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Distribution of Glyphosate in Chicken Organs and its Reduction by Humic Acid Supplementation

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Glyphosate (*N*-(phosphonomethyl) glycine) is a most popular herbicide in agricultural practices throughout the world. It is possible that glyphosate spread in the ecosystems can reach plants, animals. The present work was directed to investigate the glyphosate residue in different organs of broiler chickens using ELISA and to study the possibility of its neutralisation using humic acid, *Chlorella vulgaris* and *Saccharomyces boulardii*. Results showed that glyphosate residues could be detected in the animal feed and different organs as liver, spleen, lung, intestine, heart, muscles and kidney. Humic acid, *Chlorella vulgaris* and *Saccharomyces boulardii* showed neutralization of the antimicrobial effect of glyphosate *in vitro*. Also, feed supplementation of commercial broiler with humic acid (0.2%) leads to a significant decrease in the glyphosate content, i.e. by 53%, 28%, 44%, 50%, 56%, 16%, 63% and 0% in serum, liver, spleen, lung, gastro-intestinal tract, heart, muscles and kidney, respectively. There were no significant effects of humic acid on the production parameters. This enlightenment will help to overcome the negative effect of glyphosate residues on gastrointestinal microbiota and protect consumers from glyphosate residues in chicken meat.

Key words: broiler, *Chlorella vulgaris,* glyphosate, humic acids, *Saccharomyces boulardii* antimicrobial

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Introduction

Glyphosate (*N*-(phosphonomethyl) glycine) is a highly effective herbicide because of its potent and specific inhibition of 5-enolpyruvyl shikimate 3-phosphate synthase (EPSPS), an enzyme of the shikimate pathway that governs the synthesis of aromatic amino compounds in higher plants, algae, bacteria and fungi (Barry and Padgette, 1992). Some researchers believe that glyphosate is not harmful to humans or to any mammals because the shikimate pathway is absent in all animals. However, this pathway *is* present in gut microbiota which plays a role in human physiology. Recently a new pathway of glyphosate through inhibition of mammalian cytochrome P450 (CYP) enzymes, detoxify xenobiotics, was described (Samsel and Seneff, 2013). Glyphosate acts by chelating manganese required in the reduction of the flavin mononucleotide (FMN) co-factor EPSPS (Cerdeira and Duke, 2006; Clair *et al.*, 2012). In previous studies we concluded that the highly pathogenic bacteria as *Salmonella* Entritidis, *Salmonella* Gallinarum, *Salmonella* Typhimurium, *Clostridium (C) perfringens,* and *C. botulinum* are highly resistant to glyphosate. However, most of beneficial bacteria as *Enterococcus faecalis, Enterococcus faecium*, *Bacillus badius* and *Bifidobacterium adolescentis* were found to be highly susceptible (Shehata *et al.*, 2013a). A reduction of beneficial bacteria in the gastrointestinal tract (GIT) microbiota by ingestion of glyphosate could disturb the normal gut bacterial community. It is worthy to mention that, *E. faecalis, E. faecium and Bacillus badius* inhibited *C. botulinum* types A, B, D and E growth and botulinum neurotoxin (BoNT) expression (Shehata *et al.,* 2013b). The toxicity of glyphosate to the most prevalent *Enterococcus* spp. could be a significant predisposing factor that is associated with the increase in *C. botulinum* mediated diseases by suppressing the antagonistic effect of these bacteria on clostridia (Krüger *et al.,* 2013). It is possible that glyphosate spread in the ecosystems can reach plants, animals and the food chain (MacDonald and McBride, 2009). Data on the real presence of glyphosate in feed from glyphosate sprayed crops are sparse. Glyphosate residue differs from country to country (in some countries glyphosate is sprayed out of control) and even within a country depending on the quantity and frequency of glyphosate application. Humic acids are classified among the polyvalent weak organic acids and possess the ability to form com-

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plexes. Humic substances could adsorb glyphosate *in vitro* (Piccolo *et al.,* 1996; Banta *et al.*, 2009; Mazzei and Piccolo, 2012). Also Chlorella (microalgae) and *yeast* showed detoxifying properties (Shim *et al*., 2008; Baptista *et al*., 2008). Accordingly, the present study was directed to investigate the distribution of glyphosate residue in chicken organs and its neutralization using humic acid, *Chlorella vulgaris* and *Saccharomyces boulardii.*

Materials and Method

Distribution of Glyphosate in Feed and Tissues

A total of one hundred commercial broiler chickens collected from different farms were slaughtered at 30-day-old. Different organs as liver, spleen, lung, intestine, heart, muscles and kidney were collected and tested for presence of glyphosate using ELISA. Briefly, samples were collected from 10 chickens per farm at 39-day-old after slaughtering and cut to small pieces. In relation to its ability to retain water specimens were suspended in aqua distilled (Braun, Germany) at the rate of 1:1 (low water retention), 1:5 or 1:10 (high water retention). The specimens were heated at 100℃ for 10 min, homogenized with ULTRA-TURRAX® (IKA, Wilmington, Germany) and frozen at minus 80℃ for eight hours. Homogenized specimens were thawed at 40℃ and centrifuged at 10,000 xg for 10 min. The supernatant was filtered with an ultracentrifugal filter (3,000 Da) to remove proteins and peptides. Filtrates were centrifuged again at 10,000 xg for 10 min and the supernatant was tested for glyphosate concentration by ELISA using Glyphosate ELISA kits (Abraxis, Warminister, PA, USA) according to the manufacturer's protocol. Test validation was done with Gas Chromatography-Mass Spectroscopy (GC-MS) by Medizinische Labor (Bremen, Germany), the correlation coefficient between the two tests was 98%.

In vitro Neutralization of Glyphosate

The minimal inhibitory concentration (MIC) of glyphosate (Roundup UltraMax®, Monsanto, USA) on *E. faecalis, Bacillus badius* (isolated from algae *Chlorella vulgaris,* Ökologische Produkte Altmark Co., Germany) and *Bifidobacterium adolescentis* (isolated from chickens), as indicators, was determined according to the National Committee for Clinical Laboratory Standards (NCCLS). Briefly, the lowest concentration of glyphosate which shows bactericidal or bacteriostatic effects was determined in a 24-well microtitre plate. Serial dilutions of glyphosate (5, 2.5, 1.2, 0.6, 0.3, 0.15 and 0.075 mg/m*l*) were made in reinforced clostridial medium (RCM, Sifin, Germany). Tested bacteria was added at a final concentration of 10^4 CFU/ml and the test plates containing diluted glyphosate and tested bacteria were incubated overnight at 37℃. The MIC value was evaluated by quantitative analysis of bacterial growth on Citrat- Azid-Tween- Carbonat Agar (CATC, Oxoid, Germany). The neutralizing effect of humic acid RB4, composed of different molecular weights molecules ranged from 1,500 Da to 200,000 Da, (WH Pharmawerk Weinböhla GmbH, Weinböhla, Germany), was tested. The MIC value of glyphosate on *E. faecalis*, *Bacillus badius* and *Bifidobacterium adolescentis* in the presence of humic acid RB4 (1 mg/m*l*), *Chlorella vulgaris* extract (Ökologische Produkte Altmark Co., Germany) at a concentration of 1 mg/m*l* and *Saccharomyces boulardii* at a concentration of 10^9 CFU/m*l* (UCB Pharma GmbH, Monheim, Germany) determined.

In vivo Neutralization of Glyphosate Using Humic Acid

The experiment was performed in two chicken broiler barns, designated A and B, each barn accommodated for 22,000 broiler chicks. Chickens kept in house A were fed the basic diet without supplementation of humic acid, while chickens kept in house B were fed the same diet with humic acid RB4 (WH Pharmawerk Weinböhla GmbH, Weinböhla, Germany) supplementation (0.2%) from the first day till slaughtering. The ration was formulated as follow: starter (21% corn, 40% wheat, 29% soya bean and 4.5% fat), grower (22% corn, 47% wheat, 19% soya bean and 5% fat), and finisher (17% corn, 48% wheat, 17% soya bean and 4.9 % fat). Chickens were allowed to have free access to feed and water until the end of experiment. All chickens were vaccinated against infectious bronchitis (IB) at 12-day-old, Newcastle disease (ND) and infectious bursal disease at 18-days-old. The total mortality and body weight (BW) were calculated at the end of the experiments. Glyphosate residues were determined in serum, liver, spleen, lung, GIT, heart, muscles and kidney using ELISA as mentioned above.

Statistical Analysis

The statistical analysis was carried out with GraphPad Prism 4 (GaphPad Software, La Jolla, USA). Two-way analysis of variance followed by unpaired Student t-test was used to identify significant differences between means.

Results

Distribution of Glyphosate in Feed and Tissues

The glyphosate residues could be detected in feed, liver, spleen, lung, intestine, heart, muscles and kidney using ELISA in the concentrations of 370, 9.8, 21.1, 24.2, 98.3, 20.4, 5.0 and 16.0 ng/gm, respectively, (Table 1).

Neutralisation of Glyphosate in vitro

The MIC value of glyphosate for *E. faecalis*, *Bacillus badius* and *Bifidobacterium adolescentis* were 300, 300 and 150 μg/m*l*, respectively. The RB4 and *Chlorella vulgaris* in concentrations of 1 mg/ml showed the higher neutralization

Table 1. **Distribution of glyphosate in feed and chickens tissues**

Sample N=30	Glyphosate (ng/gm)		
	Minimum	Maximum	Mean±SD
Feed	190.0	400.0	370.0±92.0
Liver	6.0	13.6	9.8±3.0
Spleen	11.8	25.0	21.1±17.0
Lung	12.0	25.0	24.2±9.0
Intestine	20.0	120.0	98.3±42.0
Heart	17.0	20.0	20.4±0.6
Muscles	3.6	4.9	5.0±0.3
Kidney	0.4	17.6	16.0±13.0

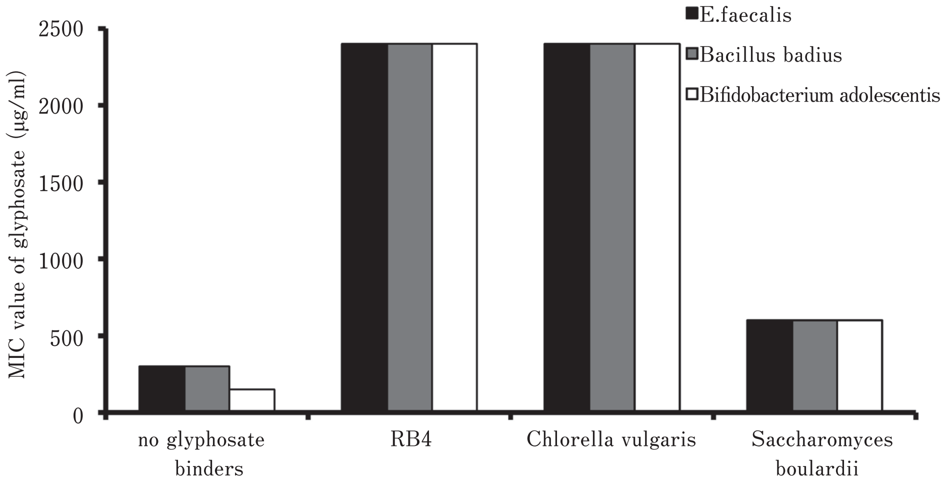

Fig. 1. **Changes in the MIC values of glyphosate on *E. faecalis*, *Bacillus badius* and *Bifidobacterium adolescentis* using different glyphosate binders.**

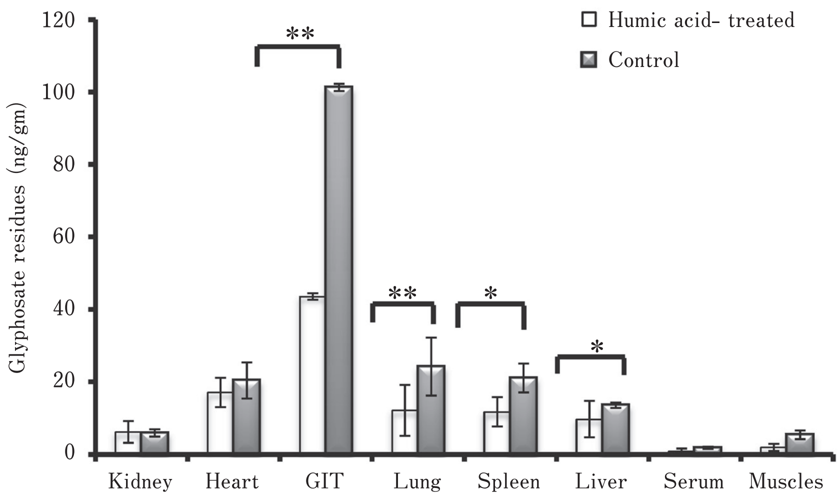

Fig. 2. **Effect of humic acid supplementation on glyphosate accumulation in chickens.** Glyphosate was measured using ELISA and expressed as ng/gm. Asterisks denote significant decrease of glyphosate in humic acid treated chickens (* $P=0.05$, ** $=P<0.001$).

of the antimicrobial effect of glyphosate. The MIC-values of glyphosate for *E. faecalis*, *Bacillus badius* and *Bifidobacterium adolescentis* in the presence of humic acid or *Chlorella vulgaris* were 2400 μg/m*l* (Fig. 1). However, the MIC-value of glyphosate for *E. faecalis*, *Bacillus badius* and *Bifidobacterium adolescentis* in the presence *Saccharomyces boulardii* was 600 μg/m*l* (Fig. 1).

In vivo Neutralization of Glyphosate Using Humic Acid

In untreated chickens, the glyphosate concentrations in serum, liver, spleen, lung, GIT, heart, muscles and kidney were 2, 14, 21, 24, 101, 20, 6 and 6 ng /gm, respectively, however, in humic acid treated chickens, glyphosate residues were 0.88, 9.78, 11.79, 12.20, 43.6, 17.4, 1.9 and 6.2 ng /gm, respectively. Supplementation of humic acid caused a significant decrease in the glyphosate content, i.e. by 53%, 28%, 44%, 50%, 56%, 16%, 63% and 0% in serum, liver, spleen, lung, GIT, heart, muscles and kidney, respectively, (Fig. 2). At 30-day-old, there is no significant improvement of body weight and total mortalities between humic acid-treated and untreated chickens (Table 2), the average body weight of both was 1.69 Kg. However at 39-day-old, the average body weight of 2.456 Kg while it was 2.339 Kg in untreated chickens (Table 2).

Table 2. **Effect of humic acid supplementation on the production parameters**

Parameter	Humic acid-treated chickens	Non-treated chickens
Total number	22500	23100
Slaughtered number at 30-day-old	6509	6509
Slaughtered number at 39-day-old	14581	15573
Body weight at 30-day-old (average/kg)	1.69	1.69
Body weight at 39-day-old (average/kg)	2.453	2.339
Total feed intake (kg)	76530	78690
Food conversion ratio	1.64	1.66

Discussion

Distribution of Glyphosate in Feed and Tissues

Glyphosate residues in food and feed have been on the rise, due to higher rates and frequency of application, which in turn is due to increasing weed resistance (Samsel and Seneff, 2013). In the present study glyphosate residues could be detected in liver, spleen, lung, intestine, heart, muscles, kidney and animal feed (Table 1). The maximum residue levels (MRLs) of glyphosate in soya bean, maize, cereal grains, cotton seed, alfalfa, hay, sorghum straw, wheat and wheat straw were agreed by the United Nations Food and Culture Organization's to be 20, 5.0, 30, 40, 500, 500, 50, 200 and 300 mg/kg (WHO, 1994). Data on the real presence of glyphosate and its metabolite in feed from glyphosate sprayed crops are sparse. A now common practice of crop desiccation through herbicide administration shortly before the harvest assures an increased glyphosate residues in food sources as well (Baig *et al*., 2003; Ellis *et al*., 1998). Also, the maximum daily intake (MDI) of glyphosate depends on the ration composition and the percent of each component in the ration. Glyphosate residues concentrate in approximately 80% genetically modified plants grown for food and feed up to 400 ppm, maximal residual levels.

Neutralisation of Glyphosate in vitro

Many studies have reported that glyphosate can be sorbed to humic acids (Piccolo *et al*., 1996; Banta *et al*., 2009; Mazzei and Piccolo, 2012). In the present study the humic acid RB4 neutralized the antimicrobial effect of glyphosate *in vitro*. The MIC-value of glyphosate for *E. faecalis*, *Bacillus badius* and *Bifidobacterium adolescentis* in the presence of RB4 humic acids or *Clorella vulgaris* were 2.4 mg/m*l*.

Chlorella has also useful detoxifying properties. The use of oral supplements of *Chlorella pyrenoidosa* has been reported to significantly reduce dioxin levels in breast milk of 35 nursing women in Japan (Nakano *et al*., 2007). Also Chlorella supplementation significantly reduced liver toxicity and cadmium-accumulation in cadmium poisoned rats (Shim *et al*., 2008).

Yeast has been used as general performance promoter in poultry feeds and has been shown to have beneficial effects against mycotoxins exposure (Celyk *et al*., 2003, Santin *et al*., 2003, Baptista *et al*., 2004). The absorbent ability of yeast to mycotoxins could be attributed to the presence of innumerable sites on its surface for physical adsorption of molecules (Shetty and Jespersen 2006). In the present study *Saccharomyces boulardii* showed a low absorbent ability to glyphosate (Fig. 1).

Neutralisation of Glyphosate by Humic Acid Supplementation in vivo

The use of humic acids and their sodium salt for the oral treatment of all animals on food production farms is currently permitted. Supplementing animal feeds with non-nutritive adsorbents as humic acid has proven to substantially reduce mycotoxicosis (Sabater-Vilar *et al*., 2007) and improved the performance, carcass, GIT and meat quality traits (Ozturk *et al*., 2011). In our study, the mortality was negligible with no difference between control and humic acid-treated group. Also the humic acid-treated chickens showed no improvement in feed conversion in birds and body weight at 30-day-old (Table 2). Kocabagli *et al*. (2002) reported an improvement in feed conversion in birds that were given 0.25% humic acid either from 0 to 42 d or during grow-out periods only, between d 21 to 42. A similar conclusion was drawn by Yoruk *et al*. (2004), who showed a better feed conversion in hens supplemented with 0.1–0.2% humic acid, and it did not affect body weight. On the contrary, Rath *et al*. (2006) found that humic acid-treated chickens showed a reduction in body weight, and the feed conversion ratio was numerically higher.

Conclusion

Glyphosate spread in the ecosystems can reach chickens and accumulated in different tissues. Our findings indicated that humic acid RB4, *Chlorella vulgaris* and *Saccharomyces boulardii* neutralized the antimicrobial activity of glyphosate *in vitro* with different degrees. Supplementation of humic acids in animal feed could play an important role in the retention of glyphosate residues and reduce glyphosate in animal products.

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