Effect of a Glyphosate-Containing Herbicide on *Escherichia coli* and Salmonella Ser. Typhimurium in an In Vitro Rumen Simulation System

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Glyphosate (*N*-(phosphonomethyl)glycine) is the most-used herbicide worldwide. Many studies in the past have shown that residues of the herbicide can be found in many cultivated plants, including those used as livestock feed. Sensitivity to glyphosate varies with bacteria, particularly those residing in the intestine, where microbiota is exposed to glyphosate residues. Therefore, less susceptible pathogenic isolates could have a distinct advantage compared to more sensitive commensal isolates, probably leading to dysbiosis.

To determine whether the ruminal growth and survival of pathogenic *Escherichia coli* or *Salmonella* serovar Typhimurium are higher when glyphosate residues are present in the feed, an *in vitro* fermentation trial with a "Rumen Simulation System" (RUSITEC) and a glyphosate-containing commercial formulation was performed.

Colony forming units of *E. coli* and *Salmonella* ser. Typhimurium decreased steadily in all fermenters, regardless of the herbicide application. Minimum inhibitory concentrations of the studied *Salmonella* and *E. coli* strains did not change, and antibiotic susceptibility varied only slightly but independent of the glyphosate application.

Overall, application of the glyphosate-containing formulation in a worst-case concentration of 10 mg/L neither increased the abundance for the tested *E. coli* and *Salmonella* strain in the *in vitro* fermentation system, nor promoted resistance to glyphosate or antibiotics.

Keywords: glyphosate, roundup, rumen simulation system, RUSITEC, glyphosate resistance, microbial community, fermentation

Introduction

The non-selective herbicide glyphosate (*N*-(phosphonomethyl)glycine) is the active ingredient in the formulation Roundup®. Since the introduction of glyphosate-resistant crops in 1996, it became the most-used plant protection product worldwide [1–3]. Glyphosate disrupts the synthesis of aromatic amino acids by inhibiting the enzyme 5-enolpyruvylshikimic acid-3-phosphate synthase (EPSPS) in the shikimate pathway, which is present in plants and microorganisms but not in humans [4, 5] and was patented as a broad-spectrum antimicrobial [6]. Various glyphosate-containing products have been approved and are currently available on the market. These formulations usually consist of an active ingredient (glyphosate, often as the isopropylamine salt, IPA), a surfactant to enhance physical and chemical properties (e.g., spreading and absorption), and water [7]. After application, the glyphosate IPA salt dissociates, and the free glyphosate acid is transported into the plant, where it becomes active [8].

Intensive use of glyphosate has been associated with increased resistance in plants, while glyphosate residues are routinely detected along the food production chain and in the environment. The herbicide has been detected in soybeans [9–14], maize [15, 16], canola [17], and poultry and cattle feed [18], as well as in urine samples of humans and cows [19–21]. Data regarding the amount of residues vary depending on the time of harvest, particular pesticide regulations in different countries, and the applied formulation. In soybeans, the detected amount of glyphosate ranges from 100 ng/g in seeds or 780 ng/g in leaves up to 450 ng/g or 7790 ng/g, respectively [14]. For maize, a maximum of 40 ng/g in seeds and about 420 ng/g in leaves has been detected, whereby residues on fields with a history of previous glyphosate treatment had higher levels compared to first-treatment fields [16]. In barley and oats, 5.85 mg/kg glyphosate has been measured [13]. Overall, Reuter et al. saw the possibility of crops to accumulate up to 252 mg glyphosate per kg [17], but data about the level of glyphosate residues in prepared livestock feed are sparse. Shehata et al. estimated 0.4–0.9 mg/kg in poultry and cattle feed in Germany [22]. In order to identify how much glyphosate remains in cattle feed after the harvest, Schnabel et al. treated wheat and peas with the formulation Roundup® Record according to the legal European Union (EU) regulations and determined an intake of 73.8 or 84.5 mg glyphosate per cow per day, depending on the proportion of concentrate in the total mixed ration [23]. A small amount of glyphosate is potentially degraded to aminomethylphosphonic acid (AMPA) in the rumen [24].

Considering the shared metabolic pathway in plants and bacteria, which is targeted by glyphosate, it is conceivable that glyphosate may further influence bacterial communities that come in contact with it. Indeed, it has been demonstrated that pathogenic bacteria are likely to be more resistant to glyphosate than commensals [22, 25]. *E. coli* and *Salmonella enterica* are two zoonotic bacterial species commonly found in livestock animals, as well as in meat samples after slaughtering [26]. Transmission of multidrug-resistant bacteria such as

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Table 1. Overview of the strains used to infect the RUSITEC fermentation vessels with the inoculum quantity and the used resistances to detect the isolates on our agar plates. Minimum inhibitory concentration (MIC) was determined for isopropylamine glyphosate in the formulation Roundup LB Plus (RU, registration number 024142-00) with and without pH adjustment with NaOH

Species	MIC RU	MIC RU pH7	Original host	Selectivity resistances	Inoculum
Salmonella ser. Typhimurium	80 mg/mL	80 mg/mL	Pig	Nalidixic acid	8.42E+08 cfu
E. coli	40 mg/mL	80 mg/mL	Cow	Enrofloxacin, cefotaxime	1.25E+09 cfu

extended-spectrum-beta-lactamase (ESBL) producers along the food production chain has attracted a lot of attention in recent years [27]. However, little is known about the effects of glyphosate residues on colonization and/or infection of farm animals with *E. coli* or *Salmonella spp*.

Varying sensitivities to glyphosate are likely to result in bacterial composition shifts in favor of more resistant pathogenic isolates, leading to dysbiosis and a possible loss of protecting opportunistic bacteria [17, 25, 28, 29], along with a potential risk of increased shedding and zoonotic transmission. It has been shown in bees that glyphosate can interfere with gut colonization as well [30].

Sub-lethal glyphosate concentrations could further induce resistances and lead to changing antibiotic susceptibility profiles [31–33], with the possibility of transferring antibiotic resistances between isolates from livestock and humans as another major concern [34]. Our own recent studies showed small but significant increases in minimum inhibitory concentrations (MICs) of glyphosate and a commercial glyphosate-containing formulation in *Salmonella enterica* isolated in recent years in Germany, when compared to historic isolates [35]. Similarly, this was indicative for glyphosate and *E. coli* [36].

Therefore, in the present study, we sought to understand whether the presence of glyphosate residues in feed may give an advantage to pathogenic enteric bacteria in colonization and infection of livestock, particularly cattle. For this, the *in vitro* effects of a glyphosate-containing formulation on growth, survival, and resistance of *E. coli* and *Salmonella* ser. Typhimurium at a worst-case glyphosate concentration [23] were investigated using the "Rumen Simulation Technique" (RUSITEC) [37].

Materials and Methods

The used *in vitro* fermentation system (RUSITEC) was run as described by Riede et al. [37].

RUSITEC Set-up. For inoculation of the RUSITEC fermenter, ruminal content from 3 ruminally fistulated, non-lactating Holstein-friesian cows, fed with 25% grass silage, 25% maize silage, and 50% concentrate, was obtained. The liquid and solid contents were separated by gauze filtration. Six fermentation vessels (V = 700 mL) were filled with the rumen liquid. Seventy grams of solid digesta were inserted into a nylon bag (11.5 m × 6.5 cm, pore size 150 μm). A second nylon bag was filled with 15 g of fresh substrate (49.5% grass silage, 39.7% maize silage, 5% wheat meal, 5% soy cake, and 0.8% mineral feed). Both nylon bags were introduced into each fermentation vessel. On the next day, the bag with the original rumen solid content was replaced with another substrate bag, and the day after that, the former feeding bag was exchanged, leading to a retention time of 48 h for each bag.

The pH and redox potential (mV) were measured daily prior to feeding, as well as the effluent volume. Concentrations of NH_3 and short chain fatty acids (SCFA) were determined at the end of the equilibration period on day 6.

Infection of the Fermenters. After 7 days of equilibration, each fermentation vessel was inoculated with 1 mL of an *E. coli* and a *Salmonella* ser. Typhimurium strain, respectively. Therefore, overnight cultures of the isolates were subcultured in Mueller Hinton I (CM0405 Oxoid Ltd., Hampshire) and grown to a concentration of 10^9 colony forming units (cfu)/mL each to obtain 10^6 cfu/mL in the fermenter (Table 1).

The *E. coli* strain was initially isolated from a lactating cow with acute mastitis and provided by the German Federal Office of Consumer Protection and Food Safety. It is classified as an ESBL-*E. coli* and, among others, resistant to enrofloxacin and cefotaxime. To recover this isolate from the rumen fluid, CHROMagar™ Orientation (Merck KgaA, Darmstadt) supplemented with 4 μg/mL enrofloxacin and 2 μg/mL cefotaxime was thus used. The MIC for Roundup® LB Plus (RU, registration number 024142–00) was 40 mg/mL isopropylamine glyphosate (IPA).

The *Salmonella* Typhimurium DT104 strain used in this study was initially isolated from a pig and was provided by the German Federal Institute for Risk Assessment. Selective XLD media (Oxoid GmbH, Wesel, Germany) was used to re-isolate the strain from the fermenter. The initial MIC for RU was 80 mg/mL IPA.

After inoculation of the strains, 3 out of 6 fermenters (fermenter numbers 2, 4, and 6) were challenged with the common glyphosate-based herbicide RU containing 360 g/L glyphosate (RU), whereas the other fermenters (fermenter numbers 1, 3, and 5) served as controls (CTRL).

Schnabel et al. determined a daily glyphosate intake of up to 84.5 mg per day for lactating dairy cows [23]. Rounding this value to 100 mg per day and taking the rumen content volume (about 100 L) into account, we established a daily glyphosate exposure level of 1 mg/L rumen content. To create a worst-case scenario, RU was added to obtain 10 times of this concentration (10 mg/L) daily.

Strains were enumerated from the rumen fluid by standard dilution plating on respective selective agar plates at different time points after inoculation (0, 0.5, 2, 4, 8, 12, 24, 48, 72, 96, 120, 144, and 168 h). If the strains were no longer quantitatively detectable, rumen samples were enriched overnight in buffered peptone water (DM494D Mast Group Ltd., Merseyside) and streaked out for qualitative analysis on the respective selective agar, as described above.

Susceptibility Testing. Three isolates of each strain from each fermenter and the last sampling time point from which bacteria could be recovered were further assessed for changes in antimicrobial susceptibility relative to the original parent strains. Prior to the fermenter experiments, the initial MICs of RU and RU supplemented with NaOH (to achieve pH7) for these isolates were determined as described previously [35, 36]. In short, serial twofold dilutions of RU in Mueller Hinton broth ranging from 160 mg/mL to 2.5 mg/mL IPA were prepared in conical 96-well plates and stored at −80 °C until use.

For one of the isolates each, antibiotic susceptibility testing via VITEK® system (bioMérieux Deutschland GmbH, Nürtingen, Germany) with the test card VITEK® 2 AST N-248 with common relevant antibiotics (piperacillin, piperacillin–tazobactam, cefotaxime, ceftazidime, cefepime, aztreonam, imipenem, meropenem, amikacin, gentamicin, tobramycin, ciprofloxacin, moxifloxacin, tigecycline, fosfomycin, and trimethoprim/sulfomethoxazole) was further performed.

E. coli isolates were further tested for the presence of beta-lactamase genes $bla_{\text{CTX-M}}$, bla_{SHV}, bla_{TEM} and the CIT-type

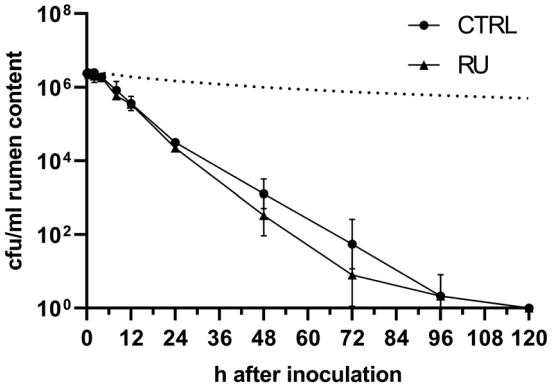

Figure 1. *E. coli* in the fermentation vessels measured by standard dilution plating on CHROMagar supplemented with 4 μg/mL enrofloxacin and 2 μg/mL cefotaxime. Control group (CTRL) without any glyphosate compared to the group treated with a worst-case amount of glyphosate in the formulation Roundup LB Plus (10 mg/L, RU). The dotted line represents the theoretical loss of the *E. coli* due to the wash-out effect of the buffer if bacteria would be in a steady state.

pAmpC genes (blaCMY), following the protocol described by Roschanski et al. [38].

Statistical Analysis. All statistical analyses were performed using IBM® SPSS® Statistics Version 24. All fermenters were compared at each time point individually with a *t*-test. To compare vessels with and without Roundup®, the median of the bacterial counts in each fermenter group was calculated and compared with either a non-parametric Wilcoxon test or a t-test. Further, to determine potential statistical differences in qualitative analysis, a chi-squared test was performed when possible (i.e., where not all results were the same).

Ethics. With the study being *in vitro*, working with an artificial fermentation system in the lab, no ethical approval needed to be obtained. Rumen fluid extraction was executed in accordance with the German Animal Welfare Act approved by the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES, Oldenburg, Germany).

Results

To determine the effects of the glyphosate-containing formulation Roundup® LB Plus on growth and survival of *E. coli* and *Salmonella* ser. Typhimurium, we enumerated bacterial counts of the isolates after inoculation in vitro by means of the "Rumen Simulation Technique" (RUSITEC) and standard dilution plating.

E. coli in the Fermenters. After inoculation of 1.27E+09 cfu *E. coli*, the median starting concentrations in the fermenters were 2.73E+06 cfu/mL in the CTRL group and 3.12E+06 cfu/mL in the vessels, where RU equivalent to 10 mg/L glyphosate was added.

In both groups, the concentration of *E. coli* did not vary significantly within the first 2 h. After 12 h, one logarithm step less was detectable, followed by a steady decline of about one to one and a half logarithm steps each day. At day 4 no more *E. coli* were quantitatively detectable in two out of three fermenters of each group (CTRL and RU). Qualitatively *E. coli* was still present in 5 out of 6 fermenter vessels on day 4 but not anymore on day 5. An overview of the cfu/ml rumen content can be found in Figure 1.

***Salmonella* Ser. Typhimurium in the Fermenters.** In addition to *E. coli*, vessels were simultaneously co-inoculated with 1.02E+09 cfu of the *Salmonella* ser. Typhimurium strain. Initial median starting concentrations were 1.50E+06 cfu/mL in the CTRL and 1.43E+06 cfu/mL in the RU group. After 30 min in both groups, the bacterial counts declined slightly followed by an increase after 2 and 4 h, where approximately the double amount of *Salmonella* compared to the starting concentrations could be detected (3.24E+06 cfu/mL after 2 h in the RU treated group and 3.22E+06 cfu/mL after 4 h in the CTRL group). This was followed by a steady decline in both groups (Figure 2). At the end of the experiment after 7 days, only 10 cfu/mL in the CTRL and 90 cfu/mL in the RU group were still present.

Comparison of the Treated and Non-treated Fermenters. Comparing the median from the control and the worst-case group, no statistically significant differences could be found in *Salmonella* ser. Typhimurium ($P = 0.753$) and *E. coli* ($P = 0.678$) using Wilcoxon-test analysis or $P = 0.967$ and $P = 0.825$ using a *t*-test, respectively. More detailed statistical comparisons of all vessels at each sampling point are presented in Table 2.

Ruminal metabolism in the system was checked via pH and redox potential measurement (Table 3). Values were constant during the experiment in all fermentation vessels. SCFA and NH_3 have been checked after adaptation of the ruminal system and before the start of the experiment to ensure proper ruminal settings (data not shown).

Susceptibility Testing. MIC measurements were carried out for 3 isolates of each strain and fermenter from the last sampling point, which displayed bacterial growth. For *E. coli*, isolates recovered at day 2 from fermenters 2, 4, and 5 and at day 3 from the fermenters 1, 3, and 6 were investigated. *Salmonella* Typhimurium isolates were examined after 5 days for all fermenters. The MIC values for RU did not change compared to the ancestor (Table 4).

Further, for one isolate of each strain and fermenter, antibiotic susceptibility testing by VITEK® was performed. Individual strains differed in MIC for single antibiotics compared to the ancestor (Table 5). Differences were, in general, in the dimension of 1 or 2 dilution steps except for *E. coli* in cefepime, where ancestor showed a MIC of ≥64 μg/mL, and the isolates from Fermenter 1, 4, and 5, a MIC of 4 μg/mL.

In addition, the *E. coli* isolates were tested for ESBL genes using multiplex real-time polymerase chain reaction (PCR). Isolates from all fermenters as well as the ancestor were positive for CTX and negative for SHV, TEM, and AmpC (data not shown).

Discussion

In recent years, glyphosate residues have been detected in plants that are commonly used as animal feed, especially in soy [9–13], in farm animal feed [18], and in animals themselves [20, 39]. Therefore, intestinal bacteria of livestock are exposed to these residues, whereby in general, pathogenic bacteria seem to be more resistant to glyphosate than commensals [22], leading to dysbiosis with corresponding effects on health [25, 28, 40].

This study thus aimed to determine possible effects or advantages of glyphosate residues on growth and survival for *E. coli* and *Salmonella* ser. Typhimurium isolates *in vitro* by means of the Rumen Simulation System (RUSITEC).

The Number of inoculated *E. coli* decreased steadily in all fermenters until after 120 h, where no quantitative or qualitative detection was anymore possible on the selective agar plates. No difference has been detected between the CTRL and the RU group, neither quantitatively nor qualitatively.

In an artificial rumen experiment inoculated with sheep content by Bach et al., the amount of *E. coli* O157:H7 similarly decreased over time [41]. After 120 h, no quantitative detection was possible. Qualitative analyses were negative,

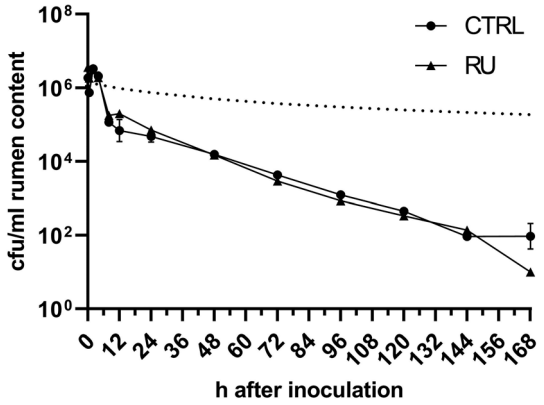

Figure 2. *Salmonella* ser. Typhimurium in the fermentation vessels measured by standard dilution plating XLD agar. Control group (CTRL) without any glyphosate compared to the group treated with a worst-case amount of glyphosate in the formulation Roundup LB Plus (10 mg/L, RU). The dotted line represents the theoretical loss of the *Salmonella* due to the wash-out effect of the buffer if bacteria would be in a steady state.

168 h after inoculation, respectively. The decline in the amount of *E. coli* is slightly slower but comparable to the results in this study, indicating a normal process for an *in vitro* ruminal setting.

With a first small initial drop and a following short peak, the amount of *Salmonella* ser. Typhimurium in the fermenters declined as well. In contrast to *E. coli*, Salmonella was quantitatively detectable until the end of the experiment on day 7. However, only a few bacteria survived regardless of the RU treatment.

As seen in an *in vivo* study by Brownlie and Grau, elimination of different *Salmonella spp*. in the rumen is common [42]. Twenty-four hours after inoculation, the bacteria were no more detectable, when cows were fed normally. The numbers of *Salmonella spp.* remained the same or increased only when the daily feed intake was reduced. The following starvation was accompanied by a decreased amount of volatile fatty acids and an increased pH. Although the amount of *Salmonella* in the *in vivo* study from Brownlie and Grau decreased faster compared to our *in vitro* results, the trend is comparable.

The addition of RU did not cause changes in basic rumen fermentation parameters (pH and redox potential), in agreement with other studies [37, 43].

Bacterial exposure to glyphosate or similar biocides is known to facilitate emergence of resistance against the agents themselves [33, 44–48]. Furthermore, a shift in antibiotic susceptibility can be associated with sub-inhibitory concentrations of glyphosate [31, 32] or biocides [33, 46, 49, 50]. Most adaptions are based on non-specific mechanisms, such as an increase in efflux pump activity [32, 44, 49, 50]. To test the possibility of increased resistance following the exposure to RU, strains from the last time point with detectable bacterial growth in each fermenter have been tested for changes in their MIC for RU using broth microdilution and a panel of antibiotics using VITEK®.

Even though some authors suggest that exposure to glyphosate can lead to increased expression of efflux pumps [32, 44], all tested strains did not vary in MIC for RU compared to their ancestral strain. This corroborates the results of an evolutionary mutagenesis study of Tincher et al., in which an *E. coli* K-12 wild-type and mutant strain had been exposed to the formulation Roundup® concentrate Plus for longer terms without detecting any mutagenesis [51]. Considering the MIC of 40 mg IPA per mL for *E. coli* or 80 mg IPA per mL for *Salmonella* ser. Typhimurium, respectively, the used strains require a large amount of active ingredient to be overcome until a change in MIC via broth microdilution is visibly detectable. Additionally, the worst-case glyphosate dosage of 10 mg/L is substantially lower than the MIC of the inoculated strains. It is therefore possible that isolates were not challenged enough to adapt.

However, regarding antibiotic susceptibility, few changes could be found by VITEK® analysis. Most of the changes seemed negligible, having been only within the range of 1 dilution step for *Salmonella* ser. Typhimurium or 2 dilution steps for *E. coli*, respectively. The sole exception was the susceptibility against the fourth-generation cephalosporin cefepime in *E. coli*, where in the tested isolates of fermenter 1 (CTRL), 4 (RU), and 5 (CTRL), the MIC decreased within 4 dilution steps.

The influence of glyphosate-based herbicides on antibiotic susceptibility is supported by Kurenbach et al. [31], who

Table 2. Statistical analysis of the differences between the control vessels and the vessels with 10 mg/L Roundup as a worst-case scenario for each sampling point quantitatively with the *t*-test. Further, a qualitative analysis with a chi-squared test for *E. coli* was performed (x: incalculable, because all fermenters are equal). No significant difference between the groups at any sampling point

Time point		*t*-test		Chi-squared test
		E. coli	*Salmonella* ser. Typhimurium	*E. coli*
P0	Inoculation	$P = 0.244$	$P = 0.855$	x
P1	0,5 h	$P = 0.558$	$P = 0.503$	x
P2	2 h	$P = 0.456$	$P = 0.309$	x
P3	4 h	$P = 0.706$	$P = 0.970$	x
P4	8 h	$P = 0.275$	$P = 0.540$	x
P5	12 h	$P = 0.687$	$P = 0.539$	x
P6	24 h	$P = 0.151$	$P = 0.792$	x
P7	48 h	$P = 0.178$	$P = 0.339$	x
P8	72 h	$P = 0.257$	$P = 0.355$	$P = 0.273$
P9	96 h	$P = 1.000$	$P = 0.534$	$P = 0.273$
P10	120 h	–	$P = 1.000$	x
P11	144 h	–	$P = 0.729$	$P = 0.273$
P12	168 h	–	$P = 0.163$	x

Table 3. Control of ruminal metabolism. Means of the treated (RU) and non-treated (CTRL) vessels on each day of the experiment

Days after inoculation	CTRL		RU	
	pH	Redox potential (mV)	pH	Redox potential (mV)
0	6.66	−273	6.65	−274
1	6.70	−281	6.64	−279
2	6.66	−261	6.68	−278
3	6.67	−277	6.69	−282
4	6.69	−279	6.74	−281
5	6.71	−281	6.70	−272
6	6.66	−264	6.67	−282
7	6.67	−265	6.63	−264
Mean	6.68	−273	6.68	−276
	6.69 ± 0.025	271 ± 10	6.68 ± 0.055	273 ± 9

Table 4. Minimum inhibitory concentrations (MIC) of isolated bacteria at the time point of the experiment with still solid growth on agar plates in comparison to the ancestral strain. MIC for IPA was tested in Roundup (RU) and RU adjusted to pH 7 (RU pH 7) (F: fermentation vessel)

F	RU	*E. coli*				*Salmonella* ser. Typhimurium			
		Sample number	Day	MIC RU (mg/mL)	MIC RU pH 7 (mg/mL)	Sample number	Day	MIC RU (mg/mL)	MIC RU pH 7 (mg/mL)
1	−	P8	3	40	80	P10	5	80	80
2	+	P7	2	40	80	P10	5	80	80
3	−	P8	3	40	80	P10	5	80	80
4	+	P7	2	40	80	P10	5	80	80
5	−	P7	2	40	80	P10	5	80	80
6	+	P8	3	40	80	P10	5	80	80
Ancestor				40	80			80	80

Table 5. Minimum inhibitory concentrations in μg/mL tested with the VITEK® system and the test card AST N-248 with common relevant antibiotics. Shown in bold are the differences compared to the ancestor strain (R: resistant; S = susceptible)

	Ceftazidime	Cefepime	Aztreonam
E. coli Ancestor	16 R	≥ 64	16 R
E. coli Fermenter 1	16 R	**4**	**≥ 64 R**
E. coli Fermenter 4[a]	**4 S**	**4**	**≥ 64 R**
E. coli Fermenter 5	16 R	**4**	16 R
	Piperacillin/Tazobactam	Moxifloxacin	
Salmonella ser. Typhimurium Ancestor	8 S	0.5 S	
Salmonella ser. Typhimurium Fermenter 1	**≤ 4 S**	**1 R**	
Salmonella ser. Typhimurium Fermenter 2[a]	**≤ 4 S**	0.5 S	
Salmonella ser. Typhimurium Fermenter 3	**≤ 4 S**	0.5 S	

[a]Fermenter belonging to the RU treated group.

measured enhanced and decreased tolerances for different antibiotics after exposure to Roundup® weed killer in an *in vitro* experiment with single cultures. In their study, however, the *Salmonella* strain used was less susceptible to ampicillin, ciprofloxacin, and kanamycin and more susceptible to chloramphenicol and tetracycline. Similarly, changes in antibiotic susceptibility in bacteria have been found after biocide exposure. Molina-González et al. identified differences in susceptibility testing for antibiotics, depending on the *Salmonella* strain and the substance [49]. Likewise, an adaptation to biocides can be accompanied by a resistance to some antibiotics in *E. coli* [33]. An increase in resistance is detected in most cases. In contrast to these findings, there are also reports showing no change in antibiotic susceptibility after biocide exposure [47, 48, 50]. With conditions similar to our study, Karatzas et al. exposed *Salmonella* ser. Typhimurium as well to steady sub-inhibitory biocide concentrations for a week with no effect on antibiotic susceptibility. Only when the biocide concentration was increased gradually, a change in susceptibility for some antibiotics could be observed [50]. Condell et al. examined 189 *Salmonella enterica* strains with 7 commercially available biocides, observing an impact on the tolerance against the active compounds of the biocides but not against complex formulations or different antibiotics [47]. Likewise, this has been shown for other enteric bacteria such as *E. coli* [48].

Considering the accumulated evidence in the literature, resistances against a biocide or a herbicide such as glyphosate are often but not always accompanied by a change in antimicrobial susceptibility. As indicated by Wales and Davies, controlled laboratory studies may not be the most suitable way to draw conclusions for biocides and microorganism interactions [52]. Nonetheless, using the RUSITEC fermentation system provided more realistic conditions than sole laboratory *in vitro* studies. No adaptive resistance mechanisms leading to increased MIC for RU, and only slight changes in antibiotic susceptibility have been observed. Notably, the tolerance variations in the latter were equally measured in control and RU fermenters, regardless of the added herbicide.

Overall, no benefits for growth and survival of the tested pathogenic *E. coli* and *Salmonella* ser. Typhimurium strains with a worst-case glyphosate concentration of 10 mg/L present in the formulation Roundup® LB Plus could be detected in the *in vitro* rumen simulation system. Bacterial counts decreased equally in all fermenters. The MIC against RU did not change and antibiotic susceptibility only changed slightly for some antibiotics and strains regardless of glyphosate exposure.

Considering that there are various glyphosate-containing formulations on the market available worldwide, our findings are restricted to our experimental setup, where complete formulation Roundup® LB Plus and specific *E. coli* and *Salmonella* ser. Typhimurium isolates were used. We demonstrated that the worst-case concentration of Roundup has no effect on the pathogenic Enterobacteriaceae under our experimental conditions within a RUSITEC system. It therefore remains to be shown whether other formulations or pure glyphosate would influence the bacterial community in a fermenter model or in monogastric animals *in vivo*.

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Authors' Contributions

K.B. performed the experiments, collected, analyzed and interpreted the data, and drafted the manuscript and figures, with critical evaluation and support of all other authors. J.P. performed the experiments and collected the data. S.R. helped in designing the experiment and gave advice during the whole execution. U.R. and G.B. conceived and designed the study and critically revised the manuscript. All authors approved the final version to be published.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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