

## ALTERATION OF AVIAN HEPATIC CYTOCHROME P450 GENE EXPRESSION AND ACTIVITY BY CERTAIN FEED ADDITIVES

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We investigated the effect of four feed additives, namely  $\beta$ -glucan, a drinking water acidifier (DWA), a sanguinarine-containing product (SN) and fulvic acid, on hepatic cytochrome P450 (CYP) mRNA expression and CYP enzyme activity in chickens. The test substances were given to the chickens in the recommended dose or in tenfold dose. The administration of 5 mg/kg body weight (bw)  $\beta$ -glucan and 0.1 ml/kg bw DWA for five days decreased the relative gene expression of CYP1A4 and CYP2C23a. The dosing of 50 mg/kg bw  $\beta$ -glucan, 5 and 50 mg/kg bw SN, 1 ml/kg bw DWA and 250 mg/kg bw fulvic acid doubled the hepatic CYP1A4 activity. The activity of CYP2C and CYP3A remained unchanged. Avoidance of CYP1A-mediated feed–drug interactions requires accurate dosing of  $\beta$ -glucan, DWA and fulvic acid. According to our results, no treatment resulted in excessive or less CYP2C and CYP3A protein formation, which reduces the risk of potential feed additive–drug interactions in chickens. However, the administration of feed additive SN containing a plant alkaloid should be avoided concomitantly with CYP1A-metabolised medicines.

**Key words:** Chicken, cytochrome P450 mRNA and enzyme activity, feed additives

Domesticated chickens, as one of the most important food protein sources, should remain healthy and antimicrobial-free during the entire raising period. Feed additives may improve the immunity and feed utilisation of chickens. The effect of these feed additives on drug-metabolising enzymes is unknown, although the flock may need to be treated at any time during the growing period, and it is questionable whether the additives cause any alteration in cytochrome P450 (CYP) enzyme activity, which may modify the therapeutic effect of medicines.

Xenobiotic-metabolising CYPs are known to be expressed primarily in the liver, and the most important CYP isoenzymes belong to the families CYP1-3. The mRNA expression levels of CYPs are in correlation with their enzymatic activity, but the degree of correlation depends on the CYP isoform (Temesvári et

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al., 2012). Watanabe et al. (2013) found that CYP2C45 showed the highest basal mRNA expression in chicken liver, and CYP2C23b gene was the most induced gene by phenobarbital followed by CYP2C23a and CYP2C45, although CYP2C23a showed higher enzyme activity compared to CYP2C45 after phenobarbital exposure. The high abundance of CYP2C subfamily in chickens indicates the importance of these enzymes in avian drug metabolism. Several substances are available for use as feed additives or drinking water supplementation in livestock farming. In our study we investigated the effect of  $\beta$ -glucan, a sanguinarine-containing product (Sangrovit<sup>®</sup>), a drinking water acidifier (Immunofort<sup>®</sup>), and fulvic acid.

The (1-3), (1-6)  $\beta$ -glucans, the cell wall constituents of fungi, are recognised by mammalian cells as pathogen-associated molecular patterns and thus act as biological response modifiers. The fungal  $\beta$ -glucans act as broad-spectrum enhancers of host defence mechanisms, positively influencing the immunological response of mammals to bacterial, viral, and fungal infections (Palócz and Csikó, 2014). Sangrovit<sup>®</sup> is a natural plant-derived (*Macleaya cordata*) phytogetic feed additive containing many benzophenanthridine alkaloid compounds, the most abundant of which is sanguinarine (Kantas et al., 2015). Dietary supplementation with Sangrovit<sup>®</sup> significantly improved body weight and feed conversion ratio in broiler chickens (Vieira et al., 2008). Immunofort<sup>®</sup> 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 pathogenic microorganisms. Fulvic acid is one of the most active fractions of humic substances which are commonly found in soil. Fulvic acid contains many reactive functional groups, including 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 mucosal epithelium of the gastrointestinal tract, protected against infections and toxins, and improved the utilisation of nutrients in animal feed (Islam et al., 2005). The chelating ability of humic substances might enhance the intestinal uptake of microelements (Szabó et al., 2017). Dietary supplementation with fulvic acid improved feed efficiency and immunity as well (Chang et al., 2014).

There is a lack of knowledge about how the applied additives act on the pathways of xenobiotic metabolism. If drug therapy becomes necessary in a chicken flock, it is important to know how the administered substances alter the cytochrome P450 expression, leading to interactions with the drugs or their metabolites.

The aim of this study was to investigate the effect of four feed additives, namely  $\beta$ -glucan, Immunofort<sup>®</sup> (a drinking water acidifier, DWA), Sangrovit<sup>®</sup> (a sanguinarine-containing product, SN) and fulvic acid on hepatic CYP mRNA expression level and CYP enzyme activity in chickens (*Gallus gallus domesticus*).

## Materials and methods

### *Housing and treatments of chickens*

Forty-five clinically healthy 31-day-old broiler chickens (Ross 308, Herbro Ltd., Hernád, Hungary) were used in this study. The birds were housed in stainless steel cages, five birds per cage, acclimated at  $20 \pm 2$  °C on a 12-h light/12-h dark schedule. Non-medicated finisher feed (Purina<sup>®</sup>) and water were provided *ad libitum*. The chickens (mean weight  $749 \pm 24$  g) were randomly divided into 9 groups (5 chickens/group) comprising one control group and 8 experimental groups. Four of the 8 experimental groups received the feed additive at the recommended dose, while the other four were given a tenfold dose of the additive. The groups were as follows: groups receiving  $\beta$ -glucan (5 and 50 mg/kg bw), groups receiving the sanguinarine-containing product (SN) (5 and 50 mg/kg bw), groups receiving the drinking water acidifier (DWA) (0.1 and 1 ml/kg bw), and groups receiving fulvic acid (25 and 250 mg/kg bw). The test substances were freshly dissolved in water daily and were administered via crop tube (2 ml/kg bw) to the birds individually for five consecutive days. The controls received the same amount of water via crop tube (placebo). One day after the last treatment day the chickens were euthanised (Euthasol<sup>®</sup> 1 ml/kg bw intracoelomally) and their livers were perfused *in situ* with physiological saline solution until the blood was drained. Then the livers were collected, shock-frozen in liquid nitrogen and stored at  $-80$  °C until further processing.

### *Feed additives applied*

Baker's yeast  $\beta$ -glucan (Wellmune WGP<sup>®</sup>, Biothera Company, USA; main component: water-soluble  $\beta$ -(1-3), (1-6)-D-glucan; recommended dose: 5–10 mg/kg body weight).

Sanguinarine-containing product (Sangrovit<sup>®</sup> WS, Phytobiotics GmbH, Germany; main components: quaternary benzophenanthridine alkaloids and propidine alkaloids; recommended dose: 10–100 g/1000 l drinking water).

Drinking water acidifier (Immunofort<sup>®</sup>, Europharmavet Ltd., Hungary; main components: formic acid, propionic acid, phosphoric acid, methionine hydroxy analogue, zinc, copper; recommended dose: 1 l/1000 l drinking water).

Fulvic acid (Fulvix pulvis<sup>®</sup>, Alpha-Vet Ltd./Organit Ltd., Hungary; main component: fulvic acid: 70%; recommended dose: 250 g/1000 l drinking water) (Palócz et al., 2019).

### *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 homogeniser (Schuett Biotec GmbH, Göttingen, Germany). Microsomes were isolated by two-step differential

ultracentrifugation (Beckman L7-65 Ultracentrifuge, Beckman-Coulter) according to Nebbia et al. (2001). Protein concentrations were measured by Pierce™ BCA protein assay kit (ThermoFisher Scientific, Waltham, MA, USA).

#### *Quantitative real-time PCR*

The liver samples (100 mg) were homogenised in 1 ml of ice-cold RNeasy lysis reagent (Qiagen, Crawley, UK) by a Potter-Elvehjem homogeniser (Schuett Biotec GmbH, Göttingen, Germany). Total RNA was isolated from the tissue samples 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). Quantitative real-time PCR (qPCR) was performed as described previously (Palócz et al., 2017). The tested genes of interest were the avian cytochrome P450 1A4 (CYP1A4, formerly known as CYP1A1), cytochrome P450 2C23a (CYP2C23a, 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. The primer sequences are listed in Table 1. 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.

#### *Cytochrome P450 activity*

The CYP1A4, CYP3A and CYP2C activities of liver microsome samples were measured using the P450-Glo™ substrates (Luciferin-CEE, Luciferin-IPA, Luciferin-ME; Promega, Madison, USA) (Palócz 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).

#### *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.

**Table 1**  
Primer sequences used for qPCR

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

F: forward, R: reverse, B-actin: beta-actin, GAPDH: Glyceraldehyde 3-phosphate dehydrogenase, CYP: cytochrome P450, bp: base pair

### Ethical approval

The animal trials were conducted according to approved laboratory animal experimentation ethics in conformity with the national and European law, in a manner 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 the Faculty of Veterinary Science, Szent István University (no. 27/2015).

### Results

The microsomal protein concentration was practically equal ( $23.17 \pm 1.52$  mg/ml) in each sample. The activities of CYP1A4, CYP2C and CYP3A are shown in Figs 1, 2 and 3, 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 CYP1A4 activity ( $P = 0.002$ ,  $P = 0.010$ ,  $P = 0.015$ ,  $P < 0.001$  and  $P < 0.001$ , respectively; Fig. 1). The water-soluble feed additives applied orally for five consecutive days did not alter the activity of chicken hepatic CYP2C and CYP3A enzymes (Figs 2 and 3).

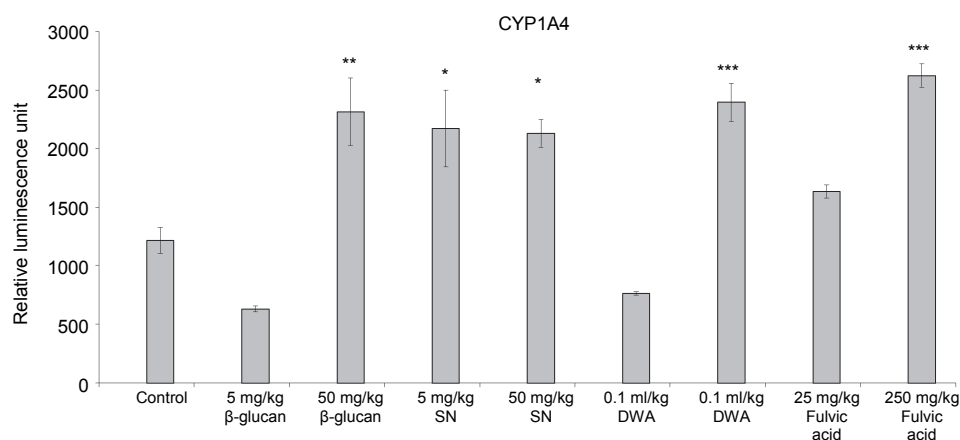


Fig. 1. Changes in cytochrome P450 1A4 enzyme activity in chicken liver microsomes ( $n = 5$ /group; \* $P < 0.05$ ; \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; each treatment group compared to control). Data are shown as mean  $\pm$  SD. SN – Sanguinarine-containing product, DWA – drinking water acidifier. The chickens were treated orally for 5 days

The results of chicken CYP450 gene expression levels normalised to two housekeeping genes are shown in Fig. 4. Treatment with 5 mg/kg bw  $\beta$ -glucan and 0.1 ml/kg bw DWA downregulated the relative gene expression of CYP1A4 and CYP2C23a. The level of CYP1A4 mRNA decreased to 36%, 60% and 53%

after 50 mg/kg bw  $\beta$ -glucan, 5 mg/kg bw SN and 1 ml/kg bw DWA administration, respectively. Furthermore, the administration of 50 mg/kg bw SN resulted in a 64% downregulation of the CYP2C23a gene.

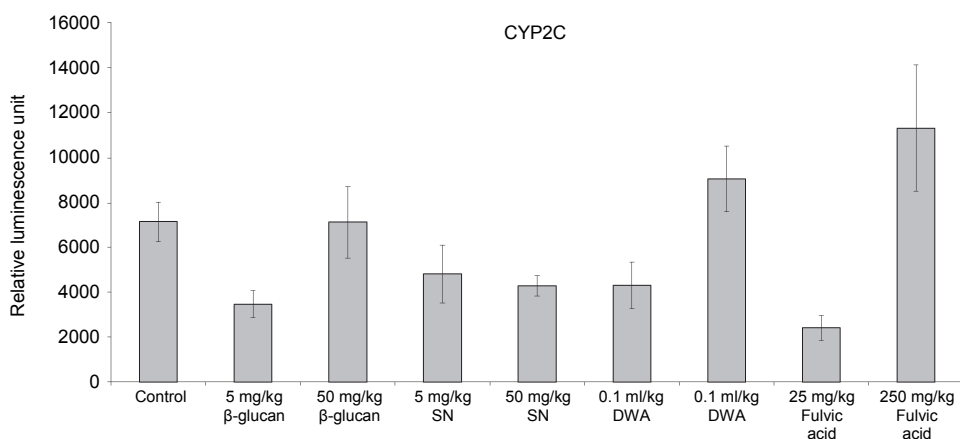


Fig. 2. Changes in cytochrome P450 2C enzyme activity in chicken liver microsomes (n = 5/group; the level of significance was set at  $P < 0.05$ ; each treatment group compared to control). Data are shown as mean  $\pm$  SD. SN – Sanguinarine-containing product, DWA – drinking water acidifier. The chickens were treated orally for 5 days

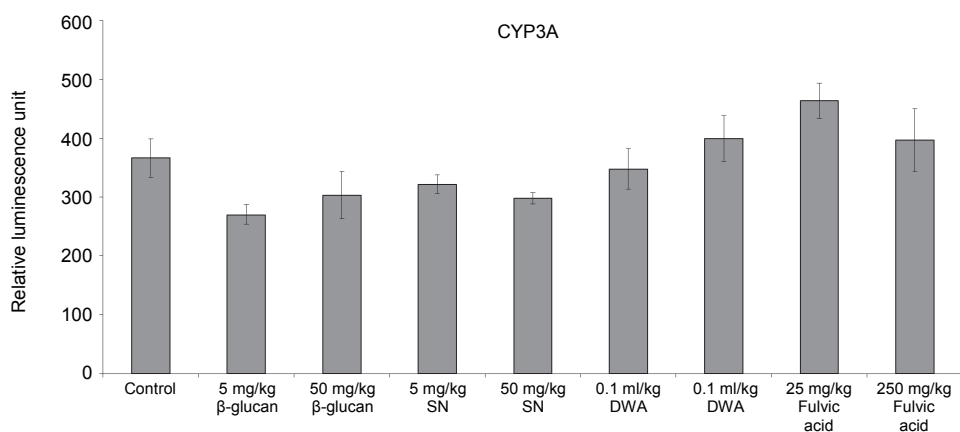


Fig. 3. Changes in cytochrome P450 3A enzyme activity in chicken liver microsomes (n = 5/group; the level of significance was set at  $P < 0.05$ ; each treatment group compared to control). Data are shown as mean  $\pm$  SD. SN – Sanguinarine-containing product, DWA – drinking water acidifier. The chickens were treated orally for 5 days

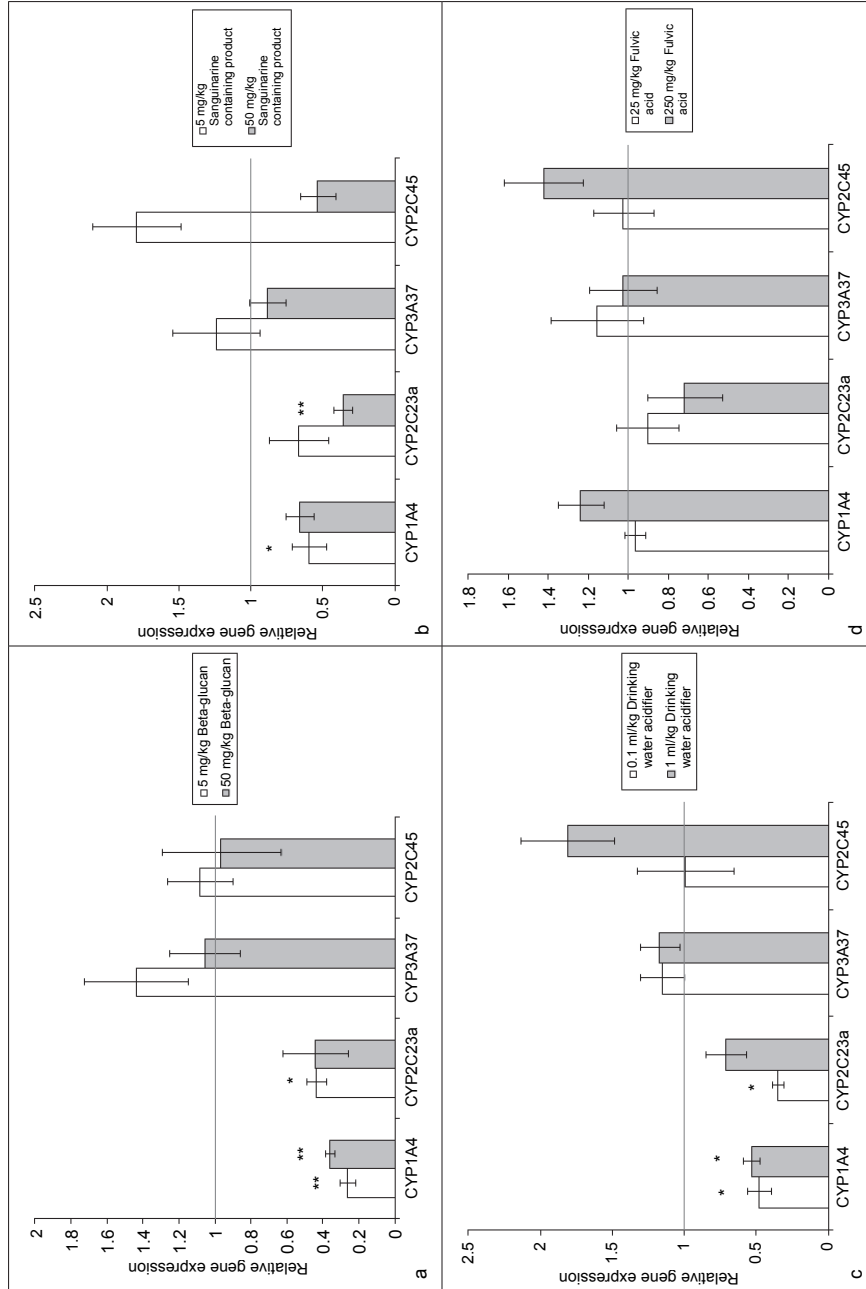


Fig. 4. Relative gene expression of the CYP genes in chicken liver (n = 5/group; \*P < 0.05; \*\*P < 0.01). Results are expressed as mean mRNA expression ratio relative to controls (represented by a horizontal line). Data are shown as mean ± SD. (a) β-glucan, (b) sanguinarine-containing product, (c) drinking water acidifier, and (d) fulvic acid treatment. The chickens were treated orally for 5 days



## Discussion

In general, the administration of feed additives to chicken flocks is proven to be effective for promoting their health or weight gain during the growing period. Although they are suspected to be safe, it is presumed that orally absorbed feed additives may influence the metabolism of drugs through the enzymes of phase I and/or phase II reaction; however, few studies have defined the actual extent of these effects (Fink-Gremmels, 2008; Csikó et al., 2014; de Boer et al., 2015). The altered metabolic pattern of medicines may influence their therapeutic activity in the treated flocks and their residue levels in foods.

In this study the activity of CYP1A enzyme was enhanced after the administration of both the recommended and the tenfold dose of SN which can be explained by the fact that polycyclic aromatic substances are metabolised via this enzyme family (Sridhar et al., 2017), hence SN, a product containing quaternary benzophenanthridine alkaloids and protopine, provoked the activity of CYP1A enzymes. The other three tested feed additives increased the activity of CYP1A enzymes in the tenfold dose but not in the recommended dose, a finding that draws attention to the importance of dosing these substances in a precise manner.

There are several differences in catalytic profiles of the chicken CYP3A37 and the human and canine CYP3A enzymes. Interestingly, CYP2C23a behaves, in regard to regulation, very similarly to CYP3A37 (Ourlin et al., 2000). The chicken CYP3A37 has relatively low abundance, which suggests that this is not the most important isoenzyme in the metabolism of xenobiotics (Watanabe et al., 2013). CYP2C enzymes play major roles in the detoxification of xenobiotics (Carre et al., 2002) and their high abundance in the liver makes them the primary drug-metabolising factors. In our study, the gene expression of CYP2C23a was decreased on some occasions; however, the activity of CYP2C enzymes did not change following the administration of the four feed additives examined. Interestingly, the gene expression level of CYP1A4 was decreased after the administration of  $\beta$ -glucan, SN and DWA; in 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, so it is possible that the gene expression phase has been down-regulated due to a feedback resulting from excess protein formation. Downregulation of the CYP2C23a gene may also be a consequence of a negative feedback as the formed CYP2C23a mRNA may inhibit the transcription of its own gene, since the activity of CYP2C remained unaltered after the treatments. Nonetheless, to prove this assumption further experiments would be necessary with more frequent sampling periods to monitor the continuity of changes in gene expression.

In conclusion, supplementation of the chickens' drinking water with  $\beta$ -glucan, SN, DWA or fulvic acid may cause cytochrome P450-mediated interactions, particularly with substances which are metabolised via the CYP1A en-

zymes. This subfamily metabolises a smaller proportion of medicines and none of them are antimicrobials. Benzimidazole anthelmintic drugs have the highest possibility of interaction with feed additives on CYP1A enzymes (Diani-Moore et al., 2006) in the chicken industry, especially when certain feed additives are overdosed or supplements containing plant alkaloids are used. Care should be taken during antiparasitic therapy of the flock if the applied feed additive contains polycyclic aromatic amines such as quaternary benzophenanthridine alkaloids and protopine alkaloids.

The activity of the avian CYP2C, which is considered the most significant in avian drug metabolism, remained unchanged after the administration of each feed additive in either dose; consequently, clinically important alterations in the CYP-mediated antimicrobial metabolism have low probability. Especially the application of fulvic acid seems to be the safest with regard to possible feed–drug interactions, since neither the activity nor the gene expression of CYP2Cs was altered after five-day administration of the substance. Further investigations are needed to clarify the changes in the gene expression profile of CYP genes. Based on our results none of the treatments resulted in excess CYP2C or CYP3A protein, which reduces the emerging risk of interactions between the tested feed additives and drugs.

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