

The Influence of Glyphosate on the Microbiota and Production of Botulinum Neurotoxin During Ruminal Fermentation

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Abstract The aim of the present study is to investigate the impact of glyphosate on the microbiota and on the botulinum neurotoxin (BoNT) expression during in vitro ruminal fermentation. This study was conducted using two DAISY^{II}-incubators with four ventilated incubation vessels filled with rumen fluid of a 4-year-old non-lactating Holstein–Friesian cow. Two hundred milliliter rumen fluid and 800 ml buffer solution were used with six filter bags containing 500 mg concentrated feed or crude fiber-enriched diet. Final concentrations of 0, 1, 10, and 100 µg/ml of glyphosate in the diluted rumen fluids were added and incubated under CO₂-aerated conditions for 48 h. The protozoal population was analyzed microscopically and the ruminal flora was characterized using the fluorescence in situ hybridization technique. *Clostridium botulinum* and BoNT were quantified using most probable number and ELISA, respectively. Results showed that glyphosate had an inhibitory effect on select groups of the ruminal microbiota, but increased the population of pathogenic species. The BoNT was produced during incubation when inoculum was treated with high doses of glyphosate. In conclusion,

glyphosate causes dysbiosis which favors the production of BoNT in the rumen. The global regulations restrictions for the use of glyphosate should be re-evaluated.

Introduction

An increasing number of nonspecific diseases in dairy farms have been described in Germany and other countries in recent years. The observed symptoms in general were indigestion (constipation alternating with diarrhea), non-infectious chronic laminitis, engorged veins, oedemas, retracted abdomen, cachexia, and apathy. Most of the cases occurred during the peripartur period and often resulted in sudden death [7]. Böhnel et al. [7] assumed that the botulinum neurotoxin (BoNT) produced by *Clostridium* (*C.*) *botulinum* in the intestines was responsible for the symptoms described as a disease complex named ‘visceral botulism.’ *C. botulinum* is ubiquitous in the environment and, although it is not a member of the normal gastrointestinal flora of ruminants, the bacterium can occur sporadically or through the oral intake of contaminated silage [15, 25, 27, 28]. The ingestion of *C. botulinum* or BoNT does not automatically lead to intoxication since small amounts of BoNT in the gastrointestinal tract of ruminants can be degraded by proteolytic bacteria [3].

Bacteriocines produced by lactic acid bacteria can degrade *C. botulinum* bacteria. *Enterococcus* spp. in particular have an inhibitory effect on the growth of *C. botulinum* and inhibit the production of BoNT [26, 36]; however, changes in composition of the gastrointestinal microbiota could favor the establishment of *C. botulinum* and the production of BoNT. An important factor possibly affecting the gastrointestinal microbiota in ruminants is the broad-spectrum herbicide glyphosate.

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Table 1 Ingredients of the experimental diets

| | diet 1 | diet 2 |
|--|--------|--------|
| Hay (%) | 84.17 | 59.17 |
| Grass pellets (%) | 5 | 0 |
| Sugarbeet pellets (%) | 10 | 0 |
| Mineral feed ^a (%) | 0.83 | 0.83 |
| Concentrate feed for lactating cows ^b (%) | 0 | 40 |

^a Contains (%) Ca (4), P (6), Na (6.5), Mg (8); (IU/g) Vit A (1,000), Vit D3 (125); (mg/g) Vit E (5), biotin (0.15), β -Carotin (1.5), Cu (1.1); Mn (3.7), Zn (5), J (0.16), Se (0.04), Co (0.022)

^b Contains (%) sugarbeet molasses pulp (20), rapeseed meal (14), rye bran (12), corn gluten (10), rye (10), wheat bran (10), wheat semolina bran (9), sunflower meal (7), CaCO₃ (3.5), soybean cups (3), sugarbeet molasses (1), NaCl (0.29); (IU/g) Vit A (7.5), Vit D3 (0.95)

The substance is well known for its antimicrobial effects on intestinal bacteria such as *Enterococcus* sp., whereas *C. botulinum* remains resistant to glyphosate [26]. The herbicide is used in plants, where it prevents the production of the aromatic amino acids tyrosine, phenylalanine, and tryptophan through competitive inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) in the shikimate pathway [17]. Besides plants, rumen bacteria also have EPSPS and produce aromatic amino acids via the shikimate pathway [38]. It has been demonstrated that glyphosate showed cytotoxic effects on different cells in vitro [5, 6, 14]. Recently, it has found that the major pesticides are more toxic to human cells than their declared active principles [35]. Adjuvants in pesticides increased the cytotoxicity of glyphosate 100–1,000 times [26, 35]. The herbicide enters the gastrointestinal tract of ruminants through glyphosate-contaminated feed [4] where it could have a deleterious effect on specific microbiota. The objective of this study was to investigate the influence of glyphosate on the ruminal microbiota in an artificial system (DAISY) using a crude fiber-accentuated and a concentrated feed-accentuated ration.

Materials and Methods

Animals and Feeding

A 4-year-old non-lactating Holstein–Friesian cow was used as the donor animal for rumen fluids. The cow was fed 2 kg dry mass/100 kg body mass/day of a crude fiber-rich feed distributed over three feeding times per day and had ad libitum access to water. The composition of the feed corresponds to the experimental diet 1 (Table 1) used in this study. Two weeks prior to the initial collection of rumen fluids, the cow was adapted to the feed to ensure

stable conditions in the rumen at the beginning of the experimental period.

In Vitro Fermentation of Diluted Rumen Fluids

The study was conducted using two DAISY^{II}-incubators (ANKOM Technology, Fairport, NY, USA), each of them containing four ventilated incubation vessels (volume 4 l).

Influence of Glyphosate on Rumen Microbiota

All chemicals and reagents were purchased from Sigma–Aldrich (Germany) unless otherwise stated. Before incubation, each vessel was filled with 800 ml buffer solution (10 g/l KH₂PO₄, 0.5 g/l MgSO₄ × 7 H₂O, 0.5 g/l NaCl, 0.1 g/l CaCl₂ × 2 H₂O, 0.5 g/l urea, 15 g/l Na₂CO₃ and 1 g/l Na₂S × 9 H₂O) and six filter bags (pore size 50 μ m, R 510, ANKOM Technology, Fairport, NY, USA) which contained 500 mg each of either diet 1 or 2 (a maximum 3 mm particle size of the diet ingredients and crude nutrients (see Tables 1, 2). Glyphosate (*N*-(phosphonomethyl) glycine; Sigma-Aldrich, St. Louis, MO, USA) was added from a 2 % (w/v) base solution to reach final concentrations of 0, 1, 10, and 100 μ g/ml in the diluted rumen fluids. Subsequently, the vessels were aerated for 1 min with CO₂ and pre-warmed for 2 h in the DAISY^{II}-incubators to ensure a temperature of 39 °C at the beginning of the incubation.

Rumen fluid was orally collected from the donor cow 3 h after the morning feeding and passed through four layers of cheese cloth. Each incubation vessel received 200 ml of the filtrate, mixed gently, aerated with CO₂ for another 2 min and incubated for 48 h at 39 °C. After incubation, aliquots of the incubation medium were immediately fixed with a 1:10 methylgreen-formalin-saline-solution (contains 0.6 g/l methylgreen, 100 ml/l formalin, 8 g/l NaCl, 900 ml/l distilled water) in centrifuge tubes for protozoal analysis, 1:2 with 96 % (v/v) ethanol or 1:4 with 4 % (w/v) paraformaldehyde for bacterial analysis. The tubes for protozoal analysis were stored at 4 °C and the tubes for bacterial analysis were stored 1 h at 4 °C and then at –20 °C (ethanol fixed vials) or 24 h at 4 °C and then at –20 °C (paraformaldehyde fixed vials) until further processing. Subsamples of the inoculum for the 0 h values were taken after the addition of rumen fluid and fixed as described above.

Influence of Glyphosate on *C. botulinum* Type B

C. botulinum type B (7273) was obtained from the National Collection of Type Cultures (NCTC, Salisbury, UK), cultured anaerobically in cooked meat medium (Oxoid, Wesel, Germany) at 37 °C for 5 days and thereafter cultivated in Reinforced Clostridial Medium (RCM; Sifin, Berlin, Germany) anaerobically at 37 °C for 3 days. The cultures

Table 2 Composition of the crude nutrients of the experimental diets

| | Diet 1 | Diet 2 |
|---------------------------------|--------|--------|
| Dry matter (% of DM) | 94.35 | 92.57 |
| Crude ash (% of DM) | 9.33 | 8.62 |
| Crude protein (% of DM) | 9.07 | 13.9 |
| Crude fiber (% of DM) | 27.07 | 21.27 |
| Crude fat (% of DM) | 0.88 | 1.47 |
| Nitrogen free extract (% of DM) | 53.65 | 54.74 |
| Organic matter (% of DM) | 90.67 | 91.38 |
| Nitrogen (% of DM) | 1.45 | 2.22 |

were then heated at 80 °C for 10 min, analyzed for sporulation using a Gram and Rakette stain and left aerobically at room temperature until further processing.

Preparation of the incubation vessels and buffer solution for experiment 2 was as described for experiment 1, except that smaller, rubber plugged gastight vessels were used (volume 125 ml) which were filled with 80 ml buffer solution that was added directly (without filterbags) with 1 g of either diet 1 or 2. Glyphosate was added to provide final concentrations of 0, 1, 10, 100, or 1,000 µg/ml.

Twenty milliliter of orally collected, filtrated rumen fluid as described in experiment 1 was added to the vessels and preheated spores of *C. botulinum* type B were added to reach a final concentration of 10⁵ cfu/ml in the inoculum. The vessels were aerated with CO₂ for 2 min, closed gastight and incubated for 48 h at 39 °C. Gas produced during incubation was released with a gastight syringe after 3, 6 h and then every 6 h of incubation. After incubation, aliquots of the incubation medium were taken to estimate the concentration of *C. botulinum* and botulinum neurotoxin. Subsamples of inoculum for the 0 h values were taken after the addition of rumen fluid.

Analysis of the Protozoal Population

The protozoal population was analyzed using a modified McMaster slide [13] and light microscope according to phenotypical criteria of Williams and Coleman [46]. Each of the two counting chambers were filled with 1 ml of methylgreen-formalin-saline-fixed rumen fluid from experiment 1 and the population of *Entodinium* spp., *Diplodinium* spp., *Epidinium* spp., *Isotricha* spp., *Dasytricha* spp., *Ophryoscolex* spp. and total ciliates (as the sum of all previous species) were counted at 400× magnification and estimated as cells/ml according to the following formula:

$$\text{cells/ml} = \frac{n \times d}{v \times a \times c},$$

where *n* is the number of counted protozoa, *d* the dilution factor (100 entodinium, 10 for all others), *v* the sample

volume (1 ml), *a* the chamber volume (0.15 cm³), and *c* the number of chambers counted (2).

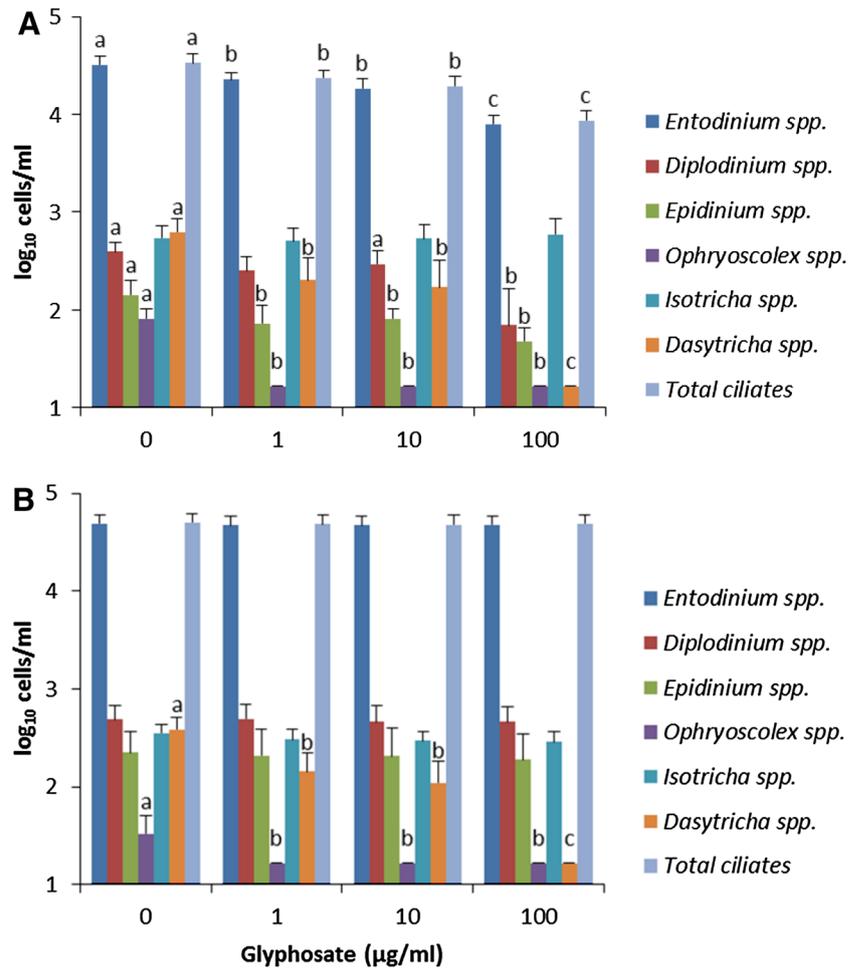
Analysis of the Ruminal Flora

The ruminal flora was characterized using the fluorescence in situ hybridization technique using the following 16S rRNA/23S rRNA-targeted oligonucleotide probes (sequences of the probes are available at probeBase [29]:

(1) an equimolar mixture of five bacteria-directed probes (EUB338, EUB785, EUB927, EUB1055, EUB1088) referred to as EUBmix [42] to detect all bacteria; (2) Bac303 for the detection of *Bacteroidaceae* and *Prevotellaceae* [32]; (3) Bfi826 for *Butyrivibrio fibrisolvens*-related clones [24]; (4) Rfla729 for *Ruminococcus albus* and *Ruminococcus flavefaciens* [23]; (5) Str for *Streptococcus* spp. [43]; (6) Eury498 for Euryarchaeota [8]; (7) Clit135 for the *Clostridium lituseburense* group [18]; (8) Chis150 for the *Clostridium histolyticum* group [18]; (9) Clept1240 for the *Clostridium leptum* subgroup [40]; (10) Erec482 for the *Clostridium coccoides/Eubacterium rectale* group [18]; (11) Lab158 for Lactobacilli and Enterococci [22]; and (12) DSS658 for Desulfobacteraceae [34].

The oligonucleotides were purchased 5' labeled with the indocarbocyanine dye Cy3 (BioTeZ, Berlin, Germany). The ethanol and paraformaldehyde fixed aliquots from experiment 1 were hybridized on silanized slides, modified from Maddox and Jenkins [30], with eight wells. Hybridization, washing procedures, and enumeration were performed in the dark by transferring 10 µl of the fixed sample to the well, drying (37 °C for 1 h) and dehydrating for 3 min in 50, 70, and 96 % ethanol each. After air drying, 10 µl of lysozyme buffer (100 mM-Tris-HCl (pH 8,0), 50 mM-EDTA, 1 mg lysozyme (130,000 U/mg; Boehringer, Mannheim, Germany) was added to each well and incubated in humidity chambers at 37 °C for 30 min to obtain improved permeability of the cell walls of Gram-positive bacteria during hybridization. After another drying process (45 min at 37 °C), the samples were hybridized for 45 min at 46 °C (50 °C Lab158) in a humidity chamber after adding 10 µl hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl and 0.05 % (w/v) sodium dodecyl sulfate, pH 7.2) containing 5 ng probe/ml. The hybridization buffer for the Clept1240 and DSS658 probes also contained 10 and 60 % (v/v) formamide, respectively. After hybridization, the slides were gently rinsed with distilled water (DW) and washed for 10 min in washing buffer (0.9 M NaCl, 20 mM Tris-HCl and 0.05 % (w/v) sodium dodecyl sulfate, pH 7, 2) at the same temperature as the hybridization process. The washing buffer for the Clept1240 and DSS658 probes contained only 0.45 M and 10 mM NaCl, respectively. EDTA (50 mM) also was added to the DSS658. Thereafter, the slides were again rinsed with DW and air dried.

Fig. 1 Cell counts of ruminal protozoa with diet 1 (a) and diet 2 (b) after 48 h in vitro incubation with different concentrations of glyphosate. Values are mean \pm SD ($N = 6$). Different letters within a species represent significant differences ($p < 0.05$)



To exclude false hybridization and counting of artifacts, the samples were counter-stained 5 min with 10 μl DAPI solution [0.9 M NaCl, 20 mM Tris-HCl, 1 $\mu\text{g/ml}$ 4,6-diamidino-2-phenylindole [Sigma-Aldrich, St. Louis, MO, USA]], rinsed with DW, air dried and embedded in antifading solution (233 mg of 1,4-diazobicyclo(2,2,2) octane) (DABCO, Sigma-Aldrich, St. Louis, MO, USA), 10 ml 1 \times phosphate buffered saline [130 mM sodium chloride, 10 mM sodium phosphate, pH 7.4 (PBS)]. Enumeration of bacteria was done with an epifluorescence microscope (eclipse Ni-U; Nikon, Düsseldorf, Germany) equipped with a mercury lamp (HBO 100 W/3, Osram, Munich, Germany) and a 12 bit CCD camera (ProGres CF, Jenoptik, Jena, Germany). The cells were counted at 1,000 \times magnification (100 \times immersion oil objective) equipped with a DAPI (UV-2A), a Cy3 (G-2A) filter and a counting net (1 cm^2 , Nikon, Düsseldorf, Germany) installed between the ocular and the objective. The concentration of bacteria was calculated as cells/ml according to the following formula:

$$\text{cells/ml} = \frac{n \times d \times a \times 100}{c}$$

where n is the number of counted cells, d the dilution factor (paraformaldehyde fixed bacteria 4 \times for DSS658 or 40 \times

for EUB mix, Bac303; ethanol fixed bacteria (all others) 2 \times), a the area factor for the total area of the well (2,827.4) and c the number of counted nets per well (10).

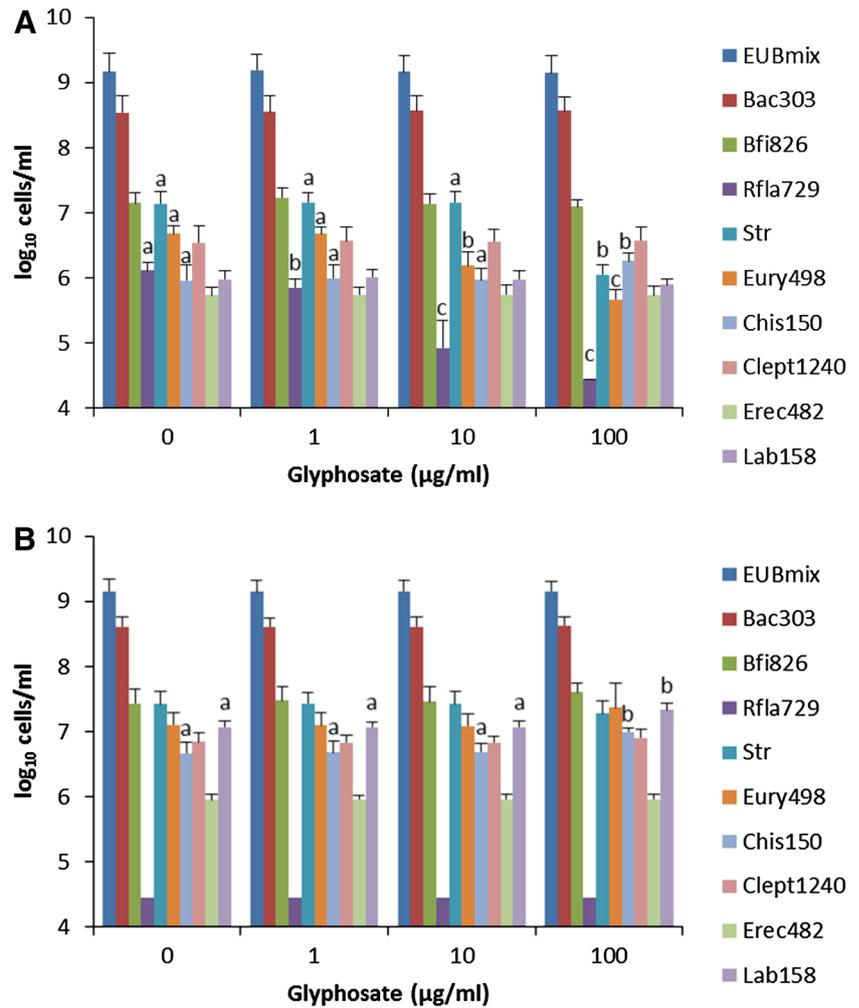
Determination of *C. botulinum* and Botulinum Neurotoxin

To estimate the population of *C. botulinum*, the samples from experiment 2 were heat treated (10 min at 80 $^{\circ}\text{C}$) and incubated anaerobically in Differential Reinforced Clostridial Medium (DRCM, Sifin, Berlin, Germany) at 37 $^{\circ}\text{C}$ for 6 days followed by quantification of *C. botulinum* as colony forming units (cfu)/ml using the three-tube most probable number (MPN) method [33]. Before the heat treatment, botulinum neurotoxin was detected using an ELISA as described by [26].

Statistical Analysis

To determine if variables differed among the treatment groups, the values before and after incubation were compared using the Statistical Package for Social Science (SPSS version

Fig. 2 Cell counts of ruminal flora incubated 48 h in vitro with diet 1 (a) and diet 2 (b) after 48 h in vitro incubation with different concentrations of glyphosate. Values are mean \pm SD ($N = 6$). Different letters within a species differ significantly ($p < 0.05$)



18.0; SPSS Inc., Chicago, IL, USA). The values of the flora and fauna were log₁₀-transformed. For non-detectable parameters, a half detection limit was used which was 1.67×10^2 /ml for protozoa and 2.82×10^4 /ml for ruminal flora. Normality was tested using the Kolmogorov–Smirnov-test. One-way ANOVA was performed for normally distributed variables. The Kruskal–Wallis- H test and Mann–Whitney U test were used for non-normally distributed variables. Differences were considered to be significant at $p < 0.05$.

Results

Influence of Glyphosate on Rumens Microbiota

Glyphosate was toxic to all ciliates except *Isotricha* spp. and cell counts decreased after 48 h in vitro incubation with diet 1 (Fig. 1a). The population of all species except *Isotricha* spp. and *Diplodinium* spp. were significantly lower when treated with 1 and 10 µg/ml glyphosate, and of *Diplodinium* spp. treated with 100 µg/ml glyphosate. Only

Ophryoscolex spp. and *Dasytricha* spp. were inhibited by glyphosate in diet 2 where glyphosate inhibited *Ophryoscolex* spp. at the lowest concentration and *Dasytricha* spp. at the highest (Fig. 1b).

The ruminal bacterial flora was also affected by glyphosate (Fig. 2a, b). After incubation with diet 1, the cell counts of Rfla729 were significantly lower with 1 µg/ml glyphosate, for Str with 100 µg/ml and for Eury498 with 10 and 100 µg/ml (Fig. 3a, b). In contrast, cell counts of Chis150 were increased at the highest concentration of glyphosate (100 µg/ml) (Fig. 4a, b). Chis150 and Lab158 were significantly higher with 100 µg/ml glyphosate in diet 2. Before incubation, the population of all organisms was similar.

Influence of Glyphosate on *C. botulinum* Type B

Botulinum neurotoxin type B could not be detected at 0, 1, 10, and 100 µg/ml glyphosate after 48 h in vitro incubation. However, it was detected at 1,000 µg/ml glyphosate after 48 h in vitro incubation with both diets even though

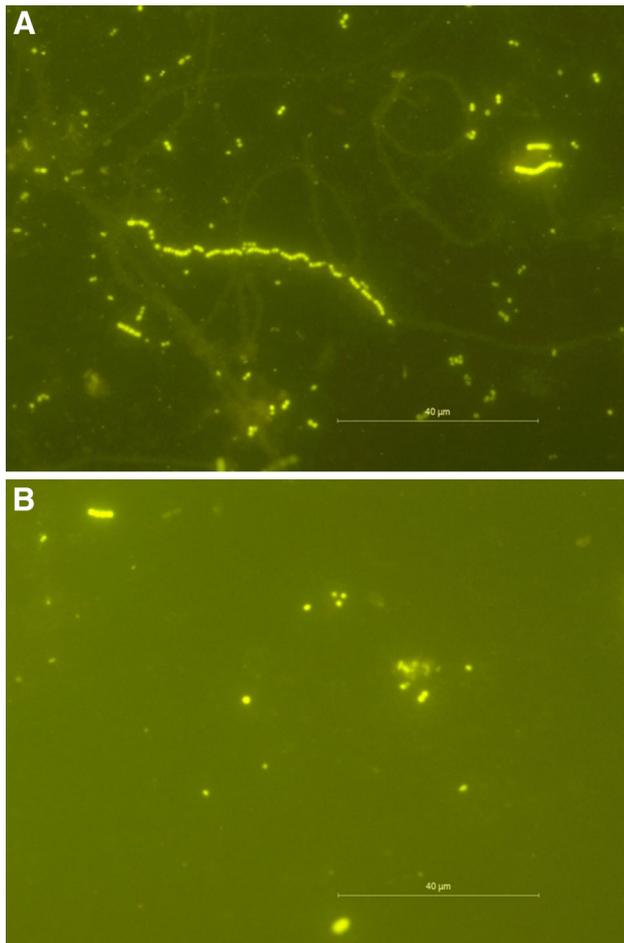


Fig. 3 Appearance of Rfla729 in diet 1 supplemented with 0 µg/ml (a) and 10 µg/ml (b) glyphosate incubated 48 h in vitro

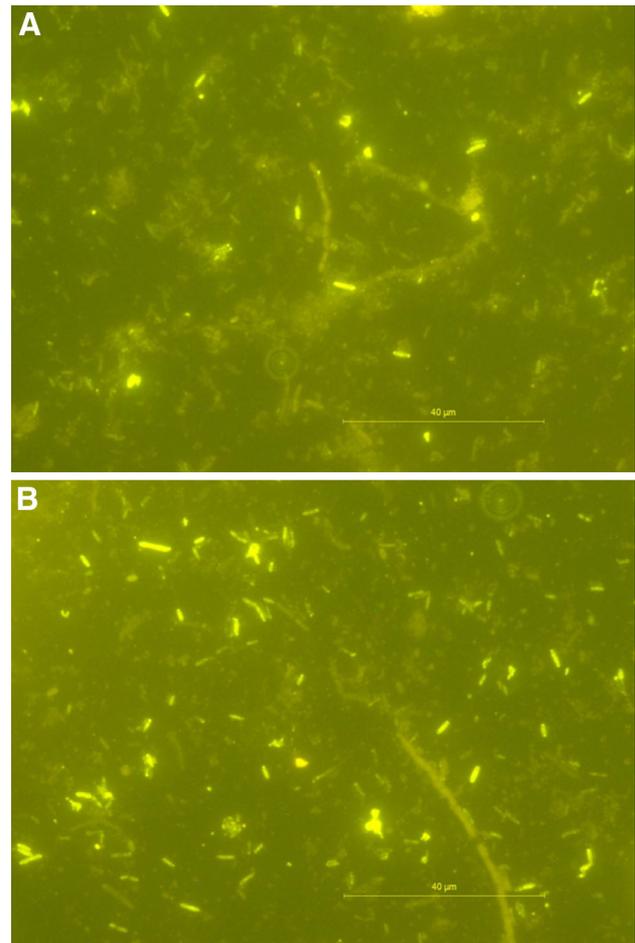


Fig. 4 Appearance of Chis150 in diet 1 supplemented with 0 µg/ml (a) and 10 µg/ml (b) glyphosate incubated 48 h in vitro

the population of *C. botulinum* type B did not differ significantly before or after incubation (Table 3). The toxin production was significantly higher with diet 1. It was 38–98 and 25–45 ng/ml for diet 1 and 2, respectively.

Discussion

This in vitro study shows that glyphosate causes a shift in the microbial population of rumen fluids to favor the production of BoNT within 48 h even though the population of *C. botulinum* spores was not significantly changed (Table 3). Several groups of bacteria and protozoa were inhibited by glyphosate. It was noted that more species were inhibited with the crude fiber-rich diet (Figs. 1, 2, 3, 4) than the lower fiber one to indicate there may be an inhibitory effect on the microbiota responsible for fiber degradation. This could be significant since 30–40 % of microbial fiber degradation in the rumen is performed by entodiniomorphid protozoa [12, 16] and all entodiniomorphid protozoa were highly susceptible to

glyphosate. *Ruminococcus* (*R.*) *albus* and *R. flavefaciens*, the most important ruminal bacteria for fiber degradation [20], also were strongly inhibited by glyphosate in trials with crude fiber-rich diets.

Among the carbohydrate-specific ruminal subpopulations, the cellulolytic bacteria are especially important. These bacteria convert the major plant polysaccharide (cellulose) and nitrogenous compounds to hydrolytic products of cellulose, volatile fatty acids, and microbial cell products which can be used by other microbes and the host animal. The cellulolytic ruminal bacteria are more sensitive than other ruminal bacteria to certain stress conditions such as low pH [45]. These results are comparable to those of Zebeli and Metzler-Zebeli [48] who reported lowered fiber digestion with metabolic stress of gastrointestinal microbiota. These results suggest that glyphosate leads to ruminal disorders influencing the whole body system of the animal. Inhibition of pH sensitive fibrolytic enzymes through pH changes [10, 11] or the binding of cations necessary as cofactors for microbial enzymes through the

Table 3 The effect of glyphosate on the growth of *C. botulinum* type B (\log_{10} cfu/ml) at 0 and 48 h in vitro incubation with diet 1 and 2

| Diet | Incubation time (h) | Glyphosate concentration ($\mu\text{g/ml}$) | | | | |
|------|---------------------|---|-----------------|-----------------|-----------------|-----------------|
| | | 0 | 1 | 10 | 100 | 1,000 |
| 1 | 0 | 5.14 \pm 0.18 | 5.16 \pm 0.15 | 5.06 \pm 0.32 | 5.14 \pm 0.18 | 5.11 \pm 0.11 |
| | 48 | 6.25 \pm 0.33 | 6.15 \pm 0.26 | 6.11 \pm 0.32 | 6.19 \pm 0.28 | 6.29 \pm 0.26 |
| 2 | 0 | 4.98 \pm 0.03 | 5.14 \pm 0.18 | 4.98 \pm 0.23 | 5.14 \pm 0.18 | 5.09 \pm 0.18 |
| | 48 | 6.04 \pm 0.23 | 5.93 \pm 0.54 | 6.24 \pm 0.34 | 6.30 \pm 0.30 | 6.28 \pm 0.28 |

Values are mean \pm SD ($n = 3$)

chelating properties of glyphosate [9, 31] could be possible mechanisms. Follow-up in vivo investigations are needed. Fiber fermentation provides important nutrients for many non-fibrolytic microbial species. These nutrients include salts of short-chained fatty acids such as acetate, propionate, butyrate, and lactate as well as glycerol and gases such as carbon dioxide, hydrogen, and methane, products of fiber degradation that are released in the rumen [1]. Inhibition of fiber degrading microbes by glyphosate, as shown in our study, could also affect non-fibrolytic microbial species indirectly to explain the reduction of *Streptococcus* spp. during incubation with the crude fiber-rich diet.

Euryarchaeota were also reduced which could be due to direct effect of glyphosate or indirectly due to dysbiosis of protozoa, hence the rumen protozoa influence the population and composition of its microbiota [37]. Also several symbiotic associations between anaerobic protozoa and methanogenic bacteria have been found, and the episympiosis of bacteria and protozoa has been described [44].

While certain groups were inhibited, the population of members of the *C. histolyticum* group increased with both diets when treated with glyphosate (Fig. 2). The *C. histolyticum* group consists of cluster I and II of the *Clostridiaceae* including many species with pathogenic potential [47]. Therefore, the glyphosate-induced dysbiosis of crude fiber-rich diets during the dry period of cows could induce a higher susceptibility for pathogens or support such microorganisms that normally occur in small numbers. Glyphosate stimulation of lactate producing bacteria with the concentrate rich diet (Fig. 2) could result in decreased pH to induce a sub-acute acidosis during the lactation period [21]. Although BoNT/B was produced with both diets at the highest glyphosate concentration, the *C. botulinum* type B spore population did not change (Table 3). This indicates that glyphosate toxicity to the ruminal microbiota avoided degradation of BoNT/B by proteases of these microorganisms.

BoNT/B was detectable only with the highest dosage of 1,000 $\mu\text{g/ml}$ glyphosate in this study possibly because glyphosate concentrations of 1, 10, and 100 $\mu\text{g/ml}$ could have been chelated cations inherent in the buffer solution that inactivate herbicide and nullify its activity on the microbiota. Freuze et al. [19] reported that 1 $\mu\text{g/l}$ of glyphosate (6 nM) in the presence of an equivalent molar

concentration of free copper (0.4 $\mu\text{g/l}$ Cu^{2+}) reduces free glyphosate to 10 %. At 1,000 $\mu\text{g/ml}$, glyphosate was inhibitory to neurotoxin degrading bacteria and their enzymes. The mechanistic effect could be explained due to dysbiosis induced by glyphosate, hence the proteolytic enzymes of the autochthonous microbiota of rumen are able to degrade BoNT. These enzymes are able to degrade BoNT [2, 3, 39, 41]. *Lactobacillus* suspensions are able to degrade BoNT after 48 h incubation at 37 °C [41].

This could explain how glyphosate favors toxico-infection with *C. botulinum* in the rumen and/or the whole gastrointestinal tract of cows. Although BoNTB was only detectable at the highest concentrations of the herbicide, negative effects could accumulate in younger animals through the chronic ingestion of residual glyphosate in the milk replacer made with genetically modified (GM) soy, that is administered shortly after finishing the colostrum milk period or in the milk of nursing cows. We believe that glyphosate will have a greater influence on cattle herd health in the near future due to increasing application rates of glyphosate to crops and much higher residual levels especially in glyphosate-resistant GM crops.

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