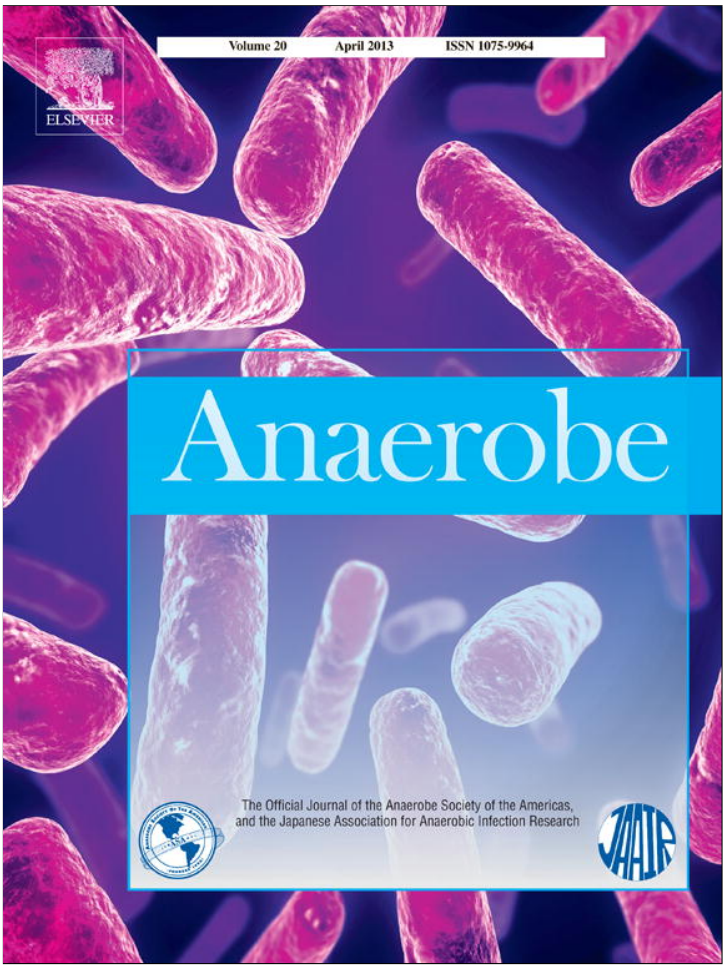

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Pathogenesis and toxins

Glyphosate suppresses the antagonistic effect of *Enterococcus* spp. on *Clostridium botulinum*

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ABSTRACT

During the last 10–15 years, an increase of *Clostridium botulinum* associated diseases in cattle has been observed in Germany. The reason for this development is currently unknown. The normal intestinal microflora is a critical factor in preventing intestinal colonisation by *C. botulinum* as shown in the mouse model of infant botulism. Numerous bacteria in the gastro-intestinal tract (GIT) produce bacteriocines directed against *C. botulinum* and other pathogens: Lactic acid producing bacteria (LAB) such as lactobacilli, lactococci and enterococci, generate bacteriocines that are effective against *Clostridium* spp. A reduction of LAB in the GIT microbiota by ingestion of strong biocides like glyphosate could be an explanation for the observed increase in levels of *C. botulinum* associated diseases. In the present paper, we report on the toxicity of glyphosate to the most prevalent *Enterococcus* spp. in the GIT. Ingestion of this herbicide could be a significant predisposing factor that is associated with the increase in *C. botulinum* mediated diseases in cattle.

1. Introduction

Clostridium botulinum associated diseases in cattle appear in two forms, acute or chronic. The acute form of botulism occurs after the uptake of preformed botulinum neurotoxin (BoNT) in feeds causing flaccid paralysis and death by respiratory failure. Graham and Schwarze, [1] described the acute form of botulism as developing of paralysis without previous symptoms, leading to sudden death or slow recovery after complete muscle relaxation. In contrast, the chronic form of the disease is characterized by weakness, local paralysis, emaciation, muscular stiffness and recumbency of varying degrees. A neurologic manifestation may be highly aggressive behaviour resulting in damage to feeding troughs, mangers or fences. Animals may also display visual disturbances [1]. The causal bacterium, *C. botulinum*, is an ubiquitous Gram-positive, spore-forming obligate anaerobic bacterium. Strains generate neurotoxins that block the release of acetylcholine at the neuromuscular junctions. Amino acid variation within the BoNT results in seven immunologically distinct BoNT serotypes (A–G) which are further divided into subtypes [2–5]. Two other bacteria, *C. baratii* and *C. butyricum*, can also produce BoNT F and E, respectively. The factors that determine the severity of the disease and the factors that prevent intestinal colonisation by *C. botulinum* spores are incompletely characterized [6]. In the mouse model of infant botulism, the normal intestinal microflora has been shown to be a critical factor in preventing intestinal colonisation by *C. botulinum* [7]. Wang and Sugiyama [8] reported that mice developed symptoms of botulism when they were treated with metronidazole before oral application of *C. botulinum* A and B spores. Some mice harboured relatively large amounts of toxin in the large bowel without displaying any clinical signs, possibly bound to phospholipids and gangliosides [9]. This is in line with other intestinal diseases, where the normal enteric microflora has been found to protect against colonization by a variety of bacterial pathogens [6]. The microflora 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 [10].

Numerous bacteria of the GIT produce bacteriocines directed against some other bacterial species including pathogens. Among others, lactic acid bacteria like lactobacilli, lactococci and enterococci may generate such bacteriocines. Specifically, *Enterococcus faecalis* (*E. faecalis*) may generate an enterocin 1146 that was shown to be very effective against *C. butyricum* and *C. perfringens* [11–14]. Glyphosate, N-(phosphonomethyl) glycine, the most widely used

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herbicide in the world, is the active ingredient in Roundup® and contains also adjuvants such as polyethoxylated tallowamine [15]. Glyphosate is a strong systemic metal chelator and was initially patented for that purpose in 1964 [16]. It is also a selective and potent, microbiocide. Its herbicidal action is generated by chelating manganese required in the reduction of the FMN co-factor for the 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase enzyme in the shikimate pathway, inhibiting this metabolic pathway of plants and also many microorganisms [17]. The extensive use of glyphosate as a broad-spectrum herbicide in agriculture, and especially the direct application of glyphosate to Roundup Ready® soya, corn, rapeseed, cotton, sugar beets, and alfalfa fed to animals, leads to incorporation of residual glyphosate into the GIT. Moreover, glyphosate showed differences in sensitivity between microorganisms [18,19] which could disturb the normal gut bacterial community. The aim of this paper is to document the inhibitory effect of glyphosate on *Enterococcus* spp. which antagonises *C. botulinum.*

2. Materials and methods

2.1. Isolation and identification of Enterococcus strains

Enterococcal isolates were isolated from cattle, horses, and algae (*Chlorella vulgaris*, (Ökologische Produkte Altmark Co, Germany) by plating specimens on citrate- acid-tween-carbonate (CATC agar, Oxoid, Germany) and incubated aerobically at 37 °C for 48 h. Typical red colonies were sub-cultured on Caso agar (3.5% Casein-Soya, 0.3% yeast extract, 0.1% glucose, 1.5% Agar Agar). These colonies were examined for Gram reaction and cellular morphology. All Gram positive, catalase-negative cocci isolated on this medium were presumptively identified as *Enterococcus* spp. The sugar fermentation profiles of these isolates were checked for glucose, trehalose, arabinose, mannitol, salicin, raffinose, dulicit, xylose and lactose utilization. Haemolysis of these isolates was checked by culturing on blood agar medium. Species identification of isolated strains was based on their matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) profile (Table 1). Briefly, 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 followed by addition of 1 μl α-cyano-hydroxy-cinnaminic acid (Bruker Daltonik Co, Bremen, Germany) in a standard organic solvent mixture (2.5% trifluoroacetic acid, 50% AN in water). 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 (Ardeypharm GmbH, Herdecke, Germany) 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, Bremen, Germany) and the spectra were automatically identified using the Bruker BioTyper™ 1.1 software.

2.2. C. botulinum strains

C. botulinum type A (7272), type B (7273), *C. botulinum* type C (8264) and type E (8266) were obtained from National collection of type culture (NCTC), while *C. botulinum* type D (Pasteur 1873-D) was obtained from Institute of Pasteur, Paris, France. *C. botulinum* strains were cultured anaerobically in a cooked meat medium at 37 °C for 5 d, followed by cultivation in reinforced clostridial medium (RCM, Sifin, Germany) anaerobically at 37 °C for 3 d. *C. botulinum* types A and B were heated at 80 °C for 10 min while types C, D and E were heated at 60 °C for 30 min and left aerobically at room temperature. Cultures were analysed daily for sporulation using a Gram or Rakette stain.

Table 1
Effect of different *Enterococcus* spp. on neurotoxin production in a co-culture with *C. botulinum* types A, B, D and E.

Enterococcus spp.[a]	Lab. designation	Source	Neurotoxin determination by ELISA[b]			
			BotNt A	BotNt B	BotNt D	BotNt E
E. faecium	E1	*Chlorella vulgaris*	−	−	−	−
E. fecalis	E1	*Chlorella vulgaris*	−	−	−	−
E. faecium	E3	Cattle	−	−	−	−
E. hirae	E4	Cattle	−	−	−	−
E. faecalis	E5	Horse	−	−	−	−
E. malomaoldoratus	E6	Horse	−	−	−	−
E. derrisei	E7	Horse	+/−	−	−	+
E. durans	E8	Cattle	−	−	−	−
E. faecium	E9	Cattle	−	−	−	−
E. feacalis	E10	Cattle	−	−	−	−
E. faecalis	E11	Cattle	−	−	−	−
E. faecalis	E12	Horse	+/−	−	−	−
E. hirae	E13	Horse	−	−	−	−
E. casseliflavus	E14	Cattle	+/−	−	−	−
E. coli strain (Nissle 1917)			++	++	++	++
−			++	+++	++	++

Enterococcus spp. prevent toxin production of *C. botulinum* types A, B, D and E while *E. coli* 1917 strain Nissle has no effect.
[a] Species identification performed by MALDI-TOF.
[b] ELISA results expressed as negative (−), low positive (+), positive (++) and highly positive (+++) compared with standards *C. botulinum* neurotoxins with known concentrations.

2.3. Glyphosate

- Roundup UltraMax (Monsanto, USA) which contains 450 mg/ml of glyphosate was used in this study.
- N-(Phosphonomethyle)glycine (Sigma Aldrich, Taufkirchen, Germany).

2.4. Effect of Enterococcus on C. botulinum types A, B, C, D, and E

To study the effect of *Enterococcus* spp. 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 different *Enterococcus* spp. was studied by addition of different bacterial dilution (10^1–10^9 cfu/ml to *C. botulinum* culture medium. The mixture was incubated anaerobically at 37 °C for 5 d. *C. botulinum* was quantified using the most probable number (MPN) estimation method using Differential Reinforced Clostridial broth (DRCM, Sifin, Germany) and the neurotoxins were tested using an ELISA.

2.5. C. botulinum neurotoxins ELISA

C. botulinum neurotoxins were tested using ELISA as described by Krüger and co-workers [20]. All ELISAs were performed in flat-bottomed ELISA plates (96 wells, high binding; Costar, Corning, New York, USA). Standard volumes were 100 μl per 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 $NaHCO_3$ 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, IgG from rabbit against the botulinum neurotoxines type A to E (Institute of Bacteriology and Mycology, University of Leipzig) overnight at 4–6 °C, the wells of the 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.

Type A and B were detected with 2.5 μg/ml horse ($[Fab]_2$ from IgG) against *C. botulinum* A and B (Novartis Vaccines and Diagnostics GmbH, Marburg, Germany). For detection of type C and D 0.1 μg/ml IgG from rabbit against botulinum neurotoxin C and D (Institute of Bacteriology and Mycology, University of Leipzig) were used. Type E was detected with 2.5 μg/ml IgG from horse against *C. botulinum* type E (WDT, Garbsen, 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 H_2O_2 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 H_2SO_4 (50 μL per well). The optical density (OD) was measured using a microplate ELISA reader at 450 nm. The standards were supernatants with known concentrations of the botulinum neurotoxins (Institute of bacteriology and mycology, University of Leipzig).

2.6. Inhibitory activity of glyphosate on Enterococcus isolates

2.6.1. Inhibitory and bactericidal activity

Minimal inhibitory concentration tests (MIC) and minimal bactericidal concentration tests (MBC) were done with glyphosate. The lowest concentration of glyphosate and Roundup, to show bactericidal or bacteriostatic effects was determined in a 96-well micro-titre plate. Serial dilutions of glyphosate (from 10 to 0.001 mg/ml were made in nutrient broth. *Enterococcus* isolates were added at a final concentration of 10^4 cfu/ml and the test plates containing diluted glyphosate and *Enterococcus* were incubated overnight at 37 °C before plating aliquots on CATC agar. Bacterial growth on each agar plate was evaluated.

2.6.2. Kinetics of the inhibitory effect of glyphosate on Enterococcus faecalis

To study the effect of glyphosate and Roundup on *E. faecalis*, cells were added to RCM medium at a final concentration of 10^4 cfu/ml. The inhibitory effects of different glyphosate concentrations were studied after 8 h cultivation at 37 °C under anaerobic conditions.

3. Results

All tested *Enterococcus* spp, isolated from *Chlorella vulgaris* and from faeces of cattle and horses (Table 1) inhibited neurotoxin production by *C. botulinum* types A, B, D and E reference strains. *C. botulinum* type C did not produce neurotoxin. Likewise, all enterococci co-cultivated with *C. botulinum* reduced the growth (cell numbers) of *botulinum* type A, B, C, D and E (Table 2). Even low numbers of *E. faecalis* (Fig. 1A) and *E. faecium* (Fig. 1B) inhibited BoNT production by all *C. botulinum* strains. Glyphosate itself also inhibited growth and BoNT expression of *C. botulinum* type B at relatively high concentrations (1 mg/ml). Roundup is more toxic to *C. botulinum*, the inhibitory effect of Roundup on the growth and toxin production of *C. botulinum* type B was more than 1 mg/ml (Table 3). Supplementation of the medium with 1 or 10 mg/ml glyphosate Roundup and glyphosate reduced the cell numbers of *C. botulinum* type B about 100 fold after 5 days of cultivation, respectively (Table 3). The inhibitory concentrations of glyphosate to *C. botulinum* type B were 10–100 fold higher than those that suppressed growth (0.1 mg/ml and 1 mg/ml) of *E. faecalis* (Table 3).

4. Discussion

Antagonism between *C. botulinum* and bacterial members of the micro-ecosystem are well known [6,15]. Special strains of *C. perfringens*, *C. sporogenes* and *Bacillus cereus* were able to antagonize *C. botulinum* Types A, B, F and E [21]. The growth of non-proteolytic strains of *C. botulinum* (Type 17B, Beluga and 202 F) was reduced by *Lactobacillus* spp., *Lactococcus* spp., *Streptococcus* spp. and *Pediococcus* spp. [22]. Okereke and Montville [23] reported that bacteriocine producers of the lactate producing group of bacteria

Table 2
Effect of different *Enterococcus* spp. on the growth of *C. botulinum* types A, B, C, D and E incubated anaerobically at 37 °C for 5 days.

Enterococcus spp.	Log_{10} cell counts of *C. botulinum*[a] (Mean ± SD, $n = 3$)				
	Type A	Type B	Type C	Type D	Type E
E1	2.24 ± 0.49	2.75 ± 0.68	3.10 ± 0.22	3.04 ± 0.15	3.08 ± 0.42
E2	2.32 ± 0.63	3.02 ± 0.19	3.70 ± 0.36	2.99 ± 0.21	2.37 ± 0.10
E3	2.68 ± 0.39	3.52 ± 0.35	3.40 ± 0.17	2.68 ± 0.39	2.84 ± 0.13
E4	2.22 ± 0.47	2.53 ± 0.89	2.21 ± 0.62	3.48 ± 0.18	2.21 ± 0.62
E5	2.38 ± 0.62	3.71 ± 0.77	3.0 ± 0.40	3.22 ± 0.30	3.11 ± 0.89
E6	2.75 ± 0.68	2.81 ± 0.60	3.20 ± 0.63	2.83 ± 0.64	2.67 ± 0.48
E7	3.54 ± 0.31	3.66 ± 0.69	2.80 ± 0.45	3.71 ± 0.64	3.91 ± 0.65
E8	2.12 ± 0.67	2.39 ± 0.29	3.30 ± 0.88	2.90 ± 0.74	2.30 ± 0.28
E9	2.33 ± 0.25	3.87 ± 0.50	2.60 ± 0.68	3.00 ± 0.23	3.06 ± 0.95
E10	2.83 ± 0.52	2.76 ± 0.54	3.30 ± 0.46	2.69 ± 60	3.37 ± 0.41
E11	2.18 ± 0.33	3.03 ± 0.32	2.80 ± 0.38	2.18 ± 0.33	2.26 ± 0.36
E12	3.37 ± 0.89	3.67 ± 0.73	3.54 ± 0.40	3.75 ± 0.68	3.49 ± 0.58
E13	2.75 ± 0.58	3.10 ± 0.43	2.90 ± 0.34	3.83 ± 0.41	2.79 ± 0.37
E14	3.02 ± 0.19	3.14 ± 0.15	3.60 ± 0.16	3.08 ± 0.10	2.60 ± 0.16
E. coli 1917 strain Nissle	6.60 ± 0.26	6.62 ± 0.40	6.28 ± 0.23	5.74 ± 0.58	5.28 ± 0.75
–	6.67 ± 0.11	6.45 ± 0.32	6.80 ± 0.50	6.28 ± 0.27	6.16 ± 0.50

[a] *C. botulinum* Type A, B, C, D and type E (10^4/ml) cultured anaerobically in a co-culture with *Enterococcus* spp. (10^4/ml) for 5 d. *C. botulinum* quantified using the most probable number (MPN) estimation method. Data expressed as reciprocal log_{10}. *Enterococcus* spp. prevent the growth of *C. botulinum* types A, B, C, D and E. *E. coli* 1917 strain Nissle has no effect on the growth of *C. botulinum* types A, B, C, D and E.

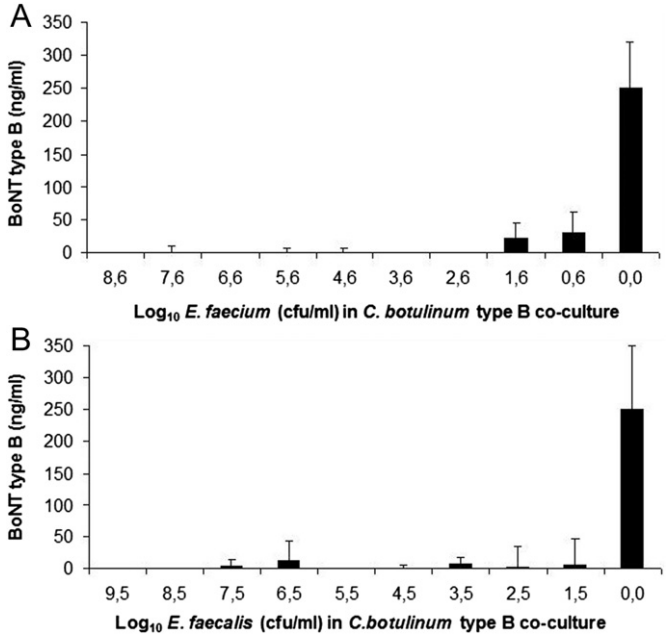

Fig. 1. Influence of *Enterococcus faecalis* (A) and *E. Faecium* (B) populations on neurotoxin production by 10^4/ml *C. botulinum* type B in a co-culture incubated for 5 days anaerobically. *C. botulinum* neurotoxin was determined by ELISA.

inhibited *C. botulinum*; especially *Enterococci* were able to inhibit *C. botulinum* [13]. 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 *Bacteroides fragilis* group. *C. ramosum* was the only member of the genus *Clostridium* to inhibit *C. botulinum* type G and F strains. Most of the 14 *C. botulinum* investigated strains were inhibited in co-cultural experiments with intact faecal samples. Sullivan et al. [7] 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 infant intestine. We believe that the same interactions can be assumed in relation to *C. botulinum* associated diseases in cattle.

Our own results with cattle showed a negative correlation of the presence of enterococci in faeces of cows on farms with *C. botulinum* associated diseases (Data are not published). Cows that were poorly colonized by enterococci showed in 68% symptoms of *C. botulinum* associated diseases while those with good colonization by enterococci rarely displayed signs of disease (9%). 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. In the present study enterococci are able to suppress growth and toxin production of *C. botulinum* types A, B, C, D and E and prevent the BoNT production types A, B, D and E (Tables 1 and 2). The fact that no BoNTs were detected in enterococci-botulinum co-cultures could be due to prevention of botulinum growth and thus prevent toxin production. The BoNTs were only detectable by ELISA when the culture medium contains 10^4 CFU/ml of *C. botulinum* or more [24]. However other experiments are necessary to test the modulatory effect of enterococci on BoNT expression. Although there are several factors in agriculture that influence the composition of gastrointestinal microbiota, glyphosate is well-known for its bactericidal effects against enterobacteria and can, therefore, change the composition of gastrointestinal microbiota. Abraham [25] patented the herbicidal agent glyphosate, in combination with the polyvalent anion oxalic acid and precursors, for the prevention and therapy of pathogenic infections including all members of the *Enterococcaceae*. The mechanism of action has been described as by chelating with Manganese, a cofactor involved with the EPSP synthase enzyme in the shikimate pathway and thus inhibiting this important metabolic pathway of many microorganisms [26,27]. Since the introduction of glyphosate-resistant (GR) in the mid- to late-1990s many consequences associated with production of GR crops were soon reported based on field observations of apparent increased disease and nutritional deficiencies relative to conventional or transgenic cultivars [28]. The responses to glyphosate vary among soil bacteria based on sensitivity of intracellular EPSPS to the herbicide. Subsequent research revealed that antagonistic interactions between the fungal species were eliminated by glyphosate suggesting that the herbicide might influence overall soil fungal community structure [19]. Glyphosate can disturb also the fresh water microbial communities directly or indirectly and reduces the biodiversity of species in the aquatic community [29]. In our study glyphosate concentrations at 0.1 mg/ml inhibited growth of *E. faecalis* but did not inhibit growth or BoNT production of *C. botulinum* Type B. The MIC of glyphosate on the growth of *C. botulinum* type B was 1 mg/ml. The loss of enterococci could be induced by contamination of feeds with residual glyphosate during crop production as suggested by the in-vitro toxicity of glyphosate to *E. faecalis*. It is worthy to mention that the effects Roundup is more toxic to *C. botulinum*, at 1 mg/ml there were an inhibitory

Table 3
Influence of glyphosate and roundup on the growth of *C. botulinum* type B and *E. faecalis*.

Herbicide concentration (mg/ml)	Glyphosate[a]			Herbicide formulation[b]		
	C. botulinum type B (cfu/ml) (mean ± SD)[c]	BoNT (ng/ml)[d]	*E. faecalis* (cfu/ml) (mean ± SD)[e]	*C. botulinum* type B (cfu/ml) (mean ± SD)	BoNT (ng/ml)	*E. faecalis* (cfu/ml) (mean ± SD)
0	6.9 ± 0.34	300 ± 47	8.2 ± 0.87	6.9 ± 0.34	270 ± 120	8.2 ± 0.87
0.1	5.3 ± 0.78	312 ± 20	0	5.1 ± 0.78	337 ± 50	0
1	5.4 ± 0.45	319 ± 60	0	3.3 ± 0.80	0	0
10	3.2 ± 0.43	0	0	3.0 ± 0.65	0	0

[a] Glyphosate (N-Phosphonomethyl) glycine).
[b] Herbicide formulation (Roundup).
[c] *C. botulinum* type B (10^4/ml) cultured anaerobically in reinforced clostridial medium (RCM) containing different concentrations of glyphosate or herbicide formulation for 5 d. *C. botulinum* quantified using the most probable number (MPN) estimation method. Data expressed as reciprocal $\log_{10}$.
[d] *C. botulinum* type B quantified by ELISA.
[e] *E. faecalis* cultured aerobically in RCM containing different concentrations of glyphosate or herbicide formulation for 8 h and quantified on citrate-acid-tween-carbonate (CATC) agar. Data expressed as reciprocal $\log_{10}$.

effect of Roundup on the growth and toxin production of *C. botulinum* type B (Table 3). Richard and co-workers mentioned that Roundup adjuvant enhances glyphosate bioavailability and/or bioaccumulation [30].

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 [31]. 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 [32]. 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. In conclusion our results suggest that the herbicide glyphosate could be a significant predisposing factor responsible for the increase in *C. botulinum* associated diseases currently being experienced on dairy farms due to loss of the antagonistic bacteria as enterococci from the GIT. This hypothesis needs to be confirmed by further tests and in-vivo experiments.

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