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ORIGINAL ARTICLE

Investigations on the possible impact of a glyphosate-containing herbicide on ruminal metabolism and bacteria *in vitro* by means of the 'Rumen Simulation Technique'

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Abstract

Aims: This study was performed in a well-established *in vitro* model to investigate whether the application of a glyphosate-containing herbicide might affect the bacterial communities and some biochemical parameters in a cow's rumen.

Methods and Results: The test item was applied in two concentrations (high and low) for 5 days. In a second trial, fermentation vessels were inoculated with *Clostridium sporogenes* before the high dose was applied. Effluents were analysed by biochemical, microbiological and genetic methods. A marginal increase in short-chain fatty acid production and a reduction in NH_3-N were observed. There were minor and rather equivocal changes in the composition of ruminal bacteria but no indications of a shift towards a more frequent abundance of pathogenic Clostridia species. *Clostridium sporogenes* counts declined consistently.

Conclusions: No adverse effects of the herbicide on ruminal metabolism or composition of the bacterial communities could be detected. In particular, there was no evidence of a suspected stimulation of Clostridia growth.

Significance and Impact of the Study: Antibiotic activity of glyphosate resulting in microbial imbalances has been postulated. In this exploratory study, however, intraruminal application of concentrations reflecting potential exposure of dairy cows or beef cattle did not exhibit significant effects on bacterial communities in a complex *in vitro* system. The low number of replicates (n = 3/dose) may leave some uncertainty.

Introduction

The total herbicide glyphosate is one of the most frequently applied active ingredients in plant protection products worldwide. Its unique herbicidal mode of action is by the inhibition of the enzyme 5-enolpyruvlyshikimate-3-phosphate synthase (EPSPS) that is involved in the aromatic amino acid biosynthesis pathway in plants (Boocock and Coggins 1983; Duke and Powles 2008). This enzyme is not produced by mammals but is common in many bacteria and may be found also in fungi and some other micro-organisms (Roberts *et al.* 1998; Priestman *et al.* 2005; Clair *et al.* 2012). Therefore, a potential of glyphosate to alter the gastro-intestinal microflora in humans or in farm animals may be suspected leading to imbalances (sometimes called 'dysbiosis' or 'dysbacteriosis') with subsequent clinical signs or symptoms. In fact, Shehata *et al.* (2013) reported that some beneficial bacteria of the poultry microbiota such as *Bifidobacterium adolescentis*, *Enterococcus* and *Lactobacillus* species were moderately to highly susceptible to a glyphosate-containing herbicide when their minimal inhibitory

concentrations were added to the bacterial cultures *in vitro*. In contrast, pathogenic species such as *Salmonella enteritidis* or *Clostridium perfringens* appeared to be more resistant. Since it has been shown that complex and interacting microbial communities, as present in the digestive tract, are more difficult to be changed (Preidis and Versalovic 2009), it should be verified if the above mentioned effect can be found here, too. The available data from literature are scarce and partly contradictory. Schrödl *et al.* (2014) concluded from their investigations on four species of fungi a modulating effect of glyphosate on the fungal community in the rumen of dairy cows. However, they did not perform a controlled feeding study. Instead, blood, urine and rumen fluid samples were taken from 14 German dairy farms. The individual feeding regimes were not reported and the amounts of glyphosate that were, in fact, taken up by the animals remained unknown. In contrast, Hüther *et al.* (2005) did not find an adverse effect of high amounts of glyphosate (0·77 g kg^{-1} dry mass) on rumen fermentation in sheep under controlled feeding study conditions. Altogether, it remains to be seen if concentrations of glyphosate resulting from dietary intake of residues in foodstuffs were of concern for the rumen microbial community.

In this study, possible effects of the glyphosate-based herbicide (GBH) Plantaclean® 360 on rumen metabolism and on the qualitative and quantitative composition of the ruminal bacterial communities were investigated by means of the 'Rumen Simulation Technique (RUSITEC)'. This method was first described by Czerkawski and Breckenridge (1977) and has become a well-established model in nutritional physiology (Martínez *et al.* 2010).

In trial A, the GBH alone was applied in two concentrations (low and high) reflecting the expected dietary exposure of cattle. Furthermore, Krüger *et al.* (2013) had hypothesized that exposure to glyphosate might lead to an overgrowth of Clostridia. Therefore, in trial B, effects of the high dosage of GBH in combination with the addition of *Clostridium sporogenes* were investigated. *Clostridium sporogenes* was applied as a surrogate for pathogenic bacteria from this group such as *Clostridium botulinum*.

Materials and methods

Test material

The herbicide Plantaclean® 360 (Plantan GmbH, Buchholz, Germany) served as test material. It is a commercially available, water-soluble concentrate containing 486 g glyphosate isopropylamine salt (equivalent to 360 g glyphosate acid) per litre.

In vitro experiments

The RUSITEC experiments were carried out similar as described by Czerkawski and Breckenridge (1977). Two trials (A, B) were conducted with six fermentation vessels (V = 700 ml) inoculated with rumen contents collected in the morning (approx. 3 h after feeding) from a ruminal-fistulated nonlactating cow (3 years of age, 450 kg body weight) that had been fed 9 kg hay and 200 g concentrate (provided by DEUKA Schaffutter, Erfurt, Germany) per day. Separation of the liquid from the solid phase of the rumen content was obtained by squeezing the fresh material through gauze. Rumen contents were kept at 39°C during this process. A schematic overview of a fermentation vessel is provided in Fig. 1. Starting the experiments, one nylon bag (11·5 × 6·5 cm, pore size 150 μm) was filled with the solid phase of the rumen content (70 g), another one with a total of 10 g substrate

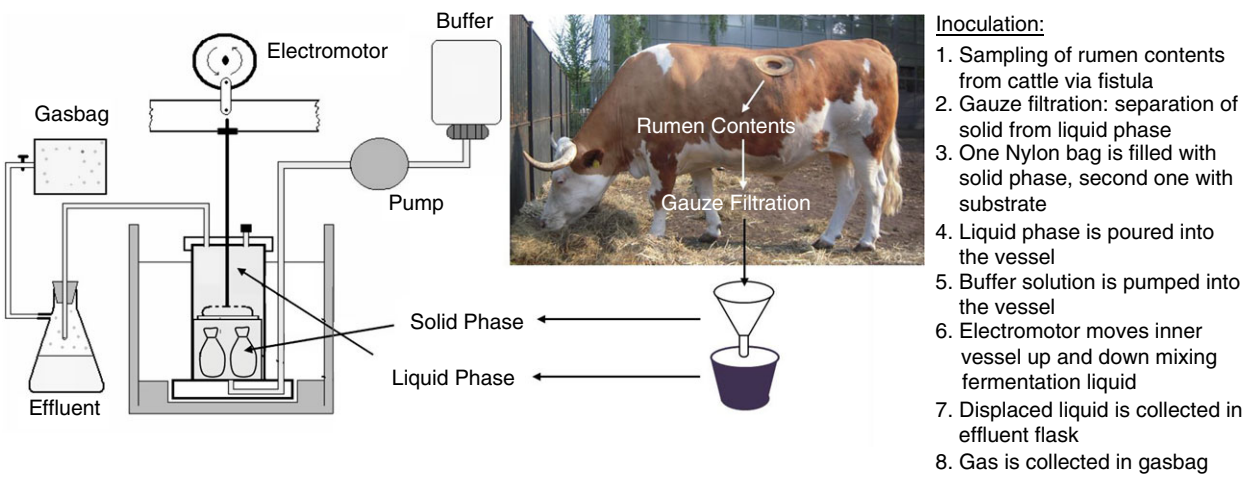

Figure 1 One fermentation vessel of the RUSITEC system.

Table 1 Composition of the diet fed to the donor cow and the substrate in the RUSITEC

Hay		Concentrate	
Ingredient	Amount (% of DM)	Ingredient	Amount (% of DM or IU kg^{-1})
Ash	7·1	Ash	0·69
Crude protein	7·1	Crude protein	19·62
Crude fat	1·5	Crude fat	4·24
Crude fibre	31·9	Crude fibre	9·48
Acid detergent fibre	36·7	Acid detergent fibre	14·94
Neutral detergent fibre	60·4	Neutral detergent fibre	30·55
Acid detergent lignin	3·1	Acid detergent lignin	5·57
Nonfibre carbohydrates	23·9	Nonfibre carbohydrates	44·9
Organic matter	84·6	Organic matter	9·31
		Calcium	1·50
		Phosphorous	0·55
		Sodium	0·25
		Vitamin A	20 000 IU kg^{-1}
		Vitamin D_3	1600 IU kg^{-1}

DM, dry matter; IU, international units.

(6 g hay, 4 g concentrate, as described above) and the pure liquid phase was poured into the vessels. The next day (24 h later), the nylon bag containing the solid phase was replaced by a substrate- filled one. From that day onwards, changing of nylon bags was carried out in an alternating way with a 24 h-interval, leading to a retention time of 48 h for each nylon bag in the vessel.

Crude nutrient content of the diet is given in Table 1. The rumen liquid was analysed prior to each run (means ± SEM) for pH (6·48 ± 0·05), redox potential (−348·5 mV ± 6·5) and concentrations of acetate (77·63 mmol l^{-1} ± 8·00), propionate (22·80 mmol l^{-1} ± 2·02), isobutyrate (0·70 mmol l^{-1} ± 0·00), butyrate (10·05 mmol l^{-1} ± 0·72), isovalerate (1·60 mmol l^{-1} ± 0·00) and valerate (1·08 mmol l^{-1} ± 0·00).

A buffer solution (for composition, see Table 2) similar to ruminant saliva was infused continuously into the vessels to reach a liquid turnover of once a day. Effluents of the vessels were collected in glass flasks on ice with the simultaneous collection of fermentation gases in gas-tight bags (Plastigas; Linde AG, München, Germany).

In both trials (A, B), an equilibration period of 6 days (day 0 to day 6) was followed by a control (day 7 to day 11) and then by an experimental period (day 12 to day 16) of 5 days each. That way, ruminal metabolism and microbiological parameters could be compared during control and experimental period for the same vessel.

Table 2 Chemical composition of the buffer solution (in mmol l^{-1})

Ingredient	Amount
NaCl	28·00
KCl	7·69
$CaCl_2{\cdot}2H_2O$	0·22
HCl (1N)	0·50
$MgCl_2{\cdot}6H_2O$	0·63
NH_4Cl	5·00
$Na_2HPO_4{\cdot}12H_2O$	10·00
$NaH_2PO_4{\cdot}H_2O$	10·00
$NaHCO_3$	97·90

In trial A, during the experimental period the GBH was added daily to fermentation vessels in triplicates at two different concentrations. The low glyphosate dose (LG) was chosen to reflect the estimated maximum dietary glyphosate intake of dairy cattle according to model assumptions (Germany 2013), that is, 43·4 mg kg^{-1} dry mass per day. Beef cattle might ingest higher residues since their diet might contain up to 103 mg kg^{-1} dry mass. The high glyphosate dose (HG) did not directly correspond to that dose but was higher to ensure a sufficient margin between the two concentrations. The actual average amounts of glyphosate acid in the vessels ($n = 3$ per dose) were analytically determined to be 0·42 mg (LG) or 2·92 mg (HG).

In trial B, on the last day of the control period (day 11), each of the six fermentation vessels was inoculated with *Cl. sporogenes* in a concentration of 10^4 CFU ml^{-1}. Thereafter, three vessels received the HG daily throughout the experimental period, whereas the other three vessels served as controls and remained untreated. The concentration of 10^4 CFU ml^{-1} was chosen to detect either an increase or a decrease in CFU of Clostridia in response to the application of GBH. For safety reasons, *Cl. sporogenes* (provided by Ripac-Labor GmbH, Potsdam-Golm, Germany) was applied as a surrogate for *Cl. botulinum* to study its growth behaviour in the *in vitro* system. *Clostridium sporogenes* and *Cl. botulinum* exhibit morphological similarity and demonstrate a high degree of relatedness but *Cl. sporogenes* is not pathogenic. Therefore, this germ may be used as a nontoxigenic alternative (Bradbury *et al.* 2012) to facilitate research under normal laboratory conditions.

Sampling and analytical procedures

Glyphosate analysis

In trial A of the RUSITEC experiments, effluents from the control (day 11) and from the experimental period (pooled sample of day 12 to day 16) were analysed for concentrations of glyphosate in a state laboratory

(LAVES, Oldenburg, Germany) by means of an LC-MS/MS method (Quick Method for the Analysis of Highly Polar Pesticides in Food of Plant Origin, QuPPe, Ver. 07) to exclude or verify its presence in the test system and to determine the total recovery. The limit of detection (LOD) for glyphosate by this method is 0·05 mg kg^{-1} and the limit of quantification (LOQ) is 0·1 mg kg^{-1}.

Ruminal metabolism

In trial A, the anaerobic status of the system, pH and redox potentials were monitored daily. Concentrations of short-chain fatty acids (SCFA) and NH_3-N in effluents were measured daily and degradation of organic matter (OM) was determined every 48 h in both, the control and experimental period. In trial B, in contrast, these examinations were performed only in the control period to avoid contamination of the laboratory with *Cl. sporogenes*. The analyses for SCFA concentrations, NH_3-N concentrations and for degradation of OM were carried out as described previously (Koch *et al.* 2006; Meibaum *et al.* 2012; Riede *et al.* 2013). Daily production of SCFA was calculated by multiplying their measured concentrations by the effluent volume.

Analysis of microbial communities

Single strand conformation polymorphism (SSCP) is a simple and powerful technique for identifying sequence changes in amplified DNA. In this study, it was used for the characterization of the composition and changes in the microbial community. For this purpose, the microbial communities of 'Total bacteria' and the '*Clostridium* cluster I' (i.e. proteolytic Clostridia) according to Collins *et al.* (1994) were considered.

First, differential centrifugation was performed as described by Brandt and Rohr (1981). The SSCP procedure for 16S rRNA genes of bacterial sequences was carried out as published by Meibaum *et al.* (2012). Briefly, after isolation of genomic DNA, polymerase chain reaction (PCR) was used for amplification of 16S rRNA genes. The total reaction volume was 25 μl with a final concentration of 1× PCR buffer with 2·5 U μl^{-1} HotStar HiFidelity DNA polymerase (Qiagen, Hilden, Germany). The F27 forward primer (AGA GTT TGA TC(A/C) TGG CTC AG; Lane 1991) and the R1492 reverse primer (TAC GG(C/T) TAC CTT GTT ACG ACT T; Weisburg *et al.* 1991) were also obtained from Qiagen and applied at a concentration of 50 μmol l^{-1}. PCR conditions were as follows: initial denaturation at 95°C for 5 min, followed by 25 cycles of denaturation at 94°C for 60 s, annealing at 50°C for 60 s and elongation at 72°C for 70 s. Final elongation was for 10 min at 72°C. For the subsequent nested PCR, the Com1 forward primer (CAG CAG CCG CGG TAA TAC) and the Com2-Ph reverse primer (CCG TCA ATT CCT TTG AGT TT; Schwieger and Tebbe 1998) were used.

For PCR amplification of *Clostridium* cluster I sequences, according to Dohrmann *et al.* (2011), the forward primer P930 (GTG AAA TGC GTA GAG ATT AGG AA) and reverse primer P932-Ph (GAT (C/T)(C/T) G CGA TTA CTA G(C/T)A ACT; Le Bourhis *et al.* 2005) were used (source as above). Reverse primers were phosphorylated at the 5′ end for further single strand digestion. PCR reaction mixture and conditions were the same as for total bacteria with the exception that annealing took place at 58°C but for only 50 s.

Gel electrophoresis of single strand DNA was carried out at 20°C and 300 V for 22·5 h (total bacteria) or 30 h (Clostridia). Polyacrylamide (0·625%) SSCP gels were air-dried and scanned (ScanMaker i800; Mikrotek, Willich, Germany).

Microbial profiles obtained by SSCP analysis of the total bacteria and *Clostridium* cluster I communities in pooled fermentation liquid of the control period (days 9, 11) were compared with those from the experimental period (days 13, 16) in trial A. In trial B, comparison to samples of days 13 and 16 was carried out separately to gain insight into possible temporal changes.

Bacteriological analysis

In samples taken on days 9 and 11 (control period, trials A and B), and on days 13 and 16 (experimental period, trial A) or 12–16 (experimental period, trial B), respectively, qualitative and quantitative determination of micro-organisms was carried out.

For the culturing and isolation of the aerobic and anaerobic bacteria, different protocols were used. The preparation of samples was performed according to ASU L 06.00-16, that is, an officially approved method according to the German food and feed legislation. For quantitative analysis, dilution series of samples were cultivated at 37°C on Columbia agar with 5% sheep blood, whereas moulds and yeasts were kept at 30°C on Sabouraud agar plates (Oxoid, Wesel, Germany). Cultivation time was 48 h.

For qualitative analysis of all present Clostridia, isolation was carried out additionally by enrichment using liver broth obtained also from Oxoid. After over-night cultivation of the dilution series at 37°C, 10 μl of the liver broth was transferred onto a Columbia-agar plate and Clostridia cultivated again in its presence for 48 h under anaerobic conditions at 37°C. This protocol was applied to quantifying *Cl. sporogenes* in trial B, too.

Once *Cl. perfringens* was detected, further differentiation was tried by genotyping its major toxins. For this purpose, a multiplex PCR as described by Meer and Songer (1997) was used. Quantification of the *alpha* toxin was done according to Beer and Al-Khatib (1968).

All colonies with different morphologies, including all cultivated bacteria, yeasts and moulds, were identified by means of the 'Matrix-assisted linear desorption/ionzation time-of-flight mass spectrometry' (MALDI-TOF MS) technique (Shimadzu/Kratos, Manchester, UK) as described by Kallow *et al.* (2010). The strains were analysed on a stainless steel target plate (MABRITEC AG, Basel, Switzerland), using a whole-cell protocol with 1 μl matrix solution of saturated α-cyano-4 hydroxy-cinnamic acid in a mixture of acetonitrile, ethanol and water (1 : 1 : 1) acidified with 3% (v/v) trifluoroacetic acid. For each strain, mass spectra were prepared in duplicate and analysed in the linear positive ion extraction mode. Mass spectra were accumulated from 100 profiles, each from five nitrogen laser pulse cycles, by scanning the entire sample spot. Ions were accelerated with pulsed extraction at a voltage of 20 kV. Raw mass spectra were processed automatically for baseline correction and peak recognition. Resulting mass fingerprints were exported to the SARAMIS (Spectral Archiving and Microbial Identification System; AnagnosTec GmbH, Potsdam, Germany) analysis program and compared to reference superspectra and spectra to identify the species. The available open database allows identification of more than 1500 different bacteria species and more than 300 yeasts and moulds.

Statistical analysis

Statistical analysis was carried out with GRAPHPAD PRISM 4.0 (GraphPad Software Inc., La Jolla, CA). For evaluation of ruminal metabolism parameters, mean values obtained from the control and experimental periods were calculated for each fermentation vessel. Control values were based on a 5-day observation period. In the experimental period, the data included were confined to the last 3 days in order to guarantee stable temporal conditions. One-way ANOVA was performed then to compare the means from the control period (six fermentation vessels) with the means obtained in three fermentation vessels receiving either the LG or HG during experimental period. Two-way ANOVA was applied for data on *Cl. sporogenes* counts. *Post hoc*, the Bonferroni test was applied. Differences were regarded as significant at $P < 0{\cdot}05$, but trends at $P < 0{\cdot}1$ already.

Automatically detected SSCP band patterns of digitalized images were compared using the software GELCOMPAR II (Applied Maths, Sint-Martens-Latem, Belgium). Cluster analyses (clustering algorithm: UPGMA) with dendrograms were performed on the basis of similarity matrices using the Pearson product-moment correlation coefficient. A nonparametric multivariate analysis of variance that is suitable for statistical analysis of not normally distributed and discontinuous data was carried out using dissimilarity matrices as described previously by Anderson (2001). Calculations were conducted using the software PERMANOVA (ver. 1.6) leading to *P*-values obtained by a permutation procedure. Differences between treated ($n = 3$) and control vessels ($n = 6$) were regarded as significant at $P < 0{\cdot}05$ (Anderson and Robinson 2003).

Results

Recovery of glyphosate

During the control period before treatment, glyphosate could not be detected in the effluents. Application of the GBH into the fermentation vessels during the experimental period of trial A led to mean concentrations of 0·34 and 3·31 mg l^{-1} glyphosate in effluents for LG and HG respectively. The resulting mean recovery of 0·26 or 2·31 mg glyphosate per day accounted for 61·9 and 79·0%, respectively, of the actually applied LG or HG.

Impact on rumen metabolism

The application of the GBH in the experimental period of trial A did not alter pH or redox potentials in fermentation vessels as compared to the control period (Table 3). However, the addition of HG led to a significant decrease in NH_3-N concentrations from 8·51 to 7·07 mmol l^{-1} ($P < 0{\cdot}01$). While the production of propionate, butyrate and valerate remained unaffected in response to the addition of GBH, there was a trend of an increasing acetate and total SCFA production after the addition of GBH ($P < 0{\cdot}1$). In contrast, isovalerate concentration was significantly higher after administration of HG ($P < 0{\cdot}01$). For the isobutyrate concentration, a significant increase after addition of HG ($P < 0{\cdot}05$) was only observed when compared with LG but not with the control. The addition of HG resulted in a significant decrease in molar proportion of propionate ($P < 0{\cdot}05$) and in an increase for isovalerate compared with the control and LG-treated fermentation vessels ($P < 0{\cdot}01$). For valerate, there was a trend of a decrease in molar proportion in response to the addition of GBH ($P < 0{\cdot}1$). In addition, a trend of an increased degradation of OM was observed after the addition of GBH ($P < 0{\cdot}1$).

Impact on microbial communities

Dendrograms with estimates of similarity for microbial communities of total bacteria and Clostridia, based on SSCP profiles, are presented in Fig. 2 for trial A and in Fig. 3 for trial B.

In trial A, SSCP profiles of the bacterial communities obtained from each fermentation vessel during the

Table 3 Impact of the GBH on ruminal metabolism in the RUSITEC system (Trial A, means with standard deviation; six vessels in the control and three in LG and HG groups each)

Parameter	Control	LG	HG	*P*-value
pH	6·75 ± 0·05	6·75 ± 0·03	6·73 ± 0·02	ns
Redox potential (mV)	−292 ± 36·2	−304 ± 12·3	−278 ± 36·6	ns
NH_3-N (mmol l^{-1})	$8·51^{a}$ ± 0·62	$7·49^{a,c}$ ± 0·28	$7·07^{b,c}$ ± 0·41	**
Total SCFA (mmol day^{-1})	28·3 ± 2·26	29·3 ± 2·31	32·2 ± 2·21	†
Acetate	15·9 ± 1·17	16·8 ± 1·32	17·0 ± 1·95	†
Propionate	5·97 ± 0·56	5·74 ± 0·36	6·42 ± 0·53	ns
Isobutyrate	$0·14^{a,b}$ ± 0·05	$0·11^{a}$± 0·01	$0·22^{b}$ ± 0·04	*
Butyrate	4·17 ± 0·45	4·52 ± 0·63	5·04 ± 0·49	ns
Isovalerate	$0·93^{a}$ ± 0·09	$0·93^{a}$ ± 0·12	$1·21^{b}$ ± 0·04	**
Valerate	1·42 ± 0·10	1·32 ± 0·04	1·42 ± 0·18	ns
Molar proportions (%)				
Acetate	56·1 ± 0·87	57·3 ± 1·18	55·8 ± 0·94	ns
Propionate	$20·7^{a}$ ± 0·67	$19·6^{a}$ ± 0·43	$19·9^{b}$ ± 0·29	*
Isobutyrate	$0·49^{a,b}$ ± 0·12	$0·4^{a}$ ± 0·03	$0·63^{b}$ ± 0·07	*
Butyrate	14·8 ± 0·94	15·4 ± 1·31	15·6 ± 0·54	ns
Isovalerate	$3·27^{a}$ ± 0·18	$3·17^{a}$ ± 0·19	$3·79^{b}$± 0·22	**
Valerate	5·04 ± 0·44	4·54 ± 0·27	4·39 ± 0·27	†
Degradation of OM (%)	47·4 ± 2·46	48·2 ± 0·23	51·2 ± 1·54	†

P-values obtained by One-way ANOVA; **P* < 0·05, ***P* < 0·01, †trend: *P* < 0·1.
[a–c]Means within a row with different superscripts differ significantly (*P* < 0·05).

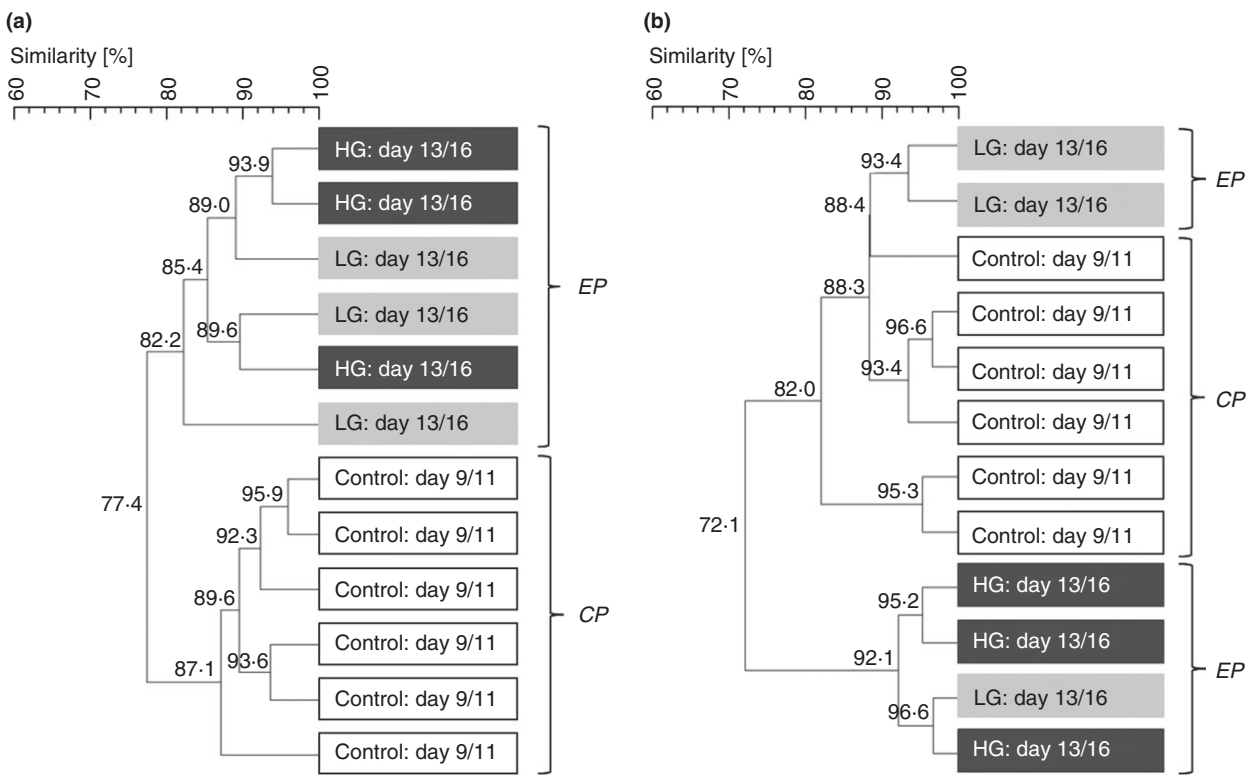

Figure 2 Trial A: Dendrograms of the 16S rRNA gene-based SSCP profiles for the microbial communities of total bacteria (a) and Clostridia (b) in the control period (CP: □, pooled sample of days 9/11) and the experimental period (EP: pooled sample of days 13/16) after the addition of a low (LG: ■, 0·42 mg) or a high daily dose of glyphosate (HG: ■, 2·92 mg). For the cluster analysis, Pearson's correlation and the unweighted pair group method with arithmetic mean (UPGMA) algorithm were applied.

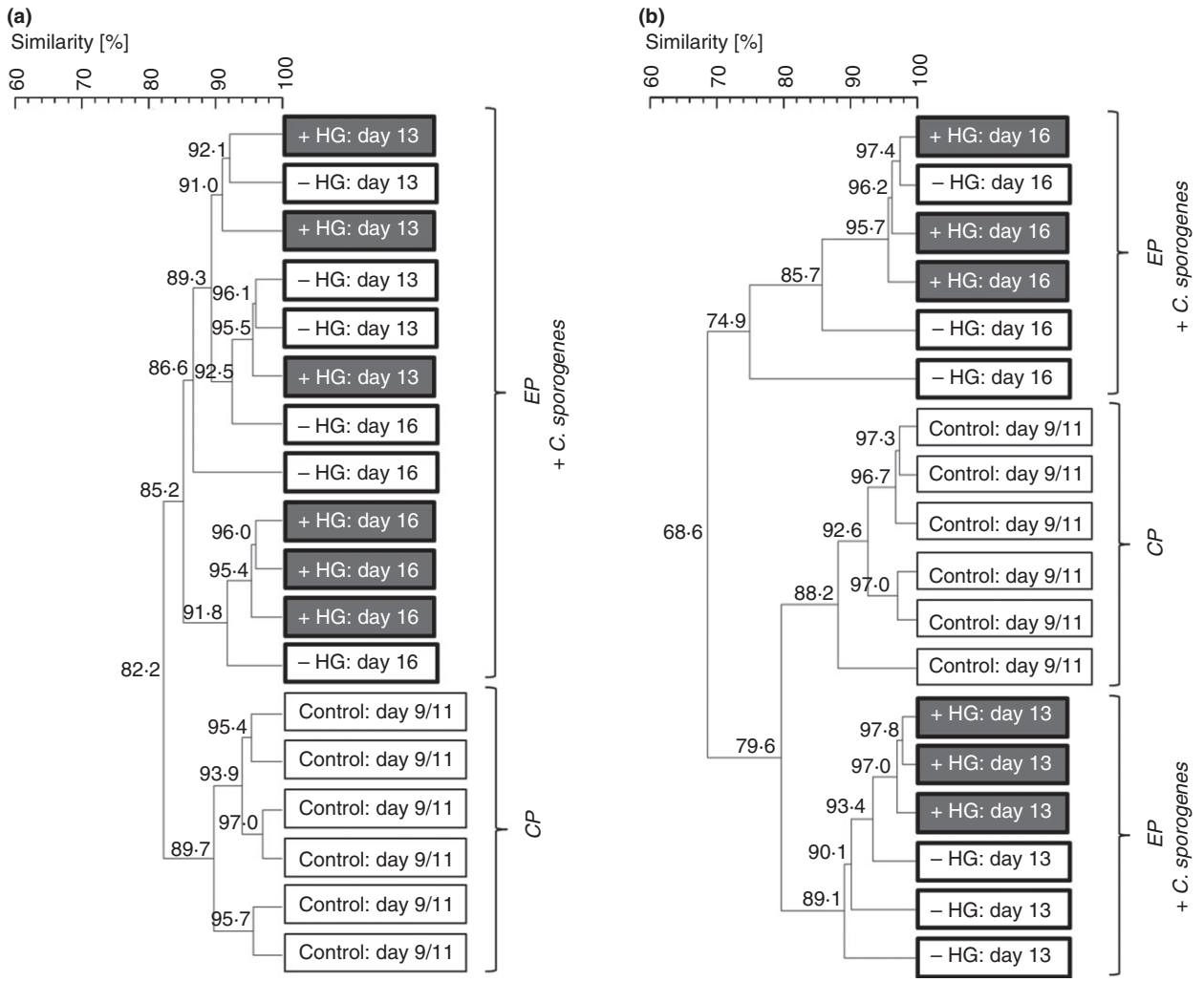

Figure 3 Trial B: Dendrograms of the 16S rRNA gene-based SSCP profiles for the microbial community of total bacteria (a) and Clostridia (b) in the control period (CP: □, pooled sample of days 9/11) and the experimental period (EP: pooled sample of days 13/16) after the addition of 10^4 CFU g^{-1} *Clostridium sporogenes* (–HG: □ = without high dose of glyphosate; +HG: ■ = with high dose of glyphosate giving 2·92 mg glyphosate per day). For the cluster analysis, Pearson's correlation and the unweighted pair group method with arithmetic mean (UPGMA) algorithm were applied.

control period (pooled sample of day 9 and 11) resembled each other quite well with an average agreement of 87·1% (Fig. 2a). SSCP profiles from the experimental period (pooled sample of day 13 and 16) formed a separate cluster. If directly compared, profiles from control period showed only a 77·4% agreement with profiles from the experimental period on average. This difference was statistically significant ($P < 0{\cdot}01$) suggesting a treatment-related effect. However, a dose-dependent influence on SSCP profiles of total bacteria was not observed. For SSCP profiles of the Clostridia community, there were no defined clusters in response to the treatment in the experimental period (Fig. 2b) and no statistical difference was obtained when compared to the control period.

In trial B, SSCP profiles of the bacterial communities from each fermentation vessel during the control period (pooled sample of day 9 and 11) were similar with an average agreement of 89·7% (Fig. 3a). After the addition of *Cl. sporogenes* at the beginning of the experimental period, we found significant differences in SSCP profiles from the control period compared to those from all six vessels of day 13 ($P < 0{\cdot}001$) and day 16 ($P < 0{\cdot}01$) of the experimental period. In addition, profiles from day 13 compared with profiles from day 16 were significantly different ($P < 0{\cdot}01$) suggesting a time-dependent influence. However, the application of the HG apparently had no impact on the SSCP profiles of the bacteria community.

As to be expected, the SSCP profiles of the Clostridia community were clearly altered by introduction of *Cl. sporogenes* (Fig. 3b) at the beginning of the experimental period. SSCP profiles were significantly different on days 13 and 16 when compared to the previous control period ($P < 0{\cdot}001$). A significant difference between SSCP profiles from day 13 and day 16 was also identified ($P < 0{\cdot}01$). SSCP profiles of the Clostridia community were not affected by the addition of HG.

Bacteriological analysis

The following genera/species were identified by MALDI-TOF MS technology in trial A: *Aerococcus viridans, Bacillus licheniformis, Enterococcus faecalis, Escherichia hermannii, Lactobacillus* spp., *Leucobacter* spp., *Morganella* spp., *Pseudomonas* spp., *Psychrobacter* spp., *Riemerella* spp., *Staphylococcus* spp. and *Streptococcus* spp. Interestingly, *Bifidobacterium* spp. was only detectable during the experimental period in response to the addition of both LG and HG but not during the control periods. Following treatment, concentrations of up to 2400×10^4 CFU g^{-1} were observed. There were no other differences in bacterial numbers in relation to treatment or time.

Because of artificial adding of *Cl. sporogenes* to the test system in trial B, spontaneous occurrence of Clostridia was of particular interest. In fact, in trial A, *Cl. perfringens* was identified on day 9 in the control period in one fermentation vessel in a low concentration of <10 CFU g^{-1} but could not be typed. Following application of HG, *Cl. perfringens* type A was detected on day 13 in the same vessel at the same concentration. This bacterium produced small amounts of α-toxin (≤4 NE) but no β2-toxin. In a further fermentation vessel treated with HG, *Cl. perfringens* type A and *Clostridium bifermentans* were found in concentrations below 10 CFU g^{-1} on day 13. After application of LG, *Cl. perfringens* type A was found in one vessel and some production of α-toxin (≤4 NE) could be shown.

In trial B, *Clostridium sartagoforme* was identified in a concentration of 6×10^8 CFU g^{-1} in one fermentation vessel prior to HG addition. In the same fermentation vessel, after addition of *Cl. sporogenes* in a concentration of 10^4 CFU ml^{-1} at the beginning of the experimental period and HG, *Cl. sartagoforme* occurred on day 14 in a concentration of 3×10^7 CFU g^{-1}, on day 15 in a concentration of 1×10^6 CFU g^{-1} and on day 16 in a concentration of 2×10^7 CFU g^{-1}. In the remaining fermentation vessels, further Clostridia species were not detected.

Further identified genera/species in trial B were *Actinomyces viscosus*, species from the *Bacillus cereus* group, *Corynebacterium* spp., *Enterococcus* spp., *Escherichia coli, Gemella* spp., *Globicatella sulfidifaciens, Lactobacillus mucosae, Leucobacter* spp., *Paenibacillus odorifer, Pseudomonas* spp., *Staphylococcus* spp., *Streptococcus* spp. and *Veillonella* spp. Again, there were no differences in bacterial numbers in relation to treatment or time.

Fate of *Clostridium sporogenes* in trial B

Following the addition of *Cl. sporogenes* (10^4 CFU ml^{-1}), a similar significant time-dependent reduction ($P < 0{\cdot}001$) in the number of CFU in the fermentation vessels treated with HG and in the untreated vessels was observed (Fig. 4). It became obvious that there was no evidence of growth stimulation of *Cl. sporogenes* by HG.

Discussion

Farm animals such as cattle may become exposed to glyphosate via their diets. Beside the general increase in glyphosate use, this intake is most likely due to application conditions that have considerably changed for some years. Residues could mainly arise from two sources. On the one hand, forage may contain glyphosate when the ripe crops have been treated only a few days before harvest to make subsequent collection easier. On the other hand, concentrates may contain imported soy or maize obtained from genetically modified crops which are widely cultivated in particular in the USA and South America. One of the main intentions of genetic modification is to make plants resistant to glyphosate. In a very

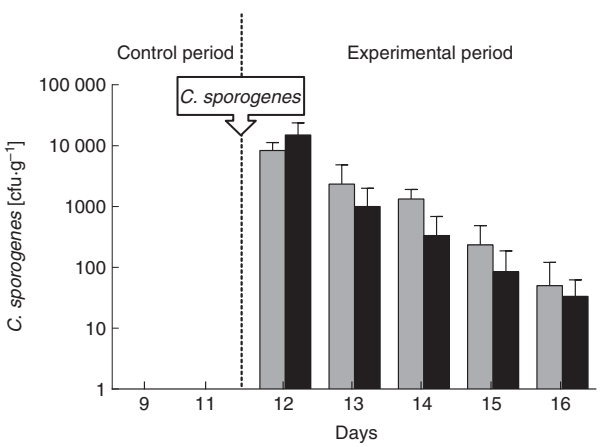

Figure 4 Trial B: Time-dependent decline of colony forming units of *Clostridium sporogenes* during experimental period after addition of 10^4 CFU g^{-1} *Cl. sporogenes* in fermentation vessels treated with the high glyphosate dose of 2·92 mg day^{-1} (■) and without glyphosate (▩) (means with standard deviation; control $n = 6$; +HG $n = 3$; −HG $n = 3$; Two-way ANOVA, treatment: not significant, time: $P < 0{\cdot}001$, treatment x time: not significant).

recent publication, Von Soosten *et al.* (2016) could show that concentrates are the main source of dietary glyphosate exposure of dairy cows in Germany.

Actual dietary exposure of farm animals in Europe was confirmed by Krüger *et al.* (2014) who measured not only the urinary excretion of this compound in lactating cows from dairy farms in Denmark and Germany but also detected glyphosate residues in various organs of slaughtered cattle. Von Soosten *et al.* (2016) reported an extremely variable glyphosate intake with mean values ranging from 0·08 to 6·67 mg per day in six independent feeding studies including 32 dairy cows.

Although the estimated daily oral intake of glyphosate by farm animals is low and of no toxicological relevance (Germany 2013), a possible impact on the bacteria in the gastrointestinal tract should be investigated because inhibition of the enzyme EPSPS by glyphosate has been shown in different bacteria species (Priestman *et al.* 2005; Clair *et al.* 2012). Consistent with that, a transient effect of glyphosate on soil micro-organisms has been described (Roslycky 1982; Locke *et al.* 2008).

Shehata *et al.* (2013) measured the influence of different concentrations of a glyphosate-containing herbicide (Roundup UltraMax®) on 23 bacterial species and strains *in vitro*. They found lower minimum inhibitory concentrations (0·075–0·6 mg ml^{-1}) for beneficial bacteria. In contrast, some pathogenic germs such as *Cl. perfringens* and several *Salmonella* species appeared much less sensitive. In these species, growth inhibition was seen only at the highest tested concentration of 5 mg ml^{-1} although it was not evident from the article if these concentrations related to the herbicide or the contained glyphosate. However, separate testing of isolated bacterial cultures may not reflect the situation, for example, in the gut, where hundreds and thousands of species and strains form large and ever interacting microbial communities. Ackermann *et al.* (2015) found only a marginal impact on ruminal bacteria in a batch system although concentrations of up to 100 mg glyphosate per litre had been applied that was by 20–25 times higher than the maximum concentration in our experiments. However, also this test system does not reflect realistic conditions in the rumen. Therefore, it was decided to study effects on bacteria in a more complex *in vitro* system that we consider a better model for what may happen in ruminants after ingestion of glyphosate residues. The RUSITEC is a scientifically well established, semi-continuous incubation method in nutrition physiology to study the impact of different diets (Koch *et al.* 2006; Martínez *et al.* 2010) or various additives as, for example, fumaric acid (Riede *et al.* 2013) on rumen microbial metabolism and microbial populations *in vitro*. It allows incubations of up to 3 weeks.

The herbicide Plantaclean® 360 was applied instead of glyphosate itself because it is the formulation that is sprayed and not the pure active ingredient. In case of adverse findings in the study described here, such as a positive impact on Clostridia growth, it had been intended to repeat the experiment with technical grade glyphosate to allocate the effects clearly either to the active substance or to the plant protection product with various co-formulants. However, there was no need for this second step.

Glyphosate analysis in the effluents of the fermentation vessels by a sensitive and specific LC-MS/MS method confirmed successful application but the rather poor recovery in particular from the vessels receiving LG leaves the fate of a considerable amount in the experimental system open. Several reasons seem to be conceivable. First, glyphosate might have been degraded by micro-organisms to sarcosine, inorganic phosphate (Dick and Quinn 1995) or aminomethylphosphonic acid (AMPA) (Rueppel *et al.* 1977; Balthazor and Hallas 1986). Determination of these substances was not included in this study. In addition, a strong adsorption behaviour of glyphosate to soil particles has been described (Sprankle *et al.* 1975) and its adsorptive characteristic seems to be applicable to glassware as well as mentioned by Hao *et al.* (2011) and Moye and Deyrup (1984). Thus, the lacking amount of glyphosate may have adsorbed, at least partly, to the glassware surface of the flasks in which effluents were collected. Moreover, it is conceivable that glyphosate may have adsorbed to feed particles of the substrate, such as hay or concentrate. Last, the fact that glyphosate is a chelating agent binding trace elements (Sprankle *et al.* 1975; Glass 1984) might have partially contributed to its poor recovery.

The two concentrations had been calculated to reflect worst-case assumptions for glyphosate intake by cattle (Germany 2013). In fact, a study by Von Soosten *et al.* (2016) showed that, under practical feeding conditions, these doses are unlikely to be reached. There was a sufficient margin between LG and HG to allow meaningful comparison and to detect a potential dose response.

The classical RUSITEC application is to measure influences on physiological processes in the rumen. In our study, the pH that may considerably affect the proportion of the formed fatty acids (Gürtler 1980) was not altered by treatment with Plantaclean® 360. Likewise, the redox potential was not changed confirming stable anaerobic conditions for bacterial growth in the vessels.

The application of HG led to a significantly increased isovalerate concentration. Moreover, there were numerical increases in propionate and butyrate concentrations and statistical trends suggesting an increase in acetate and, as a result, in SCFA in total after addition of GBH. Unfortunately, data on SCFA production was available only

from trial A and, thus, an intra-study confirmation for these results was lacking. The increased isovalerate concentration might be linked to the decreased NH_3-N concentration after HG application. Isovalerate is a fermentation product of microbial protein degradation (Hungate 1966) on the one hand. On the other hand, it is used for the synthesis of microbial protein (Allison *et al.* 1962; Dehority *et al.* 1967). NH_3-N is derived from degradation of nonprotein-nitrogen compounds or protein as well (Owens and Zinn 1988). Thus, it can be speculated about the GBH having caused a stimulation of microbial growth with a simultaneous increase in protein degradation.

The significant changes in molar proportions of propionate and isovalerate in response to HG addition reflect the trend for increased concentrations of total SCFA in combination with the numerical changes in propionate and isovalerate concentrations themselves.

Since the rumen is a sensitive system, the biochemical processes going on there are prone to react promptly to changes in the diet (Ramos *et al.* 2009). Altogether, all these findings were in the physiological range found for rumen SCFA and NH_3-N concentrations (Hungate 1966; Gürtler 1980; Czerkawski 1986) and there was no clear evidence of an adverse effect of treatment with GBH regarding these parameters in our study. Our results confirm previous research *in vivo*. In rumen-fistulated sheep, Hüther *et al.* (2005) did not find any indications of an impact on ruminal fermentation parameters or bacteria if the animals had been fed a diet containing glyphosate concentrations as they might be expected to occur from recommended agricultural use.

Biotransformation in the rumen results from microbial activity. Thus, based on the marginal biochemical findings, not more than a small shift in microbial communities might be expected. Altogether, the bacterial analysis confirmed the abundance of those bacteria species and genera that have been described previously for the rumen (Stewart and Bryant 1988). The only difference between control and experimental period of trial A was an increase in *Bifidobacterium* spp. A concentration of up to 2400×10^4 CFU g^{-1} was determined after application of both LG and HG. Prior to treatment, that bacterial genus had not been detected at all. However, the abundance of *Bifidobacterium* species in the rumen can vary from complete absence to presence in high numbers, depending on the substrate type (Trovatelli and Matteuzzi 1976). Moreover, *Bifidobacterium* spp. was not found in trial B during the experimental period. This finding would rather support the assumption that growth of *Bifidobacterium* spp. was not related to the addition of GBH and arose in trial A by chance. In any case, an increase in *Bifidobacterium*, even if treatment-related, would not be regarded as adverse. *Bifidobacterium* spp. is not pathogenic and even thought to exhibit beneficial effects (Bottacini *et al.* 2014; Bunesova *et al.* 2014).

An impact of the treatment with the GBH on the microbial community in the rumen may be indicated in SSCP profiles by revealing significant differences between control period and experimental period. Two groups were considered, that is, the more general one comprising total bacteria (including beneficial and pathogenic species as well) and Clostridia belonging to the cluster I. The genus *Clostridium* is phylogenetically extremely heterogenous. Based on studies regarding 16S rRNA gene sequences, it is subdivided into different clusters (Rainey and Stackebrandt 1993; Collins *et al.* 1994) with cluster I comprising more than half of the pathogenic species including those considered the major pathogenic ones, that is, *Clostridium barati, Cl. botulinum, Clostridium haemolyticum, Clostridium novyi, Cl. perfringens, Clostridium tetani, Clostridium septicum* and *Clostridium chauvoei* (Stackebrandt *et al.* 1999). The cluster I is recognized as the genus *Clostridium sensu stricto* (Collins *et al.* 1994).

Indeed, in trial A, a significant difference between the control and experimental period was observed for the microbial community of total bacteria suggesting an influence of the GBH. However, the effect was not dose-related and it remains open which bacterial species has mediated the observed shift in the microbial community of total bacteria in the first experiment. The increase in *Bifidobacterium* spp. in the experimental period could have contributed to that change but, since the SSCP analysis is a qualitative method, it is not likely that it would be responsible alone.

The microbial community of *Clostridium* cluster I was not affected by addition of GBH. This is in accordance with the results of the bacteriological analysis which did not detect differences in the absence or presence of identified species in relation to treatment.

Altogether, a clear-cut and dose-dependent effect of GBH on the microbial communities could not be shown.

The application of *Cl. sporogenes* at the beginning of the experimental period in Trial B resulted in significant changes of the microbial community of total bacteria and *Clostridium* cluster I as compared to the control period. This was to be expected because *Cl. sporogenes* was not only included in the SSCP analysis of *Clostridium* cluster I but also of total bacteria by using more degenerate primers. Within the experimental period, the significant differences between SSCP profiles on day 13 and day 16 of both microbial communities indicated an impact of time of *Cl. sporogenes* on the overall community profile as *Cl. sporogenes* had been introduced into the vessels only once at the beginning of the experimental period. However, there were no significant differences in the SSCP

profiles of both microbial communities between the presence and absence of HG in the vessels. This finding was fully consistent with the similar decline in *Cl. sporogenes* counts between days 12 and 16 in the experimental period, both with and without HG treatment. *Clostridium perfringens* was detected at very low concentrations (<10 CFU g^{-1}) which are not regarded as dangerous.

Based on this data, it may be concluded that exposure to the GBH or glyphosate at realistic dietary concentrations does not result in Clostridia overgrowth due to imbalances in the bacterial population of the rumen *in vitro*. This latter finding is of particular interest since a causal link to a disease of so far unknown aetiology in cattle has been suspected. There are reports on severe health deterioration in particular in lactating cows from dairy farms in Northern Germany. It was speculated that the described clinical signs (mainly neurological and gastro-intestinal in nature, accompanied by losses in milk yield, cachexia and, sometimes, death) in cattle and even symptoms in farmers who take care of the animals might be due to chronic exposure to *Cl. botulinum* or its toxins (Böhnel *et al.* 2001; Rodloff and Krüger 2012). Glyphosate was blamed for being the external factor to affect benign and beneficial bacteria and to cause *Cl. botulinum* overgrowth (Krüger *et al.* 2013). Our data suggest that this is not likely and, thus, is consistent with recent information (Seyboldt *et al.* 2015) according to which a contribution of *Cl. botulinum* can be excluded since the neurotoxin could not be detected in excreta.

In contrast, the RUSITEC findings suggested that marginal and by nature not adverse effects on bacteria other than Clostridia and on few parameters in ruminal metabolism might occur. The latter effects could be further examined in similar experiments using more than one cow as donor of ruminal contents, more replicates (vessels) to reduce uncertainty, more dose levels and perhaps a different glyphosate-containing herbicide with other co-formulants. It might be also considered to include protozoa which contribute a lot to ruminal microflora along with bacteria and which at least partly depend on the shikimate cycle, too (Roberts *et al.* 1998). Ackermann *et al.* (2015) reported an impact of glyphosate on the protozoal population in a batch test system incubating rumen fluid only for 48 h at high concentrations of 10 or 100 mg l^{-1}, in particular, a decrease in *Ciliata* species. However, such findings would be of concern only if they were confirmed to occur under realistic exposure conditions.

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Conflicts of Interest

The authors declare no competing interests. The senior author actively participated in the regulatory evaluation of glyphosate under Regulation (EC) 1107/2009 but this paper does not necessarily reflect the regulatory position of the German Federal Institute for Risk Assessment although the project was mentioned and preliminary findings briefly reported in the 'Renewal Assessment Report' on glyphosate (Germany, 2013).

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