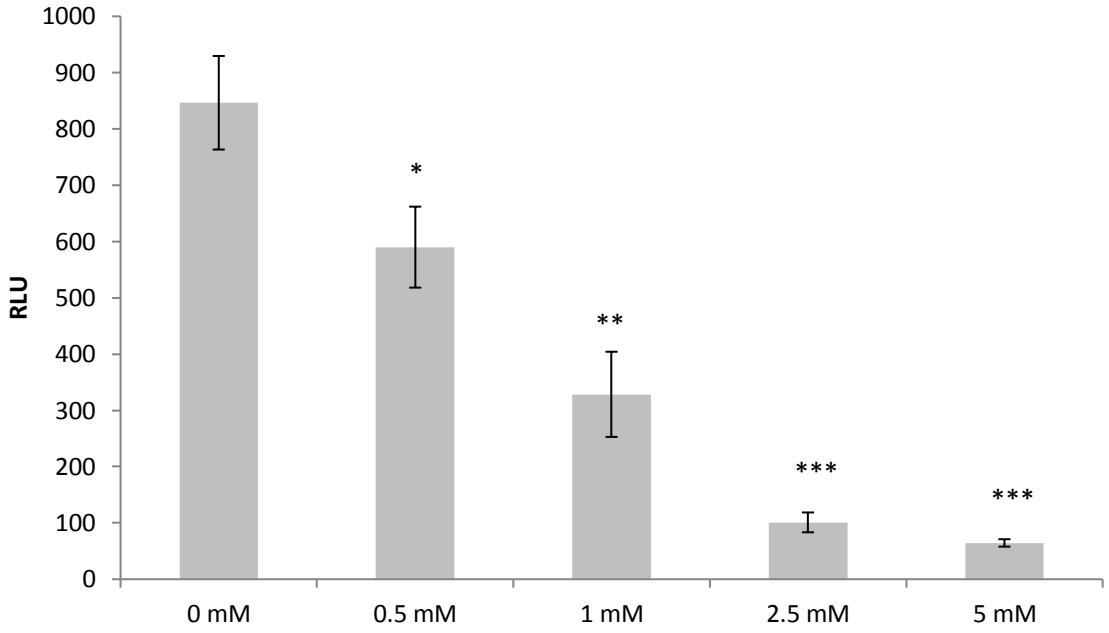


Figure 18: The effect of tiamulin fumarate on the chicken hepatic CYP1A activity. Data are shown as mean±SD. RLU – Relative luminescence unit; *p<0.05, **p<0.01, ***p<0.001

Figure 19 illustrates the simultaneous effect of the different feed supplements and tiamulin on the CYP1A enzymes. The enzyme activity decreased due to the added tiamulin fumarate in the same proportion as without the applied additives.

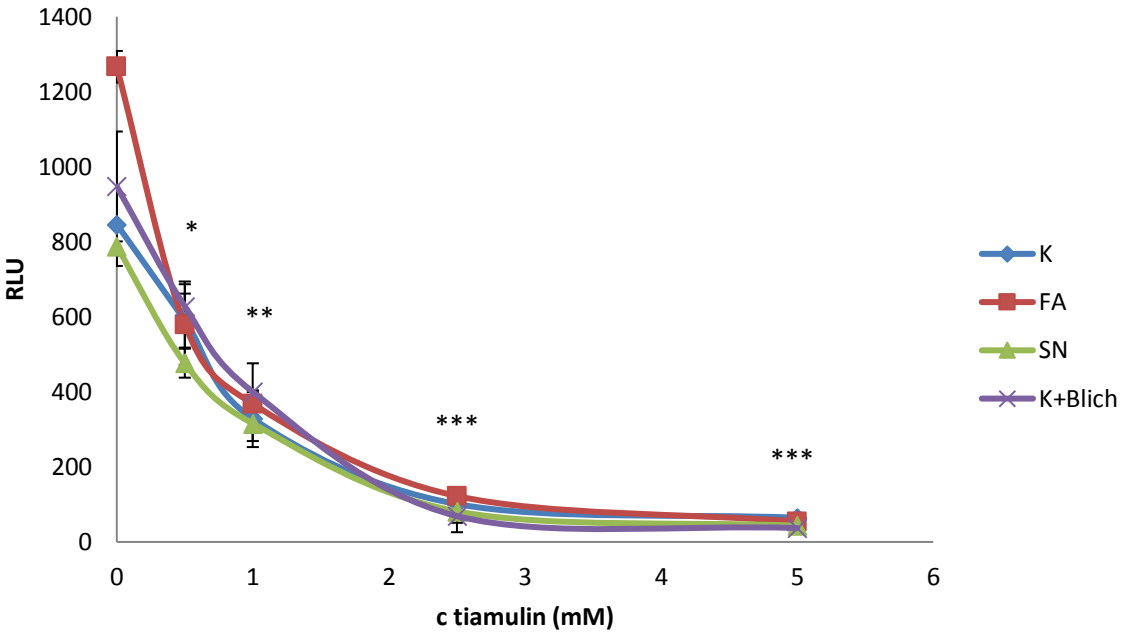


Figure 19: The effect of tiamulin fumarate and supplements on the chicken hepatic CYP1A enzymes. FA= 250 mg/kg bw. fulvic acid, SN= 50 mg/kg bw. sanguinarine, Blich= 5% supernatant of *Bacillus licheniformis*, K= control group; tiamulin alone, Data are shown as mean±SD. RLU – Relative luminescence unit; *p<0.05, **p<0.01, ***p<0.001

4.2.6 Tylosin's effect on rabbit CYP450 activity *ex vivo*

While tylosin at 5 mM concentration exhibits a great effect on the CYP2C enzymes' activity, fulvic acid and SN have no significant influence on the enzyme activity or on the effect of tylosin tartrate (Figure 20).

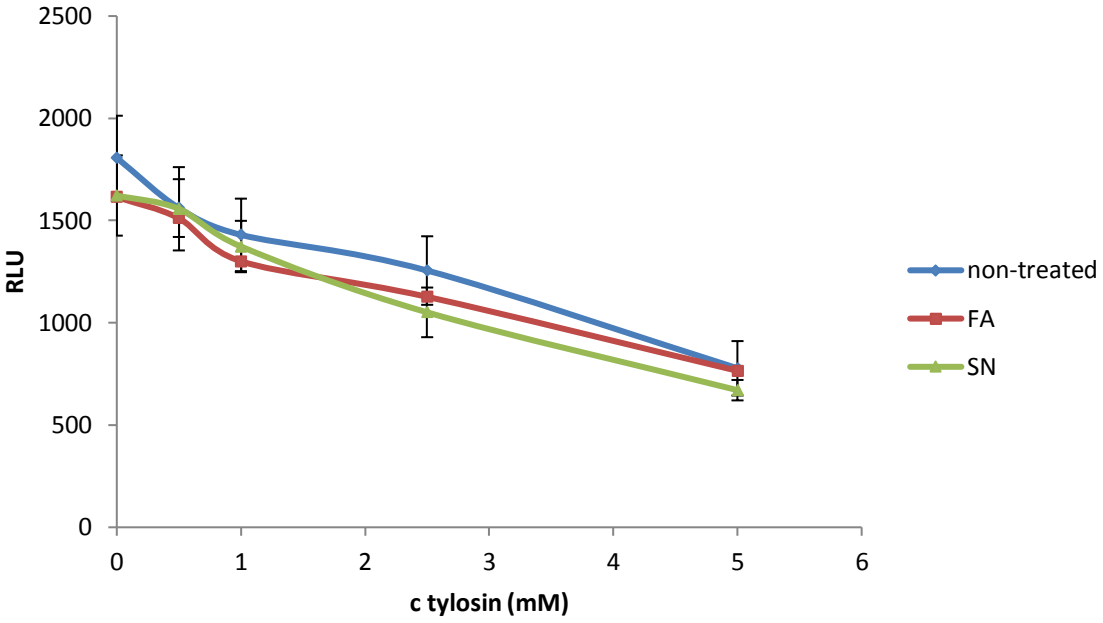


Figure 20. The activity of rabbit hepatic CYP2C9: Tylosin-trated sample in comparison to supplemented fulvic acid (FA, 25 µg/ml) and sanguinarine containing product (SN, 5 µg/ml) with tylosin treatment. Data are shown as mean±SE. RLU = Relative luminescence unit

The simultaneous treatment of fulvic acid and SN with various concentrations of tylosin does not have a different effect on the CYP3A6 activity compared to the tylosin alone (Figure 21).

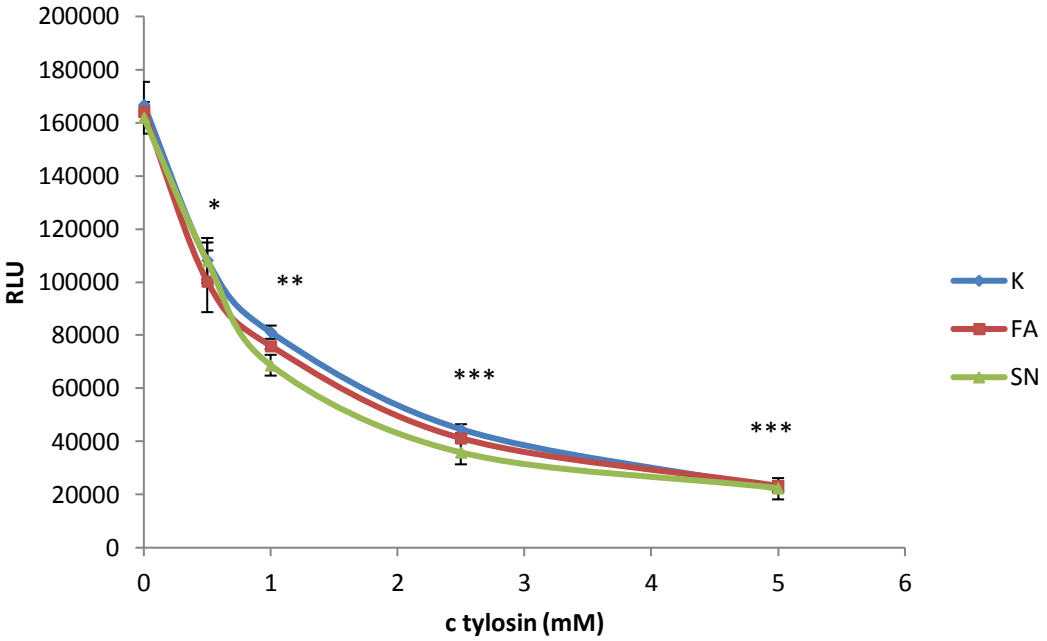


Figure 21. The activity of rabbit hepatic CYP3A6: Control, tylosin-treated (K) samples in comparison to supplemented fulvic acid (25 µg/ml) (FA) and sanguinarine (5 µg/ml) (SN) in combination with tylosin tartrate. Data are shown as mean±SE. RLU = Relative luminescence unit, *p<0.05; **p<0.01; ***p<0.001

Tylosin causes a significant inhibition on the enzymes' activity of both CYP2C9 (Figure 20) and CYP3A6 (Figure 21), adding sanguinarine containing or fulvic acid drinking water additive do not have significant influence on the activity of the investigated enzymes.

4.3 Results of the semi-field trial

4.3.1 *P. multocida* serum titer after one vaccination

The serum samples collected before the first vaccination did not contain *P. multocida* antibody, the obtained titer values were 0 in each individual.

The serum *P. multocida* antibody titer of the untreated controls was 4532±7432 after the vaccination. Neither the 5 day-long nor the 10 day-long β-glucan treatments altered the blood serum antibody level of the animals after inoculation (Figure 22). The value of the standard deviation of 5 mg/kg bw. for ten days and the 50 mg/kg bw. for five days groups were considerably smaller than the control group and no zero titer values were gained in these groups.

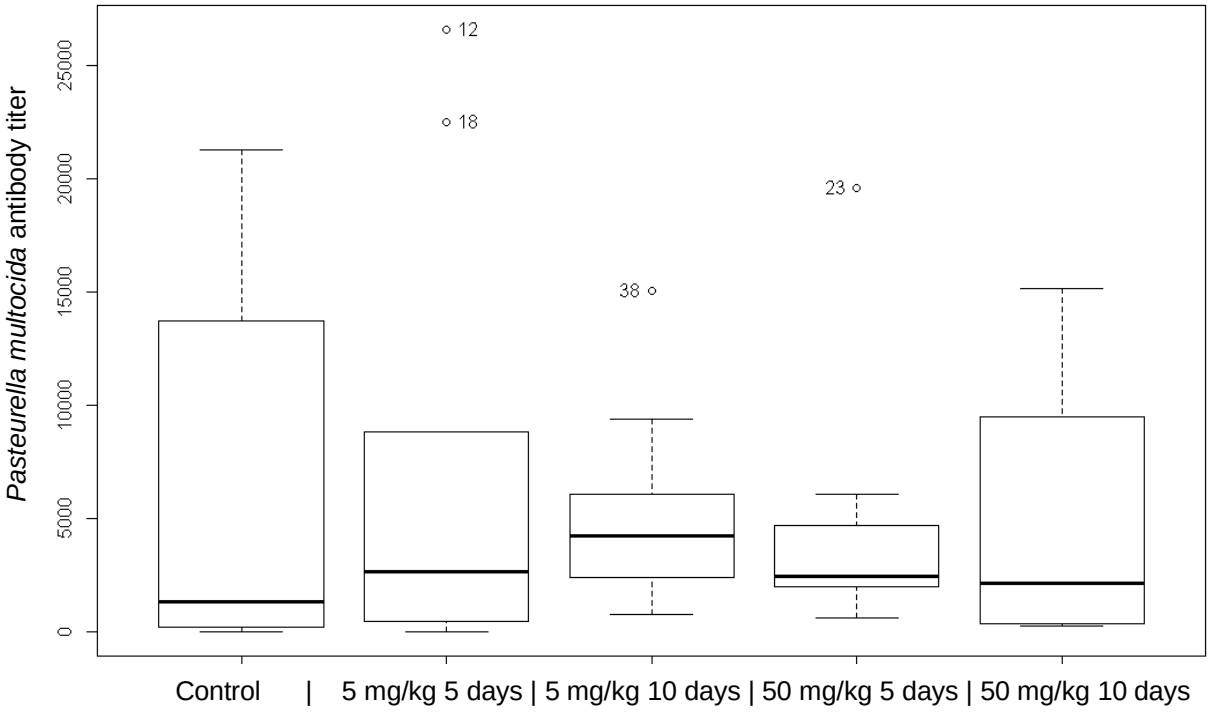


Figure 22. Box-plot diagram of the level of *Pasteurella multocida* antibody titer in chicken plasma after beta-glucan supplementation.

4.3.2 *P. multocida* serum titer of the repeated vaccination protocol

The serum samples collected before the first vaccination did not contain *P. multocida* antibody, the obtained titer values were 0 in each individual. The serum *P. multocida* antibody titer of the untreated controls was 5382±4454 after the first and 6742±4218 after the second vaccination. Neither the 5 day-long nor the 10 day-long β-glucan treatments altered the blood serum antibody level of the animals after both inoculations (Figure 23).

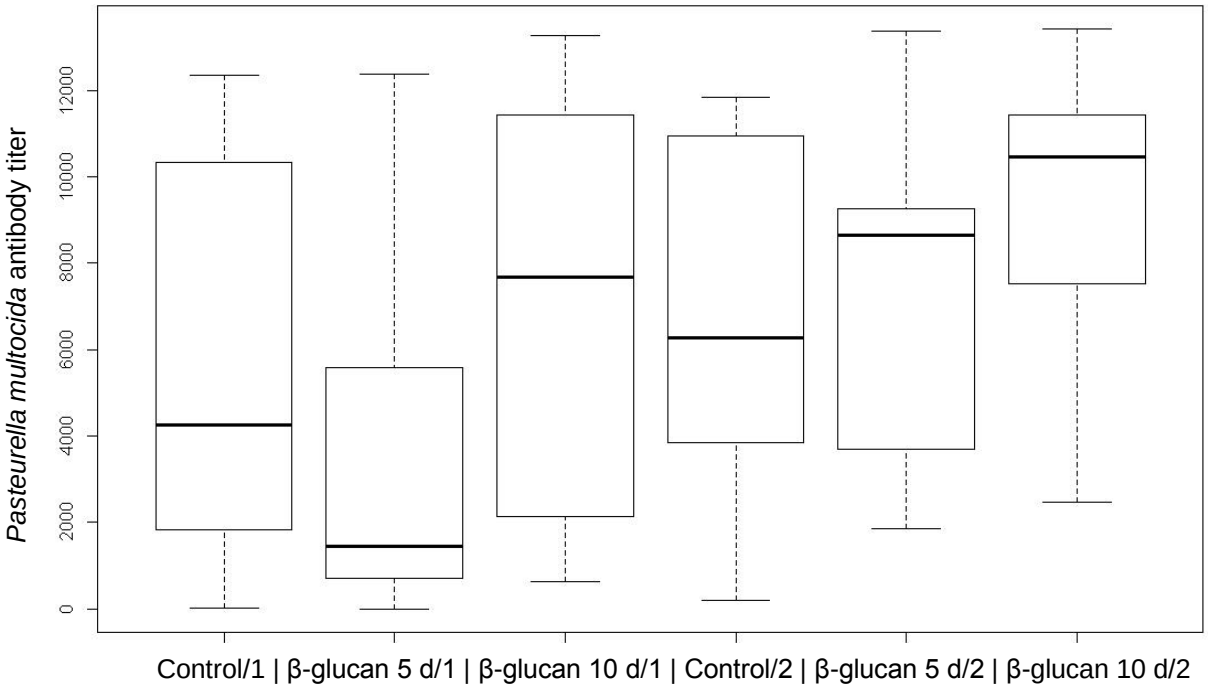


Figure 23. Box-plot diagram of the level of *Pasteurella multocida* antibody titer in chicken plasma after 5 mg/kg bw. beta-glucan supplementation. 5 d – five days of treatment, 10 d – ten days of treatment, /1 after the first *Pasteurella multocida* vaccination, /2 after the second *Pasteurella multocida* vaccination

The fulvic acid supplementation in both the five- and ten-days treatment period and both after the first and second vaccination increased the *P. multocida* antibody level of the chickens ($p<0.01$) (Figure 24).

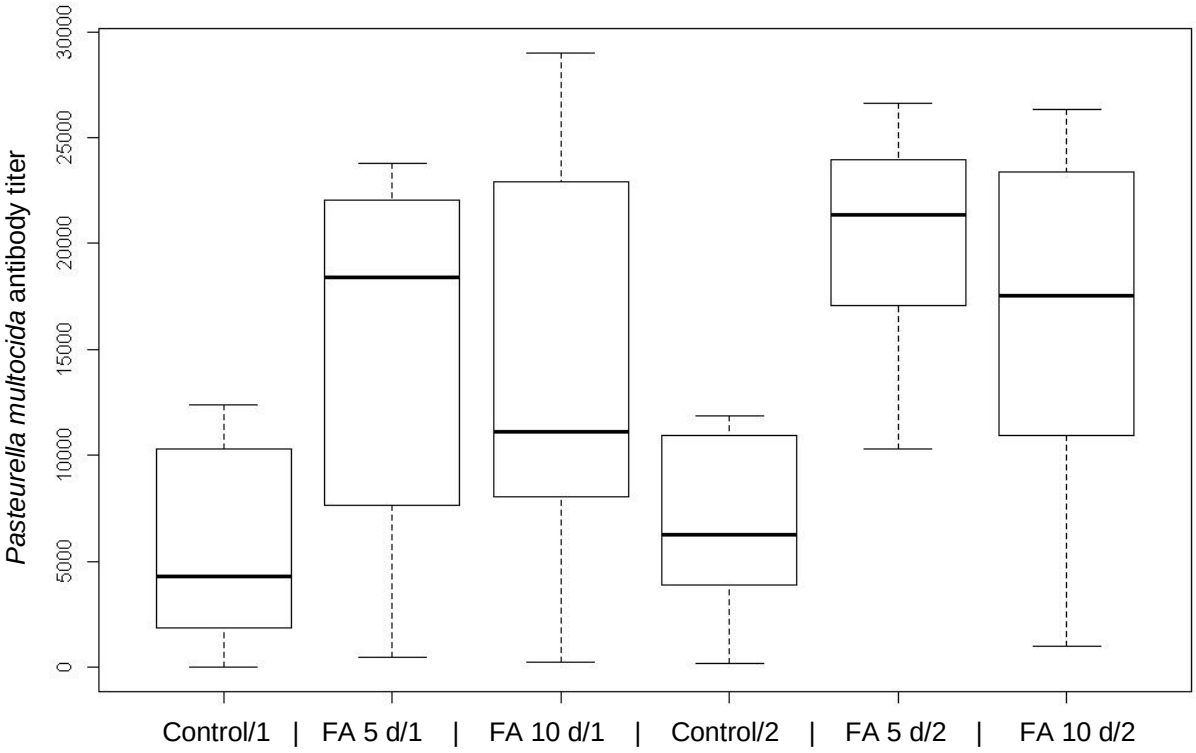


Figure 24. Box-plot diagram of the level of *Pasteurella multocida* antibody titer in chicken plasma after 25 mg/kg bw. fulvic acid (FA) supplementation. 5 d – five days of treatment, 10 d – ten days of treatment, /1 after the first *Pasteurella multocida* vaccination, /2 after the second *Pasteurella multocida* vaccination

The five and ten-day-long SN supplementation enhanced the antibody level after the second immunization ($p<0.05$ and $p<0.01$, respectively) (Figure 25).

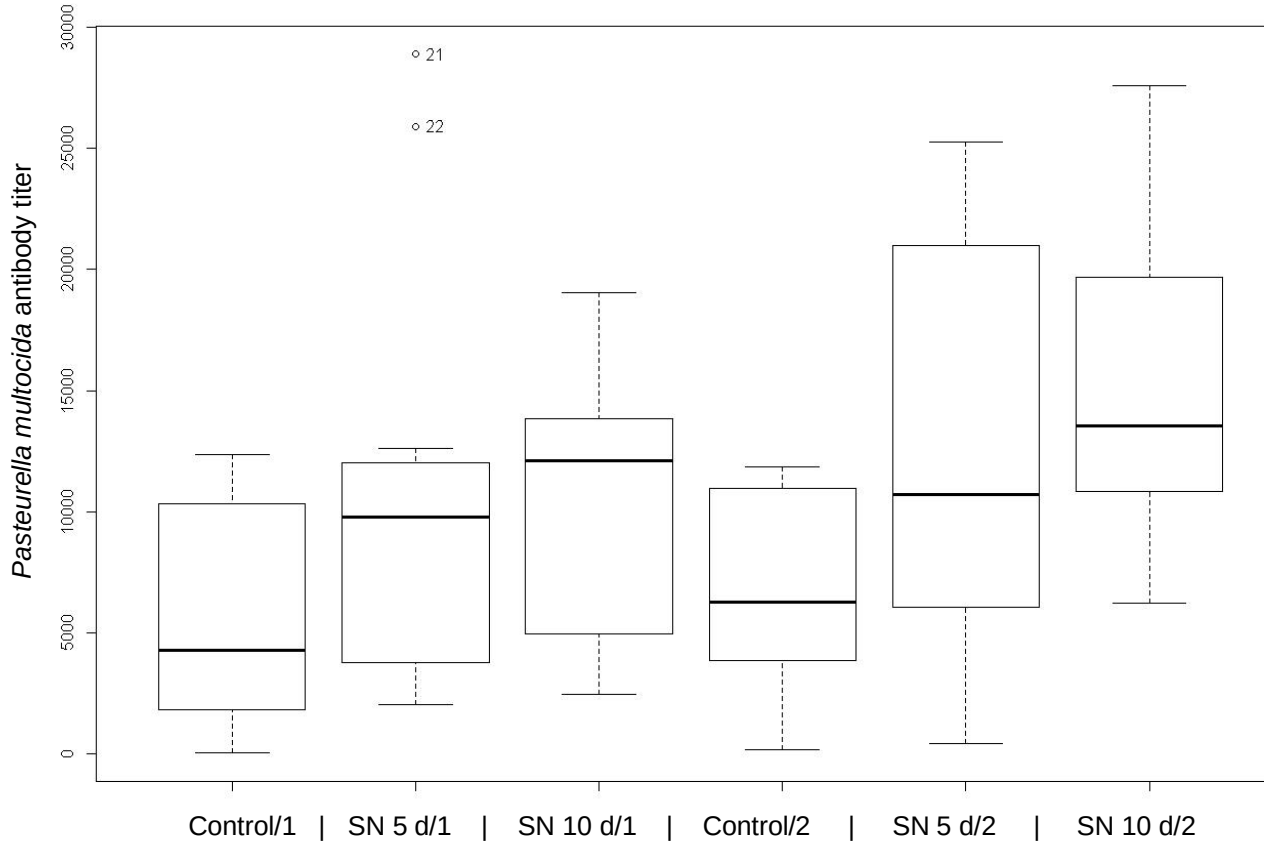


Figure 25. Box-plot diagram of the level of *Pasteurella multocida* antibody titer in chicken plasma after 5 mg/kg bw. sanguinarine containing product (SN) supplementation. 5 d – five days of treatment, 10 d – ten days of treatment, /1 after the first *Pasteurella multocida* vaccination, /2 after the second *Pasteurella multocida* vaccination

The supplementation with DWA for 10 days increased the level of *P. multocida* antibody both after the first and the second vaccination ($p<0.05$ and $p<0.01$, respectively) (Figure 26).

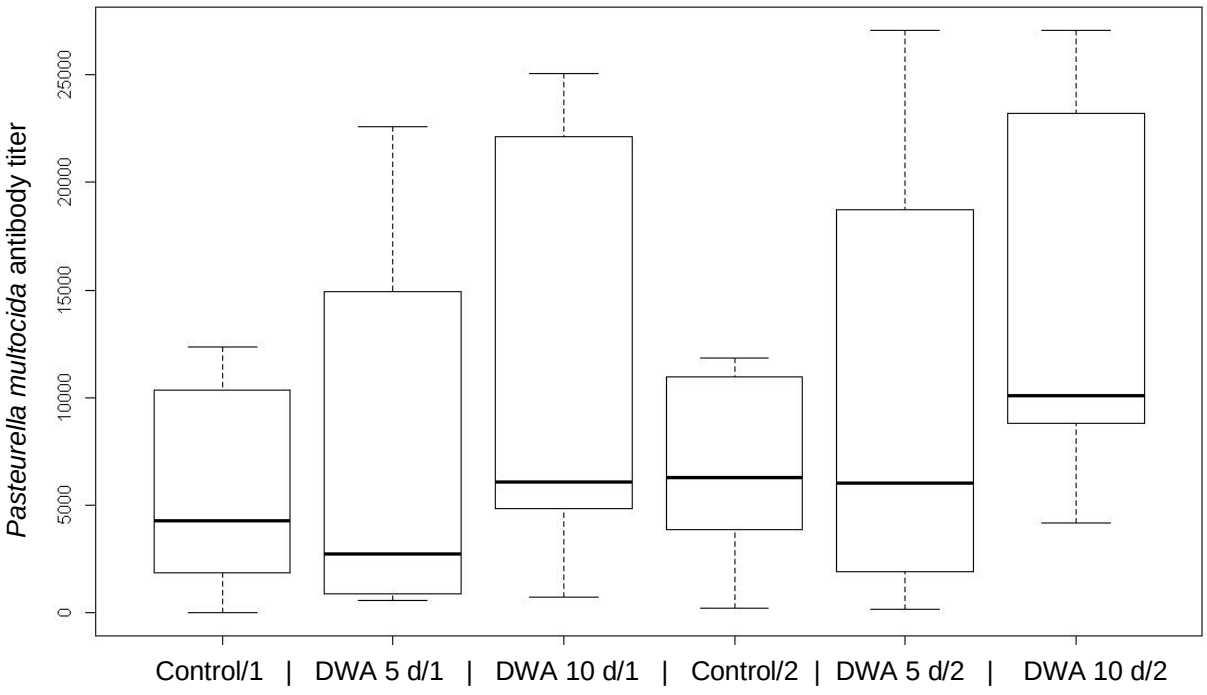


Figure 26. Box-plot diagram of the level of *Pasteurella multocida* antibody titer in chicken plasma after 0.1 ml/kg bw. drinking water acidifier (DWA) supplementation. 5 d – five days of treatment, 10 d – ten days of treatment, /1 after the first *Pasteurella multocida* vaccination, /2 after the second *Pasteurella multocida* vaccination

5. Discussion

The drinking water supplements prevented the upregulation of the pro-inflammatory cytokine TNF-alpha in LPS-treated enterocytes. Together with LPS the β-glucan and fulvic acid treatment further enhanced the level of IL-8 mRNA. This equivocal act may be explained by the different functions of the mediators. Unlike the pro-inflammatory cytokine TNF-alpha that activate inflammatory mediators, IL-8 is classified as chemokine, and its main effector role is to recruit neutrophils to the site of inflammation (Akdis et al., 2011; Cho et al., 2016). Hsp70 was downregulated after the simultaneous treatment with LPS and the supplements. Heat shock proteins have been reported to stimulate the production of pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, and IL-12 (Tsan and Gao, 2009), decreased Hsp70 level may lead to reduced pro-inflammatory cytokine level. The immunomodulatory effect of β-glucan, SN, fulvic acid and DWA in enterocytes was demonstrated *in vitro*, although, these results should be substantiated by live animal studies.

The majority of the veterinary drugs are decomposed through the CYP3A29 enzyme in swine (Howard et al., 2015), which was not influenced by any of the treatments; consequently their concomitant use with veterinary medicinal substances is safe. However, the enhanced CYP mRNA levels at higher doses of the supplements call the attention of the importance of the accuracy of dosing. The relative gene expression of CYP1A1 was substantially elevated (35 times) due to the high dose fulvic acid treatment, which presumes that the fulvic acid is a ligand of a nuclear receptor of the CYP1A1. It was demonstrated in rat hepatoma cells that humic substances can induce the aryl-hydrocarbon receptor (Bittner et al., 2006). Interestingly, all test substances which have acidic characteristic enhanced the gene expression level of CYP1A1; all of them might serve as a ligand of nuclear receptor of CYP1A1 gene. Among them fulvic acid has the highest affinity to the nuclear receptors. Among the tested substances β-glucan appears to be the safest additive in terms of drug metabolism since it is not exerted any effect on the investigated CYP genes.

In conclusion, the administration of the investigated additives in parallel with veterinary pharmaceuticals is safe because the level of the most abundant porcine CYP mRNA; CYP3A29 was not affected by any of the treatments. Although, the supplements with acidic characteristics increased the intestinal gene expression of certain CYP genes, especially at higher doses; consequently, it may lead to elevated CYP protein level, potentiating possible drug interactions, in particular with those substances which are metabolized via the CYP1A subfamily. Although their stimulating effect on the CYP enzymes should be supported by *in vivo* studies and CYP protein levels should be monitored. The modulating effect of all test feed additives was demonstrated in endotoxin-evoked inflammation in intestinal epithelial

cells; enhanced chemokine IL-8 and repressed cytokine TNF-α synthesis may prevent the body from systemic damage due to chronic inflammation.

Rabbits provide an excellent model for investigating the CYP450 system as they have high initial enzyme activity compared to other species such as rodents (Fink-Gremmels, 2008; Nebbia et al., 2003). Primary hepatocyte cell cultures are a suitable tool for assaying enzyme induction and inhibition, and the rapid degradation of CYP enzymes in culture (Eeckhoutte et al., 2002; Paine, 1996) can be obviated by utilising 4 h cultures (Beigel et al., 2008; Noel et al., 2013).

Phenobarbital-mediated induction is regulated at the transcriptional level (Moore et al., 2002), while ketoconazole (Ekroos and Sjögren, 2006) and α-naphthoflavone (Cho et al., 2003) cause conformational changes in the enzyme structure due to binding to the enzyme's active site. Our results showed that ketoconazole acts as a transcriptional inducer in rabbit CYP1A1 and CYP3A6, *in vitro*. Our findings are in accordance with Korashy et al. (Korashy et al., 2007) where ketoconazole was found to enhance CYP1A1 both at the transcriptional and translational level in human and murine cell lines. Others have found that ketoconazole is an effective CYP1A1 inhibitor in humans (Paine et al., 1999) and rats (Elsherbiny et al., 2008); our experiments are concordant, showing that ketoconazole is also a highly effective inhibitor of rabbit CYP1A1, *in vitro*. In our study, ketoconazole had opposite effects at the transcriptional and protein levels on CYP450 enzymes, *in vitro*. *In vivo*, however, ketoconazole increased CYP1A1 activity. According to the qPCR results, ketoconazole treatment increased rabbit hepatocyte CYP1A1 and CYP3A6 gene expression of hepatocytes *in vitro*, but the gene expression level of CYP1A1 remained unchanged *in vivo* after three days of ketoconazole treatment. Ketoconazole upregulated the gene expression of CYP3A6 both *in vitro* and *in vivo*, in line with previous findings that ketoconazole increased the gene expression of CYP3A4 in human cell lines and human primary hepatocytes (Novotna et al., 2014). The inducer effect of ketoconazole on CYP1A1 enzyme activity *in vivo* in rabbits was not described before our studies.

The inducer effect of phenobarbital was most pronounced in the CYP3A6 enzyme *in vitro*, and is in line with phenobarbital's known stimulating effect on the CYP3A subfamily (Ohno et al., 2009). Phenobarbital treatment caused increased gene expression and increased CYP enzyme activity in all examined isoenzymes both *in vitro* and *in vivo*.

Luciferin-IPA is the most sensitive substrate for CYP3A4 (Cali et al., 2006). This substrate worked well with the rabbit CYP3A6 enzyme, providing further evidence of the functional similarity of the two enzymes. The obtained RLU values for all three CYP activities in rabbit hepatocytes are very comparable with RLU values found in human hepatocytes; HepG2 (Yueh et al., 2005), HepaRG (Mueller et al., 2014), and in freshly isolated and cryoplateable

hepatocytes (Moeller et al., 2011). The Luciferin-PFBE substrate did not produce detectable signal with the rabbit CYP3A6 enzyme. Others have used the HepG2 human hepatoma cell line with the same cell numbers as used in our study and found RLU of approximately 1000 in 2D cell cultures (Lan et al., 2010). In another study, recombinant (yeast) CYP3A4 at a concentration of $5*10^7$ cells/ml in 200 µl medium was used, producing an RLU value of approximately 300 (Neunzig et al., 2011). According to these and our studies, the Luciferin-PFBE substrate did not produce strong luminescent signal with either human CYP3A4 or rabbit CYP3A6.

Compared to other cytochrome assays, the P450-Glo™ Assays have key advantages. For example, the Masters method (Masters et al., 1967) is not specific for individual isoenzymes and can only provide indirect measurement of overall cytochrome activity. While the similarly indirect Nash method (Nash, 1953) does provide information regarding specific CYP isoenzymes, it is insufficiently sensitive to register most xenobiotic-induced changes. Indirect but isoenzyme-specific measurement is possible with high-performance liquid chromatography (Lahoz et al., 2008; Lee et al., 2013) and Western blot (LeCluyse, 2001), but these methods are complex and problematic because the amount of CYP protein is not directly proportional to CYP activity. Analytical methods based on ^{3}H- or ^{14}C-labeled drug substrates are accurate but require protective equipment, subsequent decontamination and specialised waste disposal (Zlokarnik et al., 2005). The fluorescence methods suffer from optical interference between test substances and CYP substrates (Cali et al., 2006). In contrast, the P450-Glo™ CYP assay provides a simple and specific method for the detection of cytochrome activity.

Our *in vitro* findings were largely consistent with the *in vivo* results, validating the short-term primary hepatocyte cell culture method in xenobiotic metabolism investigations and in short-term toxicity screening. This therefore appears to be an effective and cost-effective way to progress much-needed new medicines to registration in rabbits.

Furthermore, the changes in rabbit CYP1A1 and CYP3A6 enzymes after phenobarbital and ketoconazole exposure ran in parallel with those previously described for human CYP1A1 and CYP3A4 enzymes. These results suggest that the rabbit is a good model for human pharmacology investigations.

Fulvic acid and a sanguinarine-containing product were investigated during our examinations in laboratory experiments, as they can be used for rabbits to prevent infectious diseases, especially in rabbit farming. Metabolic interactions between therapeutic agents and feed additives are examined less intensively. It has been described that pharmacokinetic parameters of the macrolide antibiotic erythromycin were significantly altered when butyrate was added to the feedstuff (Csiko et al., 2014). In veterinary medicine, another macrolide – tylosin, is used for rabbits in various diseases. Our study focused on the CYP2C and CYP3A

enzymes. The *in vitro* application of fulvic acid or the sanguinarine-containing supplement did not change the activity of hepatic CYP2C and CYP3A6 enzymes. According to these results, it can be hypothesized, that the use of the two investigated substances in rabbits is safe. The tylosin tartrate considerably inhibited the activity of CYP3A6 and only higher concentrations of tylosin reduced the activity of CYP2C. This inhibitory effect was not influenced by either sanguinarine or fulvic acid, thus it can be postulated that their use in rabbits in combination with tylosin is safe. There are many further feed additives that are used in rabbits which could exhibit influence on the CYP450 enzyme system and therefore, their possible metabolic interactions should be investigated when they are given in combination with therapeutic drugs.

In general, administration of supplements to the chicken flock is proven to be effective for promoting their health during the growing period. It is commonly known that feed additives influence the metabolism of drugs through the enzymes of phase I and/or phase II reaction; however few studies define the actual extent of these effects (Csiko et al., 2014; de Boer et al., 2015; Fink-Gremmels, 2008). There is a lack of knowledge about how the applied additives act on the pathways of xenobiotic metabolism especially in poultry species. Nonetheless, in case a necessary drug therapy takes places in the chicken flock, it is important to know, how the administered substances alter the cytochrome P450 expression, leading to interaction with the drugs or their metabolites.

Surprisingly, CYP2C23a behaves, in regard to regulation, very similarly to CYP3A37 (Ourlin et al., 2000). The chicken CYP3A37 has relatively low abundance, it follows that this isoenzyme is not the most important one in the xenobiotic metabolism (Watanabe et al., 2013). CYP2C enzymes play major roles in detoxification of xenobiotics (Carre et al., 2002) their high abundance in liver makes them the primary drug metabolizing factors. In our findings gene expression of CYP2C23a was decreased in some occasions; however, the activity of CYP2C enzymes did not changed following the administrations of the four examined supplements.

Interestingly, the gene expression level of chicken CYP1A4 decreased after administration of beta-glucan, SN and DWA by contrast the same treatments increased the activity of CYP1A4. This contradiction can be the consequence of the sampling time; both the mRNA samples and the microsome samples were collected at the same time point, so it is possible, that the gene expression phase was completed by the time the mRNA samples were gained.

Our evaluation showed that using one reference gene to normalize the CYP gene expression levels produces unreliable results. Although, completely opposite results were never occurred; a downregulation of a gene never appeared as upregulation with a different

calculation method. Altogether, with the more reliable multiple control genes method more differences in expression levels of the genes of interest were revealed.

Upregulation of the investigated CYP genes did not occur following the administration of the examined drinking water additives to the chicken.

Overall, the four applied drinking water supplementers did not cause clinically important alterations in the gene expression level and the activity of the avian CYP2C, which considered the most significant in avian drug metabolism. Consequently, the risk of cytochrome mediated interactions between the examined supplements and pharmaceuticals used in the chicken industry are unlikely.

During the laboratory experiments, we tested the effects and interactions of the feed supplements sanguinarine, fulvic acid and *Bacillus licheniformis* and the antibiotic tiamulin on the activity of the two chicken hepatic microsomal CYP450 enzymes – CYP1A and CYP2C. The presence of the probiotic supernatant or SN or FA in addition to tiamulin did not influence its inhibitory effect on the activity of CYP enzymes. The practical importance of our investigation is that the use of these feed supplements in the chicken flock is safe, even during an additional medication of other drug substances such as our model inhibitor agent tiamulin.

The investigated commercially available supplements are only examples of the various number of available feed additives applied for different purposes in the poultry industry and further trials might be useful, considering their possible interactions with antimicrobials and other pharmaceuticals or feed supplements.

During our study, we were focusing on the avian CYP1A and CYP2C enzymes. The CYP2C23 and CYP2C45 isoenzymes are the most significant ones in the first phase of drug metabolism of chickens. A possible participation of other enzymes during the metabolism of antimicrobials in chickens cannot be excluded and their role during the metabolism of drugs is rarely studied, so this area could also be of further experimental interest. It is well known that simultaneous administration of tiamulin and some other antibiotics of the ionophore group (polyether compounds such as salinomycin or monensin) may lead to a toxic interaction in pigs and poultry. Tiamulin is able to cause a 'macrolide-type' inhibition. Drugs like triacetyloleandomycin, erythromycin and other macrolides inactivate P450 via formation of an intermediate (nitroso-) metabolite complex, which is partly reversible (Witkamp et al., 1994).

The hypothesis that tiamulin may cause an inhibition of enzymes belonging to the cytochrome P450 family was supported by the study of Anadon et al. (Anadon et al., 1989). According to our results, therapeutic levels of tiamulin only inhibited the activity of CYP1A, the activity of CYP2C was reduced only in higher (supratherapeutic) doses. Tenfold doses of fulvic acid and sanguinarine-containing supplements were used to reveal their possible

effects on the CYP1A and CYP2C enzymes. Only the tenfold dose of fulvic acid caused a small inducing effect on the activity of CYP1A. It can be suggested that the normal dose of the investigated supplements can be safely used in chicken flocks even together with drug substances.

The facultative pathogen *Pasteurella multocida* can be eliminated from the flock by most antimicrobial groups, although fowl cholera should be managed rather than treated, because of the subsequent risk of the emergence of bacterial resistance (Palocz et al., 2014). The prophylactic use of bacterial vaccines in animal herds prevents the outbreak of bacterial diseases, consequently decreasing the antibiotic consumption in farm animals reducing the selective drug pressure that can result in resistant strains (Jansen et al., 2018). During our study some control chickens produced low antibody level both after the first and second inoculation. Among the tested feed additives; Fulvic acid, SN and DWA would be a reliable choice for the support of *P. multocida* immunization. Compared to controls the SN and the 10 day long DWA treatments doubled, while the fulvic acid treatments tripled the amount of produced *Pasteurella* antibody in chickens. Achieving high specific antibody levels at population level prevents the onset of the disease, which provides economical production without the risk of spreading resistance, furthermore it ensures food safety. The cost benefit ratio of the vaccination protocol combined with a dietary feed additive under field conditions should be evaluated. Presumably the cost of prevention would not exceed the mutual cost of the antimicrobial treatment, the production loss due to the bad condition of the individuals and because of the necessary withdrawal period. Interestingly, among the four applied feed additives, the three of them, which have acidic properties enhanced the specific antibody level in chickens. Their mechanism of action is unknown, but according to our results it can be deduced, that they have positive influence on the adaptive immunity pathways. The acidic environment provided by the acidic feed additives may facilitate entry of vaccine antigens into these signaling pathways. It supported by the fact that the low-pH phagolysosomal system prepares peptide antigens for the initiation of most cellular adaptive immune responses, and the generation of the antigenic peptides presented by antigen presenting cells is acid dependent (Criscitiello et al., 2013). Dietary acidifiers (organic and inorganic acids) have the potential to reduce the gastrointestinal pH, which protects the gastrointestinal tract from pathogenic bacteria invasion and proliferation (Ahmed et al., 2014). Consequently, the constant struggle with the facultative pathogenic bacteria does not exhaust the energy of immune system and it will reserve energy to the elimination of entering pathogens. Beta-glucan has shown to be priming the innate immune response of chickens (Lowry et al., 2005), but it failed to enhance the antibody production after the *P. multocida* vaccination during our study. Dietary beta-glucan has strong effect on the innate immune system, it has

proliferative activities of professional phagocytes; granulocytes, monocytes, macrophages, and dendritic cells (Novak and Vetvicka, 2008). The promising outcome of this animal trial should be justified under field conditions in a large animal farm.

In conclusion, the simultaneous use of fulvic acid, drinking water acidifier or a sanguinarine containing product with the inoculation of *Pasteurella multocida* vaccine leads to higher specific antibody level in the chicken flock, which may provide reliable protection for the animals and leads to reduced antibiotic consumption.

6. New scientific results

I. Possible effects on infection and inflammatory processes

1. The inhibitory effect of all test feed additives (Wellmune WGP®, Sangrovit® WS, Immunofort®, and Fulvix pulvis®) was demonstrated in endotoxin-evoked inflammation in porcine intestinal cell culture; Hsp70 and TNF-alpha mRNA levels were reduced after the simultaneous LPS and feed additive treatment.

2. The immunomodulatory activity of beta-glucan and fulvic acid was exhibited in LPS-induced inflammation in IPEC-J2 cells; IL-8 gene expression was increased while TNF-alpha gene expression was decreased after the cell cultures were concurrently exposed to LPS and the feed additive.

3. Administration of beta-glucan via drinking water along with *Pasteurella multocida* vaccine to chickens did not influence the amount of serum *Pasteurella multocida* immunoglobulin formed.

4. The simultaneous use of fulvic acid or drinking water acidifier or the sanguinarine containing product with the inoculation of *Pasteurella multocida* vaccine leads to higher specific antibody level in the chicken flock.

II. Effects on drug metabolism

1. Porcine CYP3A29 mRNA levels were not affected by any of the feed additives tested; Wellmune WGP®, Sangrovit® WS, Immunofort®, and Fulvix pulvis®.

2. The supplements with acidic properties increased the intestinal gene expression of porcine CYP1A1 and CYP1A2 genes, especially at higher doses.

3. The adapted cytochrome P450 luminescent method is a fast, safe, simple, and sensitive tool for testing the effect of active substances on the rabbit's cytochrome P450 system.

4. The *in vitro* and *in vivo* findings about rabbit CYP450 pattern were largely consistent, validating the short-term primary hepatocyte cell culture method in xenobiotic metabolism investigations.

5. The investigated drinking water supplements did not cause any alteration in the level of the measured rabbit hepatic microsomal CYP activity alone or in combination with tylosin tartrate ex *vivo*.

6. The *in vivo* applied water-soluble feed additives did not cause clinically important alterations in the gene expression level and the activity of the avian CYP2C.

7. Co-administration of investigated feed additives with *ex vivo* tiamulin exposure had no relevant effect on the action of both enzymes tested – neither CYP1A nor CYP2C in chickens.

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8. Own scientific publications

8.1 Publications related to the topic of the present dissertation

8.1.1 Full text papers in peer-reviewed journals:

Palócz Orsolya, Csikó György: Az antibiotikumok túlzott mértékű használatának csökkentését célzó szerek az állattenyésztési és klinikai gyakorlatban: Irodalmi áttekintés: Reduction of the excessive use of antibiotics in animal husbandry and in clinical practice, Literature review, MAGYAR ÁLLATORVOSOK LAPJA 136:(3) pp. 177-183. (2014), IF 0,185

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Palócz Orsolya, Farkas Orsolya, Szentmiklósi Diána, Nagy Tamás és Csikó György: Xenobiotikumok hatása házinyúl eredetű citokróm P450 enzimrendszerre *in vivo vs. in vitro,* 42. Állatorvostudományi Akadémiai Beszámolók, 2016. január

Somogyi Zoltán, Palócz Orsolya, Csikó György: Baromfikolera vakcina védőhatásának támogatása béta-glükánnal, 42. Állatorvostudományi Akadémiai Beszámolók, 2016. január

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8.2.1 Full text papers in peer-reviewed journals:

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Palócz Orsolya, Benedekné Major Katalin, Csigó Brigitta, Csikó György: Az ABCB1/MDR1 génmutáció elterjedtsége bizonyos magyarországi kutyaállományokban/Prevalence of ABCB1/MDR1 gene mutation in certain Hungarian canine population, MAGYAR ÁLLATORVOSOK LAPJA 140:(8) pp. 495-500. (2018)

8.2.2 Conference presentations

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Palócz Orsolya, Farkas Orsolya, Pásztiné Gere Erzsébet és Gálfi Péter: LPS és reaktív oxigén vegyületek hatása IPEC-J2 sejtek által termelt gyulladásos citokinek, valamint toll-like receptorok génexpressziójára, 39. Állatorvostudományi Akadémiai Beszámolók, 2013. január

Farkas Orsolya, Palócz Orsolya, Csikó György, Pásztiné Gere Erzsébet, Mátis Gábor, Kulcsár Anna, Kulcsárné Petrilla Janka, Neogrády Zsuzsanna, Gálfi Péter: Oxidatív stressz és gyulladás hatásának vizsgálata *in vitro* bélhám- és májsejt ko-kultúra modellen, 39. Állatorvostudományi Akadémiai Beszámolók, 2013. január

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Mátis Gábor, Pászti-Gere Erzsébet, Farkas Orsolya, Kulcsár Anna, Palócz Orsolya, Petrilla Janka, Csikó György, Neogrády Zsuzsanna, Gálfi Péter: Effects of LPS challenge and the role of probiotics in IPEC-J2 cell monoculture and a novel porcine enterohepatic co-culture system, Proceedings of the International Scientific Conference on Probiotics and Prebiotics IPC 2013. Konferencia helye, ideje: Kassa, Szlovákia, 2013.06.11-2013.06.13., 2013. p. 33.

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Palócz Orsolya, Farkas Orsolya, Csikó György, Pásztiné Gere Erzsébet, Mátis Gábor, Kulcsár Anna, Kulcsárné Petrilla Janka, Neogrády Zsuzsanna és Gálfi Péter: Probiotikumok hatása LPS kiváltotta gyulladás mellett in vitro bélhám-májsejt ko-kultúra modellen, 40. Állatorvostudományi Akadémiai Beszámolók, 2014. január

Farkas Orsolya, Palócz Orsolya, Gálfi Péter: Metoxiflavonok gyulladáscsökkentő hatásának vizsgálata sertés vékonybélhám modellen, 40. Állatorvostudományi Akadémiai Beszámolók, 2014. január

O. Farkas, O. Palócz, P. Gálfi: Effect of gut microbiota on anti-inflammatory activity of apigenin and apigenin-trimethylether, 8th ISANH Congress on Polyphenol Applications, Konferencia helye, ideje: Lisszabon, Portugália, 2014.06.05-2014.06.06.

Farkas Orsolya, Palócz Orsolya, Pásztiné Gere Erzsébet, Gálfi Péter: Klorogénsav gyulladáscsökkentő hatásának *in vitro* vizsgálata vékonybélhám modellen, 41. Állatorvostudományi Akadémiai Beszámolók, 2015. január

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Kovács Dóra, Palócz Orsolya, Karancsi Zita, Csikó György, Farkas Orsolya: Flavonoidok hatása a citokróm P450 enzimrendszerre sertés bélhám sejtkultúrán, 42. Állatorvostudományi Akadémiai Beszámolók, 2016. január

Palócz Orsolya, Csikó György: Prevalence of ABCB1 gene mutation in affected dog breeds in Hungary, In: Zsuzsanna Heiszler, Róbert Hohol, Nóra Éles-Etele (szerk.), Hungarian Molecular Life Sciences 2017: Programme and Book of abstracts 2017. p. 266. 1 p. Konferencia helye, ideje: Eger, Magyarország, 2017.03.31-2017.04.02.

Farkas O., Karancsi Z., Kovács D., Lubov B., Csikó Gy., Palócz O.: Effect of flavonoids on cytochrome P450 activity in porcine intestinal epithelial cells - *in vitro* interaction study, JOURNAL OF VETERINARY PHARMACOLOGY AND THERAPEUTICS 41: Suppl 1, 14th Congress of the European Association for Veterinary Pharmacology and Toxicology in Wrocław, Poland, 2018.06.23-2018.06.27.

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8.3 Supervisor of theses

Lencse Zsófia: Probiotikumok és nátrium-n-butirát gyulladáscsökkentő hatásának modellezése vékonybélhám sejtkultúrán, TDK dolgozat, **2013.** Témavezetők: Farkas O., Palócz O.

Szentmiklósi Diána: *In vitro* gyógyszer-metabolizmus házinyúlban, TDK dolgozat, **2014.** Témavezető: Palócz O.

Somogyi Zoltán: Antibiotikum helyettesítők nyúl pasteurellózis megelőzésére és kezelésére, TDK dolgozat, **2014.** Témavezető: Palócz O.

Nagy Tamás: Gyógyszer-metabolizmus vizsgálatok házinyúlban *in vitro* és *in vivo* módszerekkel, TDK dolgozat, **2015.** Témavezető: Palócz O.

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Csigó Brigitta: A német juhászkutyák érintettsége az MDR1 génmutációval kapcsolatban Magyarországon, TDK dolgozat, **2017.** Témavezetők: Csikó Gy., Palócz O.

Katharina Lethaus: Cytochrome P450 mediated feed additives and antimicrobial interactions in rabbits, TDK thesis, **2017.** Témavezetők: Csikó Gy., Palócz O.

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Giricz Márton: Itatóvíz savanyító és β-glükán metabolikus és gyulladáscsökkentő hatása sertés bélhámsejt tenyészeten, TDK dolgozat, **2017.** Témavezető: Palócz O.

Töltési Júlia: Féregellenes szerekkel szembeni rezisztencia elterjedtsége juhok nematodáiban, szakdolgozat, **2017.** Témavezetők: Csikó Gy., Palócz O.

Pia Hartwig: Disinfecting effect of chlorine-dioxide on duck eggs originated from an intensive poultry farm, TDK thesis, **2018**. Supervisors: Csikó Gy., Palócz O.

Nathalie Hafner: Characterisation of fungi isolated form porcine microbiota and examination of their synergism with probiotic bacteria, TDK thesis, **2018**. Supervisors: Csikó Gy., Palócz O.

Regina Remsei: A magyar kutyafajták érintettsége az ABCB1 génmutációban, TDK dolgozat, **2019.** Témavezetők: Csikó Gy., Palócz O.

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