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

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Received: 13 May 2019; accepted: 28 May 2019

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-enolpyruvyl-shikimic 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...
Effect of Glyphosate on Pathogens in the RUSITEC

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.

<table>
<thead>
<tr>
<th>Species</th>
<th>MIC RU</th>
<th>MIC RU pH7</th>
<th>Original host</th>
<th>Selectivity resistances</th>
<th>Inoculum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salmonella ser. Typhimurium</td>
<td>80 mg/mL</td>
<td>80 mg/mL</td>
<td>Pig</td>
<td>Nalidixic acid</td>
<td>8.4E+08 cfu</td>
</tr>
<tr>
<td>E. coli</td>
<td>40 mg/mL</td>
<td>80 mg/mL</td>
<td>Cow</td>
<td>Enrofloxacin, cefotaxime</td>
<td>1.25E+09 cfu</td>
</tr>
</tbody>
</table>

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 genic isolates, leading to dysbiosis and a possible loss of protective function of the gut microbiome 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 NH₃ 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 10⁸ colony forming units (cfu)/mL each to obtain 10⁹ 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 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 (bioMeérieux Deutschland GmbH, Nürtingen, Germany) with the test card VITEK® 2 AST N-248 with common relevant antibiotics (pipercillin, pipercillin−tazobactam, cefotaxime, cefazidime, cefepime, aztreonam, imipenem, meropenem, amikacin, gentamicin, tobramycin, ciprofloxacin, moxifloxacin, tigecycline, fosfomycin, and trimethoprim/sulfamethoxazole) was further performed.

*E. coli* isolates were further tested for the presence of beta-lactamase genes *blaCTX-M*, *blaSHV*, *blaTEM* and the CIT-type gene.
Initial median starting concentrations were 1.50E+06 cfu/mL with 1.02E+09 cfu of the fermenters of each group (CTRL and RU). Qualitatively E. coli addition to but not anymore on day 5. An overview of the cfu/ml rumen was still present in 5 out of 6 fermenter vessels on day 4 coli more one to one and a half logarithm steps each day. At day 4 no step less was detectable, followed by a steady decline of about significantly within the first 2 h. After 12 h, one logarithm reduction of the inoculated numbers 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 = 0.875) and E. coli (P = 0.767) 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 NH3 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,
known to facilitate emergence of resistance against the agents

ment with other studies [37, 43].

accompanied by a decreased amount of volatile fatty acids and
daily feed intake was reduced. The following starvation was
remained the same or increased only when the
monella spp.
detectable, when cows were fed normally. The numbers of
Twenty-four hours after inoculation, the bacteria were no more
Salmonella (10 mg/L, RU). The dotted line represents the theoretical loss of the
E. coli

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 fermentation vessels
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

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

As seen in an in vivo study by Brownlie and Grau, elimina-
tion 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 Sal-
monella 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
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pared to our in vitro results, the trend is comparable.

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fermentation parameters (pH and redox potential), in agree-
ment with other studies [37, 43].

Bacterial exposure to glyphosate or similar biocides is
known to facilitate emergence of resistance against the agents

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

<table>
<thead>
<tr>
<th>Time point</th>
<th>t-test</th>
<th>Chi-squared test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incoculation P0</td>
<td>P = 0.244</td>
<td>P = 0.855</td>
</tr>
<tr>
<td>P1 0.5 h</td>
<td>P = 0.558</td>
<td>P = 0.503</td>
</tr>
<tr>
<td>P2 2 h</td>
<td>P = 0.456</td>
<td>P = 0.309</td>
</tr>
<tr>
<td>P3 4 h</td>
<td>P = 0.706</td>
<td>P = 0.970</td>
</tr>
<tr>
<td>P4 8 h</td>
<td>P = 0.275</td>
<td>P = 0.540</td>
</tr>
<tr>
<td>P5 12 h</td>
<td>P = 0.687</td>
<td>P = 0.539</td>
</tr>
<tr>
<td>P6 24 h</td>
<td>P = 0.151</td>
<td>P = 0.792</td>
</tr>
<tr>
<td>P7 48 h</td>
<td>P = 0.178</td>
<td>P = 0.339</td>
</tr>
<tr>
<td>P8 72 h</td>
<td>P = 0.257</td>
<td>P = 0.355</td>
</tr>
<tr>
<td>P9 96 h</td>
<td>P = 1.000</td>
<td>P = 0.534</td>
</tr>
<tr>
<td>P10 120 h</td>
<td>–</td>
<td>P = 1.000</td>
</tr>
<tr>
<td>P11 144 h</td>
<td>–</td>
<td>P = 0.729</td>
</tr>
<tr>
<td>P12 168 h</td>
<td>–</td>
<td>P = 0.163</td>
</tr>
</tbody>
</table>

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

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

days after inoculation. The 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 sus-
ceptibility can be associated with sub-inhibitory concentrations
of glyphosate [31, 32] or biocides [33, 46, 49, 50]. Most adap-
tions are based on non-specific mechanisms, such as an in-
crease in efflux pump activity [32, 44, 49, 50]. To test the pos-
sibility 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 antibi-
otics using VITEK™.

Even though some authors suggest that exposure to glypho-
sate 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 evolu-
tionary 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 with-
out detecting any mutagenesis [51]. Considering the MIC of
40 mg IPA per mL for E. coli or 80 mg IPA per mL for Sam-
monella ser. Typhimurium, respectively, the used strains re-
suire 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 dilu-
tion 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 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)

<table>
<thead>
<tr>
<th>Sample DayMIC</th>
<th>RUMIC RU pH 7 Sample DayMIC RUMIC RU pH 7</th>
<th>E. coli</th>
<th>Salmonella ser. Typhimurium</th>
</tr>
</thead>
<tbody>
<tr>
<td>F RU</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample number</td>
<td>(mg/mL)</td>
<td>(mg/mL)</td>
<td>number</td>
</tr>
<tr>
<td>1 – P8 3 40 80 80</td>
<td>P10 5 80 80</td>
<td>E. coli</td>
<td>Salmonella ser. Typhimurium</td>
</tr>
<tr>
<td>2 + P7 2 40 80 80</td>
<td>P10 5 80 80</td>
<td>E. coli</td>
<td>Salmonella ser. Typhimurium</td>
</tr>
<tr>
<td>3 – P8 3 40 80 80</td>
<td>P10 5 80 80</td>
<td>E. coli</td>
<td>Salmonella ser. Typhimurium</td>
</tr>
<tr>
<td>4 + P7 2 40 80 80</td>
<td>P10 5 80 80</td>
<td>E. coli</td>
<td>Salmonella ser. Typhimurium</td>
</tr>
<tr>
<td>5 – P7 2 40 80 80</td>
<td>P10 5 80 80</td>
<td>E. coli</td>
<td>Salmonella ser. Typhimurium</td>
</tr>
<tr>
<td>6 + P8 3 40 80 80</td>
<td>P10 5 80 80</td>
<td>E. coli</td>
<td>Salmonella ser. Typhimurium</td>
</tr>
<tr>
<td>Ancestor</td>
<td>40 80 80 80</td>
<td>E. coli</td>
<td>Salmonella ser. Typhimurium</td>
</tr>
</tbody>
</table>
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, Karatas et al. exposed Salmonella ser. Typhimurium as well as E. coli. 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 biocide.

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.

### Funding Sources

The project was supported by funds of the Federal Ministry of Food and Agriculture (BMEL) based on a decision of the Parliament of the Federal Republic of Germany via the Federal Office for Agriculture and Food (BLE) (Grant number: 314–06.01-2815HS015).

We acknowledge support by the German Research Foundation and the Open Access Publication Fund of the Freie Universität Berlin.

### Authors' Contributions

K.B. performed the experiments, collected, analyzed and interpreted the data, and drafted the manuscript. U.R. and G.B. conceived and designed the study 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.

### Acknowledgments

We gratefully acknowledge Dr. Olga Makarova of Freie Universität Berlin for her valuable comments on experimental design and the manuscript. We would like to further thank the colleagues at the Institute for Animal Hygiene, Animal Welfare and Farm Animal Behaviour, the University of Veterinary Medicine Hannover for excellent technical support, and the Institute of Animal Nutrition at the Federal Research Institute for Animal Health as well as Dr. Dirk von Soosten for providing ruminal content and support. We acknowledge the Federal Office of Consumer Protection and Food Safety for providing the E. coli isolate and the Federal Institute for Risk Assessment for providing the Salmonella ser. Typhimurium isolate for the study.

### References


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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).

<table>
<thead>
<tr>
<th></th>
<th>Ceftazidime</th>
<th>Cefepime</th>
<th>Aztreonam</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli Ancestor</td>
<td>16 R</td>
<td>≥ 64</td>
<td>16 R</td>
</tr>
<tr>
<td>E. coli Fermenter 1</td>
<td>16 R</td>
<td>4</td>
<td>≥ 64 R</td>
</tr>
<tr>
<td>E. coli Fermenter 4*</td>
<td>4 S</td>
<td>4</td>
<td>≥ 64 R</td>
</tr>
<tr>
<td>E. coli Fermenter 5</td>
<td>16 R</td>
<td>4</td>
<td>16 R</td>
</tr>
</tbody>
</table>

*Fermenter belonging to the RU treated group.

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* | ≤ 4 S | 0.5 S
Salmonella ser. Typhimurium Fermenter 3 | ≤ 4 S | 0.5 S

≤ 64 R

≥ 64 S

≤ 64 S

≥ 64 S
Effect of Glyphosate on Pathogens in the RUSITEC


