

The Effect of Glyphosate on Potential Pathogens and Beneficial Members of Poultry Microbiota In Vitro

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Abstract The use of glyphosate modifies the environment which stresses the living microorganisms. The aim of the present study was to determine the real impact of glyphosate on potential pathogens and beneficial members of poultry microbiota in vitro. The presented results evidence that the highly pathogenic bacteria as *Salmonella* Enteritidis, *Salmonella* Gallinarum, *Salmonella* Typhimurium, *Clostridium perfringens* and *Clostridium botulinum* are highly resistant to glyphosate. However, most of beneficial bacteria as *Enterococcus faecalis*, *Enterococcus faecium*, *Bacillus badius*, *Bifidobacterium adolescentis* and *Lactobacillus* spp. were found to be moderate to highly susceptible. Also *Campylobacter* spp. were found to be susceptible to glyphosate. A reduction of beneficial bacteria in the gastrointestinal tract microbiota by ingestion of glyphosate could disturb the normal gut bacterial community. Also, 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.

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 [2]. Its herbicidal action is generated by chelating manganese required in the reduction of the flavin mononucleotide (FMN) co-factor EPSPS [12]. It is possible that glyphosate can spread in the ecosystem and reach plants, animals and food chain. Glyphosate and its metabolite aminomethylphosphonate (AMPA) could be detected in green immature seed [33], harvested seeds [16] and in ground water [47]. Glyphosate and AMPA are amongst the first major pollutants of surface waters [25]. In the literature, different opinions exist about the safety of this herbicide. Some reports have not found any significant risk to human and animal health by the use of glyphosate, since this EPSPS enzyme is absent in animals [15, 52]. However, other inhibition pathways are involved, like cytochrom P450 aromatase inhibition. It has also been shown that glyphosate in vertebrates is genotoxic [44] and teratogenic [40]. Glyphosate is cytotoxic to human placental, embryonic kidney and liver cell lines at very low sub-agricultural dilutions [4, 5, 20]. In vivo, the adverse effects of glyphosate on male reproductive system of ducks and rabbits [39, 59] as well as liver of rats [6, 7] were reported. The use of such herbicides modifies the environment which

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stresses the living organisms [35, 55]. In addition, they can disturb also the fresh water microbial communities directly or indirectly and reduces the biodiversity of species in the aquatic community [46]. Moreover, glyphosate showed differences in sensitivity between microorganisms [9, 13, 23]. Normal enteric microbiota has been found to protect the gastrointestinal tract (GIT) against colonisation by a variety of pathogens [11]. The microbiota of the GIT of domestic animals consists of a balanced composition of facultative and obligatory anaerobic bacteria. The mature microbiota profile varies considerably along the length of the GIT and may be specific to animal species and individuals [22]. Numerous bacteria of the GIT produce bacteriocins directed against some other bacterial species including pathogens. Lactic acid bacteria like lactobacilli, lactococci and enterococci may generate such bacteriocins, specifically; *Enterococcus faecalis* may generate an enterocin 1146 that was shown to be very effective against *Clostridium butyricum* and *Clostridium perfringens* [28, 41, 42]. In the present study, the effect of glyphosate on growth and viability of potential pathogens and beneficial members of poultry microbiota were investigated in vitro.

Materials and Methods

Glyphosate

Roundup UltraMax® (Monsanto, USA) which contains 450 mg/ml of glyphosate was used in this study.

Strains and Cultural Conditions

Experiments were carried out with *Bacillus badius*, *Bacillus cereus*, *Bacteriodes vulgatus*, *Bifidobacterium adolescentis*, *Campylobacter coli*, *Campylobacter jejuni*, *C. perfringens*, *C. botulinum* type A, *C. botulinum* type B, *Escherichia coli*, *E. coli* 1917 strain Nissle, *E. faecalis*, *E. faecium*, *Lactobacillus buchneri*, *L. casei*, *L. harbinensis*, *Riemerella anatipestifer*, *Salmonella* Enteritidis, *Salmonella* Gallinarum, *Salmonella* Typhimurium, *Staphylococcus aureus*, *Staphylococcus haemolyticus* and *Staphylococcus lentus* were used. Cultural conditions and origin of these strains are described in Table 1.

Bacterial Identification

Bacterial strains were tested using matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF). In brief, about 10 mg of cell material of the cultured strains were suspended in 300 µl of sterile water. 900 µl of absolute ethanol was added, and the mixture was centrifuged at 10,000 rpm for 2 min. The supernatant was discarded, and

the pellet was suspended in 50 µl formic acid (70 % v/v). After adding 50 µl acetonitrile (AN), the mixture was centrifuged at 10,000 rpm for 2 min. 1 µl of the clear supernatant was transferred to the MALDI target and allowed to dry. A saturated solution of α -cyano-hydroxy-cinnamic acid (Bruker Daltonik GmbH) in a standard organic solvent mixture (2.5 % trifluoroacetic acid to AN 50 % in water) was added to resuspended 1 µl of the dried material. All chemicals used were of the highest quality (Merck, designated to be especially suitable for HPLC or MALDI-based techniques). Before each MALDI run, *E. coli* 1917 strain Nissle was analysed to serve as the positive control and calibration standard. The MALDI-TOF MS analysis was performed using a Bruker microflex LT mass spectrometer (Bruker Daltonik Co), and the spectra were automatically identified using the Bruker BioTyper™ 1.1 software. *C. perfringens* was identified by MALDI-TOF and multiplex PCR [3], data are not shown.

Effect of Glyphosate on Different Bacteria

The minimal inhibitory concentration (MIC) of Roundup UltraMax® was determined in triplicate in a 24-well microtiter plate. 100 µl of tested bacteria (10^5 cfu/ml) was added to 900 µl broth media containing different concentrations of glyphosate (5.0, 2.40, 1.20, 0.60, 0.30, 0.15 and 0.075 mg/ml). Plates containing diluted glyphosate and bacteria were incubated at 37 °C (Table 1). Bacterial growth was evaluated on suitable agar medium. Culture condition for each bacterium is shown in Table 1. The MIC value was evaluated by quantitative analysis of bacteria on agar plate. The morphology of bacteria was examined microscopically.

Effect of Glyphosate on *C. botulinum* Type A and B

Clostridium botulinum was cultured anaerobically in a cooked meat broth at 37 °C for 5 days, followed by cultivation in reinforced clostridial medium (RCM, Sifin, Germany) anaerobically at 37 °C for 3 days. *C. botulinum* types A and B were heated at 80 °C for 10 min and left at room temperature under aerobic condition. Cultures were tested daily for sporulation using a Gram or Raketite stain. To study the effect of glyphosate on *C. botulinum* strains, heat treated spores or vegetative cells were added to RCM medium at a final concentration of 10^4 cfu/ml. The inhibitory effect of glyphosate was determined using the following concentrations: 5, 2.40, 1.20, 0.60, 0.30, 0.15 and 0.075 mg/ml. The mixture was incubated anaerobically at 37 °C for 5 days. *C. botulinum* was quantified using the most probable number (MPN) estimation method. *C. botulinum* type A and B neurotoxins (BotNT) were analysed using ELISA [30]. In brief, ELISA was performed in flat-

Table 1 Target strains used, their origin and medium used for glyphosate experiments

Genus/species	Origin	Reference no. and related collections	Lab. no.	Culture media		Incubation conditions
				Agar	broth	
<i>Bacillus badius</i>	Green algae (Altmark Co, Germany)	Isolated in this study	1/12	Blood agar ^a	RCM ^b	37 °C/24 h aerobic
<i>Bacillus cereus</i>	Green algae (Altmark Co, Germany)	Isolated in this study	2/12	Blood agar	RCM	37 °C/24 h aerobic
<i>Bacteriodes vulgatus</i>	Chickens	Isolated in this study	3/12	Blood agar	RCM	37 °C/48 h anaerobic
<i>Bifidobacterium adolescentis</i>	Chickens	Isolated in this study	4/12	MRS ^c	MRS	37 °C/48 h anaerobic
<i>Campylobacter coli</i>	Institute of Bacteriology, Leipzig University	11151/03	11151/03	Caso-modified agar	Caso-modified broth	37 °C/48 microaerophilic
<i>Campylobacter jejuni</i>	Institute of Bacteriology, Leipzig University	188	188	Caso-modified agar	Caso-modified broth	37 °C/48 microaerophilic
<i>C. perfringens</i>	RIPAC-LABOR GmbH, Germany	884/2	884/2	NeoP ^d	RCM	37 °C/24 h anaerobic
<i>C. botulinum</i> type A	National collection of type culture (NCTC)	7272	7272	Blood agar	RCM	37 °C/48 h anaerobic
<i>C. botulinum</i> type B	National collection of type culture (NCTC)	7273	7273	Blood agar	RCM	37 °C/48 h anaerobic
<i>E.coli</i>	Chickens	Isolated in this study	5/12	Blood agar	RCM	37 °C/24 h aerobic
<i>E. coli</i> 1917 strain Nissle	Ardeypharm GmbH, Herdecke, Germany	Nissle	Nissle	Blood agar	RCM	37 °C/24 h aerobic
<i>Enterococcus faecalis</i>	<i>Chlorella vulgaris</i>	Isolated in this study	6/12	CATC ^e	RCM	37 °C/24 h aerobic
<i>Enterococcus faecium</i>	Chickens	Isolated in this study	7/12	CATC	RCM	37 °C/24 h aerobic
<i>Lactobacillus buchneri</i>	Effective microorganisms (Multikraft Co, Austria)	12/25	12/25	MRS	MRS	37 °C/48 h anaerobic
<i>Lactobacillus casei</i>	Effective microorganisms (Multikraft Co, Austria)	12/26	12/26	MRS	MRS	37 °C/48 h anaerobic
<i>Lactobacillus harbinensis</i>	Chickens	Isolated in this study	8/12	MRS	MRS	37 °C/48 h anaerobic
<i>Riemerella anatipestifer</i>	Institute of Poultry Diseases, Berlin University	12109	12109	Blood agar	RCM	37 °C/24 h aerobic
<i>Salmonella</i> Enteritidis	Chickens	Isolated in this study	9/12	Caso agar ^f	RCM	37 °C/24 h aerobic
<i>Salmonella Gallinarum</i>	Institute of Poultry Diseases, Berlin University	Z34/11	Z34/11	Caso agar	RCM	37 °C/24 h aerobic
<i>Salmonella Typhimurium</i>	Chickens	Isolated in this study	10/12	Caso agar	RCM	37 °C/24 h aerobic
<i>Staphylococcus aureus</i>	Chickens	Isolated in this study	11/12	Blood agar	RCM	37 °C/24 h aerobic
<i>Staphylococcus haemolyticus</i>	Chickens	Isolated in this study	12/12	Blood agar	RCM	37 °C/24 h aerobic
<i>Staphylococcus lentus</i>	Chickens	Isolated in this study	13/12	Blood agar	RCM	37 °C/24 h aerobic

^a Blood agar (Fluka, Germany)^b Reinforced clostridial medium (RCM, SIFIN, Germany)^c deMan, Rogosa and Sharpe Lactobacillus Agar (Oxoid, Germany)^d Neomycin-polymyxin blood agar (neomycin (100 mg/l, Roth, Germany)/polymyxin B (50 mg/l Fluka, Germany)^e Citrat-Azid-Tween-Carbonat agar (Oxoid, Germany)^f Caso agar (3.5 % casein-soya, 0.3 % yeast extract, 0.1 % glucose, 1.5 % agar)

bottomed ELISA plates (96 wells, high binding; Costar, Corning, New York, USA). All standard volumes were 100 µl/well and the standard incubation condition was 1 h

at room temperature (1 h at RT) on a microtitre plate shaker (400 rpm). The coating buffer was 0.1 M NaHCO₃ and the wash solution (WS) was 0.9 % NaCl with 0.1 %

Tween 20 (Sigma-Aldrich, Taufkirchen, Germany). After coating the wells with capture antibodies (3 µg/ml, rabbit IgG against *C. botulinum* neurotoxin type A or B, Institute of Bacteriology and Mycology, University of Leipzig), the plates were incubated overnight at 4–6 °C. ELISA plates were washed twice with WS and loaded with the diluted specimens. Supernatants of the cultures were diluted 1:10 or higher in assay buffer (assay buffer: 20 mM Tris, pH 8.00 [adjusted with 1 M HCl], 0.9 % NaCl, 5 mM EDTA, 1.0 % gelatine from cold water fish skin, 0.2 % bovine serum albumin, 0.1 % rabbit IgG and 0.2 % Tween 20 (all from Sigma-Aldrich or Fluka, Taufkirchen, Germany)). The plates were incubated for 1 h at RT, washed five times with WS and loaded with the detection antibodies conjugated with horseradish peroxidase, diluted in assay buffer. BotNT types A and B were detected with 2.5 µg/ml horse ([Fab]₂ from IgG) against *C. botulinum* types A and B, respectively (Novartis Vaccines and Diagnostics GmbH, Marburg, Germany). After 1 h incubation at RT, the plates were washed four times with WS. All washing steps were done by a Nunc-Immuno-Washer 12 (Nunc, Wiesbaden, Germany). The antibody bound marker enzyme, horseradish peroxidase, was detected by adding 3 mM H₂O₂ and 1 mM 3,3',5,5'-tetramethylbenzidine (TMB) in 0.2 M citrate-buffer to each well (pH 4.0). The substrate reaction was stopped with 1 M H₂SO₄ (50 µl/well). The optical density (OD) was measured using a microplate ELISA reader at 450 nm. The supernatant with known concentrations of the BotNT types A and B (Institute of Bacteriology and Mycology, University of Leipzig) were used as a standard.

Effect of Glyphosate on *Campylobacter* spp.

The effect of glyphosate on *C. coli* and *C. jejuni* was tested in a 10 ml tissue culture flask in Caso-modified broth media containing 3.5 % caso broth 0.3 % yeast extract, 0.3 % casein hydrolysate, 0.03 % FeSO₄ and 0.03 % natrium pyruvate. Glyphosate was added with different concentrations (5.0, 2.40, 1.20, 0.60, 0.30 and 0.15 mg/ml). Tested bacteria was added at a final concentration of 10⁴ cfu/ml under microaerophilic conditions at 37 °C for 48 h. Bacterial growth in the presence of different concentrations of glyphosate was tested on Caso-modified agar (3.5 % caso agar, 0.3 % yeast extract, 0.3 % casein hydrolysate, 0.03 % FeSO₄ and 0.03 % natrium pyruvate, 0.4 % active charcoal and 1 % agar).

Effect of Glyphosate on Sporozoites

Sporulated *Eimeria tenella* oocysts (isolate LE-01 Eten-05/1) were kindly supplied by Institute of Parasitology, Faculty of Veterinary Medicine, Leipzig University,

Leipzig, Germany. Sporozoites of *E. tenella* were excysted as described [56]. In brief, after surface sterilization with bleach, the oocyst walls were broken using 0.5-mm glass beads (Biospec product, Bartleville, OK, USA). Thereafter, the sporozoites were recovered from sporocysts by enzymatic excystation using 0.025 trypsin (w/v) (Carl Roth, Karlsruhe, Germany), 10 mM MgCl₂ (w/v) and 1 % sodium taurocholic acid (w/v) (Sigma, Taufkirchen, Germany) at 41 °C for 60–90 min. The excysted sporozoites were purified by anion exchange method [48]. The pellet sporozoites were collected carefully from the bottom of the micro-tubes and washed three times with phosphate-buffered saline (PBS). To study the effect of glyphosate on *E. tenella*, sporozoites were incubated at 37 °C overnight with 1.20, 0.60, 0.30, 0.15 and 0.075 mg glyphosate in 1 ml Dulbecco's modified Eagle's medium (DMEM) containing 5 % newborn calve serum. The viability of sporozoites was determined by the trypan blue staining method [56]. In short, sporozoites suspensions were exposed to the same volume of 0.5 % trypan blue in 0.1 M phosphate buffer, pH 7.0. The non-stained parasites were regarded as viable and those stained as dead. Examination of morphological alterations and/or viability was done and images were captured using an inverted microscope (Leica DM IRB, Bensheim, Germany).

Results

Effect of Glyphosate on Potential Pathogens and Beneficial Bacteria

Most of tested pathogenic bacteria were highly resistant to glyphosate; however, most of tested beneficial bacteria were found to be moderate to highly susceptible (Table 2). The herbicide formulated glyphosate at a concentration of 1.2 mg/ml inhibited the growth and BotNT expression (Tables 2, 3). Supplementation of the medium with 1.2 mg/ml glyphosate reduced the cell numbers of *C. botulinum* type A and B after 5 days of cultivation. *C. perfringens*, *Salmonella* Gallinarum, *Salmonella* Typhimurium, *Salmonella* Enteritidis and *E. coli* showed to be highly resistance to glyphosate (MIC value 5 mg/ml) (Table 2). *L. casei*, *L. buchneri*, *L. harbinensis*, *Staphylococcus aureus* and *Staphylococcus lentus* were moderately resistant to glyphosate (MIC value 0.60 and 0.30 mg/ml, respectively). On the other hand, with the exception of *Lactobacillus* spp., all tested beneficial bacteria including *E. faecalis*, *E. faecium* and *B. badius*, *B. cereus* and *B. adolescentis* were highly sensitive to glyphosate with MIC value of 0.15, 0.15, 0.30 and 0.075 µg/ml, respectively. Colonies morphology showed no significant differences between control and glyphosate-treated

Table 2 Inhibitory effect of glyphosate on different bacteria

Genus/species	MIC value of glyphosate (mg/ml) ^a	Bacterial count ^b	
		Treated at MIC value (mean $\pm$ SD, $n = 3$)	Untreated (mean $\pm$ SD, $n = 3$) ^c
<i>Bacillus badius</i>	0.150	2.24 $\pm$ 0.49	8.90 $\pm$ 0.44
<i>Bacillus cereus</i>	0.300	2.75 $\pm$ 0.68	8.08 $\pm$ 0.12
<i>Bacteriodes vulgatus</i>	0.600	3.54 $\pm$ 0.31	7.37 $\pm$ 0.10
<i>Bifidobacterium adolescentis</i>	0.075	3.87 $\pm$ 0.50	8.67 $\pm$ 0.48
<i>Campylobacter coli</i>	0.150	3.07 $\pm$ 0.50	9.00 $\pm$ 0.70
<i>Campylobacter jejuni</i>	0.150	3.90 $\pm$ 0.50	9.54 $\pm$ 0.97
<i>C. perfringens</i>	5.000	3.37 $\pm$ 0.89	8.30 $\pm$ 0.28
<i>C. botulinum</i> type A	1.200	4.00 $\pm$ 0.50	8.16 $\pm$ 0.32
<i>C. botulinum</i> type B	1.200	3.56 $\pm$ 0.45	7.60 $\pm$ 0.57
<i>E. coli</i>	1.200	3.15 $\pm$ 0.24	8.00 $\pm$ 0.34
<i>E. coli</i> 1917 strain Nissle	1.200	2.35 $\pm$ 0.24	7.26 $\pm$ 0.21
<i>Enterococcus faecalis</i>	0.150	2.00 $\pm$ 0.45	8.49 $\pm$ 0.58
<i>Enterococcus faecium</i>	0.150	2.01 $\pm$ 0.34	7.06 $\pm$ 0.95
<i>Lactobacillus buchneri</i>	0.600	4.00 $\pm$ 0.88	8.00 $\pm$ 0.22
<i>Lactobacillus casei</i>	0.600	4.74 $\pm$ 0.56	8.28 $\pm$ 0.35
<i>Lactobacillus harbinensis</i>	0.600	5.30 $\pm$ 0.44	8.40 $\pm$ 0.32
<i>Riemerella anatipestifer</i>	0.150	4.00 $\pm$ 0.50	7.88 $\pm$ 0.50
<i>Salmonella</i> Enteritidis	5.000	2.35 $\pm$ 0.26	8.28 $\pm$ 0.16
<i>Salmonella</i> Gallinarum	5.000	2.15 $\pm$ 0.33	8.68 $\pm$ 0.20
<i>Salmonella</i> Typhimurium	5.000	2.75 $\pm$ 0.68	8.03 $\pm$ 0.16
<i>Staphylococcus aureus</i>	0.300	5.74 $\pm$ 0.58	9.00 $\pm$ 0.10
<i>Staphylococcus haemolyticus</i>	0.300	5.74 $\pm$ 0.32	8.08 $\pm$ 0.16
<i>Staphylococcus lentus</i>	0.300	3.90 $\pm$ 0.44	8.08 $\pm$ 0.14

^a Minimal inhibitory concentration (MIC) of Glyphosate^b Mean of quantitative bacterial counts expressed as reciprocal log₁₀^c Bacterial counts without glyphosate treatment (control)

bacteria with glyphosate. Microscopically, *Lactobacillus* spp. *B. vulgatus* and *E. tenella* showed swelling and corrugated cell wall.

Effect of Glyphosate on *E. tenella* Sporozoites

In comparison to untreated control, glyphosate at concentrations of 0.6 mg/ml clearly affected the sporozoites morphology after exposure to glyphosate for 24 h. *E. tenella* sporozoites showed morphological changes including swelling and corrugated cell wall. At concentration of 0.3 mg/ml, few sporozoites were affected. However, at 0.15 mg/ml, there was no visible effect on sporozoites morphology. At 1.20 mg and 2.4 mg/ml glyphosate, all sporozoites were destroyed.

Discussion

Glyphosate is believed to be the major active principle in the herbicide through its inhibition of EPSPS. It blocks EPSPS, a key enzyme involved in the biosynthesis of aromatic amino acids, naturally present in plants, fungi and some bacteria [49]. The present investigation was carried out to determine the effects of glyphosate on different potential pathogens and beneficial bacteria in the aim to highlight its impact on chicken GIT microbiota. Glyphosate showed different effects according to the tested microorganisms, similar results were described [9, 13, 49]. Most of beneficial poultry microbiota as *E. faecalis*, *E. faecium* and *B. adolescentis* are highly sensitive to glyphosate (Table 2). However, these concentrations did not inhibit growth or BotNT production of *C. botulinum* type A and B. It is worthy to mention that enterococci are able to suppress growth and toxin production of *C. botulinum*. The loss of most enterococci from the GIT microbiota may lead to an increase of BotNT production in animals [27]. Special strains of *C. perfringens*, *C. sporogenes* and *B. cereus* were able to antagonize *C. botulinum* types A, B, F and E [51]. Enterococci were able to inhibit *C. botulinum* growth [54]. The authors investigated 14 strains of *C. botulinum* (three type A, four type B, three type E, two type F and two type G), 88 anaerobic and 64 facultative anaerobic isolates using zone inhibition procedures. Strains of bifidobacteria, lactobacilli, propionibacteria and enterococci inhibited the growth of *C. botulinum*, as did several strains of the *Bacterioides fragilis* group. *C. ramosum* was the only member of the genus *Clostridium* which inhibits *C. botulinum* type G and F strains. Most of the investigated *C. botulinum* strains were inhibited in co-cultural experiments with intact faecal samples. Sullivan et al. [54] thought that the inhibition of *C. botulinum* spores or vegetative cells indicated that the normal flora of healthy individuals had a bacteriostatic, rather than a bactericidal effect on the growth of *C. botulinum* and concluded that infant botulism may result, in part, from the absence of inhibitory organisms in the normal flora of the

Table 3 Effect of different glyphosate concentrations on *C. botulinum* types A and B growth and BotNT expression

Glyphosate concentration (mg/ml)	<i>C. botulinum</i> type A ^a		<i>C. botulinum</i> type B	
	CFU ^b (mean $\pm$ SD, <i>n</i> = 3)	BotNT type A ^c (mean $\pm$ SD, <i>n</i> = 3)	CFU (mean $\pm$ SD, <i>n</i> = 3)	BotNT type B (mean $\pm$ SD, <i>n</i> = 3)
0	8.16 $\pm$ 0.32	2,400 $\pm$ 378	7.60 $\pm$ 0.57	1,350 $\pm$ 270
0.6	6.12 $\pm$ 0.67	2,250 $\pm$ 126	5.50 $\pm$ 0.18	470 $\pm$ 60
1.2	4.00 $\pm$ 0.50	130 $\pm$ 89	3.56 $\pm$ 0.45	70 $\pm$ 19
2.4	<10 ³	0.0	<10 ³	0.0
5.0	<10 ³	0.0	<10 ³	0.0

^a *Clostridium botulinum* spores (10⁵ cfu/ml) in reinforced clostridial medium (RCM) broth incubated with different glyphosate concentrations anaerobically for 5 days at 37 °C

^b *Clostridium botulinum* quantified using the most probable number (MPN) estimation method and expressed as reciprocal log₁₀

^c Botulinum neurotoxines measured by ELISA [30]

infant intestine. Recently, we confirmed that *E. faecalis*, *E. faecium* and *B.adius* inhibited the *C. botulinum* types A, B, D and E growth and BotNT expression [50]. The loss of such microbiota could be induced by contamination of feeds with residual glyphosate. In vitro toxicity of 0.075–0.30 mg/ml glyphosate inhibited *E. faecalis*, *B.adius* and *Bifidobacter adolescentis* (Table 2). Such concentrations could directly or indirectly disturb the normal gut bacterial community. The maximum residue levels (MRLs) of 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 [58]. The residue levels of glyphosate in dairy cattle, swine and poultry feeds are 381, 11.5 and 11.4 ppm, respectively [19]. However, data on the real presence of glyphosate and its metabolite AMPA 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. Also, the maximum daily intake (MDI) of glyphosate depends on the ration composition and the percent of each component in the ration. Some poultry and cattle feed samples in Germany were found to have 0.4–0.9 mg glyphosate/kg (Data not published). On the other hand, glyphosate were determined in water samples from a transgenic soybean cultivation area located near to tributaries streams of the Pergamino–Arrecifes system in the north of the province of Buenos Aires, Argentina ranged from 0.10 to 0.70 mg/l [43]. Glyphosate daily intake could be hazardous if feed and/or water contain high glyphosate residues; further work is urgently required to determine the real glyphosate residues in animal feed originated from different countries. Also, it is worthy to mention that, glyphosate affects bacterial community indirectly. It has high ability to complex several divalent

cationic nutrients such as calcium, magnesium, manganese and iron [1, 12, 32, 37]. These cationic nutrients easily bind to the glyphosate molecule via the carboxyl and phosphonate groups forming poorly soluble or very stable complexes. The maintenance of intracellular homeostasis of metal ions is crucial for survival of these bacteria. In particular, zinc, manganese and copper are very important metal ions, as they are co-factors of many enzymes, are involved in oxidative stress defence and have a role in the immune system of the host. DNA microarray experiments revealed that these minerals regulate the growth of *E. faecalis* V583 [14]. In a study performed in our laboratory on a cattle farm infected with *C. botulinum*, there were a correlation between the presence of enterococci in cow faeces and *C. botulinum*-associated diseases. Cows that were poorly colonized by enterococci showed in 68 % symptoms of *C. botulinum*-associated diseases. Also, these cows showed a significant reduction of trace elements in blood. On the other hand, cows with good colonisation by enterococci rarely displayed signs of botulinum diseases (9 %) (Data not published). Thus, the suppression of *C. botulinum* by enterococci and the exquisite toxicity of glyphosate to enterococci but not *C. botulinum* observed in this study may be important in the understanding of what is termed chronic botulism in farm animals.

The microbiota of the chicken's GIT has received increased attention as the focus of efforts to minimise food-borne illness in humans, to improve animal nutrition and to reduce dependence non-therapeutic antibiotic growth promotions [8, 21, 26, 38]. Bifidobacteria are considered beneficial microorganisms and thought to create conditions unfavourable to the growth of pathogens [26]. In the present study, bifidobacteria showed to be highly sensitive to glyphosate which disturb the gut microbiota. On the other hand, *Salmonella* infections are one of the most important public health concerns worldwide. In general, *Salmonella* species are widely distributed in the

environment and cause a diverse spectrum of diseases in human and animals. Poultry products, eggs and meat are considered to be one of the main sources of human food-borne infections caused by *Salmonella* [17]. In the present study, *Salmonella* Enteritidis, *Salmonella* Typhimurium and *Salmonella* Gallinarum were highly resistant to glyphosate (MIC value = 5 mg/ml). However, glyphosate may affect on *Salmonella* indirectly by reducing beneficial microorganisms as bifidobacter which create unfavourable conditions for *Salmonella* [26]. Also pathogenic *E. coli* and *E. coli* 1917 strain Nissle were found also resistant to glyphosate (MIC value = 5 mg/ml). There are several mechanisms by which bacteria and plants are naturally resistant or become resistant to glyphosate: overproduction of EPSPS via gene amplification or increased mRNA biosynthesis, amino acid alterations of EPSPS or natural resistance seen with bacterial class II EPSPS enzymes. This last mechanism of resistance has been utilized for the development of Monsanto's genetically modified Roundup Ready® crops, which have been successfully transformed with the naturally glyphosate resistant EPSPS from *Agrobacterium tumefaciens* spp. strain CP4 [10]. It was shown that two single-site amino acid alterations could confer resistance to glyphosate: G96A [18, 53] and P101S [53] (numbering according to *E. coli* EPSPS). Also, EPSPS from *Staphylococcus aureus* is insensitive to glyphosate [45]. Glyphosate inhibits the growth of *Lactobacillus* spp. at 0.6 mg/ml and showed morphological changes including swelling and corrugated cell wall. Clair et al. [13] found that glyphosate inhibited the growth of *L. delbrueckii* subsp. *bulgaricus* (food microorganism) at a concentration of 1,000 ppm and also the cell contents leaks out. Antagonism between *C. botulinum* and bacterial members of the microecosystem are well known [11, 41, 42, 57]. Campylobacters are very important cause of food-borne human diseases. Campylobacteriosis (campylobacter infection) has been described as an emerging food-borne disease [24], and they are now said to be the major cause of bacterial gastroenteritis in humans [31]. Poultry have been reported by several authors to be the leading reservoir for campylobacters, and thus, poultry meat and products are implicated as the leading source of human campylobacteriosis [36]. In the present study, *C. coli* and *C. jejuni* were found to be susceptible to glyphosate (MIC value = 0.15 mg/ml). However, it is worthy to mention that some probiotics as *L. acidophilus*, *L. fermentum*, *Bifidobacterium bifidum* and *Bifidobacterium longum* reduce the pH and create unfavourable conditions for *Campylobacter* [34]. A reduction of such beneficial bacteria could be also a predisposing factor for campylobacteriosis. In conclusion, glyphosate showed differences in sensitivity between potential pathogens and beneficial microbiota in chicken. A reduction of beneficial bacterial species in the GIT microbiota by

ingestion of glyphosate contaminated feed could disturb the normal gut bacterial community. Also, the toxicity of glyphosate to *Enterococcus* spp. and *B.adius* 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 *C. botulinum*. Investigation on the distribution of glyphosate and its metabolites in poultry feed, their kinetic and the modulatory effect on gut microbiota are in progress. Also, evaluation of genetic mutations induced by glyphosate in different bacteria should be considered.

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