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The Impact of Glyphosate on *Escherichia coli* and Bacterial Communities *in vitro* and *in vivo*

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Katrin Bote

Tierärztin

aus Völklingen

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Dekan:	Univ.-Prof. Dr. Jürgen Zentek
Erster Gutachter:	Univ.-Prof. Dr. Uwe Rösler
Zweiter Gutachter:	Univ.-Prof. Dr. Thomas Amon
Dritte Gutachterin:	PD Dr. Annemarie Käsbohrer

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ABBREVIATIONS

AI	Active Ingredient
AMPA	Aminomethylphosphonic acid
AST	Antimicrobial Susceptibility Testing
BfR	German Federal Institute for Risk Assessment
BVL	German Federal Office of Consumer Protection and Food Safety
CFU	Colony Forming Units
CLSI	Clinical and Laboratory Standards Institute
CTRL	Control group
DSMZ	German Collection of Microorganisms and Cell Cultures GmbH
E. coli	*Escherichia coli*
EC	European Commission
ECOR	Standard *E. coli* collection of reference
EFSA	European Food Safety Authority
EPSP	5-enolpyruvylshikimate-3-phosphate
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
ESBL	Extended-spectrum beta-lactamase
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GBHs	Glyphosate-based herbicides
GLY	Glyphosate
GR	Glyphosate-resistant
IARC	International Agency for Research on Cancer
IPA	Isopropylamine salt of glyphosate
MC	MacConkey

MH	Mueller Hinton
MIC	Minimum Inhibitory Concentration
MRL	Maximum Residue Limit
MSRV	Modified semi-solid Rappaport-Vassiliadis
NaOH	Sodium hydroxide
OD600	Optical density at 600nm
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reduction
PEP	Phosphoenolpyruvate
POEA	Polyethoxylated tallow amine
rRNA	Ribosomal ribonucleic acid
RU	Roundup® LB Plus
RUSITEC	Rumen Simulation Technique
S. Typhimurium	*Salmonella enterica* subsp. *enterica* ser. Typhimurium
SCFA	Short-chain fatty acids
SNPs	Single Nucleotide Polymorphisms
WT	Wild-type
XLD	Xylose Lysine Deoxycholate

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INTRODUCTION

1.1 The Herbicide Glyphosate

1.1.1 Introduction to Glyphosate

The chemical compound N-(phosphonomethyl)glycine, commonly known as glyphosate (GLY), was first synthesized by the Suisse chemist Henri Martin in 1950 without further pharmaceutical use. Its herbicidal use was only discovered twenty years later and ultimately patented by the company Monsanto in 1974 (Duke and Powles, 2008). From a chemical point of view, GLY is a small and simple molecule (Figure 1.1).

Figure 1.1: *Structural chemical formula of the herbicide glyphosate.*

Compared to other herbicides used around the time of its market introduction, agricultural usage was very simple, cost-saving, and allowed good and easy crop management (Benbrook, 2016; Duke and Powles, 2009). Moreover, GLY was less toxic than other comparable herbicides, and therefore advertised as environmentally friendly and safe (Duke and Powles, 2008, 2009). From its market launch until 2014, glyphosate usage has increased exponentially (Figure 1.2). Today, it is by far the most-used herbicide worldwide.

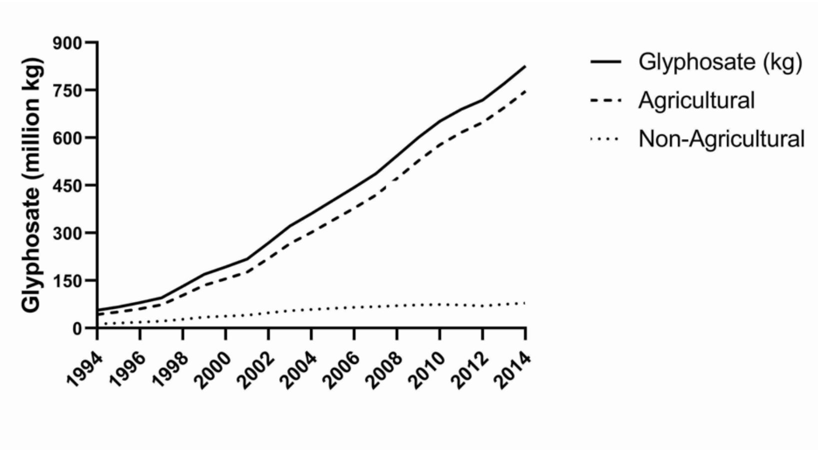

Figure 1.2: *Total volume of glyphosate used worldwide for agricultural and non-agricultural purposes in million kg per year between 1994 and 2014, adapted from Benbrook (2016).*

GLY's commercial success is closely linked to the introduction of genetically modified glyphosate-resistant (GR) plants by Monsanto in 1996, known as "Roundup Ready" (Duke and Powles, 2009). Most genetically modified GR plants possess an altered enzyme version, resulting in a changed enzyme-substrate-complex configuration more tolerant to the binding of GLY. Soybean, canola, maize and cotton were the first generated resistant plants. Sugar beets and a few others followed soon after.

However, the safety and extensive worldwide use of GLY use have been called into question during the past decades. Multiple reports show toxicity for freshwater communities (Moreno et al., 2014; Tsui and Chu, 2003; Vera et al., 2012), soil bacteria (García-Pérez et al., 2014; Lancaster et al., 2010) and other organisms (Krüger et al., 2014b; Poletta et al., 2009). Furthermore, the International Agency for Research on Cancer (IARC) classified it as "probably carcinogenic to humans" in 2015 (IARC, 2015).

1.1.2 Mode of Action

GLY acts as a non-selective competitive inhibitor (Alibhai et al., 2001), targeting an enzyme in the metabolic shikimate pathway that is responsible for the biosynthesis of aromatic amino acids (phenylalanine, tyrosine and tryptophan) and secondary plant compounds (Figure 1.3). This pathway is generally used by plants, microorganisms and some parasites, but not in animals or humans (Herrmann, 1995; Herrmann and Weaver, 1999; Roberts et al., 2002).

After foliar application, the uptake of the active ingredient (AI) proceeds almost entirely through the treated leaves (EFSA, 2015b). The glyphosate salt dissociates and the free acid translocates into the plant. Inside the chloroplasts, it inhibits the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), the sixth enzyme of the shikimate pathway, which catalyses the reaction of shikimate-3-phosphate and phosphoenolpyruvate (PEP) to 5-enolpyrovyl-3-shikimate phosphate (EPSP) (Figure 1.3) (Schönbrunn et al., 2001; Steinrücken and Amrhein, 1980). Glyphosate binds to the enzyme-substrate complex in competition to PEP and not to the enzyme itself. Thus, it does not alter other reactions involving PEP (Alibhai et al., 2001).

1.1.3 Active Ingredient vs. Commercial Formulation

Commercially available glyphosate-based herbicides (GBHs) are better known under the tradename Roundup® , Monsanto's bestselling herbicide. While there are 110 approved GBHs on the German market (July 2020), Roundup® LB Plus (registration number 024142-00) remains to be one of the best-selling products.

Due to the poor solubility of the pure substance, GBHs active ingredient GLY is used as a salt, most commonly as an isopropylamine or ammonium salt, and enhanced through additional components.

A GBH formulation usually consists of the GLY isopropylamine salt (IPA), a surfactant (15% or less) and water (Giesy et al., 2000; Mesnage et al., 2015). However, detailed compositions of the complex mixtures remain confidential as distributors are not required to declare all ingredients.

Controversially, only the AI is tested during the approval process of a new herbicidal formulation. Toxicity of added surfactants and adjuvants are not considered, even though they have the potential to enhance toxic effects and cause damage themselves (Benbrook, 2018; Clair et al., 2012; Mesnage et al., 2014). Consequently, it has been proposed to assess complete formulations during authorization processes (Cox and Surgan, 2006; Nagy et al. 2020).

Polyethoxylated tallow amine (POEA) is a surfactant that has often been mentioned in connection with GBHs (Giesy et al., 2000; Tush and Meyer, 2016). It is an example of an added substance considered to be more harmful than GLY itself (Tsui and Chu, 2003). Due to its carcinogenic potential, the use of GBHs with POEA was banned in Germany in 2014 (German Federal Government, Antwort der Bundesregierung – Drucksache 18/7232) and throughout the European Union in 2016 (Commission Implementing Regulation (EU) 2016/1313). Nevertheless, other additives and surfactants used in GBHs remain unknown to the public.

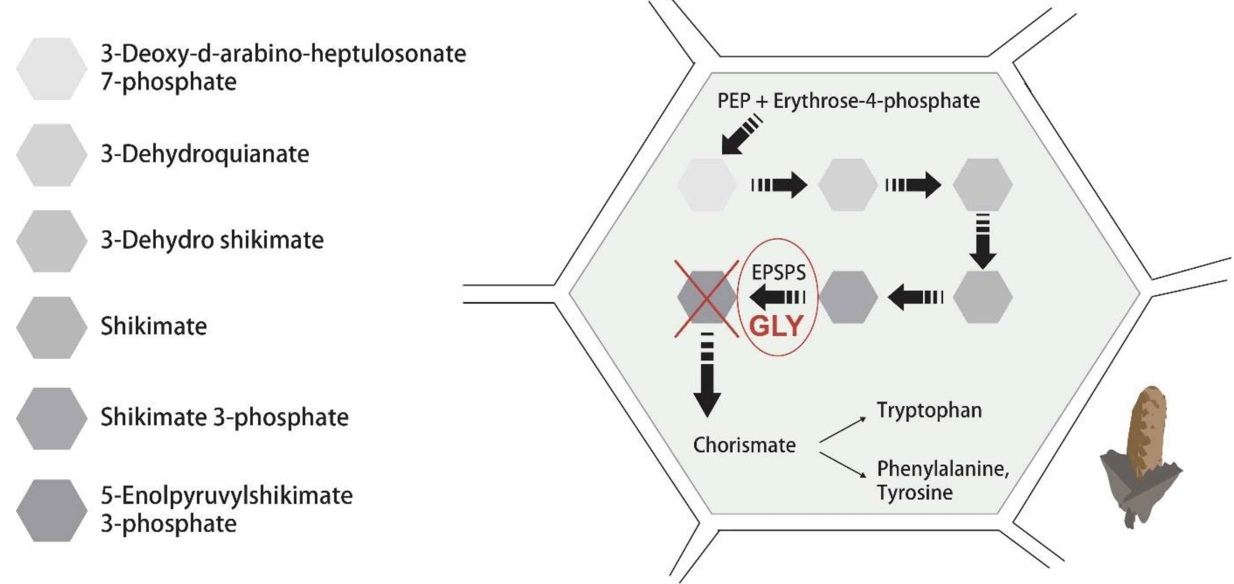

Figure 1.3*: Schematic illustration of the shikimate pathway in a glyphosate-sensitive plant. PEP = phosphoenolpyruvate, EPSPS = 5-enolpyruvylshikimate-3-phosphate synthase, GLY = glyphosate*

1.1.4 Non-Agricultural Use of Glyphosate

Considering glyphosate's specific mode of action, targeting an enzyme not present in humans, it is not surprising that the herbicide has also been considered as a new structure for drug discovery (Alibhai et al., 2001). Accordingly, it has also been patented as an antimicrobial and biocide (Abraham, 2003). GLY has been explored as potential treatment of unicellular parasites, such as *Plasmodium*

falciparum, *Toxoplasma gondii* and *Cryptosporidium parvum* (Coggins et al., 2003; Roberts et al., 2002). Moreover, as the shikimate pathway is essential to *Mycobacterium tuberculosis*, it was considered to potentially open doors to new tuberculosis treatment (Parish and Stoker, 2002).

1.2 Effects of Glyphosate on Bacteria

1.2.1 Bacterial Response to Glyphosate and Mechanisms of Resistance

Much like plants, bacteria also rely on the shikimate pathway to produce aromatic amino acids, potentially making them susceptible to GLY. Biosynthesis information for chorismate, the final product of the shikimate pathway, is stored in the *aro* section of the bacterial genome. More specifically, the EPSPS enzyme is located on the *aroA* gene (O'Connell et al., 1993).

To date, three bacterial EPSPS types are known: Class I EPSPS is the most common variant and considered to be naturally sensitive to GLY. Class II EPSPS can be isolated from some gram-positive bacteria, such as *Staphylococcus aureus*, and seems to be more GLY resistant than class I EPSPS (Funke et al., 2009). Furthermore, Fei et al. (2013) determined a novel EPSPS variant in an *Enterobacter* isolated from soil samples, which has been classified as class III. In addition, an EPSPS variant with less than 30% resemblance to the classes I and II has been discovered in a *Pseudomonas putida* strain (Sun et al., 2005).

Aside from enzyme variants, some bacteria show additional forms of GLY resistance. Cyanobacteria seem to be naturally tolerant to GLY (Powell et al., 1991). Moreover, some bacterial species, such as certain *Pseudomonas* and *Enterobacter* strains, can utilize GLY as a source of nitrogen, carbon or phosphorus (Fei et al., 2013; Schulz et al., 1985).

Similar to antimicrobials, exposure to sublethal GLY concentrations can lead to adaptation and resistance against the substance (Meyer and Cookson, 2010), which can be conferred by different mechanisms:

Overexpression of the target: Upregulation of the *aroA* gene leads to an increased amount of EPSPS, compensating the inhibiting effects of GLY (Wicke et al., 2019).

Target-site mutation: Mutations in the *aroA* gene due to amino acid substitutions can change the configuration of the EPSPS enzyme and obstruct the binding of GLY, but not of the shorter PEP (Table 1.1). Different bacteria have been utilized to produce resistant variants of the EPSPS in the effort to create genetically modified plants (Padgette et al., 1991; Stalker et al., 1985), whereby the EPSPS of *Agrobacterium tumefaciens* CP4 is the most frequently used enzyme in GR crops (Funke et al., 2006).

Chemical modification: Degradation of GLY molecules can prevent lethal effects. Bacterial transferases such as glyphosate-N-acetyltransferase (GAT) (Castle et al., 2004), oxidoreductases like glyphosate oxidoreductase (GOX) (Hadi et al., 2013) and glycine oxidase (GO) (Zhan et al., 2013) have been described in this context.

Exclusion of the target: Reduced entry or increased exit of GLY minimizes its effect. Increased amounts of efflux transporters, which lower intracellular concentrations after cell entry, have been observed (Kurenbach et al., 2017; Staub et al., 2012). Furthermore, deactivation of the *Bacillus subtilis* genes, encoding for sodium-coupled glutamate/aspartate symporter GltT and proton/glutamate symporter GltP, led to a higher tolerance for GLY. Thus, it was concluded that these transporters are major cell entry points in *Bacillus subtilis* (Wicke et al., 2019).

***Table 1.1**: Changes in the amino acid sequence of the 5-enolpyrovyl-3-shikimate phosphate synthase (EPSPS) conferring glyphosate resistances as described in the literature. Thr= Threonine, Met= Methionine, Gly=Glycine, Ala= Alanine, Ile= Isoleucine, Pro= Proline, Ser= Serine*

From	At position	To	Reference
Thr	42	Met	He et al., 2003
Gly	96	Ala	Padgette et al., 1991; Eschenburg et al., 2002
Thr	97	Ile	Funke et al., 2009 (double mutation with P101S)
Pro	101	Ser	Baerson et al., 2002; Padgette et al., 1991; Funke et al., 2009
Thr	179	Ala	Fei et al., 2013

GLY resistance can further be accompanied by co-selection of antibiotic resistances (Kurenbach et al., 2015; Wales and Davies, 2015), either as cross-resistance or co-resistance. Cross-resistance mechanisms, such as overexpression of efflux pumps or decreased cell permeability, are effective against various substances. Resistance mechanisms that are selected together, e.g. because they are linked genetically or placed on the same plasmid, are known as co-resistance. Multiple reports have shown that an overexpression of efflux pumps after GLY exposure can lead to changed antibiotic susceptibility profiles (Kurenbach et al., 2017; Staub et al., 2012).

1.2.2 Effects of Glyphosate on Bacterial Communities

The susceptibility to GLY varies between or even within bacterial species (interstrain diversity) (Moorman et al., 1992). If bacteria with diverging susceptibilities against one substance inhabit the same community, the exposure to said substance can impact community structure (Wales and

Davies, 2015). For GLY, pathogenic bacteria seem to be less sensitive than commensal bacteria (Shehata et al., 2013). This is supported by *in vitro* investigations by Ackermann et al. (2015), who detected increased numbers of pathogenic species after a ruminal fermentation trial with (pure) glyphosate in concentrations of up to 100 µg/ml.

On an intestinal microbiome level, studies in mice and female rats found a loss of *Firmicutes*, especially *Lactobacilli*, after sub-chronic and chronic exposure to a GBH (Aitbali et al., 2018; Lozano et al., 2018). While glyphosate exposure decreased the amount of *Bacteroidetes* in mice (Aitbali et al., 2018), it increased it in female rats and did not show a significant effect in male rats (Lozano et al., 2018). Furthermore, mice showed depression and anxiety-like behaviour. These changes were attributed to a decreased amount of tryptophan, one of the aromatic amino acids produced in the shikimate pathway, which is essential to neurotransmitter production (Aitbali et al., 2018). The microbiome changes in female rats were comparable to those observed in fatty liver disease, following alcohol exposure (Lozano et al., 2018).

In general, dysbiosis due to residues of herbicides, biocides or other environmental pollutants can have an important influence on health (Jin et al., 2017). Based on different susceptibilities in pathogenic and commensal bacteria, Krüger et al. attributed a loss of GLY-susceptible *Enterococci* to an increase in chronic botulism cases seen in cattle in recent years (Krüger et al., 2013b). Similarly, honeybees' intestines were colonized by less protective bacteria after GLY exposure (Motto et al., 2018).

1.3 Glyphosate Residues in Livestock Feed and Animals

1.3.1 Glyphosate Residues in Feed

Herbicide application plays an important role in modern agriculture, especially in combination with genetically engineered crops (Paarlberg, 2008). GBHs can either be applied pre-harvest to clear the field from unwanted weeds, or in combination with resistant crops as post-emergence weed management practice. The cultivation of GR soy crops is a major factor in worldwide herbicide usage. In 2011, 75% of all soy crops were GR crops (Bøhn et al., 2014).

Following application, GLY and its primary degradation product aminomethylphosphonic acid (AMPA) can accumulate in the plant. Various studies have investigated the residues in livestock feed (Table 1.2). Most studies found direct residue of GLY, but some also detected small amounts of AMPA (Arregui et al., 2004; EFSA, 2015b). Since the ingredients of GBHs besides the AI remain unknown, there is no available data on residue levels of these compounds, albeit recently the monitoring and testing of 'inert' formulation ingredients was requested (Cox and Surgan, 2006; Nagy et al. 2020). Nevertheless, it can be assumed that residues are present in equal proportion to their dosage in the applied formulation.

***Table 1.2**: Summary of glyphosate residues for livestock-related feed found in the literature. GR= glyphosate resistant, RAR = Final Addendum to the Renewal Assessment Report on Glyphosate by Rappaport Member State (RMS): Germany and Co-RMS: Slovakia.*

Residue	Type of Feed	Reference
3.3 mg/kg	Soy (genetically modified plants)	Bøhn et al., 2014
0.3 mg/kg	Soy beans (7d after spraying)	Lorenzatti et al., 2004
1.9 - 4.4 mg/kg	Soy (leaves and stems)	Arregui et al., 2004
0.1 - 1.8 mg/kg	Soy (grains)	Arregui et al., 2004
0.04 - 0.4 mg/kg	GR maize (leaves)	Reddy et al., 2018
0.024 - 0.04 mg/kg	GR maize (seeds)	Reddy et al., 2018
0.78 - 7.79 mg/kg	GR maize (leaves)	Duke et al., 2018
0.1 - 0.45 mg/kg	GR maize (seeds)	Duke et al., 2018
0.4 - 0.9 mg/kg	Poultry and cattle feed in Germany	Shehata et al., 2014
4.6 mg/kg dry matter	Chicken feed	RAR, 2015
43.4 mg/kg dry matter	Dairy cattle feed	RAR, 2015
104.9 mg/kg dry matter	Beef cattle feed	RAR, 2015
5.2 mg/kg dry matter	Pig feed	RAR, 2015
∅ 79.1 mg/d	Cow feed	Schnabel et al., 2017
342 mg/kg dry matter	Cattle feed (all diets)	EFSA, 2018
530 mg/kg dry matter	Sheep feed (all diets)	EFSA, 2018
123 mg/kg dry matter	Swine feed (all diets)	EFSA, 2018
33.4 mg/kg dry matter	Poultry feed (all diets)	EFSA, 2018

Even though the cultivation of genetically modified crops is not permitted in the European Union (EU) food and feed from third countries, where GR crops are common, are available on the European market. According to von Soosten et al. (2016), imported soy is the main source for glyphosate residues in Germany.

To ensure food safety, the European Food Safety Authority (EFSA) monitors pesticide residues and advises the European Commission (EC) on setting specific residue limits for many different food products, so-called maximum residue limits (MRLs) (EU, Pesticides Database - Maximum Residue Levels). Table 1.3 shows the current MRLs and newly proposed EFSA recommendations made in 2019 for selected crops, commonly used as livestock feed and animal products (EFSA, 2019).

***Table 1.3**: Comparison of current maximum residue levels (MRLs) and the latest recommendations (2019) , issued by the European Food Safety Authority (EFSA) for selected livestock feed and products of animal origin (EFSA, 2019).*

	Product	Existing EU MRL	Recommended MRL
Feed	Soybeans	20 mg/kg	5 mg/kg
	Maize	1 mg/kg	4 mg/kg
	Wheat	10 mg/kg	30 mg/kg
	Oat	20 mg/kg	30 mg/kg
	Barley	20 mg/kg	30 mg/kg
	Lupin	10 mg/kg	20 mg/kg
	Peas	10 mg/kg	30 mg/kg
	Rice	0.1 mg/kg	0.2 mg/kg
Animal products	Swine muscle	0.05 mg/kg	0.2 mg/kg
	Swine fat tissue	0.05 mg/kg	0.2 mg/kg
	Bovine muscle	0.05 mg/kg	0.2 mg/kg
	Bovine fat tissue	0.05 mg/kg	0.2 mg/kg
	Poultry muscle	0.05 mg/kg	0.2 mg/kg
	Poultry fat tissue	0.05 mg/kg	0.2 mg/kg
	Milk products	0.05 mg/kg	0.1 mg/kg

1.3.2 Glyphosate Residues in Animals

Unsurprisingly, as many feeds contain GLY residues, traces of the herbicide have also been found in animals. In livestock, GLY has been detected in the urine and a number of organs (kidney, liver, lung, spleen, muscles, intestine) of German and Danish cows, with higher detection levels in cows tested in Denmark and cows housed in conventional husbandry (Krüger et al., 2013a, 2014a). It has been estimated that thirty percent of orally ingested GLY is absorbed by cows (Krüger et al., 2013b). In addition, Brewster et al. (1991) detected GLY in the gastrointestinal tract of rats after oral administration and found an absorption rate of 35-40%. The excretion of predominantly unaltered GLY primarily occurs via faeces and urine (Williams et al., 2000). The concentration of glyphosate measured in urine of rats orally exposed to either glyphosate or a GBH increased with the length of the treatment but did not differ between the AI and the formulation (Panzacchi et al., 2018).

1.4 Aim of the Research

Despite glyphosate being by far the most used herbicide in the world, its impact on ubiquitous bacteria such as *Escherichia coli* (*E. coli*) as well as on microbial communities in a livestock-related context is still unclear. Until now, only a few studies with partially contradictory conclusions have been published. There is still considerable uncertainty due to the use of different methods and a low number of investigated strains.

With this in mind, the following work aimed to

(i) conduct a broad susceptibility screening of different *E. coli* isolates obtained from livestock and the corresponding environment in order to get an overview of current susceptibility levels for different isolates (commensals and pathogens from cattle, pigs and poultry), identify resistant subpopulations and the possibility of a change in susceptibility over time (Part I),

(ii) determine possible influences of residual glyphosate levels on the enrichment of zoonotic pathogens of the family Enterobacteriaceae in microbial communities *in vitro* (in a ruminal fermentation system, Part II) and *in vivo* (in pigs, Part III),

(iii) broaden the knowledge about the impact of glyphosate on both induction and selection of antimicrobial resistance (Part II and III).

During the study, a special focus was placed on possible differences between glyphosate as a single substance and as a commonly used formulation.

RESEARCH PART I: SUSCEPTIBILITY SCREENING IN *E. COLI*

2.1 Study Outline

Little is known about the levels of resistance for GLY in *Escherichia coli* (*E. coli*). To date, only few minimum inhibitory concentration (MIC) values have been determined and published. Moreover, the studies that have investigated MICs only studied individual strains. Additionally, test methods, media and formulations differ, making it nearly impossible to compare values and derive a realistic susceptibility for GLY and GBHs (Table 2.1).

***Table 2.1**: Published minimum inhibitory concentrations (MICs) for E. coli for different substances and media. n.s. = not specified; GBH= Glyphosate based herbicide, LB= Lysogeny broth, BHI= Brain heart infusion, RCM= Reinforced clostridial medium*

E. coli	GBH	Medium	MIC	Reference
n.s.	Roundup Weedkiller	LB	7400 ppm	Kurenbach et al., 2015
n.s.	Roundup Ultra Max	n.s.	1.2 mg/ml	Shehata et al., 2013
1917 strain Nissle	Roundup Ultra Max	n.s.	1.2 mg/ml	Shehata et al., 2013
K88	Jablo Glyfosat	BHI	20 mg/ml	Katholm, 2016
K88	Glyphosate	BHI	2.86 mg/ml	Katholm, 2016
K88	Glyphosate salt	BHI	10 mg/ml	Katholm, 2016
ATCC 25922	Glyfos 450 Plus	BHI	80 mg/ml	Nielsen et al,. 2018
ATCC 25922	Glyfos 450 Plus	RCM	20 mg/ml	Nielsen et al., 2018
DSM 18039	Glyfos 450 Plus	BHI	80 mg/ml	Nielsen et al., 2018
DSM 18039	Glyfos 450 Plus	RCM	20 mg/ml	Nielsen et al., 2018

Therefore, this study aimed for a broader screening of *E. coli* isolates from animal husbandry. A total of 173 *E. coli* isolates, gathered between 2014 and 2015 by the German Federal Institute for Risk Assessment (BfR) and the German Federal Office of Consumer Protection and Food Safety (BVL), were examined. It is noteworthy that the classification of these isolates was made according to their origin. Isolates obtained within the framework of zoonoses monitoring were categorized as commensals, whereas isolates from the German national monitoring program GERM-Vet were classified as pathogens.

Recently collected samples were compared to *E. coli* isolated before the ubiquitous glyphosate usage. Lacking historic isolates from livestock, the standard *E. coli* collection of reference (ECOR) was used as historic control. This collection from 1984 represents the variability in *E. coli* at that time. Most of the 65 tested ECOR isolates were of human origin. An overview of all the tested isolates and their original host is presented in Table 2.2.

***Table 2.2**: Isolates used for susceptibility testing: standard reference strains of E. coli (ECOR, Ochman and Selander, 1984) and isolates sampled in 2014 and 2015 from the German Federal Institute for Risk Assessment (BfR; commensals) or the German Federal Office of Consumer Protection and Food Safety (BVL; pathogens). ESBL= Extended spectrum beta-lactamase producing.*

Origin	**ECOR**	**Commensal *E. coli***			**Pathogenic *E. coli***		
	non-ESBL	ESBL	non-ESBL	In total	ESBL	non-ESBL	In total
Poultry	-	15	15	30	3	12	15
Pig	2	15	15	30	19	17	36
Cattle	3	15	15	30	15	17	32
Human	39	-	-	-	-	-	-
Primate	9	-	-	-	-	-	-
Dog	3	-	-	-	-	-	-
Sheep	2	-	-	-	-	-	-
Leopard	2	-	-	-	-	-	-
Bison	1	-	-	-	-	-	-
Giraffe	1	-	-	-	-	-	-
Goat	1	-	-	-	-	-	-
Cougar	1	-	-	-	-	-	-
Kangaroo rat	1	-	-	-	-	-	-
In total	**65**	**45**	**45**	**90**	**37**	**45**	**83**

To assess differences between the pure substance as IPA and the commercially available formulation Roundup® LB Plus (RU), MICs were determined for both GLY and RU. To detect possible resistant subpopulations, a 95% epidemiological cut-off was calculated. Further statistical analysis was performed to evaluate possible differences in susceptibility regarding time point of isolation, pathogenicity, host and resistance to β - lactam antibiotics.

2.2 Broth Microdilution Method

There are different methods for antimicrobial susceptibility testing (AST) of bacteria:

Agar Diffusion Test: Spreading a defined amount of bacteria on an agar plate with an antibiotic-containing disk. Reading of zone diameters (qualitative).

E-Test: Spreading a defined amount of bacteria on an agar plate with a graded antibiotic-containing plastic strip. Reading of MIC (quantitative).

Broth Macrodilution: Growing a defined amount of bacteria in liquid cultures > 2 ml with different concentrations of antibiotics. Reading of MIC (quantitative).

Broth Microdilution: Growing a defined amount of bacteria in liquid cultures < 500 µl with different concentrations of antibiotics. Reading of MIC (quantitative).

VITEK: Automated system by bioMérieux. Reading of MIC (quantitative).

All quantitative methods depend on the determination of growth inhibitory concentrations (MIC), which can be observed phenotypically.

Depending on the context, either epidemiological cut-offs, separating susceptible wild-type (WT) from resistant non-WT, or clinical breakpoints, separating assumingly treatable and non-treatable isolates, can be distinguished (Lockhart et al., 2017; Wiegand et al., 2008).

For clinical purposes, quantitative MICs are converted into the categories susceptible, intermediate and resistant. Breakpoints for these conversions are published by national committees like the European Committee on Antimicrobial Susceptibility Testing (EUCAST) or the Clinical and Laboratory Standards Institute (CLSI).

One of the main methods used to determine MICs for antibiotics is the broth microdilution, commonly conducted in commercially available 96-well plates (Barth Reller et al., 2009). Because there are no established methods for testing MICs for glyphosate, a protocol for susceptibility testing for antibiotics (Wiegand et al., 2008) was fitted to our needs.

2.2.1 Preparation of the 96-well Plates

First, 100 µl of Mueller Hinton broth (MH) were pipetted into rows B to G of a polystyrene 96-well plate with conical bottoms. Stock solutions made of pure GLY (monoisopropylamine glyphosate salt, Sigma-Aldrich Chemie GmbH) and RU (Roundup® LB Plus, German registration number 024142-00) were prepared, respectively (Table 2.3). To maintain solubility, the dilution range was chosen to allow for the highest concentrations with a maximum of 160 mg/ml. The dilution was started by pipetting 200 µl of the stock solution into row A. By transferring 100 µl of the mixture from row to row, a serial dilution was generated from row A through G. Each well was mixed five times with the pipette before transferring to the next. Row H, containing MH only, was used as positive and negative control (Figure 2.1). Prepared plates were frozen at -80 °C before usage. Preliminary tests with freezing at -20 °C or storing at 4 °C led to a loss of function (data not shown).

Table 2.3: *Stock solution preparation for one 96-well plate (200 µl/well in row A). GLY= monoisopropylamine glyphosate salt, RU= Roundup® LB Plus C= concentration of glyphosate isopropylamine salt (IPA) in the original solution, V= final volume needed for one plate (12×200 µl), MQ = Millipore water, 2× MH = double concentrated Mueller Hinton broth*

Substance	C	Stock	V	GLY/RU	MQ	2× MH
GLY	400 mg/ml	80 mg/ml	2400 µl	480 µl	720 µl	1200 µl
RU	486 mg/ml	80 mg/ml	2400 µl	395 µl	805 µl	1200 µl

2.2.2 Preparing the Inoculum

Bacteria were cultured overnight in 3 ml of MH (37 °C, 200 rpm, 45° angle). The optical density at 600 nm (OD600) was measured and the cultures diluted to an OD of 0.5, equivalent to 10^8 colony forming untits (CFU)/ml. The OD-adjusted cultures were further diluted 1:100 (equal to 10^6 CFU/ml) and 5 µl were added into each well, excluding the control samples (1:20 diluted). The inoculum equalled 5 × 10^4 CFU/well or 5 × 10^5 CFU/ml. Each sample was tested in technical triplicates.

2.2.3 MIC Determination

Based on Walzl et al. (2012), plates were incubated for 16-20 hours at 37 °C in a humidity chamber, comprising of a box (Ikea, Article 489.716.76) with a moist paper towel in the bottom of the box to ensure humidity, and two glass petri dishes to protect the plates from direct contact with moisture.

Growth was determined visually by examining the plates with a mirror below the plate and a light shining through from above (Sensititre). The lowest concentration at which visible bacterial growth (apparent as turbidity) in at least two out of three replicates was prevented, represented the MIC (Figure 2.1).

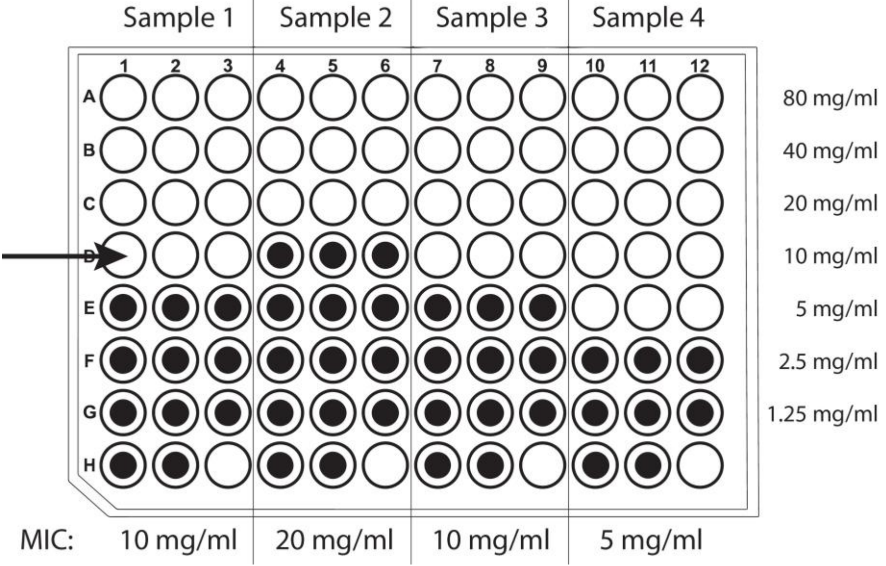

***Figure 2.1**: Determination of minimum inhibitory concentration (MIC) with a 96-well plate (broth microdilution). Serial dilution ranged from 80 mg/ml to 1.25 mg/ml with the last row as positive and negative control. Each sample was tested in technical triplicates. The MIC is defined as the lowest concentration, where visible growth is inhibited (for Sample 1 the MIC would be 10 mg/ml as indicated by the arrow).*

ORIGINAL RESEARCH
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Minimum Inhibitory Concentration of Glyphosate and of a Glyphosate-Containing Herbicide Formulation for *Escherichia coli* Isolates – Differences Between Pathogenic and Non-pathogenic Isolates and Between Host Species

Katrin Bote[1], Judith Pöppe[1], Roswitha Merle[2], Olga Makarova[1] and Uwe Roesler[1]*

[1] *Institute for Animal Hygiene and Environmental Health, Freie Universität Berlin, Berlin, Germany,* [2] *Institute for Veterinary Epidemiology and Biostatistics, Freie Universität Berlin, Berlin, Germany*

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Daniela Ceccarelli,
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New Zealand

****Correspondence:***
Katrin Bote
Katrin.Bote@fu-berlin.de;
tierhygiene@vetmed.fu-berlin.de

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Glyphosate is the most extensively used herbicide in the world. However, concerns regarding its safety, side effects, and impact on other organisms have increased in recent years. This is the first study to analyze a large set of recent and historical *Escherichia coli* isolates varying in pathogenicity and beta-lactam resistance from different host species for their susceptibility to glyphosate isopropylamine salt (IPA), the active ingredient of the herbicide, and to a complete glyphosate-containing formulation (Roundup LB Plus). For this, minimum inhibitory concentrations (MIC) were determined for 238 *E. coli* isolates by broth microdilution in Mueller Hinton I media followed by the statistical analyses using Mann-Whitney-U test, multivariable analysis of variance (ANOVA) and a multivariable proportional-odds ordinal regression model. While the overall MIC distribution was narrow and lacked a highly resistant sub-population for both substances, statistical analyses revealed small but significant associations between glyphosate resistance levels and different factors tested. Mean MIC values for the entire dataset showed a higher level of resistance to the complete glyphosate-containing formulation (40 mg/ml IPA) than to pure glyphosate (10 mg/ml IPA) in *E. coli*. Isolates that originated from poultry had significantly higher MIC values for both pure glyphosate and the complete formulation. Pathogenic and non-extended-spectrum beta-lactamase (non-ESBL) *E. coli* isolates each showed significantly higher MIC values compared to commensals and ESBL-producing *E. coli* in pure glyphosate, but not in the complete formulation. Recently sampled isolates showed statistically higher MICs than the isolates of the historic standard *E. coli* collection of reference in pure glyphosate, when tested by nonparametric Mann-Whitney-U test, but not in the multivariable model.

Further investigations are necessary to confirm whether these associations have a casual relationship with the glyphosate use or are the consequence of co-selection due to the increased application rates of antibiotics, heavy metals or other biocides. A possible accumulation of pathogenic bacteria in livestock animals fed with glyphosate-containing feed should also be considered.

Keywords: glyphosate, minimum inhibitory concentration, *Escherichia coli*, antimicrobial susceptibility testing, MIC distribution, statistical modeling

INTRODUCTION

The broad spectrum herbicide N-(Phosphonomethyl)glycine, commonly known as glyphosate, is one of the most-used pesticides in the world (Duke and Powles, 2008). It targets the enzyme 5-Enolpyruvylshikimate-3-phosphate Synthase (EPSPS) in the shikimate pathway and disrupts the formation of aromatic amino acids and other secondary plant compounds (Steinrücken and Amrhein, 1980, 1984). The pathway is present in plants, unicellular parasites, certain bacteria, and fungi but not in mammals (Herrmann and Weaver, 1999; Roberts et al., 2002). For a long time, this has been considered as a significant advantage regarding toxicity in comparison to almost all other pesticides (Benbrook, 2016). The presence of EPSPS in various microorganisms led to patenting the substance as a broad-range antimicrobial (William, 2002).

In 1996, glyphosate-resistant (GR) crops became commercially available on the market causing a paradigm shift in the herbicide use and agricultural management (Duke, 2017). From then on, glyphosate could be applied throughout the whole cultivation time without harming the desired plants and its use worldwide increased exponentially (15-fold) (Duke and Powles, 2009; Benbrook, 2016). Today, GR variants exist for example in soybean, corn, cotton, canola, alfalfa, and sugar beets (Cerdeira and Duke, 2006; Green, 2016), although none of those GR plants are cultivated in the European Union, where the release of genetically modified organisms into the environment is highly regulated (Federal Ministry of Food and Agriculture, 2013). However, the considerable utilization in other parts of the world leads to an accumulation of glyphosate residues.

In this context, glyphosate has been found in soy beans (Arregui et al., 2004; Lorenzatti et al., 2004; Bøhn et al., 2014), assumed to be one of the main sources for residues found in livestock feed (von Soosten et al., 2016).

The presence of contaminants in glyphosate-treated soy and maize exposes farm animals' microbiota to the herbicide ingredients (Krüger et al., 2013a; Katholm, 2016). The possible effects of glyphosate on the intestinal bacteria has been discussed recently. Shehata et al. (2013) state that pathogenic bacteria from the poultry microbiome are more resistant to glyphosate than beneficial members *in vitro*. Kurenbach et al. (2015) also found an increased tolerance and changed antibiotic responses in their tested *Escherichia coli* and *Salmonella enterica* serovar Typhimurium strains after exposure to sub-lethal concentrations of a herbicide formulation.

Escherichia coli is not only an important zoonotic pathogen in livestock but also ubiquitous in the environment. It represents the majority of *Enterobacteriacae* and is an intensively studied model organism in research. Additionally, *E. coli* is one of the two gram-negative bacteria species used for biocide efficacy testing as an surrogate for similar enteric bacteria (European Chemicals Agency, 2018), and has even been used for screening of bacterial metabolites with herbicidal activities (Gasson, 1980).

Contaminated food is the main source for colonization and infection of humans and a risk factor for transferring antimicrobial resistance genes (Aarestrup et al., 2008). Therefore, the question arises if an exposure to glyphosate can lead to a shift in the microbiome favoring the shedding of especially pathogenic or antibiotic-resistant *E. coli*.

Until now, there has been no detailed survey to define the susceptibility of *E. coli* to glyphosate. Therefore, our study aimed to (i) screen different *E. coli* isolates of clinical, non-clinical and environmental origin for susceptibility to glyphosate and to a glyphosate-containing formulation; (ii) compare historical and recent isolates in regards to a development of resistance over the time as glyphosate use increased; (iii) to investigate whether there is a link between host species or antibiotic resistance and glyphosate susceptibility.

MATERIALS AND METHODS

Biological Material

In total, 238 *E. coli* strains from different environments were analyzed.

We tested sixty-five *E. coli* isolates from the standard *E. coli* collection of reference (ECOR) (Ochman and Selander, 1984). This collection was established before the broad usage of glyphosate, thus representing the variations in *E. coli* at that time and is used as historic controls.

Ninety commensal *E. coli* isolates sampled in 2014 and 2015 were obtained from the German Federal Institute for Risk Assessment. They were characterized as non-pathogenic and evenly divided into poultry, pig, and cattle origin as well as into extended spectrum beta-lactamase (ESBL) and non-ESBL producing *E. coli* strains.

In addition, the German Federal Office of Consumer Protection and Food Safety provided 83 pathogenic *E. coli* isolates from clinical cases they collected in 2014 and 2015 for the GERMAP survey of antibiotic resistances of pathogenic bacteria isolates. Poultry, pig, and cattle isolates were equally represented. Forty-eight of the isolates were non-ESBL and 35 were ESBL *E. coli* (**Table 1**).

TABLE 1 | Origin and distribution of the 238 tested *E. coli* isolates divided by different collections.

Origin	ECOR	Commensal *E. coli*			Pathogenic *E. coli*		
	Non-ESBL	ESBL	Non-ESBL	in total	ESBL	Non-ESBL	in total
Poultry	–	15	15	30	3	12	15
Pig	2	15	15	30	19	17	36
Cattle	3	15	15	30	15	17	32
Human	39	–	–	–	–	–	–
Primate	9	–	–	–	–	–	–
Dog	3	–	–	–	–	–	–
Sheep	2	–	–	–	–	–	–
Leopard	2	–	–	–	–	–	–
Bison	1	–	–	–	–	–	–
Giraffe	1	–	–	–	–	–	–
Goat	1	–	–	–	–	–	–
Cougar	1	–	–	–	–	–	–
Kangaroo Rat	1	–	–	–	–	–	–
in total	65	45	45	90	37	45	83

Minimum Inhibitory Concentration (MIC) Testing

There are no standards for testing MICs of herbicides. Therefore, a susceptibility testing protocol according to Wiegand et al. (2008), which is in compliance with CLSI M07-A10 standards for antibiotic susceptibility testing, was established. Polystyrene 96-well plates with a conical bottom (Sarstedt GmbH, Nümbrecht, Germany) were used. Based on growth and killing dynamics of a representative *E. coli*, Mueller Hinton (MH) I medium was chosen (Oxoid GmbH, Wesel, Germany, CM0405). MICs for MH II can be found in the Supplements.

A 40% monoisopropylamine salt solution of glyphosate (GLY) (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) and the glyphosate-containing commercial formulation Roundup LB Plus (RU) (German registration number 024142-00) were used. Concentration is indicated in mg/ml for the isopropylamine salt (IPA) of glyphosate. Serial dilutions ranged from 80 to 1.25 mg/ml for the pure substance and from 160 to 2.5 mg/ml for the commercial formulation. The prepared plates were stored at −80°C until usage.

For testing, overnight cultures were diluted to an OD_{600} of 0.5 (10^8 cfu/ml), which were further diluted 1:100 before adding 5 μl into each well (equivalent to 5×10^4 cfu, 5×10^5 cfu/ml, respectively). Each isolate was tested in triplicates. The plates were aerobically incubated at 37°C for 16–20 h in a humidity chamber according to Walzl et al. (2012). The growth within the wells was determined visually with a mirror below the plate and a light above (SensiTouch by Sensititre).

Statistical Analysis

For statistical analyses and calculations, IBM® SPSS® Statistics Version 24 was used. All MIC data were ranked in ascending order prior to analyses and checked visually for normal distribution. As MIC values of GLY showed sufficient normal distribution, the data of GLY could be fitted by an ANOVA approach. Regarding RU, the MIC values were not normally distributed and only included the three levels 20, 40, and 80. Thus, it was decided to regard these levels as ordinal categories and to fit a proportional-odds ordinal regression model. The influences in terms of isolation time (ECOR and recent isolates), collection (commensals and pathogens), ESBL-status and host (poultry, pig, cattle) on MIC values of GLY or RU, respectively, were tested using

(i) univariable nonparametric Mann-Whitney-U tests for not normally distributed data, and
(ii) a multivariable analysis of variance (ANOVA) for GLY, or
(iii) a multivariable proportional-odds ordinal regression model for RU

to determine different factors.

Two different statistical models for each substance were adapted containing different parameters. In the first model (Model A) the influence of the time of isolation, the ESBL-status and the host on either GLY or RU were investigated.

In the ECOR collection, there were only few livestock associated isolates (two *E. coli* from pigs and three from cattle). Most of the isolates originated from humans or exotic animals (**Table 1**). Therefore, we created a second model (Model B) without the ECOR collection, which investigated the influence of the collection (pathogen or commensal), the ESBL-status and the host (poultry, pig, cattle) on either GLY or RU.

All two-way-interactions between influence factors were included in the initial models and removed if not statistically significant.

P-values below 0.05 were regarded as statistically significant. Model diagnostics included check for normality and homoscedasticity of residuals. For analysis of variance, the assumption of equal variances was also investigated. For proportional odds ordinal regression models, the assumption of proportionality as well as the assumption of parallel lines were additionally checked.

To obtain an epidemiological cutoff, MIC_{95} was calculated for GLY and for RU each.

RESULTS

Overall, MICs of glyphosate isopropylamine salt (GLY) and of the commercial herbicide formulation Roundup LB Plus (RU) were narrowly distributed with a clear segregation between both. In most of the isolates, growth was inhibited at a concentration of 10 mg/ml GLY (equating 7.41 mg/ml pure glyphosate) or 40 mg/ml RU (equating 29.63 mg/ml pure glyphosate), both representing the mean and the mode (**Figure 1**).

Most of the isolates from the ECOR collection showed a MIC of 10 mg/ml for GLY, which represented the mode and the median. For the herbicide formulation RU, the majority of the isolates had MIC values of 40 mg/ml. Overall MICs ranged from <1.25 to 20 mg/ml for GLY and 20 to 80 mg/ml for RU (**Table 2** and **Figure 2**).

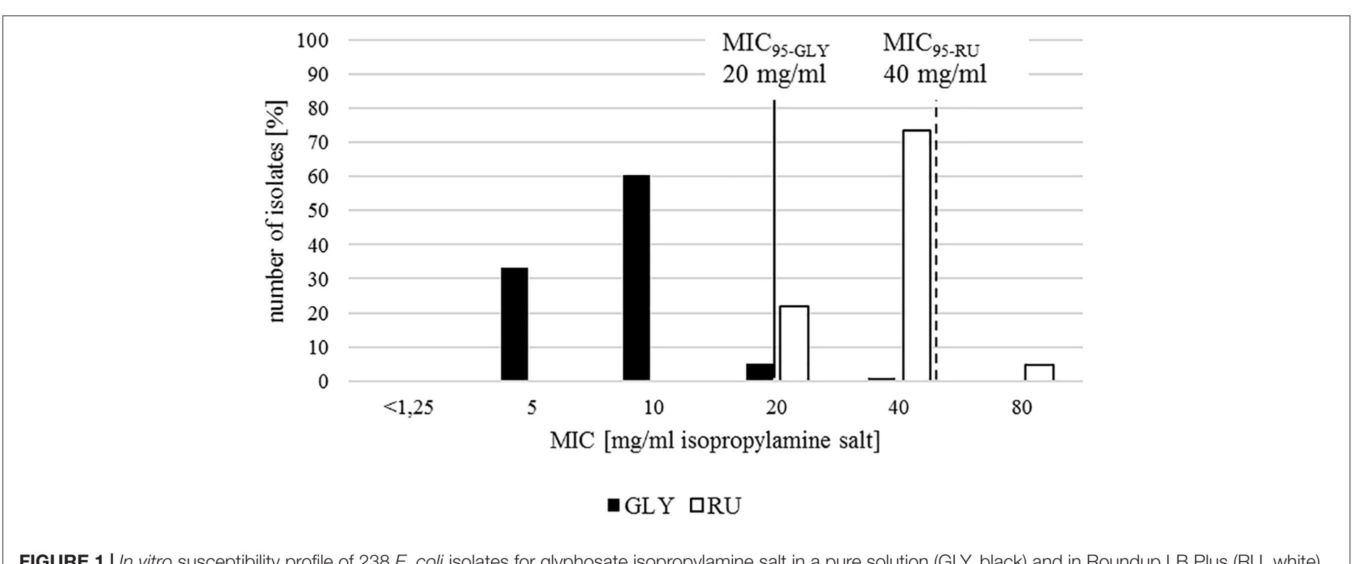

FIGURE 1 | *In vitro* susceptibility profile of 238 *E. coli* isolates for glyphosate isopropylamine salt in a pure solution (GLY, black) and in Roundup LB Plus (RU, white). Minimum inhibitory concentration including 95% of all isolates (MIC_{95}) is represented with a continuous line for GLY and a dashed line for RU.

TABLE 2 | MIC values of 238 *E. coli* for monoisopropylamine glyphosate salt (IPA) represented either as a pure solution (GLY) or as a part of the complete formulation Roundup LB Plus (RU).

MIC [mg/ml IPA]	ECOR		recent isolates		Commensal *E. coli*		Pathogenic *E. coli*		ESBL		non-ESBL		*E. coli* in total	
	GLY	RU	GLY	RU	GLY	RU	GLY	RU	GLY	RU	GLY	RU	GLY	RU
<1,25	1.5% (1)	0% (0)	0% (0)	0% (0)	0% (0)	0% (0)	0% (0)	0% (0)	0% (0)	0% (0)	0.7% (1)	0% (0)	0.4% (1)	0% (0)
5	16.9% (11)	0% (0)	39.3% (68)	0% (0)	48.9% (44)	0% (0)	28.9% (24)	0% (0)	45.8% (38)	0% (0)	26.5% (41)	0% (0)	33.2% (79)	0% (0)
10	76.9% (50)	0% (0)	54.3% (94)	0% (0)	47.8% (43)	0% (0)	61.5% (51)	0% (0)	51.8% (43)	0% (0)	65.2% (101)	0% (0)	60.5% (144)	0% (0)
20	4.6% (3)	18.5% (12)	5.2% (9)	23.1% (40)	3.3% (3)	22.2% (20)	7.2% (6)	24.1% (20)	2.4% (2)	24.1% (20)	6.5% (10)	20.7% (32)	5.0% (12)	21.9% (52)
40	0% (0)	78.5% (51)	1.2% (2)	71.7% (124)	0% (0)	74.4% (67)	2.4% (2)	68.7% (57)	0% (0)	73.5% (61)	1.3% (2)	73.6% (114)	0.8% (2)	73.5% (175)
80	0% (0)	3.1% (2)	0% (0)	5.2% (9)	0% (0)	3.3% (3)	0% (0)	7.2% (6)	0% (0)	2.4% (2)	0% (0)	5.8% (9)	0% (0)	4.6% (11)

The tested isolates were divided into different groups. The ECOR collection served as an example of historic isolates as opposed to recent isolates (consisting of commensal and pathogenic isolates gathered in 2014 and 2015) or separated according to the susceptibility against beta-lactam antibiotics. Indicated as percentage share rounded to one decimal place after the point with the number of isolates in brackets.

The commensal isolates of the investigated strains showed mostly a MIC of 5 mg/ml (representing the mode) or 10 mg/ml (representing the median) for GLY with a total range from 5 to 20 mg/ml. RU inhibited the growth of most strains at 40 mg/ml with a total range from 20 to 80 mg/ml (**Table 2** and **Figure 3**).

In contrast to commensal isolates, pathogenic *E. coli* mostly showed a MIC of 10 mg/ml for GLY with a total range of 5–40 mg/ml. For RU, the MIC was in the range of 20–80 mg/ml, whereby 40 mg/ml was the most common minimal inhibitory concentration (**Table 2** and **Figure 3**).

MIC_{95} representing 95% of the studied population was 20 mg/ml in GLY and 40 mg/ml in RU. For GLY there are two pathogenic *E. coli* isolated from cattle with a higher MIC than the cutoff. For RU 11 isolates (two from the ECOR collection isolated from humans, three commensal and five pathogenic *E. coli* from poultry and one pathogenic isolate from a pig) showed a MIC above the MIC_{95}. All of the isolates belong to the non-ESBL group.

Statistical Analysis

To test for differences between isolate parameters in glyphosate sensitivity, nonparametric Mann-Whitney-U test and depending on data distribution, two different statistical models were used.

In the Mann-Whitney-U test, both for GLY and RU, there were highly significant differences in MICs between the isolates from poultry ($P < 0.01$) compared to pig and cattle isolates which had lower MICs (**Table 3**).

Furthermore, more factors showed significant influence on GLY. Historic isolates from the ECOR collection had significantly lower MIC values ($P < 0.05$) than the isolates collected in

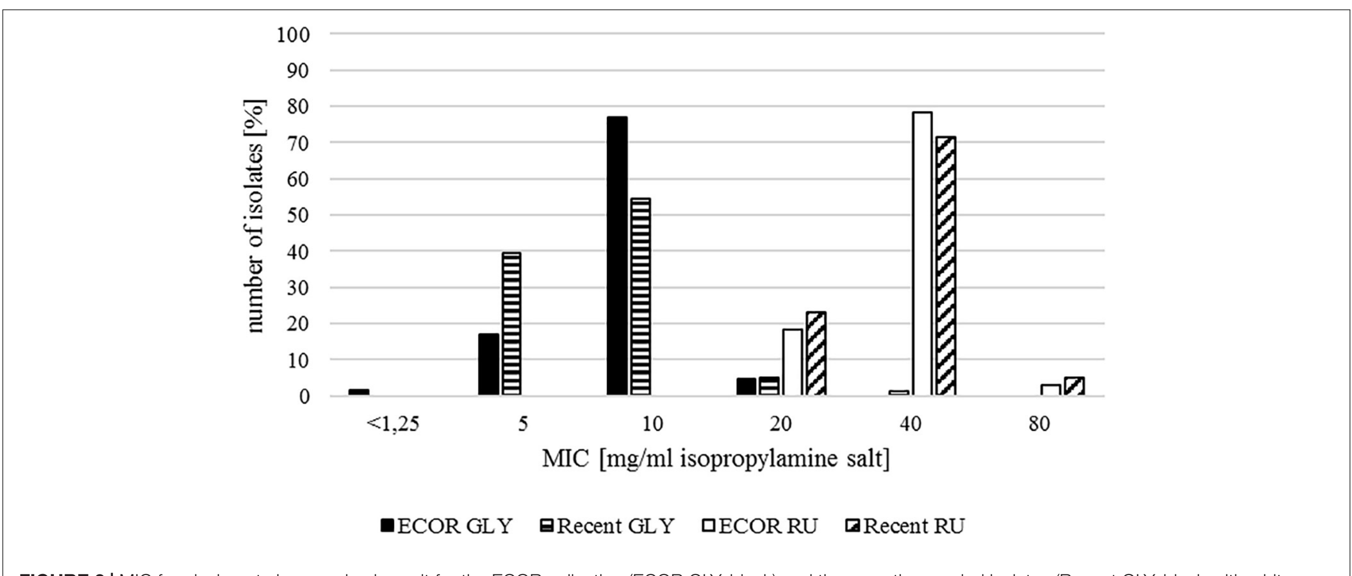

FIGURE 2 | MIC for glyphosate isopropylamine salt for the ECOR collection (ECOR GLY, black) and the recently sampled isolates (Recent GLY, black with white stripes) and for the formulation Roundup LB Plus for the ECOR collection (ECOR RU, white) and the recently sampled isolates (Recent RU, white with black oblique stripes), respectively.

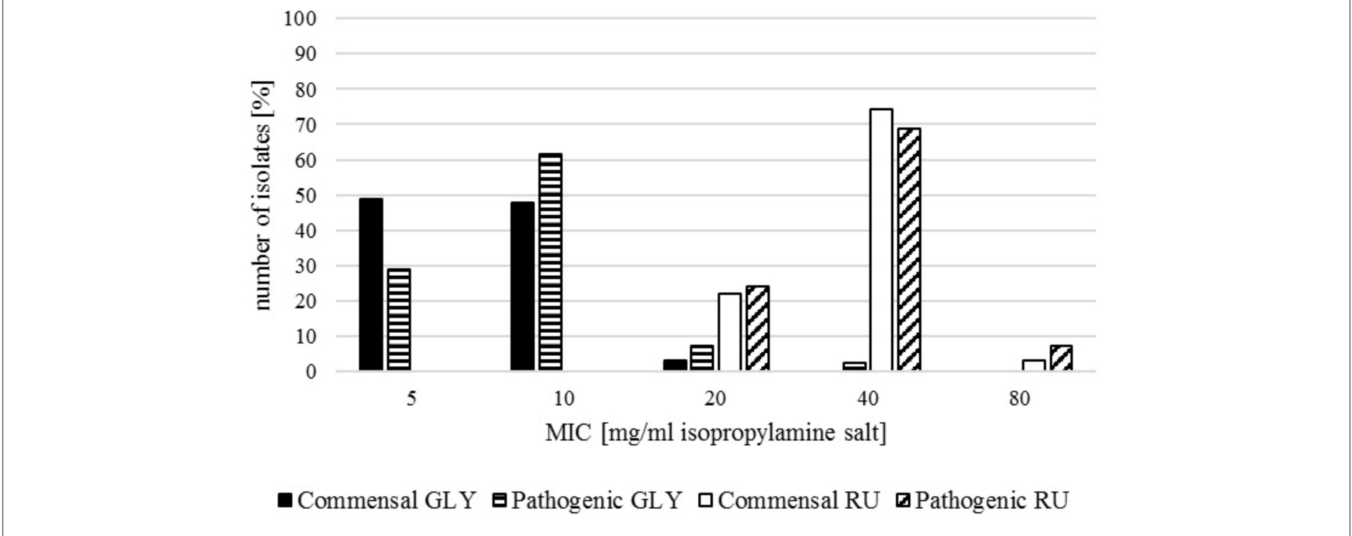

FIGURE 3 | MIC for glyphosate isopropylamine salt for the commensal *E. coli* (Commensal GLY, black) and the pathogenic *E. coli* isolates (Pathogenic GLY, black with white stripes) and for the formulation Roundup LB Plus for the commensal *E. coli* (Commensal RU, white) and the pathogenic *E. coli* isolates (Pathogenic RU, white with black oblique stripes), respectively.

the years 2014 and 2015. Pathogenic isolates differed highly significantly ($P < 0.01$) from the commensal isolates (with higher MIC values in the pathogenic group). Likewise, isolates classified as non-ESBL had statistically significantly higher MICs than the ESBL isolates ($P < 0.05$).

Model A included time of isolation (historic and recent), ESBL-status and host, whereas Model B (with the excluded ECOR strains) considered isolation as commensal or pathogen, ESBL-status and host (**Table 4**).

In contrast to the results of the Mann-Whitney-U test for GLY, no difference between the strains of the ECOR collection and recent sampled isolates was seen in model A ($P = 0.726$).

However, the ESBL-status and the host species of the isolates showed statistically significant influence on the MIC values ($P = 0.013$ and $P < 0.001$). In agreement with the Mann-Whitney-U test, non-ESBL isolates had significantly higher MIC values compared to ESBL-positive isolates.

Tukey *post hoc* analysis for the hosts revealed significant differences between isolates from poultry and pigs ($P < 0.01$) and poultry and cattle ($P = 0.01$) with higher MICs in the poultry each, as well as between isolates from pigs and human ($P = 0.019$) and pigs and other species ($P = 0.006$) with lower MICs in pigs each. There was no significant difference between the isolates from pigs and cattle ($P = 0.608$).

TABLE 3 | Effect of different parameters on MIC by means of univariable nonparametric Mann-Whitney-U test.

Comparison of		*P*-value	
		GLY	RU
Recent isolates	Historic isolates	**0.014**	0.667
ECOR collection	Commensal *E. coli*	**<0.001**	0.623
ECOR collection	Pathogenic *E. coli*	0.498	0.780
Pathogenic *E. coli*	Commensal *E. coli*	**0.004**	0.861
Non-ESBL	ESBL	**0.018**	0.362
Poultry	Pig	**<0.001**	**0.004**
Poultry	Cattle	**0.001**	**0.007**
Pig	Cattle	0.078	0.627

Statistically significant P-values <0.05 are in bold. Parameters with higher MICs are underlined.

Model B classified the differences between ESBL and non-ESBL isolates ($P = 0.035$) as well as between the hosts as significant. In accordance with model A, non-ESBL isolates and isolates from poultry had significantly higher MIC values than the ESBL isolates and isolates from cattle and pig. Additionally, a significant interaction between pathogenic and commensal isolates was present ($P < 0.001$). Pathogenic *E. coli* isolates showed significantly higher MIC values than commensals.

In the *post hoc* analysis, the differences between poultry and cattle ($P = 0.002$) as well as between poultry and pigs ($P < 0.001$) were clearly visible, with *E. coli* isolates from poultry showing significantly higher MIC values for glyphosate than isolates from other hosts.

For RU in model A, there was no significant difference between the strains of the ECOR collection and recently sampled isolates ($P = 0.293$), nor between ESBL and non-ESBL isolates ($P = 0.443$). However, a significant difference was found between poultry and human isolates (human isolates served as a reference category, $P = 0.031$). The Nagelkerke R^2 in this model was 0.088, meaning that only a small proportion of the variance could be explained with this model.

Model B also showed no significant differences between ESBL and non-ESBL ($P = 0.479$) nor between commensal and pathogenic isolates ($P = 0.314$). Nevertheless, there was a significant difference between the hosts, i.e., between cattle and poultry ($P = 0.002$) and pigs and poultry ($P = 0.001$). Poultry served as reference category and had the highest MIC values compared to cattle and pigs. With a Nagelkerke's R^2 of 0.111, it still only explained a small proportion of the variance. Obviously, the variables included in the model were not the most important influence factors on the MIC values of the investigated *E. coli* strains.

DISCUSSION

After introducing GR plants two decades ago, glyphosate is now the most used herbicide in the world. Concurrently, concerns about possible resistances to glyphosate came to the fore. However, there is little information available about the sensitivity of naturally occurring *E. coli* to glyphosate.

This is the first broad study to systematically analyze 238 different *E. coli* isolates for their susceptibility not only against GLY alone but also against a glyphosate-containing herbicide formulation.

In our study, we found differences between GLY and RU with a 4-times higher median and mode in the latter. In contrast to prior findings in the literature, where herbicidal formulations were more toxic to bacteria (Clair et al., 2012; Mesnage et al., 2014), higher concentrations of RU were needed to inhibit bacterial growth.

However, it is difficult to compare the values to the few published data. Various glyphosate formulations are used, which makes it almost impossible to compare the obtained results as Mesnage et al. (2015) also point out. Pure glyphosate acid in particular has a low solubility (12 g/l) and is therefore not commercially used, but the isopropylamine glyphosate salt is present in most of the formulations (usually combined with a surfactant and water) (Giesy et al., 2000). After application and uptake, the salt dissociates and the free glyphosate acid translocates in the plant and inhibits EPSPS (Williams et al., 2000).

Additives and surfactants in formulations vary and manufacturers are not required to declare them publicly. This leads to complex mixtures with additional effects of the supplements themselves or interactions between all ingredients.

TABLE 4 | *P*-values of the statistical models for glyphosate isopropylamine salt pure (GLY) (multivariable analysis of variance) and in Roundup LB plus (RU) (multivariable proportional-odds ordinal regression).

GLY		*P*-value		RU		*P*-value	
Comparison of		Model A	Model B	Comparison of		Model A	Model B
Recent isolates	Historic isolates	0.726	–	Recent isolates	Historic isolates	0.293	–
Pathogenic *E. coli*	Commensal *E. coli*	–	**<0.001**	Pathogenic *E. coli*	Commensal *E. coli*	–	0.314
Non-ESBL	ESBL	**0.013**	**0.035**	Non-ESBL	ESBL	0.443	0.479
Poultry	Pig	**<0.001**	**<0.001**	Poultry	Human	**0.031**	–
Poultry	Cattle	**0.01**	**0.002**	Poultry	Pig	–	**0.001**
Pig	Cattle	0.608	0.229	Poultry	Cattle	–	**0.002**

Model A investigates the time point of isolation, the ESBL-status and the host, Model B (without the ECOR collection) investigates the following categories: pathogenic or commensal, the ESBL-status and the hosts (poultry, pig, and cattle). Statistically significant P-values <0.05 are shown in bold. Group with higher minimum inhibitory concentrations are underlined.

Moreover, product compositions vary from brand to brand and regionally. Several formulations in experiments found in the literature contain the surfactant tallowamine. This substance is not used on the German market anymore and thus not present in our tested formulation (Senate Department for the Environment Transport and Climate Protection, 2012). The LD_{50} of tallowamine is much lower (oral 620 mg/kg rat) (Chemcas, 1997) than of pure glyphosate (oral 4873 mg/kg rat) (Chemcas, 2004) and indeed it has been shown that supplements in herbicide formulations can be more toxic than the active ingredient itself (Tsui and Chu, 2003; Mesnage et al., 2014; EFSA, 2015). This suggests that not only the activity of glyphosate, but rather the sum of different ingredients in a formulation or even some additives alone interact with the bacteria and influence the MIC. In response to this, a study by Clair et al. (2012) conducted with three food microorganisms observed differences between two different formulations and a glyphosate solution. In their tests, the formulations Roundup R400 and R450 were more toxic than the pure substance. Additionally, the effects were also disproportional to the amount of the active ingredient, proving the influence of additives and different mixtures.

Furthermore, in most studies only single or few isolates, which are often scarcely specified, were tested. For example, Kurenbach et al. (2015) published a MIC of 7.4 mg/ml for *E. coli* JB578 with the formulation Roundup Weedkiller in LB broth, similar to values with GLY from our experiment but not with RU. Shehata et al. (2013) published for *E. coli* a lower MIC of 1.2 mg/ml with a formulation called Roundup UltraMax. This formulation contained the surfactant tallowamine (Senate Department for the Environment Transport and Climate Protection, 2012), which, as mentioned above could be responsible for low MICs. Two *E. coli* isolates from Nielsen et al. (2018) had a comparable MIC of 20 mg/ml in reinforced clostridial medium or 80 mg/ml in brain heart infusion broth after anaerobic incubation in 96-well plates.

Besides the different MIC values, it is not always clear if stated concentrations in the literature are for glyphosate itself or the salt in a formulation and not all studies informed which media they used and how the susceptibility testing was conducted.

Specifically in nutrient rich media, bacteria may assimilate a certain amount of missing aromatic amino acids from their environment, bypassing the glyphosate-effects and thus tolerate higher concentrations. In medium lacking of aromatic amino acids, the MIC for glyphosate could be increased by adding them, which partly reversed the inhibition-effect of the herbicide (Haderlie et al., 1977; Nielsen et al., 2018).

In addition, glyphosate is known to be a chelator of bivalent cations (Madsen et al., 1978; Motekaitis and Martell, 2006). In cation-rich media, the active ingredient can be bound due to chelation leading to less free available active compounds. In MH I, the MIC was often one dilution step lower than in the cation-adjusted MH II (**Supplementary Figure 1**), with significant differences between MIC values in both media. However, differences between MIC for GLY and RU and difference between groups decreased (**Supplementary Table 1**).

Therefore, possible influences on MIC determination for glyphosate or glyphosate-containing formulations in general need further investigation, similarly concluded by Nielsen et al. (2018).

MIC_{95} has been used to distinguish different subpopulations by calculating epidemiological cutoffs values (ECV). No clear gap between isolates could be seen; nevertheless, there was a small subpopulation with less susceptibility. Besides overexpression of efflux pumps (Staub et al., 2012), changes in the EPSPS has been described as a reason for glyphosate resistance (Stalker et al., 1985; Eschenburg et al., 2002; Fei et al., 2013). Distribution of the isolates with a MIC above the cutoff reflect mostly the less susceptible categories in the statistical analysis (pathogenic, poultry origin, non-ESBL). Interestingly, the two isolates above the cutoff for GLY are not present in the group for RU, confirming again the varying behavior of formulations.

However, given the narrow distribution of all the MICs and an increase in absolute terms only one dilution step above the calculated cutoff, these isolates would need further investigation to determine a genetic basis of a glyphosate tolerance. Moreover, without a normal distribution, the calculated cutoff values might not reflect the real division between phenotypically resistant and sensitive populations (Lockhart et al., 2017).

Historical and Recent Isolates

To determine if the sensitivity to glyphosate changed over time, we included the ECOR collection in our screening (Ochman and Selander, 1984). This gave us the possibility to compare isolates prior to and after the large-scale use of glyphosate that accompanied the introduction of genetically modified crops in the nineties (Cerdeira and Duke, 2006; Duke and Powles, 2009).

In the nonparametric test, we could see significant differences between the ECOR isolates and the isolates from recent years for GLY. This gap was due to differences between the ECOR and the commensal collection, rather than the ECOR and pathogenic isolates. However, the statistically significant difference could not be confirmed in the ANOVA model, which excluded the non-livestock associated isolates of the ECOR collection. Thus, it seems that the factor of isolation time (and therefore the span of glyphosate usage in general) is not one of the important influences on MIC against glyphosate in the dataset. Nevertheless, a tendency to higher MICs in the recent isolates appeared.

Sub-lethal concentrations of biocides and herbicides can lead to adaptation and increased resistance (Thomas et al., 2000; Capita et al., 2014) and may thus explain the differences. Increased MICs could further be a result of co-induction or co-selection of applied antibiotics, other biocides or heavy metals (Karatzas et al., 2007; Capita et al., 2014; Molina-González et al., 2014; Yazdankhah et al., 2014), especially as these antimicrobials were also intensively used in the last decades. There is no possibility to review, if recent isolates were exposed to glyphosate in the intestine of the host or in the environment. Data about residues in feed are missing and would for sure vary within the data set. In addition, only very few isolates from the ECOR collection are livestock-associated.

The statistical analyses with the formulation RU showed no significant differences between the ECOR collection and recently sampled isolates. It seems to be more difficult to become less

susceptible against a complex formulation with various effects. Further, the MICs for RU are naturally higher than for GLY.

Overall, it is difficult to compare the strains of the ECOR collection and recent *E. coli* isolates in our livestock-related context, as ECOR lacks representative bacteria from livestock. Whether the observed difference in sensitivity to GLY is based on the increased use of glyphosate, antibiotics or other compounds in recent years, is yet to be established. In order to prove a change in susceptibility over time, historic isolates from farm animals should be investigated in future studies.

Commensal and Pathogenic Isolates

In previous studies, it was discussed that pathogenic bacteria are likely to be more tolerant to glyphosate (Krüger et al., 2013b; Shehata et al., 2013). Shehata et al. (2013) found higher MIC values in strains of pathogenic species like different *Salmonella* serovars and *C. perfringens* compared with, e.g., enterococci or lactobacilli. Krüger et al. (2013b) confirmed the differences in sensitivity between Clostridia and enterococci. However, pathogenic and non-pathogenic strains of the same bacteria species were never investigated.

To assess this, we compared commensal *E. coli* strains isolated from the livestock environment with *E. coli* strains responsible for clinical infections in livestock. We found that pathogenic isolates have significantly higher MIC values for GLY but not for RU, supporting the data described in the literature to some extent.

This is likely to be explained by superior stress responses in pathogenic bacteria (Chowdhury et al., 1996; McKellar and Knight, 1999). Therefore, the capacity to adapt to changes can lead to a decreased susceptibility (Chowdhury et al., 1996; Poole, 2012).

In contrast, some ingredients in the formulation seem to eliminate the advantages pathogenic bacteria have, with all isolates generally showing less sensitivity to RU.

In conclusion, higher MICs for GLY in pathogens can be a side effect of the overall benefits to adapt as mentioned before and does not necessarily imply resistance to glyphosate itself. A closer look into the genetics of the resistance mechanisms and the target structure of the herbicide are required for further studies.

Host Impact

There are statistically significant differences between the host species of the *E. coli* strains, both for GLY and RU in all tests and calculated models. Bacteria isolated from poultry showed higher MICs compared to isolates from cattle and pig.

Farm animals have individually composed feed, and accordingly, different levels of glyphosate exposure. Herbicide residues in feed can lead to the exposure of livestock-related bacteria to glyphosate and other compounds of formulations.

Little data is available about the amount of residues in feed, however, imported soybean meal seems to be the main source (von Soosten et al., 2016). Glyphosate has been found in poultry and cattle feed in Germany (Shehata et al., 2014) and in a study in cattle feed (Schnabel et al., 2017).

Poultry are typically fed with corn, wheat and barley, often supplemented with soy as a protein source. However, soy is also commonly used in pig and cattle feed. Therefore, exposure to glyphosate was possible for all hosts, though concentrations in the environment are considerably lower than in the conducted experiment.

Even though statistical analysis revealed MIC differences in the active ingredient and the formulation, there is currently no explanation for this. Data about residues in the feed and possible glyphosate exposure are lacking. Overall, reasons for varying susceptibility of *E. coli* isolates for glyphosate between the host species have to be elucidated in further investigations.

Extended Spectrum Beta Lactamase (ESBL)/Non-ESBL

In our dataset, non-ESBL isolates had higher MIC values for GLY, whereas for RU no difference between ESBL and non-ESBL could be observed.

Although ESBL isolates have a resistance to β-lactam antibiotics, it was not accompanied by a higher tolerance of glyphosate. On the contrary, the MIC for GLY is lower in ESBL isolates. This is likely to be explained by the very different mechanisms behind these two resistances. Glyphosate affects the shikimate pathway and disrupts the formation of aromatic amino acids necessary for bacterial protein synthesis (Steinrücken and Amrhein, 1980; Herrmann, 1995), whereas in ESBL 3rd and 4th generation β-lactam antibiotics are hydrolyzed (Pfeifer et al., 2010).

However, non-target site resistance can further affect other antimicrobials, as seen for example in biocide-antibiotic cross-resistances or cross-tolerance (Poole, 2012; Capita et al., 2014). Exposing *E. coli* to sub-lethal glyphosate concentrations in form of the formulation Roundup weed killer changed antibiotic susceptibility in both directions (Kurenbach et al., 2015) and adaptive resistance was mostly obtained through efflux pumps (Kurenbach et al., 2017). On the contrary, exposure to the biocide triclosan had no effects on unrelated antimicrobials (Ledder et al., 2006).

Different MICs could further be explained by fitness costs, which can accompany antibiotic resistances in bacteria (Melnyk et al., 2015).

Finally, a distortion in our isolate selection, as 12 of 15 pathogenic poultry isolates are non-ESBL (with higher MICs in pathogenic and poultry isolates as mentioned above) could explain the difference. However, the difference is not only present in the non-parametric test but indeed supported by the statistical model.

The surfactants present in RU could compensate for differences between ESBL and non-ESBL strains, explaining the similar higher MICs for the formulation.

In order to gain more clarity on the link of antibiotic and glyphosate tolerance, future studies should as well investigate effects of non-target resistances and include further antibiotics (e.g., tetracycline, macrolides, or aminoglycosides).

In conclusion, we conducted a large-scale screening for GLY and RU susceptibilities in 238 isolates of *E. coli*. We found small but statistically significant differences between the tested formulation RU and the pure glyphosate salt as well as between poultry and other host animals with higher MIC

values in Roundup and poultry. Furthermore, for glyphosate, we observed differences between non-ESBL and ESBL, and between pathogenic and commensal isolates (higher MICs in the former group). The difference between recently sampled isolates and the historic ECOR collection from 1984 was only found when the Mann-Whitney-U test for GLY was applied, but this finding was not confirmed by the modeling.

While this pilot screening yielded intriguing results indicative of the relationship between different groups of *E. coli* and changes in sensitivity to GLY/RU, further detailed investigations are required. These would ideally include inter-laboratory repetitions on a larger number of isolates to determine the precision of the susceptibility testing, and sequencing of the isolates with the MIC values above the cut-off, as well as the analysis of their gene expression.

Importantly, further investigations are needed to determine whether the observed differences are due to glyphosate use and/or application of antibiotics, other biocides and heavy metals or inter-strain diversity (Moorman et al., 1992).

Furthermore, investigations into whether the presence of glyphosate residues in feed leads to the accumulation of pathogenic bacteria in livestock animals or in livestock farms are currently ongoing.

AUTHOR CONTRIBUTIONS

KB performed the experiments, collected, analyzed, and interpreted the data, drafted the manuscript and figures, with critical evaluation and support of all other authors. JP performed the experiments and collected the data. RM contributed to the statistical data analysis, and wrote sections of the manuscript. OM and UR conceived and designed the study, and critically revised the manuscript. All the authors approved the final version to be published.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00932/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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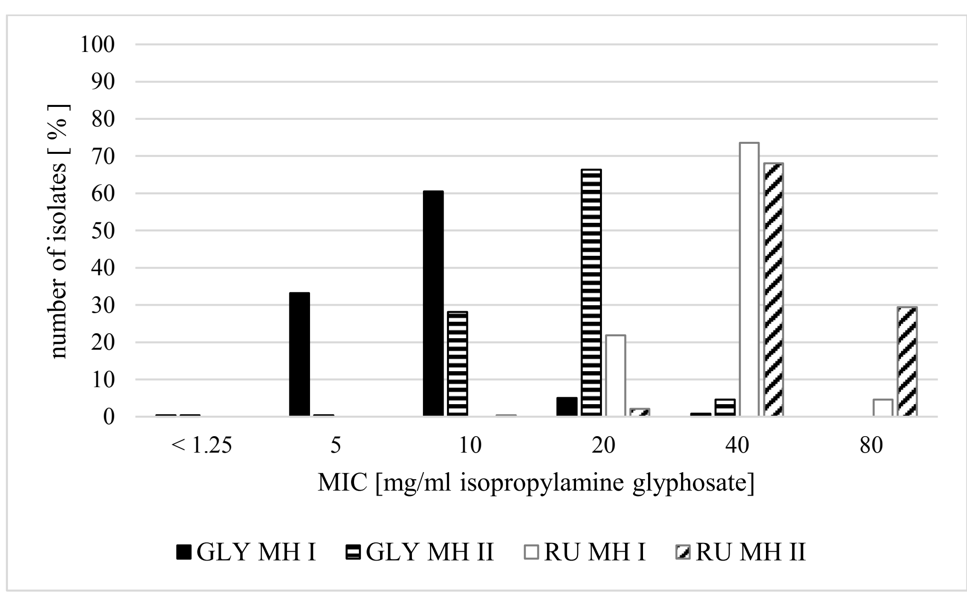
Supplementary Material



Supplementary Figure 1. MIC for glyphosate isopropylamine salt in MH I (GLY MH I, black) and in MH II (GLY MH II, black with white stripes) and for the formulation Roundup LB Plus in MH I (RU MH I, white) and in MH II (RU MH II, white with black oblique stripes), respectively.

Supplementary Table 1. Effect of different parameters in MIC by means of univariable nonparametric Mann-Whitney-U test in MH II for glyphosate isopropylamine salt (GLY) and Roundup LB Plus (RU) as well as Wilcoxon test for differences between the MIC in MH I and MH II. Statistically significant *P*-values <0.05 are in bold. Parameters with higher MICs are underlined.

comparison of		$GLY_{MH\ II}$ *P*-value	$RU_{MH\ II}$ *P*-value
		Mann-Whitney-U test	
historic isolates	recent isolates	0,051	0,961
ECOR collection	Commensal *E. coli*	0,062	0,871
ECOR collection	Pathogenic *E. coli*	0,114	0,791
Pathogenic *E. coli*	Commensal *E. coli*	0,994	0,614
non-ESBL	ESBL	0,237	0,215
<u>poultry</u>	pig	**0,032**	**0,005**
<u>poultry</u>	cattle	**0,026**	**0,005**
pig	cattle	0,965	0,939
		Wilcoxon test	
MH I	MH II	**<0,001**	**<0,001**
Median and Mode [mg/ml]		20	40
MIC_{95} [mg/ml]		20	80

RESEARCH PART II: EFFECTS OF GLYPHOSATE ON *E. COLI* AND *S.* TYPHIMURIUM IN A RUMEN FERMENTER MODEL

3.1 Study Outline

The intestinal microbiota of livestock is likely to be exposed to GLY through residues in their feed. Since bacteria have different susceptibilities to the herbicide, it can change microbial communities. Moreover, pathogenic bacteria have been described as less susceptible to glyphosate (Krüger et al., 2013b; Shehata et al., 2013), which could increase the risk for (zoonotic) diseases. From a quantitative point of view, food products are the most important cause for transmission of antimicrobial resistances from livestock to humans, according to Aarestrup et al. (2008). Preventative efforts are particularly focused on *E. coli* and *Salmonella (S.) enterica* serovar Typhimurium (*S.* Typhimurium).

To investigate effects of GLY on these two pathogenic agents, our study had two major goals:

i. Determine a possible advantage for *S.* Typhimurium or pathogenic *E. coli* after oral intake, when glyphosate in the form of RU is present.

ii. Investigate a possible resistance induction against glyphosate or antibiotics after exposure to low, sub-lethal glyphosate concentrations.

The *in vitro* system of the RUSITEC was used as described by Riede et al. (2016) and the experiment was carried out at the University of Veterinary Medicine Hannover, Foundation, Germany.

For inoculation, three rumen-fistulated, non-lactating Holstein-Friesian cows from the Friedrich-Loeffler-Institute (FLI) in Braunschweig, Germany, provided in total 5 l of fresh rumen content. After transport in a thermo box to ensure steady temperatures, the mixed content was gauze filtrated to separate liquid and solid phases. The liquid was divided into six fermenter vessels (each with V = 730 ml). Nylon bags were either filled with 70 g of solid content or 15 g of fresh substrate (49.5% grass silage, 39.7% maize silage, 5% wheat meal, 5% soy cake and 0.8% mineral feed). Each fermentation vessel was equipped with two nylon bags, one of each kind. All bags were replaced with a fresh bag after a retention time of 48 h, except for the bag with solid ingesta, which was replaced after 24 h. The bags were continuously moved up and down by an electronic motor to imitate ruminal movement. In addition, the RUSITEC contained a continuous influx of buffer solution, simulating the saliva of a cow. The used liquid was collected in a glass flask. The amounts of influx and efflux were measured daily and corresponded to approximately one turnover (730 ml) per day. The adaptation of the whole system lasted one week to ensure steady-state conditions before the experiment was started. Ruminal metabolism (pH, redox potential) was checked daily, while amounts of ammonium and short-chain fatty acids (SCFA) were assessed shortly before the experiment was started.

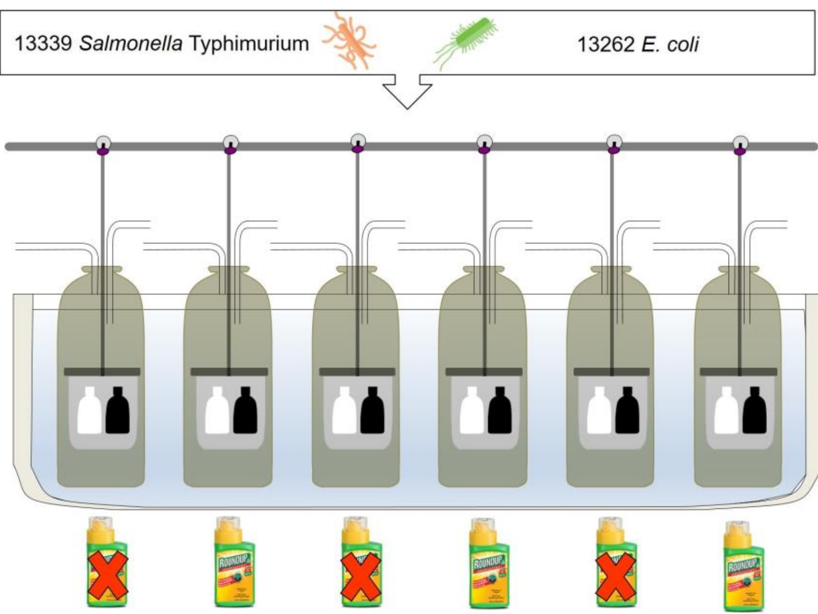


Figure 3.1*: Experimental set-up of the RUSITEC with rumen content from three fistulated cows divided into six fermentation vessels.*

For inoculation of the fermenter vessels, one *E. coli* and one *S.* Typhimurium isolate was used, respectively (Table 3.1). The *E. coli* with a MIC of 80 mg/ml IPA in RU neutralized to pH 7 was originally isolated from a cow suffering from acute mastitis and provided by the German Federal Office of Consumer Protection and Food Safety (BVL). The *S.* Typhimurium DT104 isolate with a MIC of 80 mg/ml IPA in RU at pH 7 originated from a pig and was provided by the German Federal Institute for Risk Assessment (BfR). In contrast to the MIC screening in part I of this study, the susceptibility testing for the strains was conducted in pH-adjusted media to reflect the conditions in the fermenter as well as the animal.

Table 3.1*: Isolates used to inoculate the in vitro rumen fermentation system (RUSITEC). MIC= Minimum inhibitory concentration, RU= Roundup® LB Plus*

Species	Original Host	MIC RU	MIC RU pH7	Selectivity resistances
S. Typhimurium DT104	Pig	80 mg/ml	80 mg/ml	Nalidixic acid
E. coli	Cow	40 mg/ml	80 mg/ml	Enrofloxacin, Cefotaxime

After inoculation of all fermentation vessels with approximately 10^9 CFU of each isolate, RU was added into every second fermenter to obtain a worst-case concentration of 10 mg/l (Figure 3.1). The concentration was chosen based on the tenfold increased average daily intake measured by Schnabel et al. (2017). This equalled 203 µl of a 1:10 dilution of RU containing 360 g glyphosate/l for each RU fermenter. Samples were taken directly after inoculation of the bacteria as well as after 0.5 h, 2 h, 4 h, 8 h, 12 h, 24 h, 48 h, 72 h, 96 h, 120 h, 144 h and 168 h (Figure 3.2).

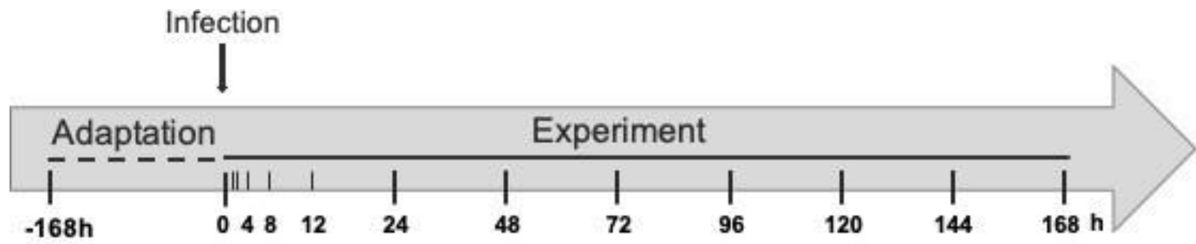


***Figure 3.2**: Overview of the sampling scheme in the fermenter experiment. One sample was taken before and immediately after inoculation. Further samples were taken during the experimental phase at 0.5 h, 2 h, 4 h, 8 h, 12 h, 24 h, 48 h, 72 h, 96 h, 120 h, 144 h and 168 h post-inoculation.*

3.2 Bacteria Selection and Susceptibility Testing

Samples were serially diluted and plated on selective agars, in order to observe the CFUs of bacteria in the fermenter.

3.2.1 E. coli Determination

CHROMagar® Orientation (Merck KgaA, Darmstadt, Germany) is a chromogenic agar, by which different classes of bacteria can easily be distinguished by colour. It has a specificity of over 99% for *E. coli*, which characteristically grows in dark pink or reddish colonies.

To prevent overgrowing on these non-selective agar plates, antibiotic resistances of the *E. coli* strain against enrofloxacin and cefotaxime were used to select for the specific isolate. The breakpoint for cefotaxime in Enterobacteriaceae is 2 mg/l (EUCAST, 2019). To screen for resistant extended-spectrum beta-lactamase (ESBL)-*E. coli*, it is recommended to use this concentration in the agar plates. In addition, 4 µg/ml of enrofloxacin were added, which corresponded to the equivalent breakpoint for enrofloxacin (CLSI, 2018). Before inoculation, rumen fluid was plated onto the agar to ensure no other resistant *E. coli* were growing on these plates.

3.2.2 *S.* Typhimurium Determination

To enumerate the *S.* Typhimurium bacteria in the fermenter, XLD media (Oxoid GmbH, Wesel, Germany) was chosen. It is commonly used for *Salmonella sp.* diagnostics, because accompanying bacterial flora is suppressed. Moreover, *Salmonella sp.* grow in characteristic black colonies and cause the agar to change from red to pink.

3.2.3 Susceptibility Testing via VITEK® 2

Automated systems for AST, such as VITEK® 2 by bioMérieux (bioMérieux Deutschland GmbH, Nuertingen, Germany) are commercially available for commonly used antibiotics. VITEK® 2 uses compact plastic reagent cards containing different antibiotics. Antibiotics commonly used in human medicine can be tested with test card AST N-248 (Table 3.2). This test was applied to determine MICs of the initially inoculated strains (ancestors) and compare them to isolates from the fermentation vessels after exposure to RU.

***Table 3.2**: Antibiotics tested with the VITEK®2 test card AST N-248.*

Amikacin	Cefotaxime	Fosfomycin	Moxifloxacin
Cefepime	Gentamicin	Tobramycin	Ciprofloxacin
Imipenem	Meropenem	Piperacillin	Piperacillin/Tazobactam
Aztreonam	Tigecycline	Ceftazidime	Trimethoprim/Sulfomethoxazole

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

Katrin Bote[1*], Judith Pöppe[1], Susanne Riede[2], Gerhard Breves[2] and Uwe Roesler[1]

[1]*Institute for Animal Hygiene and Environmental Health, Freie Universität Berlin, Berlin, Germany*
[2]*Institute for Physiology and Cell Biology, University of Veterinary Medicine, Hannover, Germany*

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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

*Author for correspondence: Institute for Animal Hygiene and Environmental Health, Robert-von-Ostertag-St. 7–13, 14163, Berlin, Germany; E-mail: Katrin.Bote@fu-berlin.de and E-mail: tierhygiene@vetmed.fu-berlin.de; Phone: +49 30 838 51845; Fax: +49 30 838 451863.

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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 $bla_{CTX\text{-}M}$, bla_{SHV}, bla_{TEM} and the CIT-type

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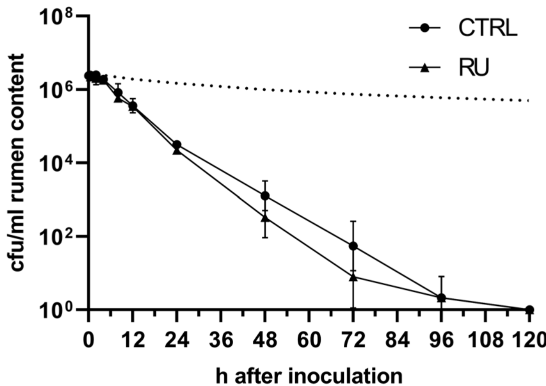

Figure 1. *E. coli* in the fermentation vessels measured by standard dilution plating on CHROMagar supplemented with 4 μg/mL enrofloxacin and 2 μg/mL cefotaxime. Control group (CTRL) without any glyphosate compared to the group treated with a worst-case amount of glyphosate in the formulation Roundup 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,

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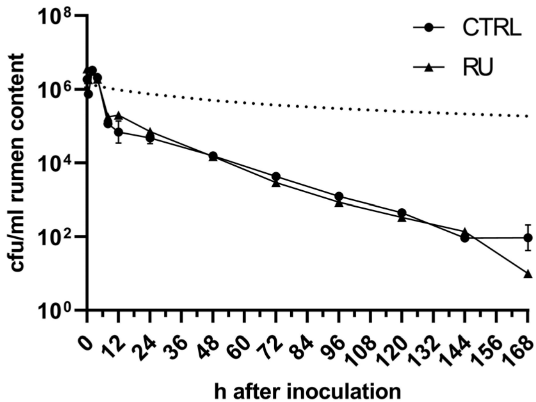

Figure 2. *Salmonella* ser. Typhimurium in the fermentation vessels measured by standard dilution plating XLD agar. Control group (CTRL) without any glyphosate compared to the group treated with a worst-case amount of glyphosate in the formulation Roundup LB Plus (10 mg/L, RU). The dotted line represents 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

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

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 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

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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)

	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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RESEARCH PART III: UNPUBLISHED DATA

4.1 MIC in pH-adjusted Media

When GLY or RU is added to a medium the pH decreases depending on the added concentration. In GLY and RU solutions with 80 mg/ml IPA the pH levels were 4.5 and 4.6, respectively. To determine possible influences of the pH on the MIC, the acidic condition in the MH I media was neutralized with a 5 molar sodium hydroxide (NaOH) solution, and susceptibility testing was repeated for a small number of isolates. In most cases, the MIC in neutral medium was higher for both GLY and RU. Almost all of the tested strains showed a MIC close to 80 mg/ml (Table 4.1).

***Table 4.1**: Comparison of minimum inhibitory concentrations (MICs) in Mueller Hinton I medium with and without pH adjustment for selected E. coli. GLY= Glyphosate isopropylamine salt, RU= Roundup® LB Plus*

Isolate	MIC GLY	MIC Gly pH7	MIC RU	MIC RU pH7
12596	<1.25 mg/ml	<1.17 mg/ml	10 mg/ml	4.7 mg/ml
12603	10 mg/ml	74 mg/ml	40 mg/ml	75 mg/ml
12604	5 mg/ml	74 mg/ml	40 mg/ml	75 mg/ml
12606	20 mg/ml	74 mg/ml	40 mg/ml	75 mg/ml
12677	20 mg/ml	74 mg/ml	20 mg/ml	37 mg/ml
12678	40 mg/ml	75 mg/ml	20 mg/ml	75 mg/ml
13227	10 mg/ml	74 mg/ml	20 mg/ml	75 mg/ml
13228	20 mg/ml	74 mg/ml	20 mg/ml	75 mg/ml

In addition, we compared the growth of a few *E. coli* strains with GLY and RU with and without pH adjustment. One example for GLY is shown in Figure 4.1. Our results suggest that GLY acts differently on bacteria depending on the pH of the medium. Since GLY is an amphoteric molecule with different pK_a-values (pK_a= the negative logarithmic acid dissociation constant), its conformation changes according to the pH, which could influence its effect on bacteria. Therefore, it is possible that the activity of GLY on bacteria depends on the pH.

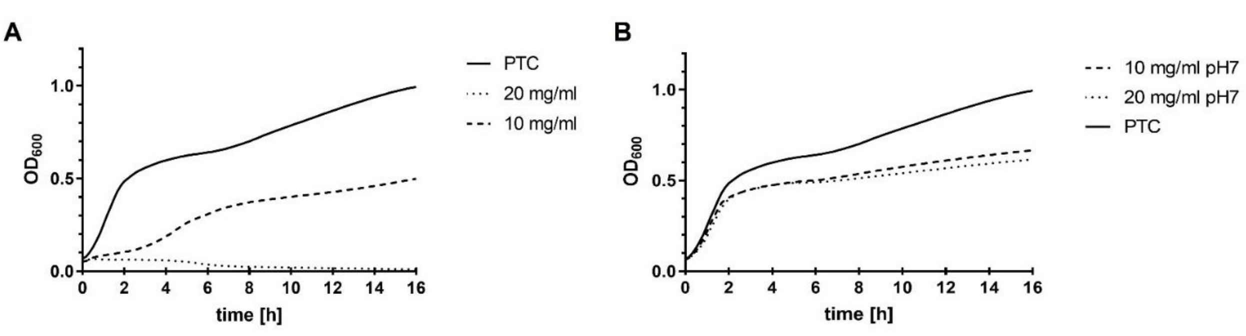

Figure 4.1: *Growth curves of E. coli 12687 in Mueller Hinton I (A) medium with 10 and 20 mg/ml of glyphosate isopropylamine salt (A) as well as in media adjusted to pH 7 (B). PTC = positive control*

4.2 MIC in other Species

As previously described, isolates seem to differ in their susceptibility for GLY and RU. We performed MIC determinations for two *Enterococcus faecalis* isolates, provided by the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ), and one *Enterobacter cloacae* isolate, isolated from a fattening turkey by the Institute for Animal Hygiene and Environmental Health, Freie Universität Berlin, Germany, for a different project (Table 4.2).

In the gram-positive *Enterococci* the MIC for GLY is below the detection limit of 1.25 mg/ml on our plates. For RU and for the gram-negative *Enterobacter* values are similar to *E. coli*. This could suggest that gram-positive bacteria are in general more susceptible to GLY. However, further investigations of different strains and species are warranted.

Table 4.2*: MIC for Enterococcus faecalis and Enterobacter cloacae. GLY= Glyphosate isopropylamine salt, RU= Roundup® LB Plus*

Isolate	MIC GLY	MIC RU
Enterococcus faecalis DSM-6134	< 1.25 mg/ml	10 mg/ml
Enterococcus faecalis DSM-20478	< 1.25 mg/ml	20 mg/ml
Enterobacter cloacae	20 mg/ml	40 mg/ml

4.3 Sequencing of Isolates of Interest

Some *E. coli* isolates from the initial MIC screening were sent to LGC (LGC Genomics GmbH, Berlin, Germany) for sequencing via Illumina NextSeq 500 V2. The *aroA* gene of the isolates above the 95% cut-off value as well as one isolate with a MIC of < 1.25 mg/ml for glyphosate, an isolate with a median MIC, and a standard K-12 MG1655 strain were compared (Table 4.3).

Table 4.3*: Isolates that had been considered interesting for comparison of the aroA gene and the origin of the sequence. MIC= Minimum inhibitory concentration, GLY= Glyphosate isopropylamine salt, RU= Roundup® LB Plus, ECOR= E.coli collection of reference*

Strain	Origin	MIC GLY	MIC RU	Info	Sequence
13233	Pig, pathogen	10 mg/ml	40 mg/ml	Median/Mode	LGC
K-12 MG1655	Standard	-	-	Standard strain	Online data base
12596	Human, ECOR	<1.25 mg/ml		Lowest MIC	Online data base
13281	Cattle, pathogen	40 mg/ml		> 95% cut-off GLY	LGC
13282	Cattle, pathogen	40 mg/ml		> 95% cut-off GLY	LGC
12657	Poultry, commensal		80 mg/ml	> 95% cut-off RU	LGC
12663	Poultry, commensal		80 mg/ml	> 95% cut-off RU	LGC
12685	Poultry, commensal		80 mg/ml	> 95% cut-off RU	LGC
13296	Poultry, pathogen		80 mg/ml	> 95% cut-off RU	LGC
13298	Poultry, pathogen		80 mg/ml	> 95% cut-off RU	LGC
13303	Poultry, pathogen		80 mg/ml	> 95% cut-off RU	LGC
13304	Poultry, pathogen		80 mg/ml	> 95% cut-off RU	LGC
13308	Poultry, pathogen		80 mg/ml	> 95% cut-off RU	LGC
13257	Pig, pathogen		80 mg/ml	> 95% cut-off RU	BioGenDV

12628	Human, ECOR		80 mg/ml	> 95% cut-off RU	Online data base
12642	Human, ECOR		80 mg/ml	> 95% cut-off RU	Online data base

The raw sequencing data from the Illumina sequencer were assembled and scaffolded via SPAdes by LGC. To obtain the gene sequences, the FASTA files delivered by the company were annotated to genes via Rapid Annotation using Subsystem Technology (RAST) Version 2.0.

Annotated sequence data were further analysed with Geneious Prime® 2020.0.2. For each isolate listed in Table 4.3, the *aroA* gene was extracted (as 3-phosphoshikimate 1-carboxyvinyltransferase (EC 2.5.1.19) CDS) and aligned to the isolate 13233, which was used as a reference for mapping due to its MIC values representing the median and mode.

All Single Nucleotide Polymorphisms (SNPs, see Annex) were checked for possible changes of the amino acid sequence after translation. SNPs leading to differences in the amino acid sequence are shown in Table 4.4.

Table 4.4*: Single nucleotide polymorphisms (SNPs) of the aroA gene with impact on amino acid sequence in comparison to E.coli isolate 13233 (with mean minimum inhibitory concentration (MIC) values). A= Adenine, C= Cytosine, G= Guanine, T= Thymine. Ala = Alanine, Arg= Arginine, Asn= Asparagine, Asp= Aspartic acid, Gln= Glutamine, Glu= Glutamic acid, Gly= Glycine, Leu= Leucine, Lys= Lysine, Phe= Phenylalanine, Pro= Proline, Ser= Serine, Thr= Threonine, Tyr= Tyrosine*

Strain	**SNP position**														
	31	172	188	256	263	404	466	489	767	862	1094	1125	1198	1276	1280
13233	A	A	G	G	T	T	G	C	A	T	A	C	C	G	C
K-12	C														
12596	C														
13281	C	G						A					G	C	G
13282	C														
12657	C		A	A	G	A			G	C	T	A	G	C	G
12663	C			A	G	A			G		T	A		C	G
12685	C	G					A								
13296	C		A	A	G	A			G						
13298	C			A	G	A			G		T	A		C	G
13303	C		A	A	G	A			G		T	A	G	C	G
13304	C		A	A	G	A			G		T	A		C	G
13308	C		A	A	G	A			G	C					
13257	C														
12628	C			A							T	A	G		
12642	C			A	G	A			G		T	A			
	Amino acid position														
	11	58	63	86	88	135	156	163	256	288	365	375	400	426	427
13233	Ser	Thr	Ser	Gly	Leu	Leu	Gly	Asp	Lys	Lys	Tyr	Asn	Pro	Ala	Ala
→	Arg	Ala	Asn	Ser	Arg	Gln	Ser	Glu	Arg	Arg	Phe	Lys	Ala	Pro	Gly

4.4 ESBL Resistance Genes in Strains from the Fermentation Experiment

E. coli isolates from each fermentation vessel, obtained at the last sampling point where detection was still possible, were further analysed for their ESBL resistance genes via multiplex real-time Polymerase Chain Reduction (PCR), according to Roschanski et al. (2014). There was no change in resistance against β-lactam antibiotics in *E. coli* strains before and after the experiment. All strains were positive for the $bla_{CTX\text{-}M}$ gene, responsible for the resistance phenotype.

4.5 *In vivo* Investigation of Glyphosate Effects on Bacteria in Pigs

The intestinal microbiota plays an important role in normal gut function and maintaining host health (Aluthge et al., 2019; Pluske et al., 2018). Due to the different susceptibilities in bacteria, glyphosate is suspected to have an impact on the microbial compositions affecting the microbial balance towards dysbiosis (Ackermann et al., 2015; Aitbali et al., 2018; Krüger et al., 2013b; Lozano et al., 2018; Mao et al., 2018; Motto et al., 2018). Numerous *in vivo* feeding studies conducted with glyphosate or GBHs in rats and mice showed differences in bacterial species composition after exposure (Aitbali et al., 2018; Lozano et al., 2018; Mao et al., 2018; Nielsen et al., 2018). In bees, glyphosate reduced the total number of bacteria in the gut and interfered with early gut colonization, leading to a loss of protective bacterial species (Motto et al., 2018). Hence, similar to antibiotics, residues of GBHs could increase the opportunities for pathogenic microorganisms to colonize and trigger disease (Fouhse et al., 2016; Schokker et al., 2014). Furthermore, they could induce changes in resistance patterns against glyphosate or other antimicrobials (Kurenbach et al., 2015, 2017).

In recent years, metagenomic approaches have been used to investigate the pig microbiome (Frese et al., 2015; Kim et al., 2011, 2015; Zhao et al., 2015) and possible changes after exposure to biological or chemical agents (Argüello et al., 2018, 2019; Bearson et al., 2013; Borewicz et al., 2015; De Rodas et al., 2018; Tilocca et al., 2017). However, changes in the microbiome due to glyphosate or GBHs have not been analysed to date. Therefore, the following questions were investigated:

i) Do residues of GLY or a ready-to-use GBH in a worst-case concentration have an impact on the shedding of *S.* Typhimurium and ESBL-*E. coli*?

ii) Could worst-case concentrations induce resistance for GLY or a GBH?

iii) How does the exposure affect the microbial community?

4.5.1 Study Outline

The animal study was permitted by the State Office of Health and Social Affairs Berlin, Germany (Landesamt für Gesundheit und Soziales Berlin, G0318/17). An overview of the experimental design and the sample collection is shown in Figure 4.2.

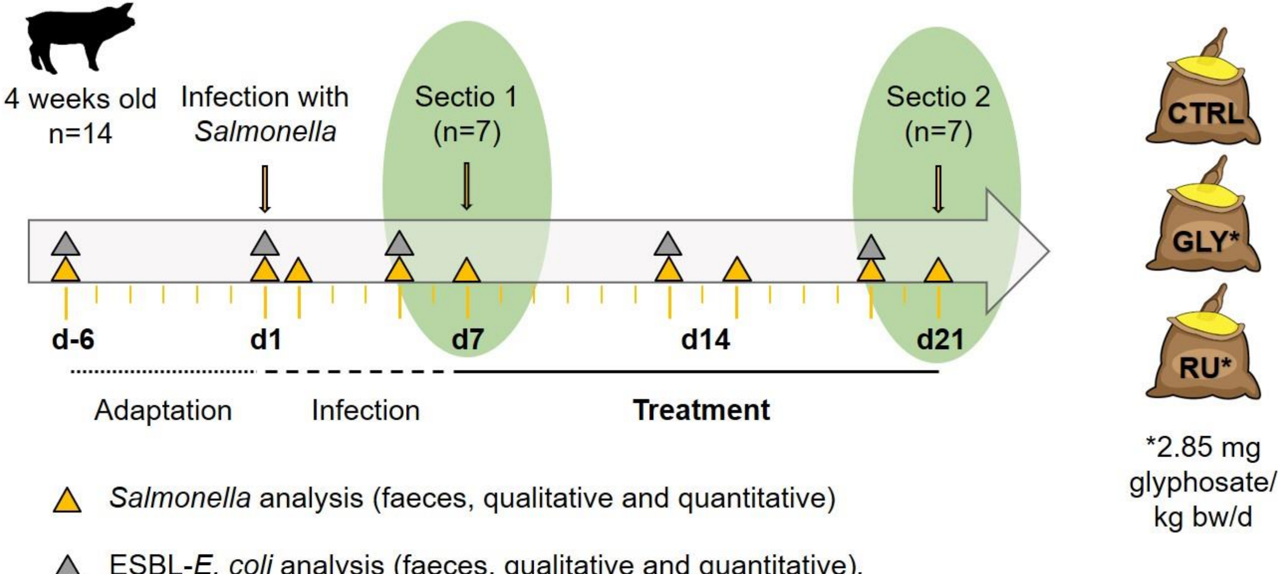

***Figure 4.2**: Timeline of the experimental animal infection, treatment period and sample collection. ESBL= extended-spectrum beta-lactamase, CTRL= control group, RU= group fed with Roundup® LB Plus, GLY= group fed with glyphosate isopropylamine salt*

In total, 42 weaning piglets (German Landrace) from serologically *Salmonella*-free breeding conducted at the Institute of Animal Nutrition at the Freie Universität Berlin, Germany, were obtained in three passes at 28 days of age. Groups consisted of 14 piglets each, with equal amounts of males and female. Piglets were littered from at least three different sows and selected for comparable weights and health status. Post-partum faeces of the mother sows as well as faeces of the piglets, collected one day before weaning, were bacteriologically tested negative for *Salmonella*.

To prevent glyphosate residues, piglets were fed with organic feed (Ferkelkorn, Meika Tierernährung GmbH, Germany) and water ad libitum, starting from two weeks before weaning as supplementary feed for familiarization, until the end of the experiment.

After weaning, all pigs were housed in the experimental animal facility of the Centre for Infection Medicine at the Department for Veterinary Medicine of the Freie Universität Berlin, Germany. Following six days of adaptation, animals were infected with about 10^8 CFU of a porcine multi-resistant *S.* Typhimurium DT104 strain with an additional nalidixic acid resistance, in conformity with methods described in the literature (Roesler et al., 2004).

For this, a single colony of the isolate was picked and incubated overnight in 5 ml of MH broth at 37 °C with 200 rpm. On the day of the inoculation, the overnight culture was diluted 1:100 and grown until OD600 reached 0.7-0.8 (equivalent to about 10^8 CFU/ml). 1 ml of the subculture was drawn into a syringe with 19 ml of physiological saline solution and kept on ice until oral inoculation via a feeding tube, which was placed after sedation with azaperone i.m. (2 mg/kg bw; Stresnil; Janssen-Cilag GmbH, Germany). CFUs were determined by plating different dilutions on MH agar.

The pig's health status was checked daily. For qualitative and quantitative *Salmonella* investigation, faecal samples were obtained through sterile dry cotton swabs at day 1, day 2, day 5, day 7, day 13, day 19 and day 21 after inoculation. In addition, samples were checked for naturally occurring ESBL *E. coli* once weekly (day 1, day 5, day 7, day 19, day 21). The rectal temperature and faeces consistency were checked during every faecal sampling. Blood sampling and weighing of the pigs were conducted weekly. Serum samples were frozen at -20 °C.

One week after infection, half of the animals of each group (n=7) were sacrificed as an internal control. For this purpose, pigs were sedated with azaperone i.m. (2 mg/kg bw; 'Stresnil'; Janssen-Cilag GmbH, Germany) and put under general anaesthesia with a mixture of ketamine (15 mg/kg bw; 'Ursotamin'; Serumwerk Bernburg, Germany), xylazine i.m. (2 mg/kg bw; 'Xylavet'; cp- pharma, Germany) and diazepam i.v. (0.5 mg/kg bw; Ratiopharm GmbH, Germany). Under deep anaesthesia, pigs were sacrificed through an intracardial injection of tetracaine hydrochloride, mebezonium iodide and embutramide (0.12 ml/kg bw; 'T61', Intervet, Germany). Once death was confirmed, samples were taken under sterile conditions. Tonsils, mandibular lymph nodes and the ileocaecal lymph node were collected in toto. Partial organ samples were taken from the spleen, ileum, caecum, jejunum (middle part) and colon (conal part), while faeces was collected from the rectum. In addition, bile samples were taken through fine needle aspiration. The same procedure was applied to the remaining pigs at the final necropsy at the end of the experiment (day 21).

Treatment of the groups

One group was treated with a worst-case GLY concentration (40% monoisopropylamine salt solution of glyphosate; Sigma-Aldrich Chemie GmbH, Germany), while a second group received a worst-case concentration of RU (monoisopropylamine glyphosate salt-containing formulation, Roundup® LB plus; German registration number 024142-00), and a third group did not receive herbicide treatment and served as a control (CTRL). Daily oral administration of a worst-case residual dosage of 2.85 mg of glyphosate/kg bw/d (EFSA, 2018) started one week after infection with *Salmonella* and lasted for two weeks. The mean weight of all pigs in the group was used for dosage calculations and adjusted weekly.

4.5.2 Bacteriological Examination

All samples were checked for *Salmonella*. Faeces and caecum content samples were additionally checked for ESBL *E. coli*.

The samples were processed according to the following protocol. Faecal samples and ileocecal lymph nodes were processed natively, while all other organs were first decontaminated externally by flaming with 96% ethanol and subsequently cut into smaller pieces. Samples were diluted 1:10 with 1% Buffered Peptone Water (DM494D; Mast Group Ltd, United Kingdom), followed by homogenization for 2 min at 200 rpm with a Stomacher (Stomacher 400 circulator; Seward Limited, United Kingdom).

For quantitative analysis, samples were further diluted and plated in pairs on Xylose Lysine Deoxycholate (XLD) agar (1.05282.0500; Merck KgaA, Germany), supplemented with 50 µg/ml of nalidixic acid (CN32.2; Carl Roth GmbH + Co, Germany) for *Salmonella,* or on MacConkey (MC)-agar, supplemented with 1 µg/ml of cefotaxime (MC+), for ESBL *E. coli* (EFSA BIOHAZ Panel, 2011). Plates were incubated at 37 °C and checked after 24 h (MC+, XLD) as well as 48 h (XLD).

For qualitative analysis according to *Salmonella* detection norm, the homogenized samples were incubated for 24 h at 37 °C before 100 µl were dropped on modified semi-solid Rappaport-Vassiliadis (MSRV) media (CM1112; Oxoid, Hampshire, United Kingdom), which was further incubated at 42 °C for 24 h. Following this, material from the border of the turbid zone was streaked out on ready-to-use XLD plates (PO5057A; Oxoid, United Kingdom). MSRV plates, as well as XLD plates, were further incubated for 24 h before final evaluation. A sample was considered *Salmonella* positive, if a significantly extended grey-white turbid zone was present on MSRV and typical black colonies were visible on the XLD plate.

For qualitative ESBL *E. coli* analysis, material from the enriched overnight cultures was streaked out as a streak dilution with a 10 µl loop on MC+ agar und incubated overnight. Samples with typical pink colonies were considered positive.

4.5.3 Minimum Inhibitory Concentration Testing

MIC testing for RU and GLY, with and without pH adjustment to pH 7, was performed according to the described method in the first part of the study. One isolate from each positive quantitative or qualitative *Salmonella* detection from the final necropsy was chosen and compared to the ancestral inoculation strain.

4.5.4 Metagenomic Analysis

The metagenome is defined as the entirety of all genetic material in an environmental sample. Metagenomics are sequence-based methods for analysing the genomes present in complex environmental samples. The advantage of these methods is direct sampling, leading to faster and more accurate results. Moreover, many isolates cannot be detected with culture-based methods. In general, there are two different approaches to metagenomics:

Amplicon sequencing: Only specific genes (marker genes) are sequenced (i.e. 16S ribosomal ribonucleic acid (rRNA)). The method is well-established and relatively cheap, although it can be biased due to a focus on specific regions. Furthermore, it does not determine functional information.

Whole-genome shotgun sequencing: All genes of all organisms contained in the sample are sequenced. The analysis is more complex and expensive. A massive amount of data is generated, requiring high computational analysis.

16S rRNA Sequencing

The 16S rRNA is a component of the small subunit of prokaryotic ribosomes. It consists of approximately 1500 bp and possesses highly conserved as well as nine unique regions (V1-V9). These unique regions make it possible to distinguish between different species.

After DNA isolation of a sample, the specific hypervariable regions from the 16S rRNA (30-100 bp) are amplified by PCR, using universal primers attaching to the conserved regions. Amplification products are cleaned from leftover free primers as well as primer dimers, and further prepared for sequencing with the respective sequencer (library preparation: attaching indices, adapters, barcodes). Reading can be single-end or paired-end, in which both ends of a fragment are sequenced and result in higher accuracy. Depending on the used V-region, the results can vary (Kim et al., 2015). According to Yang et al. (2016), amplification of the V4-V6 region leads to the best outcome.

After pre-processing and artefact removal of the sequencing data, the massive number of reads (i.e. 10,000,000) is clustered by similarity (threshold mostly 97 %) to Operational Taxonomic Unit (OTU)s. These OTUs do not have to correlate with true phylogenetic relationships.

The α-diversity can be calculated to investigate the variety of found organisms within one sample, whereas the β-diversity is a comparison between separate samples or communities. Additionally, species classification and abundance analysis were carried out.

Sample preparation

After the necropsy, faecal and caecum samples were frozen natively in 5 ml Eppendorf Tubes® at - 20 °C and later transferred to -80 °C. Next-generation sequencing library preparations and Illumina

MiSeq sequencing were conducted at GENEWIZ. Inc. (Suzhou, China) according to the company's protocol: "V3, V4, and V5 hypervariable regions of prokaryotic 16S rDNA were selected for generating amplicons and following taxonomy analysis. The QIIME data analysis package was used for 16S rRNA data analysis. The forward and reverse reads were joined and assigned to samples based on barcode and truncated by cutting off the barcode and primer sequence. Quality filtering on joined sequences was performed and sequence which did not fulfil the following criteria were discarded: sequence length <200bp, no ambiguous bases, mean quality score >= 20. Then the sequences were compared with the reference database (RDP Gold database) using UCHIME algorithm to detect chimeric sequence, and then the chimeric sequences were removed. The effective sequences were used in the final analysis. Sequences were grouped into operational taxonomic units (OTUs) using the clustering program VSEARCH(1.9.6) against the Silva 119 database pre- clustered at 97% sequence identity. The Ribosomal Database Program (RDP) classifier was used to assign taxonomic category to all OTUs at confidence threshold of 0.8. The RDP classifier uses the Silva 119 database which has taxonomic categories predicted to the species level."

Statistical analysis

SPSS (IBM Statistics, Version 24, USA) was used for statistical analysis of the bacteriological samples. Quantitative *Salmonella* detections from all sampling time points in the RU and GLY groups were compared to the CTRL group by t-test. For qualitative comparison, Fisher's exact test was used. Significance was determined as $P < 0.05$.

RStudio (Desktop Version 1.2.5, 2019) was used for further analysis of the 16S rRNA data provided by GENEWIZ Inc. Statistical comparisons were conducted via Mann-Whitney U test.

4.5.5 Results

Animals in all groups (CTRL, RU, GLY) were infected after a seven-day adaptation period with approximately 10^8 CFU of *S.* Typhimurium DT104. One animal from the GLY group sickened during the experiment and had to undergo treatment. Therefore, it was excluded from further statistical analysis.

Clinical examination

Up to half of the animals in each group suffered from semi-liquid to liquid diarrhoea one day after infection (CTRL: 4/14. RU: 7/14. GLY: 6/14). This symptom subsided during the following week, up until day seven. At the start of the feeding experiment only one pig in the GLY group and two pigs in the RU group still showed diarrhoea. The faeces consistency normalized soon after.

Elevated body temperature over or equal to 40 °C up to 41.4 °C appeared in two pigs, both in the RU and CTRL group, one day after inoculation. All pigs showed physiological body temperature before

the beginning of the feeding experiment (Figure 4.3). The weight of all piglets increased continuously, according to weekly assessment (Table 4.5).

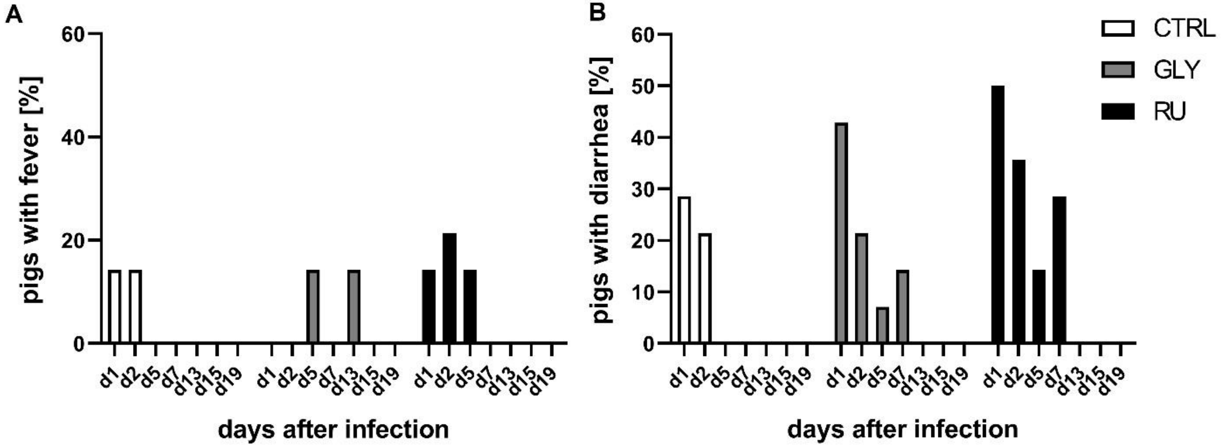

Figure 4.3: *Elevated body temperature (A) and abnormal faecal consistency (B) during the experiment, after infection with S. Typhimurium. CTRL= control group, RU= group fed with Roundup® LB Plus, GLY= group fed with glyphosate isopropylamine salt*

Table 4.5: *Mean weight and standard deviations of the pigs on different days during the experiment. CTRL= control group, RU= group fed with Roundup® LB Plus, GLY= group fed with glyphosate isopropylamine salt*

Group	Day 0	Day 5	Day 12	Day 20	Day 26
CTRL	7.41 ± 1.03 kg	7.81 ± 0.99 kg	7.97 ± 0.92 kg	10.19 ± 0.74 kg	12.39 ± 0.88 kg
RU	9.78 ± 1.05 kg	10.39 ± 0.91 kg	10.7 ± 1.00 kg	12.56 ± 1.33 kg	14.37 ± 1.51 kg
GLY	7.42 ± 1.28 kg	7.98 ± 1.25 kg	8.56 ± 1.45 kg	9.08 ± 1.30 kg	11.41 ± 1.56 kg

Faecal Shedding of *S.* Typhimurium and *E. coli*

Qualitative detection of *S.* Typhimurium was possible in the majority of pigs (86% (CTRL), 93% (RU), and 100% (GLY)) one day post-infection. All pigs tested positive by day two (RU group) or at the latest day three (Figure 4.4, A). All animals shedded ESBL *E. coli* one day after infection (Figure 4.4, B). Accordingly, quantitative detection of *E. coli* was possible for most of the pigs one day after infection. The mean of each group on the different sampling days is presented in Figure 4.5 (A,B).

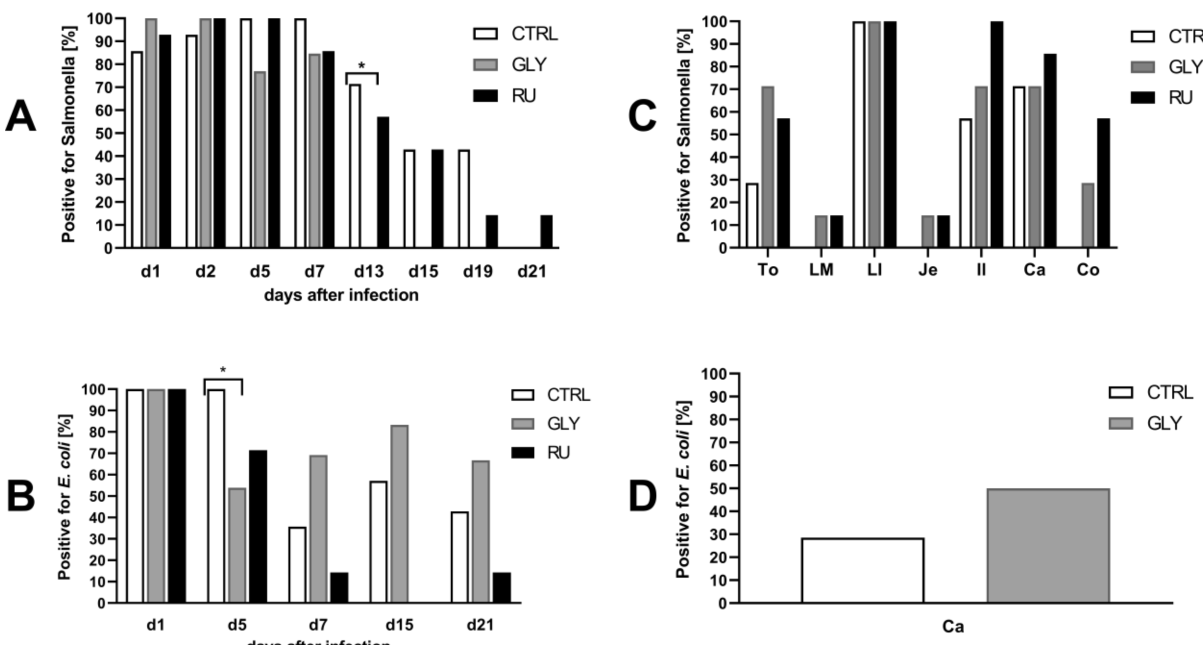

***Figure 4.4**: Qualitative evidence of S. Typhimurium (A) and E. coli (B) found in faeces at different sampling points. Comparison of positive animals (day 1-7 n=14, day 13-21 n=7) in the control (CTRL, white), the Roundup worst-case (RU, black) and the glyphosate worst-case (GLY, grey) groups. Feeding with GLY/RU started on day seven. *=Statistically significant differences (p<0.05).*

Qualitative evidence of S. Typhimurium (C) and E. coli (D) found in organs at the end of the experiment. Comparison of positive animals (n=7) in the control (CTRL, white), the RU worst-case (RU, black) and the glyphosate worst-case (GLY, grey) groups. To= tonsils, LM= mandibular lymph node, LI= ileocaecal lymph node, Je= jejunum, Il= ileum, Ca= caecum, Co= colon.

Overall, neither qualitative nor quantitative analysis revealed statistically significant differences between the RU/GLY group and the CTRL group (Table 4.6). Regarding the shedding of *S.* Typhimurium, a statistically significant difference was observed solely on day 13 post-infection. On this day, based on qualitative testing, the CTRL group had a higher number of positive pigs than the GLY group (Figure 4.4, A). In addition, increased shedding of *E. coli* was observed in the CTRL group, on day 5 based on quantitative tests (Figure 4.5, B). Moreover, qualitative tests revealed increased shedding compared to the GLY group (Figure 4.4, B).

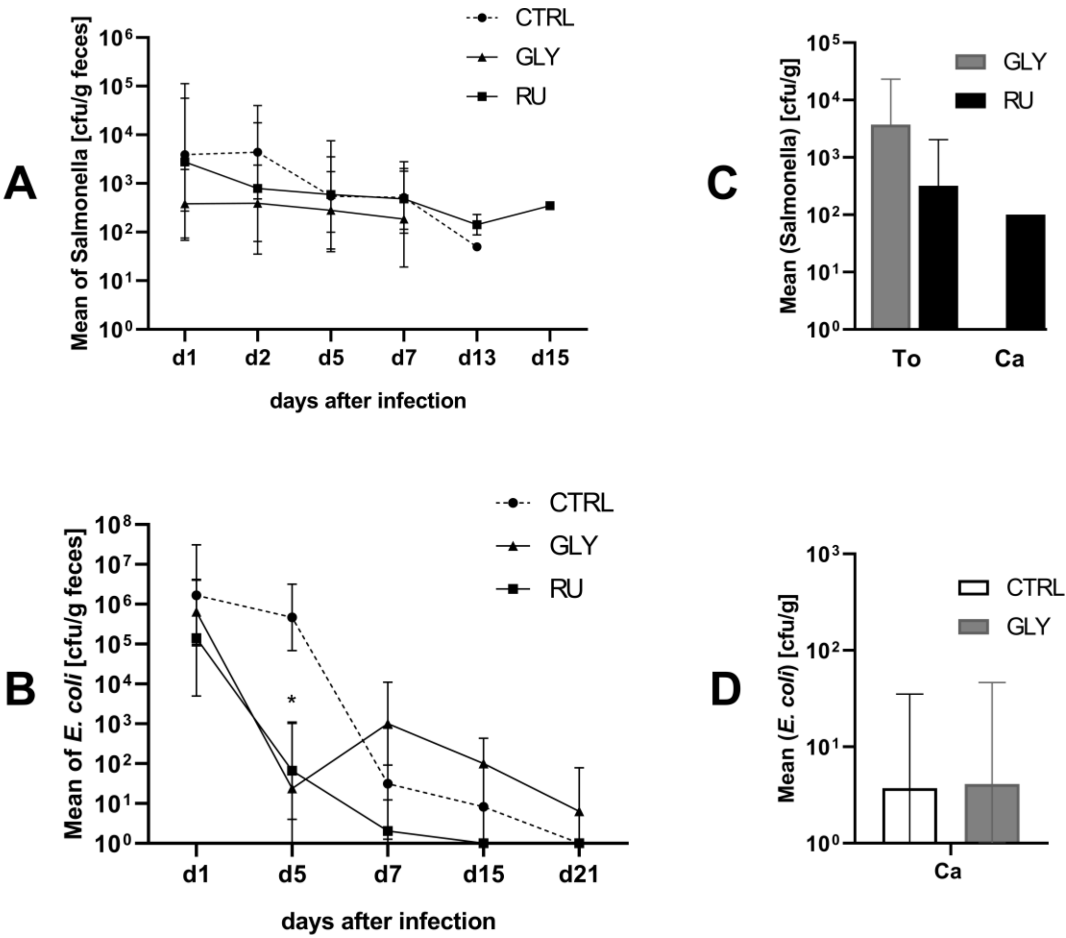

***Figure 4.5**: Quantitative evidence of S. Typhimurium (A) and E. coli (B) found in faeces after 48h (A) or 24h (B) of incubation for the control (CTRL, white), the RU worst-case (RU, black) and the glyphosate worst-case (GLY, grey) groups. Shown is log(mean) in CFU/g. *=Statistically significant differences. S. Typhimurium (C) and E. coli (D) found in organs 48h (C) of 24h (D) of incubation. To= tonsils. Ca= caecum.*

S. Typhimurium and *E. coli* in Organs

At the end of the 14-day feeding experiment, the remaining pigs were euthanized, and various organs were checked for *Salmonella* and partially for ESBL *E. coli*. Quantitatively, *Salmonella* was only detected in tonsils and caecum samples in the RU and only in tonsils in the GLY group (Figure 4.5, C). Qualitatively, *Salmonella* was detected in all ileocecal lymph nodes and, to varying degrees, in ileum, caecum, colon, jejunum, tonsil and mandibular lymph node samples (Figure 4.4, C). All spleen and bile samples were tested negative. *E. coli* was detected in a few samples from the CTRL and GLY group through quantitative and qualitative tests (Figure 4.5, D ; Figure 4.4, D). Statistically, there was no significant difference between the RU/GLY and the CTRL group (Table 4.7).

***Table 4.6**: Statistical comparison of shedded S. Typhimurium and E. coli of the RU/GLY group with the CTRL group at different time points post-infection. Quantitative analysis via t-test, qualitative analysis via chi-squared test. x = not calculable because non-existent (quantitative analysis) or equal (qualitative analysis) in both groups. Statistically significant values are displayed in bold letters (with higher values in the CTRL group). CTRL= control group, RU= group fed with Roundup® LB Plus, GLY= group fed with glyphosate isopropylamine salt*

***S.* Typhimurium**				
Days post-infection	*P*			
	RU ↔ CTRL		GLY ↔ CTRL	
	Quantitative	Qualitative	Quantitative	Qualitative
1	0.317	1	0.184	0.481
2	0.257	1	0.113	1
5	0.342	x	0.334	0.098
7	0.319	0.462	0.321	0.462
13	0.403	1	0.356	**0.021**
15	0.356	1	x	0.192
19	x	0.559	x	0.192
21	x	1	x	x
E. coli				
Days post-infection	*P*			
	RU ↔ CTRL		GLY ↔ CTRL	
	Quantitative	Qualitative	Quantitative	Qualitative
1	0.445	x	0.063	x
5	**0.003**	0.098	**0.003**	**0.006**
15	0.165	0.07	0.153	0.559
21	0.354	0.559	0.358	0.592

Table 4.7 *Statistical comparison of S. Typhimurium and E. coli in organs of the RU/GLY with the CTRL group. Quantitative analysis via t-test, qualitative analysis via chi- squared test. x = not calculable because non-existent (quantitative analysis) or equal (qualitative analysis) in both groups. To= tonsils. LM= mandibular lymph node. LI= ileocaecal lymph node. Sp= spleen. Bi= bile. Je= jejunum. Il= ileum. Ca= caecum. Co= colon. CTRL= control group, RU= group fed with Roundup® LB Plus, GLY= group fed with glyphosate isopropylamine salt*

***S.* Typhimurium**				
Organ	*P*			
	RU ↔ CTRL		GLY ↔ CTRL	
	Quantitative	Qualitative	Quantitative	Qualitative
To	0.289	0.103	0.282	0.103
LM	x	1	x	0.462
LI	x	x	x	x
Sp	x	1	x	x
Bi	x	x	x	x
Je	x	1	x	x
Il	x	0.192	x	1
Ca	0.356	1	x	1
Co	x	0.07	x	0.462
E. coli				
Organ	*P*			
	RU ↔ CTRL		GLY ↔ CTRL	
	Quantitative	Qualitative	Quantitative	Qualitative
Ca	0.172	0.462	0.577	0.592
Co	x	x	0.175	0.192

Minimum Inhibitory Concentration

The MICs for RU and GLY, with and without pH adjustment, were determined for isolates detected in caecum samples at the end of the experiment and compared to the ancestor. The MIC did not vary in any of the tested *Salmonella* variants (MIC for RU, RU pH 7, and GLY pH 7 = 80 mg/ml IPA. MIC for GLY = 40 mg/ml IPA).

4.5.6 Results of the 16S rRNA Analysis

The taxonomic categorization of the 16S rRNA conducted by GENEWIZ Inc. was further analysed. Differences after treatment with GLY or RU in samples from Sectio 2 were compared to the CTRL group. Disparities within groups were further compared to the data from Sectio 1 as internal control before treatment, to verify that found differences were caused by the treatment and not already present as naturally occurring variations. In general, a higher microbiome diversity (α-diversity) was found in treated pigs (Figure 4.6).

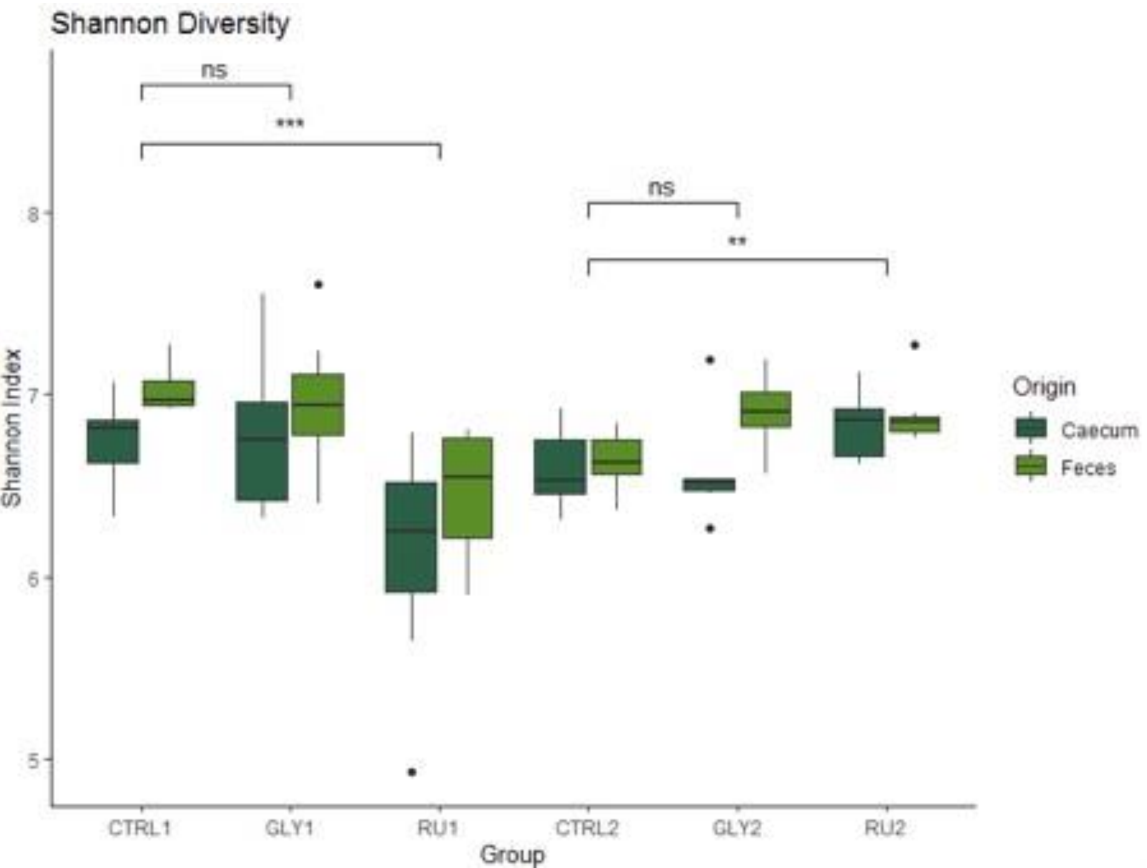

***Figure 4.6**: Alpha Diversity of the 16s rRNA analysed through a Shannon Diversity Index. CTRL= control group, RU= group fed with Roundup® LB Plus, GLY= group fed with glyphosate isopropylamine salt. The number after the group refers to the sectio (1= before starting the experimental phase; 2= final necropsy). n.s.= not significant, ** = P < 0.01, ***= P < 0.001*

On a phylum level, *Firmicutes* and *Bacteroides* accounted for almost 90% of bacteria and were most commonly identified in all samples. Including *Proteobacteria,* they account for almost 95% of all identified phyla. In the caecum samples of the GLY group, fewer *Firmicutes* and *Actinobacteria* and more *Proteobacteria* as well as Unclassified OTUs were found (Figure 4.7).

On a family level, more *Lactobacillaceae* were found in caecum samples taken after RU treatment. After GLY exposure, more *Prevotellaceae, Veillonellaceae* and *Enterobacteriaceae* and less *Lachnospiraceae, Ruminococcaceae, Clostridiaceae*, and *Streptococcaceae* were present in samples taken from the caecum. The faeces samples showed more *Lactobacillaceae* in the RU group and more *Enterobacteriaceae* in the GLY group, compared to the CTRL group. (Figure 4.8).

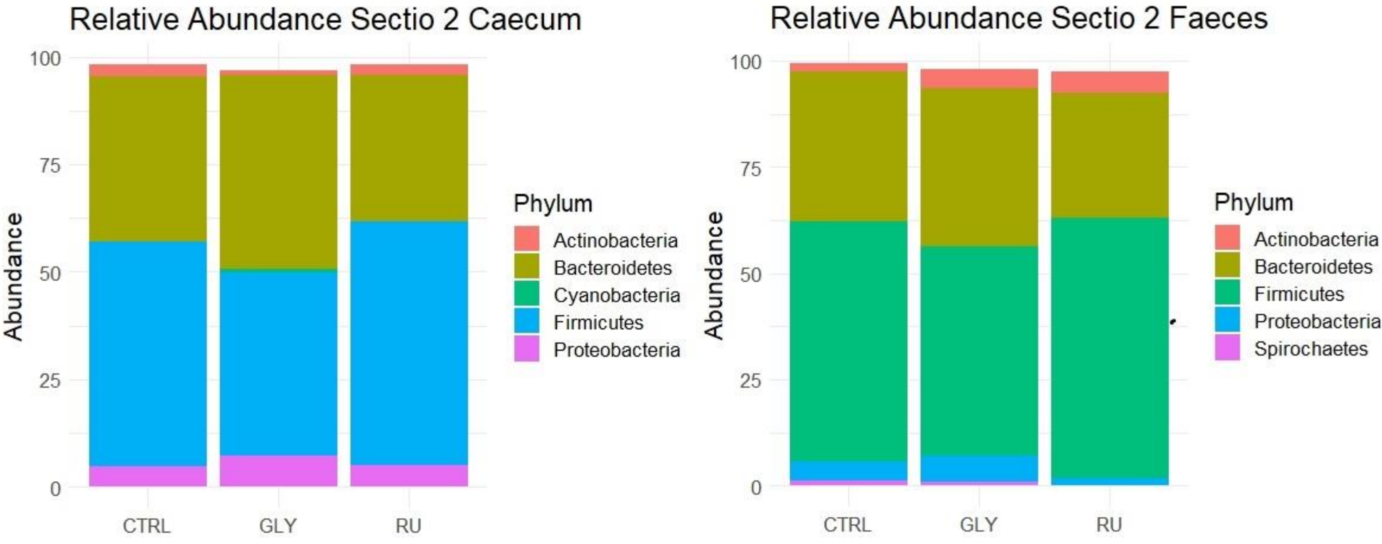

***Figure 4.7**: Medians of the relative abundances for all bacterial phyla accounting for more than 1% of the metagenome in the caecum and faecal samples of the CTRL, GLY and RU groups at the end of the experiment. CTRL= control group, RU= group fed with Roundup® LB Plus, GLY= group fed with glyphosate isopropylamine salt.*

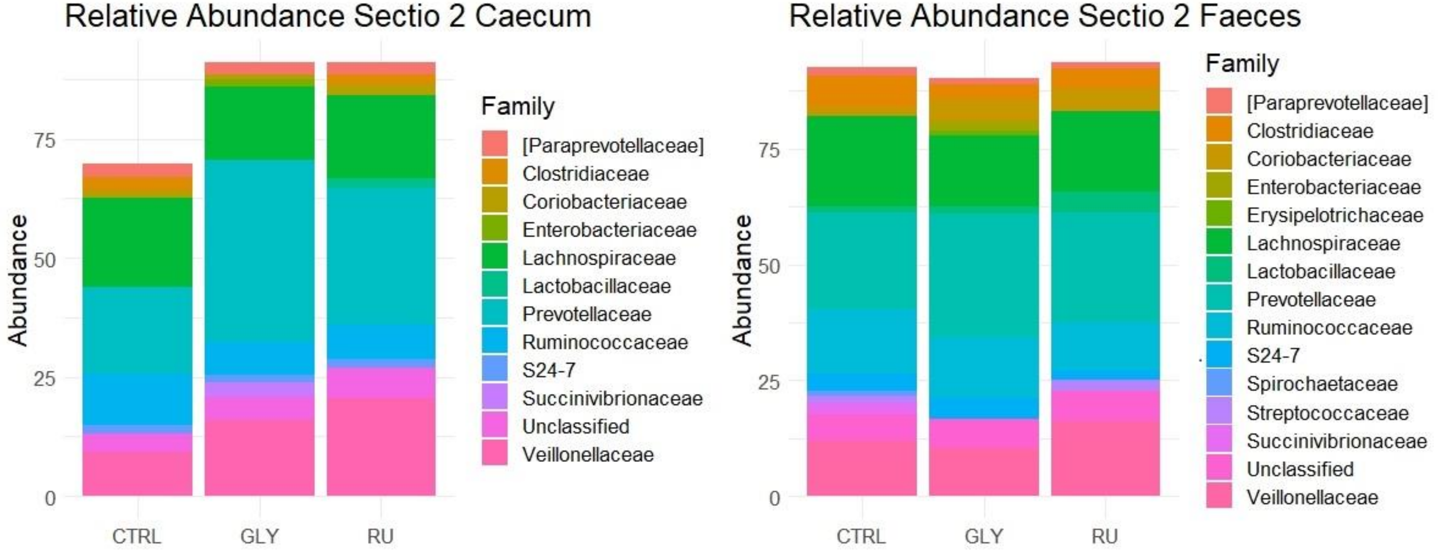

***Figure 4.8**: Medians of the relative abundances for all bacterial families accounting for more than 1% of the metagenome in the caecum and faecal samples of the CTRL, GLY and RU groups at the end of the experiment. CTRL= control group, RU= group fed with Roundup® LB Plus, GLY= group fed with glyphosate isopropylamine salt.*

Accordingly, on the genus level, more *Lactobacillus* were found in the RU group, whereas more *Escherichia* were identified in the GLY group, both in caecal und faecal samples. In the RU group, faecal samples had fewer *Clostridium* and caecal samples contained more *Lachnospira* compared to

the CTRL group. In the GLY group, more *Prevotella* and fewer *Ruminococcus* and *Streptococcus* were found in caecum samples.

Table 4.8 shows the mean relative abundances of selected phyla, families and genera at the end of the experiment. Most differences could be found in caecum samples from the GLY group.

***Table 4.8**: Mean relative abundances of selected phyla, families and genera at the end of the experiment (Sectio 2), presented in percent (%). Results that showed significant differences that were not identified in samples from Sectio 1 are highlighted in bold and grey. CTRL= control group, RU= group fed with Roundup® LB Plus, GLY= group fed with glyphosate isopropylamine salt*

Phylum	**Caecum**			**Faeces**		
	CTRL2	GLY2	RU2	CTRL2	GLY2	RU2
Firmicutes	54.96	**43.69**	56.75	58.89	54.63	63.07
Bacteroidetes	36.9	45.17	33.52	31.66	36.55	27.43
Proteobacteria	4.37	**7.37**	4.87	4.5	3.38	2.18
Actinobacteria	2.45	**1.43**	3.5	1.83	2.07	5.48
Family	**Caecum**			**Faeces**		
	CTRL2	GLY2	RU2	CTRL2	GLY2	RU2
Prevotellaceae	28.81	**38.6**	26.6	18.39	26.96	24.38
Lachnospiraceae	21.94	**15.35**	17.94	17.09	16.31	17.54
Ruminococcaceae	10.57	**7.43**	8.54	14.02	12.94	10.35
Veillonellaceae	10.82	**16.75**	20.13	10.34	11.37	14.74
Unclassified	4.38	5.12	**7.4**	6.44	6.92	6.41
Lactobacillaceae	0.65	0.42	**2.15**	1.29	2.49	**4.27**
Clostridiaceae	5.06	**1.02**	2.28	7.6	3.23	4.71
Enterobacteriaceae	0.36	**1.64**	0.74	0.38	**2.31**	0.45
Streptococcaceae	1.27	**0.08**	0.64	2.84	0.6	2.28
Genus	**Caecum**			**Faeces**		
	CTRL2	GLY2	RU2	CTRL2	GLY2	RU2
Prevotella	28.53	**38.51**	26.43	17.63	26.48	23.67
Roseburia	11.35	**7.5**	6.75	6.93	4.65	6.72
Lactobacillus	0.54	0.39	**2.08**	1.17	2.36	**4.23**
Ruminococcus	0.83	**0.57**	1.16	1.38	0.92	1.29
Escherichia	0.34	**1.55**	0.5	0.36	**2.29**	0.44
Streptococcus	1.27	**0.08**	0.64	2.84	0.6	2.28
Lachnospira	0.32	1.00	**0.88**	0.24	0.31	**0.32**
Clostridium	0.04	0.08	0.09	0.14	0.07	**0.06**
Bacteroides	0.009	0.02	0.001	0.001	0.01	**0.03**

DISCUSSION

5.1 Minimum Inhibitory Concentrations in *E. coli*

The commercial success of the herbicide glyphosate started with the introduction of GR plants in 1996. Excessive usage of GBHs in recent years, with residues being found in food and feed, started to concern large parts of the population. Since GLY's target structure is shared by several bacteria, fungi and unicellular parasites, it could have potential side-effects on many organisms besides plants.

However, sufficient data about susceptibility for GLY and formulations containing GLY (GBHs) in *E. coli* were lacking. Hence, this study aimed to screen a large amount of *E. coli* from different hosts and time points for their susceptibility regarding both the pure substance and commercially available RU.

In general, MIC values were narrowly distributed, but differed significantly for GLY and RU in MH I. For all isolates, median and mode values were 10 mg/ml IPA (7.41 mg/ml pure glyphosate) in the GLY group and 40 mg/ml IPA (29.63 mg/ml pure glyphosate) in the RU group. The significant gap between the susceptibility to the AI itself and the formulation is likely due to the complexity and variation of ready-to-use products. However, this finding is contrary to published data, where formulations have mostly been described as more toxic (Nagy et al., 2020; Mesnage et al., 2014) even disproportional to the amount of AI (Clair et al., 2012). It has been shown that some additives, especially tallow amines like POEA, show higher toxicity than the AI (EFSA, 2015a; Mesnage et al., 2014; Tsui and Chu, 2003). Due to its hazardous potency, the use of POEA has been forbidden in Europe in 2016 (European Commission Implementing Regulation (EU) 2016/1313).

Our results indicate that the surfactants and additives used in Roundup® LB Plus decrease toxicity for *E. coli*, leading to a 4-times higher MIC compared to pure glyphosate. Unfortunately, it was not possible to ascertain the individual ingredients of the RU formulation and test their individual effects or the effects of different combinations. Moreover, this finding is limited to the specific formulation used in this study. While various GBH formulations are commercially available, they do not contain a full list of ingredients with companies claiming confidentiality of their formulations (Cox and Surgan, 2006). In Germany alone, 116 different herbicides containing glyphosate as (one of) their main AIs are approved (German Federal Office of Consumer Protection and Food Safety, Online data base on plant protection products, January 2021). As pesticide regulations differ significantly between licensing areas a vast selection of GBH products is available on the global market. Products sold under the same name in different countries, aren't necessarily equal (Nagy et al., 2020). Further, GBHs are produced by many different companies for different applications, which adds to the complexity of this assessment.

Statistical Analysis

Overall, poultry isolates had significantly higher MICs for GLY and RU, both in univariable nonparametric Mann-Whitney U test and in the statistical models, compared to isolates of other origin. Furthermore, non-ESBL and pathogenic isolates showed higher MICs, although only for GLY.

This supports recently published data, concluding that pathogenic isolates were less likely to be susceptible to GLY or GBHs than commensals. Differences between pathogens like *Salmonella enterica* or *Clostridium perfringens* and regular intestinal bacteria, such as *Enterococci* or *Lactobacilli,* have been observed previously (Krüger et al., 2013b; Shehata et al., 2013). However, these studies only investigated single isolates per species and did not compare a large amount of pathogenic and non-pathogenic isolates.

The isolates classified as pathogenic *E. coli* in our screening showed higher MICs for GLY than commensal isolates. However, one should keep in mind that the classification of our strains into pathogens and commensals was based on their source of isolation, either from clinical cases or a zoonoses monitoring program. To gain more specific information on background and pathogenicity, this data should be re-analysed after strain sequencing.

The two *Enterococcus faecalis* strains available for investigation, with a MIC of <1.25 mg/ml for GLY, corroborate the differences between pathogenic and commensal intestinal bacteria described in the literature. Pathogenic bacteria are generally known for their superior stress responses (Chowdhury et al., 1996; McKellar et al., 1999), which can increase adaptation capacity and lead to higher MIC values (Chowdhury et al., 1996; Poole, 2012). These effects were not identified in the RU group. In addition to increasing MIC values, the GBH formulation used in this study appears to compensate the advantages pathogenic isolates show during GLY exposure.

Similar findings were made regarding non-ESBL and ESBL isolates. While there were significantly higher MIC values for non-ESBL isolates in GLY, there was no statistical difference between EBSL and non-EBSL isolates in RU. The glyphosate target mechanism, which inhibits the enzyme EPSPS in the shikimate pathway, differs from resistance for β-lactam antibiotics, which comprise of hydrolysing extended-spectrum cephalosporins. Therefore, ESBL *E. coli* do not benefit from a resistance targeting the structure of β-lactam antibiotics when exposed to GLY. On the contrary, some antibiotic resistances can be accompanied by fitness costs (Melnyk et al., 2015), which could explain lower MIC values. Moreover, ESBL *E. coli* from farm animals are often less virulent or even commensals. Detailed information on the specific kind of ESBL-resistance was not available for all screened isolates.

The only significant difference in all analyses, both for the AI and the formulation, was in regard to the original host. *E. coli* isolated from poultry continuously showed higher MIC values than *E. coli* isolated from pigs or cattle. Previous exposure to glyphosate residues via feed was theoretically possible for

all recent isolates tested in this study. Glyphosate residues have been documented in many feeds that are commonly used in livestock production (see Table 1.2). According to von Soosten et al. (2016), imported soy is the main source for glyphosate residues in Germany, which is commonly used as a protein source in poultry feed. However, it is also used in pig and concentrated cattle feed. In addition, glyphosate residues have also been detected in maize (Reddy et al., 2018), wheat and peas (Schnabel et al., 2017) as well as barley and oats (Stephenson and Harris, 2016), after crop or field treatment with GBHs. Retrospectively, it is not possible to obtain further information about the feed or husbandry practices used at the isolates' origin.

Considering the massively increased usage of GBHs together with GR crops, the question of resistance development not only arises for plants, but also for exposed microorganisms. To address this possibility, MICs of historic isolates from the ECOR collection (established 1984) were compared to recently sampled isolates from 2014 and 2015. Some statistically significant differences between the groups could be found for GLY, but not for RU. Using non-parametric tests, historic isolates showed higher susceptibility compared to recent ones. These differences can largely be attributed to pathogenic isolates. Interestingly, when compared to commensal isolates only, ECOR isolates showed statistically higher MIC values. However, the observed differences disappeared when the data was processed in the statistical model, which investigated the time point of isolation, the ESBL-status, and the host (meaning poultry, pig and cattle). Considering the original hosts of the historic ECOR collection, which only contained three isolates from cattle and two from pigs, the analysis of the model has to be interpreted cautiously. Furthermore, as previously mentioned, it remains unknown if or to what extent the historic isolates were exposed to glyphosate or GBHs.

Overall, statistical models suggested a tendency towards greater susceptibility to GLY in historically isolated *E. coli* for GLY. However, this tendency was not a main factor in the differences found between the groups. Ideally, more historic livestock-related isolates and isolates with proven exposure to glyphosate residues should be investigated.

Comparison with Published Literature

Only a few MIC values for specific strains have been published so far and their methods vary greatly, e.g. regarding the use of validated standards for herbicide or biocide susceptibility testing, media and tested substances. Furthermore, only a few studies specify whether their MIC values were determined for the pure AI or for the soluble salt, which is the present active form in GBHs. From this standpoint, it is almost impossible to compare different MICs, as Mesnage et al. also points out (Mesnage et al., 2015).

In a study with a similar experimental approach, Katholm et al. (2016) tested a single *E. coli* K88 strain in brain heart infusion (BHI) for its susceptibility to pure glyphosate, the equivalent salt, and the

formulation Jablo Glyfosat. With a MIC of 10 mg/ml for the salt and 20 mg/ml for the formulation, the values are in line with our results, despite differences in the chosen test media. When Nielsen et al. (2018) tested two *E. coli* strains with the formulation Glyfos 450 Plus, they determined an equal MIC in reinforced clostridial medium (RCM, 20 mg/ml) and a higher MIC in BHI (80 mg/ml), respectively. In contrast, the MIC of 1.2 mg/ml in two *E. coli* strains for the formulation Roundup® UltraMax, investigated by Shehata et al. (2013), is substantially lower.

As part of the project, the same MIC screening as described with *E.coli* has additionally been conducted with 225 *Salmonella enterica* strains, isolated from faecal samples collected from pigs and poultry (Pöppe et al., 2019). Overall, the MIC range was comparably narrow, however, the susceptibility lower (median and mode both for the AI and RU: 40 mg/ml IPA). In contrast to *E. coli*, there was no difference between the AI and the formulation and isolates from pigs showed statistically higher MICs than poultry inversing the results for *E. coli*. Statistically higher MIC values in recently sampled *Salmonella* isolates (between 2014 and 2016) in comparison to historic isolates (sampled between 1981 and 1990) corroborate the trend of an increase in MIC over the years as indicative for *E. coli*.

Susceptibility testing for antibiotics is commonly performed in nutrient-rich MH medium. Taking into account that glyphosate targets the formation of aromatic amino acids, bacteria could bypass its effects via uptake of corresponding substances from the environment. This hypothesis is supported by observations in media lacking aromatic amino acids, in which the MIC for either GLY or a GBH increased after specifically adding them (Haderlie et al., 1977; Nielsen et al., 2018). Investigating MICs in minimal medium, lacking essential nutrients, would substantially contribute to the understanding of the interactions between bacteria and glyphosate. However, it would not reflect real-life conditions, since sufficient nutrients are disposable when bacteria are exposed to glyphosate residues in the intestine of animals.

Besides the availability of aromatic amino acids, the MIC appeared to be influenced by other media components as well. The determination of MIC in MH I differed significantly from values in the cation-adjusted MH II, in which many bacteria were able to grow in a higher dilution step. Median and mode for isolates in GLY were 20 mg/ml in MH II (compared to 10 mg/ml in MH I). For RU, the MIC_{95} increased from 40 to 80 mg/ml. MIC values for GLY and RU in MH II were more closely aligned, yet differed significantly. In further statistical nonparametric analyses, only poultry isolates had significant higher MICs compared to cattle and pig isolates, both for GLY and RU. A difference between the values in MH I and MH II could be connected to glyphosate's capability to chelate bivalent cations (Madsen et al., 1978; Motekaitis and Martell, 2006), potentially affecting the availability of the active substance. Chelation might also happen to glyphosate residues in the intestine, where bivalent cations are either naturally available or additionally supplemented via feed. Furthermore, GLY could be impacted by the different intestinal buffer systems and pH levels. When susceptibility tests were

performed in neutralised medium (pH 7), the MIC for GLY increased and closely resembled the higher values determined for RU. This could be explained by different pK_a values of GLY, affecting its ability to enter bacterial cells.

95 % Cut-Off

Susceptibility screening for large amounts of bacteria commonly results in visible differences between a wild-type and a more resistant subpopulation. However, our results did not reveal a clear gap between the tested isolates. To distinguish isolates with an enhanced resistance for GLY or RU, epidemiological cut-off values (MIC_{95}, representing 95 % of the studied population) were calculated.

Two isolates from the GLY group and eleven isolates from the RU group showed MIC values above the calculated cut-off, although none of the isolates were present in both groups. All of them were non-ESBL producing *E. coli* and the majority was classified as pathogenic. Most of the strains from the RU subpopulation were obtained from poultry. In contrast, for the screening conducted with *Salmonella* isolates, no resistant subpopulation could be distinguished (Pöppe et al., 2019).

Additional sequencing was performed on all isolates above the calculated cut-off level as well as on one isolate displaying a substantially lower MIC than all others. They were then compared to an isolate with a mean MIC value as well as to the sequence of a standard laboratory *E. coli*. The results confirmed some differences in the amino acid structure of the targeted EPSPS.

In general, the enzyme EPSPS either occurs as an open form that can bind substrate 1 (shikimate-3-phosphate) or as a closed form after binding of substrate 1 and thereby forming an active reaction site that can bind substrate 2 (PEP). Glyphosate only binds to the closed formation and occupies the binding site for PEP (Schönbrunn et al., 2001).

Sequencing revealed that our isolates contained changes located close to those described in previous studies of glyphosate resistant bacteria, while none of them matched the exact positions. Most identified differences were located between the positions 63-135, at 235 and 365-375. One SNP on position 31, which causes a change of the aromatic amino acid serine to arginine, is consistent in all isolates above the MIC, but was also found in a standard *E. coli* as well as in the strain with the lowest susceptibility. Therefore, this can be considered a variation of the isolate with the mean MIC that was used for comparison, independent from the GLY/RU susceptibility.

The mechanism of the G96A GLY resistance is well investigated. The change at position 96, from the small amino acid glycine to the slightly larger alanine, results in an additional occupation of space, which mechanically affects binding at the active side of the enzyme. This prevents glyphosate from binding onto the closed enzyme form, while PEP, a slightly smaller molecule, is not inhibited (Eschenburg et al., 2002; Priestmann et al., 2005). For most of the isolates above the cut-off, changes

were identified close to previously documented positions: Gly86Ser and Leu88Arg both exchange an amino acid with a larger one, which could cause an effect similar to G96A. In addition, Fei et al. (2013) considered a change from Asp to Gly at position 120-160 important for glyphosate resistance. One isolate above the cut-off for glyphosate showed a similar change close to the suggested position (Asp163Glu).

While some findings are comparable to previously described resistance mechanisms, it is impossible to predict the effect on the binding of glyphosate as a competitor for PEP without further biochemical analysis of the exchanged amino acids' effects on structural variances of the enzyme.

Limitations

The lack of biological replicates is a major source of limitation of this study. Due to the great number of bacteria (238 isolates) and tests for two different substances (RU, MIC) in two different media (MH I and MH II), we only used technical triplicates.

In addition, commercially available plates are lacking and the manual production of stock solutions, dilutions in the 96-well plates and storage were possible sources of errors. To ensure similar conditions for each lot of produced plates, a so far not available defined quality control should be established and processed simultaneously. To better objectify MIC determination, the visual reading could be replaced by the determination of OD600 in a plate reader. Therefore, flat bottom 96-well plates should be used and a cut-off value would need to be defined.

The small number of historic isolates from livestock limits the comparison between isolates obtained before and after the introduction of GBHs. If possible, more isolates fitting that context should be investigated. Furthermore, all tested isolates should be sequenced to gain more background information, especially regarding pathogenicity and antibiotic resistances.

5.2 *In vitro* Investigation of Glyphosate Effects on Bacteria

In the first part of this project, we were able to confirm that pathogenic *E. coli* are less susceptible to GLY than commensal isolates. The same investigations for *Salmonella enterica* revealed even higher MICs (Pöppe et al., 2019). However, information about the effects of glyphosate on bacterial communities in livestock is limited. Prior research suggested that an exposure to GLY or GBHs can lead to dysbiosis (Ackermann et al., 2015; Krüger et al., 2013b; Reuter et al., 2007; Shehata et al., 2013). To determine possible advantages for a pathogenic *E. coli* or *S.* Typhimurium strain in a more complex environment, an *in vitro* ruminal fermentation experiment with worst-case residual concentrations of RU was performed. Since particularly sublethal concentrations of biocides or herbicides entail the risk of adaptation mechanisms and resistance induction (Capita et al., 2014;

Thomas et al., 2000), the susceptibility to RU and several antibiotics after exposure was further investigated.

E. coli and *S.* Typhimurium in the Fermenter

The numbers of inoculated *E. coli* steadily decreased in all fermenters until day five, independent from the addition of RU, at which point detection was no longer possible. This decrease ties well with a previous *in vitro* fermentation experiment by Bach et al. (2003), who inoculated 10^4 CFU/ml of a pathogenic *E. coli* strain into ruminal fluid from a sheep.

The number of *S.* Typhimurium in all RUSITEC fermenters dropped initially before almost doubling within 2 to 4 h post-inoculation. Following this short spike, the amount of *Salmonella* continuously declined, although the reduction was not as steep as seen in *E. coli*. After seven days, only a few *S.* Typhimurium isolates were still detectable. The survival in the RUSITEC was substantially longer than in a comparable *in vivo* study with 10^5 or 10^6 CFU of two different *Salmonella spp.* isolates, in which detection under physiological ruminal conditions became impossible after 48-72 h (Brownlie and Grau, 1967). The amount of inoculated *Salmonella* only remained stable or increased slightly in cows with increased ruminal pH, which was caused by a loss of volatile fatty acids after suffering from a period of starvation.

During prior fermentation trials with the RUSITEC system, Riede et al. (2016) did not find any changes in fermentation parameters or the bacterial composition with 0.42 or 2.92 mg glyphosate in a GBH/d after inoculation with *Clostridium sporogenes*. Similarly, the amount of *Clostridium sporogenes* decreased consistently.

In contrast to these findings, some authors found GLY to have an impact on the ruminal environment and its associated bacteria. A previous study by Reuter et al. (2007) incubated ruminal content in different GLY concentrations of up to 100 mmol/l as a batch culture. After 24 h, they reported reduced fermentation and bacterial growth as well as a shift of the microbial population with glyphosate concentrations of 10 and 60 mmol/l, which seemed to present a kind of selective pressure. This was supported by a recent study from Ackermann et al. (2015), executed with a ruminal *in vitro* fermentation system, demonstrating that the microbial community shifted in favour of pathogenic species (e.g. species belonging to the *Clostridium histolyticum* group) after exposure to 10 or 100 µg/ml of glyphosate.

Resistance Induction

Similar to antibiotic resistance, the exposure to sublethal concentrations of pesticides, including herbicides and biocides, can lead to adaptation mechanisms and resistance induction in bacteria (Condell et al., 2012; Ledder et al., 2006). However, susceptibility testing of strains isolated from each fermenter at the end of the survival period showed no differences in MICs for RU, with and without pH adjustment, in comparison to the ancestral strains. This is in line with findings from Tincher et al., who did not observe any resistance mutations after long-term exposure of *E. coli* to the GBH Roundup® Concentrate Plus (Tincher et al., 2017). A similar conclusion was reached by Condell et al., who demonstrated that exposure of 189 *Salmonella enterica* strains to sublethal concentrations of different biocides, which have comparable properties as herbicides, only increased the tolerance for some of the active ingredients themselves, but not for any formulation (Condell et al., 2012). This could likely be explained by the complexity of ingredients and their accumulating effects in ready-to-use formulations, making it more difficult to enhance tolerance compared to a single ingredient. In addition, the MIC of 40 or 80 mg/ml for RU found in the isolates used in our experiment was rather high. In order to grow in the following dilution step, bacteria would have to overcome a large amount of active ingredient. Compared to the administered worst-case concentration of 10 mg/l (= 0.01 mg/ml) RU, the MICs of our isolates were 4,000 to 8,000 times higher. Hence, the levels of RU might have simply been too low to provoke a visible effect on adaptation or selection of isolates.

A challenging problem with the application of antibiotics, biocides or heavy metals like zinc and copper is their capacity to co-select for further resistances (Capita et al., 2014; Molina-González et al., 2014; Wales and Davies, 2015; Yazdankhah et al., 2014). This has also been demonstrated for GBHs (Kurenbach et al., 2015, 2017; Staub et al., 2012). Cross-resistance mechanisms, such as overexpression of efflux pumps or decreased cell permeability, are effective against various substances. Resistance mechanisms that are selected together, e.g. because they are linked genetically or placed on the same plasmid, are known as co-resistance. Many of the described adaptations are due to unspecific mechanisms like an increased amount or activity of efflux pumps (Karatzas et al., 2007; Kurenbach et al., 2017; Molina-González et al., 2014; Staub et al., 2012). Considering glyphosates' unique target structure, this appears to be the most likely explanation for any resistance after glyphosate exposure.

Our investigation of antibiotic resistance profiles for isolates obtained from the different fermenters revealed only slight changes in a few individual isolates. Alterations in the antibiotic susceptibility profile for respective *S.* Typhimurium isolates only ranged between one dilution step, while it ranged between two dilution steps in most of the affected *E. coli* isolates. These differences could simply be attributed to measurement variability, which is common in susceptibility testing (± one dilution step). Differences in MICs for the fourth-generation cephalosporin Cefepime were more pronounced in three *E. coli* strains. Values dropped from ≥ 64 µg/ml in the ancestral strain to 4 µg/ml for strains from fermenter 1, 4 and 5. Since these isolates were not only from fermenters with RU (fermenter 4), but

also from control fermenters (fermenter 1 and 5), the change does not appear to be connected to herbicide exposure, but rather to a stress response to the unfamiliar ruminal environment. The beta-lactamase gene *bla*$_{CTX-M}$, which is present in the ESBL-*E. coli* used to inoculate the fermenters, has been linked to high resistances against Cefepime in Enterobacteriaceae (> 8 µl/ml) (Welsh et al., 2005). Accordingly, a loss of the corresponding gene could lead to a decreased resistance against cefepime. However, all *E. coli* isolates still harboured the *bla*$_{CTX-M}$ gene and except for one isolate in Ceftazidime showed resistances to multiple tested β-lactam antibiotics (Piperacillin, Ceftazidime, Aztreonam). Additionally, comparisons between MIC values of ESBL-*E. coli*, either obtained via VITEK®2 or agar dilution, revealed that susceptibility testing for Cefepime in *E. coli* using VITEK®2 is prone to errors (Rhodes et al., 2014).

Contrary to the findings described above, a number of authors have recognized no effect of biocide exposure on antibiotic resistances. For instance, a large number of *S.* Typhimurium isolates exposed to sublethal concentrations of different biocides (Condell et al., 2012) as well as enteric bacteria passaged 10 times with the biocide Triclosan (Ledder et al., 2006) did not change their susceptibility to antibiotics. Karatzas et al. (2014) exposed a *S.* Typhimurium strain to steady sub-inhibitory concentrations and to gradually increasing concentrations of different biocides for seven days each. It was reported that the consistent concentrations, similar to our experimental approach, did not lead to any change, whereas the increasing concentrations reduced susceptibility for some antibiotics.

Overall, viability of both inoculated *E. coli* and *S.* Typhimurium strains declined in the *in vitro* ruminal fermentation system, similar to the few data available from the literature. No difference between the fermenters with or without RU in worst-case concentrations of 10 mg/l were observed. The exposure to RU neither changed susceptibility to glyphosate, nor had substantial effects on the antibiotic resistance profiles. The findings are in line with previous MIC screening for RU, demonstrating that there is no link between susceptibility to β-lactam antibiotics and RU. Moreover, MICs for RU between ESBL and non-ESBL-*E. coli* did not show statistically significant differences.

Limitations

The main limitation of these results regards the test setup. Isolates were tested in an artificial *in vitro* ruminal system, which is not equivalent to their usual habitat. *In vivo E. coli* and *S.* Typhimurium enter the digestion system through the rumen, but they don't remain there. The rumen is a place of transit and selection before reaching their final destination in the intestine. Thus, controlled laboratory ruminal fermentation studies might not be ideal and further experiments and alternatives, such as *in vitro* intestinal fermentation with porcine and/or cattle ingesta and *in vivo* studies (additionally also in monogastric animals) should be considered.

5.3 *In vivo* Investigation of Glyphosate Effects on Bacteria in Pigs

The MIC screening and respective published literature suggested that effects of GLY or GBHs on the composition of the intestinal microbiome in favour of pathogenic bacteria were possible. An advantage for a *S.* Typhimurium or an ESBL *E. coli* strain could not be confirmed by the ruminal *in vitro* fermentation. However, this experiment was conducted in an artificial system and focused only on the two added Enterobacteriaceae, neither of which naturally inhabit the ruminal environment. To gain more information about the effects of GLY and RU on the shedding of zoonotic *S.* Typhimurium and ESBL *E. coli* as well as the composition of the entire intestinal microbiota, an *in vivo* experiment was conducted.

Five-week old piglets that were naturally colonized with ESBL *E. coli* were infected with 10^8 CFU of a *S.* Typhimurium DT 104 strain. After one week, either GLY or RU were orally applied for two weeks at worst-case residual levels.

Bacteriological examination

After infection, all animals started shedding *Salmonella* associated with a high amount of ESBL *E. coli*. During the experiment, the amount of CFU in faeces decreased in all groups until only a qualitative detection, if at all, was possible for both species.

Just as in the *in vitro* fermentation experiment, the exposed groups neither differed in the number of isolated bacteria (*Salmonella* as well as ESBL *E. coli*), nor in their susceptibility to GLY or RU at the end of the experiment, compared to the CTRL group.

16s rRNA analysis

To detect possible effects on the microbial community, caecum and faeces samples from the end of the experiment were analysed via NGS sequencing on Illumina Miseq.

Both caecum und faeces samples from the pigs exposed to RU showed significantly higher diversity than the CTRL group. These tendencies could also be seen in faecal samples after GLY treatment. Nielsen et al. (2018) similarly detected more species in caecum and colon samples of 4-week-old rats fed with 2.5 or 25 mg of the formulation Glyfonova/kg bw/d for two weeks, but not after being fed with GLY. They suspected that the GBH could provide nutrient sources, which in turn could be responsible for an enrichment of different bacteria. In the prior MIC screening, we noticed that GLY had a more severe impact on the growth of the tested *E. coli*, which showed significantly lower MIC values than for RU.

The majority of all bacteria in the faecal and caecum samples of all pigs were either *Firmicutes* or *Bacteroides*, accounting for approximately 90 % of the microbiome. Together with *Proteobacteria*, the three phyla constituted about 95 %. In a longitudinal study by Kim et al., over 90 % of the microbiota of pigs also belonged to *Firmicutes* or *Bacteroides* (Kim et al., 2011). In general, these two are the

most abundant phyla in the core pig microbiome (Zhao et al., 2015) regardless of the age (Aluthge et al., 2019). This also applies to pigs infected with *Salmonella* spp. (Bearson et al., 2013; Borewicz et al., 2015; Drumo et al., 2015).

Statistical analysis showed a lower amount of *Firmicutes* and *Actinobacter* and thus more *Proteobacteria* and unclassified bacteria in caecum samples from the GLY group, compared to the CTRL group. A decrease in *Firmicutes* has also been described in intestinal samples from a study on 4-week-old mice. However, they were fed with much higher concentrations of an unspecified 'Roundup' formulation (250 or 500 mg/kg bw/d) for a period of 6 or 12 weeks, corresponding to sub-chronic and chronic exposure (Aitbali et al., 2018).

A number of studies have demonstrated that exposure to GLY or a GBH decreases the number of *Lactobacillus* in the intestinal microbiome. They include studies on mice after high-level exposure to 'Roundup' (Aitbali et al., 2018), rats 31 days post-natum after exposure to GLY or 'Roundup' (1.75 mg/kg bw/d) (Mao et al., 2018), female rats in a long-term study of exposure to the GBH formulation R Grand Travaux Plus used in different concentrations of up to 5 mg/ml (Lozano et al., 2018), and honey bees (Motto et al., 2018). Furthermore, *Lactobacilli* have been described as susceptible to the GBH formulation Roundup® UltraMax (Shehata et al., 2013). Regarding the cause for these observations, Aitbali et al. (2018) suggested that a chelation of manganese with glyphosate reduced the available amount of the element that is essential to *Lactobacilli*, thereby decreasing their number. However, in our experiment, no decrease in *Lactobacilli* was found. On the contrary, we detected a statistically significant increase of *Lactobacillaceae* and *Lactobacillus* in the group exposed to RU.

In the GLY group, caecum content samples showed increased amounts of *Prevotellaceae* and *Prevotella* compared to the CTRL group. This is in line with a study on rats by Mao et al. (2018) that found an increase of *Prevotella* in postnatal dams on day 31 after exposure to GLY and 'Roundup'.

Previous studies have stated that due to different susceptibilities for glyphosate, residues can cause dysbiosis by favouring pathogenic bacteria, particularly *Clostridia sp.* (Ackermann et al., 2015; Krüger et al., 2013b; Shehata et al., 2013). Our data does not confirm these findings. While most samples did not show significant differences to the control, we found decreased amounts of *Clostridiaceae* in caecum samples from the GLY group and *Clostridiae* in faecal samples from the RU group.

Furthermore, more Enterobacteriaceae, in particular those classified as *Escherichia*, were present in caecum and faeces samples of pigs exposed to GLY, compared to the CTRL group. However, this increase was not reflected in the faecal shedding of ESBL *E. coli* measured during the experiment. The pigs in the group exposed to RU showed a higher amount of *Bacteroidetes* in faeces, although the relative abundance was quite small, with 0.03% in faeces of the RU group compared to 0.001% in faeces from the CTRL group. An increase of *Bacteroidetes* was also observed in a study by Lozano

et al. (2018), however, this finding was only made in female rats. In contrast, mice showed a decrease in *Bacteroidetes* after the exposure to a GBH (Aitbali et al., 2018).

To the best of our knowledge, the other observed differences between some samples of the GLY or RU compared to the CTRL group (such as an increase in *Lachnospira* and unclassified bacteria for RU and an increase in *Veillonellaceae* as well as a decrease in *Lachnospiraceae*, *Ruminococcaceae*, *Roseburia*, *Ruminococcus* and *Streptococcus* for GLY) have not been previously described.

Interestingly, Nielsen et al. (2018) investigated the microbiome of rats in a similar experimental setup and did not find any differences between bacterial classes. The study hypothesised that this was due to a sufficient supply of amino acids to the intestine, making it possible to evade shortages due to GLY through adequate uptake from the environment.

Overall, caecum samples showed a greater bacterial variation than faecal samples. This is comparable to findings of Argüello et al. (2018), where faecal samples were more stable than samples from the ileum after infection with *S.* Typhimurium*.* Both GLY and RU groups showed differences compared to the CTRL group, albeit independently from each other. Divergent changes to the microbiome have also been described in rats after exposure to a GBH, compared to exposure to its AI (Mao et al., 2018). Moreover, this finding complies with the differences in MICs for both substances described above.

Limitations

Findings regarding the observed microbiome changes in pigs are limited, as the piglets used in this study were merely six to eight weeks of age at the time of exposure. Hence, their microbiome was not fully developed yet. According to recent studies on pigs, microbiome diversity can stabilise as early as three weeks after weaning (Wang et al., 2019), but may increase until the age of twelve weeks (De Rodas et al., 2018). In general, early-life microbiota is more prone to changes. This was confirmed by experiments with glyphosate exposed rats (Mao et al., 2018) and honeybees (Motto et al., 2018). However, the chosen approach cannot provide data on the effects of GLY or RU exposure during the critical, more vulnerable time of weaning.

Furthermore, the finding made in this study cannot be transferred to possible influence of glyphosate residues on infections with *S.* Typhimurium only. The exposure to GLY and RU was started one week after the infection, with the intention of simulating the common case of infected, yet clinically undetectable, carrier pigs in livestock farming.

Feeding studies can be divided into a metabolic adaptation phase, in which the microbiome adapts to the new challenging diet, and an equilibrium, in which the new microbiota is stabilized. According

to a recent study, the equilibrium phase takes 3 to 4 weeks (Tilocca et al., 2017). In light of this, our results could be considered as changes at the end of the metabolic adaptation phase.

CONCLUSION

In our experiments with an representative *E. coli* strain collection, we determined significantly different MICs for GLY (median and mode: 10 mg/ml IPA in MH I) and RU (median and mode: 40 mg/ml IPA in MH I). *E. coli* Isolates from poultry were consistently more tolerant than those of pigs and cattle. Additionally, pathogenic as well as non-ESBL *E. coli* isolates had statistically higher MICs for GLY. A comparison of historic and recent isolates indicated a decrease in susceptibility in recent isolates for GLY. However, this could not be clearly substantiated due to a lack of sufficient historic samples from livestock. Sequenced isolates above a calculated 95% cut-off (20 mg/ml for GLY and 40 mg/ml for RU) revealed some SNPs, which led to changes in the amino acid sequence of the target enzyme EPSPS. However, none of them matched previously described resistance mechanisms, therefore warranting further investigation.

Additionally, there seem to be more influence factors on the susceptibility of bacteria to GLY and RU, such as the used media, including the availability of aromatic amino acids, number of bivalent cations and pH. Due to a lack of standardized methods, the use of different formulations, units and no commercially available plates, it is almost impossible to compare values of previous studies. Therefore, this screening should serve as a starting point for further investigations about the interactions between the herbicide glyphosate or other herbicides and zoonotic bacteria.

To determine if there is an advantage for pathogenic zoonotic bacteria, as indicated in the literature and corroborated by higher MICs for pathogenic *E. coli* and *Salmonella enterica*, we infected an *in vitro* RUSITEC system with one *E. coli* and one *S.* Typhimurium isolate and compared fermenters with and without a worst-case concentration of 10 mg/l glyphosate in RU. Overall, the application neither affected bacterial survival nor susceptibility to RU or other antibiotics.

Furthermore, we investigated the influence of exposure to a worst-case concentration of either GLY or RU (2.85 mg/kg bw/d) *in vivo* in pigs infected with *S.* Typhimurium and ESBL-*E. coli*. Similar to the fermentation study, no effects on shedding of *S.* Typhimurium or ESBL-*E. coli*, as well as no changes in the susceptibility to GLY or RU, were observed.

Microbiome analysis revealed greater diversity after exposure to RU. Numbers of *Lactobacillaceae* increased in the RU group as well as numbers of *Enterobacteriaceae* in the GLY group, respectively. Overall, more changes were visible in caecum samples, while faecal samples seemed to be more stable.

SUMMARY

Glyphosate (N-(phosphonomethyl)glycine) is the most-used herbicide worldwide. Many studies have found residues in feed and food. Naturally, concerns about its safety and side effects on other organisms have been raised.

With only insufficient and contradictory data about the susceptibility for the widely used herbicide glyphosate, our study is the first to systematically analyse a large amount of *E. coli* from livestock isolated at different points in time.

According to standards for antimicrobial susceptibility testing, we determined minimum inhibitory concentrations (MICs) by means of broth microdilution for the active ingredient (AI) isopropylamine salt (IPA) and the glyphosate-containing formulation Roundup® LB Plus (RU), commonly used in Germany. In total, 238 *E. coli* isolates, mainly isolated from poultry, pigs and cattle were investigated. Samples isolated between 2014 and 2015 were compared to historic samples of the standard *E. coli* collection of reference (ECOR) from 1984. For further statistical analysis, samples were divided into extended-spectrum beta-lactamase (ESBL) and non-ESBL producing *E. coli* as well as into commensal and pathogenic isolates.

Mean and mode for all isolates showed a higher level of tolerance for RU (40 mg/ml IPA) compared to GLY (10 mg/ml IPA). In general, the distribution was narrow, and a clearly resistant subpopulation was lacking. To identify less susceptible isolates, a 95% cut-off was calculated (20 mg/ml for GLY and 40 mg/ml for RU). Isolates above the cut-off were sequenced and their *aroA* gene, coding for the glyphosate target enzyme, compared. No previously known resistance mechanisms were found, however, most differences occurred close to positions described in the literature.

Isolates from poultry showed significantly higher MICs in RU and GLY, both in nonparametric Mann-Whitney U tests and statistical models (multivariable variance analysis for GLY and multivariable proportional-odds regression model for RU). In addition, both pathogenic and non-ESBL isolates showed significantly higher MICs in the GLY group, verified by both statistical methods. Solely in the nonparametric test for GLY, historic isolates were less tolerant than recently sampled isolates. However, with only very few isolates from livestock preserved in the ECOR collection, the sample size is a limiting factor of this model. Hence, future studies should include more equivalent historic isolates.

To determine whether the growth and survival of a pathogenic *E. coli* and a *S.* Typhimurium isolate in an *in vitro* ruminal experiment is influenced by 10 mg/l RU as a worst-case concentration, a 'Rumen Simulation System' (RUSITEC) was used. Fermenters were inoculated with 10^9 colony forming units (CFU) of each strain, leading to a starting concentration of 10^6 CFU/ml. Initially, the number of CFU of *Salmonella* doubled after 2 to 4h. Apart from this brief increase, the number of bacteria continuously

declined in all fermenters, without being influenced by the RU application. *E. coli* was no longer detectable in quantitative tests from day 4 and in qualitative tests from day 5 onwards. *S.* Typhimurium remained detectable until the end of the experiment on day 7, although only a few CFU survived. MICs for RU did not change after the exposure, while antibiotic susceptibility did not vary significantly. In conclusion, the exposure to RU neither increased the abundance, nor promoted resistance.

Considering the fermentation experiment focused only on two Enterobacteriaceae in an artificial environment, a more extensive *in vivo* experiment with pigs was conducted. Weaned piglets (naturally colonized with ESBL *E. coli*) were infected with 10^8 CFU of the same *S.* Typhimurium DT104 strain used in the RUSITEC experiment, at the age of five weeks. One week later, half of the animals per group (n=14/2) were sacrificed as an internal control. The other half was further exposed to nothing (CTRL), GLY or RU, in worst-case concentrations of 2.85 mg/kg bw/d, based on residue levels described in pig feed. The feeding experiment lasted for two weeks, during which faecal samples were checked twice weekly for *Salmonella* and weekly for ESBL *E. coli*. Finally, different organs were investigated and faeces and caecum contents were frozen and sent for 16S rRNA analysis via Illumina MiSeq. Neither the exposure to GLY nor to RU increased the shedding or accumulation in organs. As in the fermentation experiment, MICs of the isolates for RU or GLY did not change.

The 16S rRNA analysis revealed some differences between the microbial compositions in the different study groups. In general, the RU group showed greater diversity in both faecal and caecum samples. For the GLY group, a tendency was only observed in faeces. Overall, more differences between the CTRL and the exposed groups were found in caecum samples than in faeces. In both caecum and faeces samples, *Lactobacillaceae* (genus *Lactobacillus*) increased in pigs from the RU and *Enterobacteriaceae* (genus *Escherichia*) increased in pigs from the GLY group. In contrast to previous reports, the number of *Clostridia* did not increase, but rather decreased in some samples.

Future studies should focus on identifying reasons for inter- and intra-species susceptibility variation by taking a closer look at resistance mechanisms and target structures. Moreover, a possible link between antibiotic and glyphosate tolerance should be investigated. Effects of glyphosate on more vulnerable microbiota that are more sensitive for lack of aromatic amino acids (i.e. after birth or after weaning, infection, antibiotic treatment or immunosuppression) should be investigated.

Auswirkungen von Glyphosat auf *Escherichia coli* und Bakteriengemeinschaften *in vitro* und *in vivo*

ZUSAMMENFASSUNG

Glyphosat (N-(Phosphonomethyl)glycin) ist das am häufigsten genutzte Unkrautvernichtungsmittel der Welt. In vielen Untersuchungen konnten Spuren davon in Lebens- und Futtermitteln gefunden werden, die dementsprechend die Frage nach der Sicherheit des Produktes und der potenziellen Effekte auf die Umwelt, auf Tiere und den Menschen aufwarfen.

Aufgrund der sehr geringen und zum Teil widersprüchlichen Datenlage zur Empfindlichkeit von Nutztier-assoziierten *E. coli* gegenüber Glyphosat, wurden insgesamt 238 *E. coli*-Isolate, größtenteils aus Geflügel-, Schweine- und Rinderhaltungen, untersucht. Angelehnt an Standardverfahren zur antimikrobiellen Empfindlichkeitsprüfung, wurden minimale Hemmkonzentrationen (MHKs) mittels Mikrodilutionsverfahren bestimmt. Dabei wurde neben dem aktiven Wirkstoff (GLY) als Isopropylamin-Salz (IPA) auch die in Deutschland gängige Formulierung Roundup® LB Plus (RU) untersucht. Historische Isolate einer *E. coli* Referenzsammlung (ECOR) aus dem Jahr 1984 dienten dabei als Vergleich zu aktuellen Isolaten aus den Jahren 2014 und 2015. Weiterhin wurden die Isolate bezüglich ihres ESBL-Status sowie gemäß ihrer Isolierung als ‘Pathogen’ oder ‘Kommensale’ kategorisiert.

Die getesteten Isolate wiesen allgemein eine höhere Toleranz gegenüber RU als für GLY auf (Median- und Modalwert der MHK in RU: 40 mg/ml IPA und in GLY: 10 mg/ml IPA). Da die MHK-Verteilung gering und ohne klar abgrenzbare resistente Subpopulation war, wurde ein 95 % Cutoff-Wert berechnet. Isolate mit einer MHK über diesem Wert wurden sequenziert um die *aroA* Gene, welche das Ziel-Enzym für Glyphosat codieren, mit weniger toleranten Isolaten zu vergleichen. Dabei wurden Veränderungen gehäuft an Positionen in der Nähe zu schon bekannten Veränderungen resistenter Enzyme gefunden.

Isolate aus Geflügelbetrieben zeigten sowohl in nicht-parametrischen Mann-Whitney U Tests, als auch in statistischen Modellierungen (multivariable Varianzanalyse für GLY und multivariable Regressionsanalyse für RU) statistisch signifikant höhere MHK-Werte für GLY und RU. Dies war auch sowohl für nicht-ESBL produzierende als auch für als pathogen kategorisierte *E. coli* in GLY der Fall. Historische Isolate zeigten allein in nicht-parametrischen Tests in Glyphosat eine geringere Toleranz als aktuelle. Durch die nur geringe Anzahl an Nutztier-assoziierten Isolaten in der ECOR Sammlung ist die Aussage dieser Tests (v.a. für die Modellierungen) jedoch begrenzt und es sollten mehr historische Isolate aus dem Nutztierbereich untersucht werden.

Zur Untersuchung der Wachstumskinetik von *E. coli* und *S.* Typhimurium DT 104 unter Glyphosatwirkung wurde ein *in vitro* Pansenfermentationsexperiment mit einer worst-case

Konzentration von 10 mg/l RU durchgeführt. Die Fermenter wurden dabei mit jeweils 10^9 Kolonie-bildenden Einheiten (KbE) eines *E. coli* und *S.*Typhimurium DT 104 Isolates beimpft, was zu einer Startkonzentration von 10^6 KbE/ml in den Fermentern führte. Nach 2-4 h verdoppelte sich die Anzahl der Salmonellen, sank danach jedoch, wie auch die Anzahl der *E coli*-Isolate, in allen Fermentern, unabhängig der RU-Gabe, stetig ab. An Tag 4 post-inoculationem waren quantitativ und an Tag 5 qualitativ keine *E. coli* mehr nachzuweisen. Salmonellen waren am Ende des Versuches an Tag 7 nur noch qualititiv isolierbar. Insgesamt wurden durch die Gabe von RU weder die Erregerzahl noch deren Empfindlichkeit gegenüber RU oder Antibiotika beeinflusst.

Zur weiteren Beurteilung der Auswirkung von Glyphosat auf ESBL *E. coli* und *S.* Typhimurium sowie auf ein enterales mikrobielles Ökosystem, wurde ein *in vivo* Expositions-Versuch mit Schweinen durchgeführt. Dabei wurden 5 Wochen alte, natürlich mit ESBL-*E. coli* kolonisierte Absatzferkel mit 10^8 KbE des auch schon in den Fermenterversuchen eingesetzten *Salmonella*-Stammes infiziert. Nach einer Woche wurde die Hälfte einer Gruppe (n=14/2) als interne Kontrolle euthanasiert. Die andere Hälfte wurde jeweils entweder 2,85 mg/kg KGW/d GLY oder RU in Anlehnung an entsprechende worst-case Konzentrationen in Schweinefutter oder keinem Zusatz (Kontrollgruppe, CTRL) ausgesetzt. Während dem 2-wöchigen Fütterungsexperiment wurden Kotproben auf Salmonellen (2 mal wöchentlich) und auf ESBL-*E. coli* (wöchentlich) hin untersucht. Final wurden verschiedene Organe auf ein Vorkommen von Salmonellen getestet und Kot- und Caecumproben mittels 16S rRNA-Ganzgenomsequenzierung untersucht. Weder GLY noch RU erhöhte die Ausscheidungsrate der untersuchten Isolate oder deren Vorkommen in den bei der Sektion gewonnenen Organen. In Übereinstimmung mit dem Fermentationsexperiment änderten sich die MHK-Werte durch den Kontakt zu GLY oder RU nicht. Die 16S rRNA-Analyse zeigte einige Unterschiede in der mikrobiellen Zusammensetzung zwischen den Gruppen. Kot- und Caecum-Proben der RU Gruppe wiesen allgemein eine höhere Diversität auf, für GLY zeigte sich solch eine Tendenz hingegen nur in Kot. Im Vergleich zur Kontrollgruppe, konnten sowohl in Caecum- als auch in Kotproben der RU-Gruppe vermehrt *Lactobacillaceae* (Genus *Lactobacillus*) und in Proben der GLY-Gruppe vermehrt *Enterobacteriaceae* (Genus *Escherichia*) nachgewiesen werden. Im Gegensatz zu Hinweisen aus der Literatur, waren nicht mehr *Clostridia*, sondern in einigen Proben sogar weniger zu finden.

Die Gründe für unterschiedliche Empfindlichkeiten gegenüber GLY und RU innerhalb einer, aber auch zwischen verschiedenen Bakterienspezies, sollten in zukünftigen Studien genauer untersucht werden. Dabei sollte der Fokus auf Resistenzmechanismen und Zielstruktur sowie einem möglichen Zusammenhang zwischen Antibiotika-Resistenz und Glyphosat-Toleranz liegen. Weiterhin sollten die Auswirkungen von Glyphosat auf Mikrobiota, die für einen Mangel an aromatischen Aminosäuren empfindlich sind (z. B. nach der Geburt oder während des Absetzzeitraumes) untersucht werden.

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ANNEX

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                               ----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|
1 aroA 13233 reverse GLY10 RU40  ATGGAATCCCTGACGTTACAACCCATCGCTAGTGTCGATGGCACTATTAATCTGCCCGGTTCCAAGAGCGTTTCTAACCGCGCTTTATTGCTGGCGGCAT  100
aroA 12642 RU80                 ...........A..................C.....................................................................  100
aroA 12628 RU80 (reversed)      ..............................C.........................T.........................................T.  100
aroA 12657 reverse RU80         ...........A..................C.....................................................................  100
aroA 12663 RU80                 ...........A..................C.....................................................................  100
aroA 12685 reverse RU80         ..............................C.....................................................................  100
aroA 13257 RU80                 ..............................C.....................................................................  100
aroA 13281 GLY40                ..............................C.....................................................................  100
aroA 13282 GLY40                ..............................C.....................................................................  100
aroA reverse 13296 RU80         ...........A..................C.....................................................................  100
aroA reverse 13303 RU80         ...........A..................C.....................................................................  100
aroA reverse 13304 RU80         ...........A..................C................................................................A....  100
aroA reverse 13308 RU80         ...........A..................C.....................................................................  100
K-12                             ..............................C.....................................................................  100
aroA reverse 13298 RU80         ...........A..................C.....................................................................  100
aroA 12596 GLY 1.25              ..............................C.....................................................................  100

                               ----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|
1 aroA 13233 reverse GLY10 RU40  TAGCACACGGCAAAACAGTATTAACCAATCTGCTGGATAGCGATGACGTGCGCCATATGCTGAATGCATTAACAGCGTTAGGGGTAAGCTATACGCTTTC  200
aroA 12642 RU80                 ....................................................................................................  200
aroA 12628 RU80 (reversed)      .G........T..............T....................T..T...T.......T.....G....AG..A.......................  200
aroA 12657 reverse RU80         .......................................................................................A............  200
aroA 12663 RU80                 ....................................................................................................  200
aroA 12685 reverse RU80         .......................................................................G............................  200
aroA 13257 RU80                 ....................................................................................................  200
aroA 13281 GLY40                .......................................................................G............................  200
aroA 13282 GLY40                ....................................................................................................  200
aroA reverse 13296 RU80         .......................................................................................A............  200
aroA reverse 13303 RU80         .......................................................................................A............  200
aroA reverse 13304 RU80         .......................................................................................A............  200
aroA reverse 13308 RU80         .......................................................................................A............  200
K-12                             ....................................................................................................  200
aroA reverse 13298 RU80         ....................................................................................................  200
aroA 12596 GLY 1.25              ....................................................................................................  200

                               ----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|
1 aroA 13233 reverse GLY10 RU40  AGCCGATCGTACGCGTTGCGAAATTATCGGTAACGGCGGTCCATTACACGCAGAAGGTGCCCTGGAGTTGTTCCTCGGTAACGCCGGAACGGCAATGCGT  300
aroA 12642 RU80                 ....................................T..................A......G.....................................  300
aroA 12628 RU80 (reversed)      ........................C...........................A..A.C..........................................  300
aroA 12657 reverse RU80         ....................................T..G...............A......G.....................................  300
aroA 12663 RU80                 ....................................T..................A......G.....................................  300
aroA 12685 reverse RU80         ...T..........................................................................A....................C  300
aroA 13257 RU80                 ....................................................................................................  300
aroA 13281 GLY40                ...T....................C...........................................................................  300
aroA 13282 GLY40                ....................................................................................................  300
aroA reverse 13296 RU80         ....................................T..G...............A......G.....................................  300
aroA reverse 13303 RU80         ....................................T..G...............A......G.....................................  300
aroA reverse 13304 RU80         ....................................T..G...............A......G.....................................  300
aroA reverse 13308 RU80         ....................................T..G...............A......G.....................................  300
K-12                             ....................................................................................................  300
aroA reverse 13298 RU80         ....................................T..................A......G.....................................  300
aroA 12596 GLY 1.25              ....................................................................................................  300

                               ----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|
1 aroA 13233 reverse GLY10 RU40  CCGCTGGCGGCAGCTCTTTGTCTGGGTAGCAATGATATTGTGCTGACCGGTGAGCCGCGTATGAAAGAACGCCCGATTGGTCATCTGGTGGATGCGCTGC  400
aroA 12642 RU80                 ..............A.....C....................A...........A.....A........................................  400
aroA 12628 RU80 (reversed)      .........................................A...........A..............G.....C.........................  400
aroA 12657 reverse RU80         ..............A.....C....................A...........A.....A........................................  400
aroA 12663 RU80                 ..............A.....C....................A...........A.....A........................................  400
aroA 12685 reverse RU80         ...............................................T....................G...............................  400
aroA 13257 RU80                 ....................................................................................................  400
aroA 13281 GLY40                .....................................................A....................C.........................  400
aroA 13282 GLY40                ....................................................................................................  400
aroA reverse 13296 RU80         ..............A.....C....................A...........A.....A........................................  400
aroA reverse 13303 RU80         ..............A.....C....................A...........A.....A........................................  400
aroA reverse 13304 RU80         ..............A.....C....................A...........A.....A........................................  400
aroA reverse 13308 RU80         ..............A.....C....................A...........A.....A........................................  400
K-12                             ....................................................................................................  400
aroA reverse 13298 RU80         ..............A.....C....................A...........A.....A........................................  400
aroA 12596 GLY 1.25              ...............................................................................................T....  400
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                                ----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|
1 aroA 13233 reverse GLY10 RU40 GCCTGGGCGGGGCGAAGATCACTTACCTGGAACAAGAAAATTATCCGCCGTTGCGTTTACAGGGCGGCTTTACTGGCGGCAACGTTGACGTTGATGGCTC 500
aroA 12642 RU80                 ...A.............................................A......................................T........... 500
aroA 12628 RU80 (reversed)      .................................................A.................................................. 500
aroA 12657 reverse RU80         ...A.............................................A......................................T........... 500
aroA 12663 RU80                 ...A.............................................A......................................T........... 500
aroA 12685 reverse RU80         .................................................A...............A.................................. 500
aroA 13257 RU80                 .................................................................................................... 500
aroA 13281 GLY40                ..............................................A..A......................................A........... 500
aroA 13282 GLY40                .................................................................................................... 500
aroA reverse 13296 RU80         ...A.............................................A......................................T........... 500
aroA reverse 13303 RU80         ...A.............................................A......................................T........... 500
aroA reverse 13304 RU80         ...A.............................................A......................................T........... 500
aroA reverse 13308 RU80         ...A.............................................A......................................T........... 500
K-12                            .................................................................................................... 500
aroA reverse 13298 RU80         ...A.............................................A......................................T........... 500
aroA 12596 GLY 1.25             .........................................................................C.......................... 500

                                ----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|
1 aroA 13233 reverse GLY10 RU40 CGTTTCCAGCCAATTCCTCACCGCACTGTTAATGACTGCGCCTCTTGCGCCGGAAGATACGGTGATTCGTATTAAAGGCGATCTGGTTTCTAAACCTTAT 600
aroA 12642 RU80                 ............G....................................................................................... 600
aroA 12628 RU80 (reversed)      T...........G..............A........................................................................ 600
aroA 12657 reverse RU80         ............G....................................................................................... 600
aroA 12663 RU80                 ............G....................................................................................... 600
aroA 12685 reverse RU80         ............G..............A...................................T.................................... 600
aroA 13257 RU80                 .................................................................................................... 600
aroA 13281 GLY40                ............G......................................................................................C 600
aroA 13282 GLY40                .................................................................................................... 600
aroA reverse 13296 RU80         ......A.....G..........................A............................................................ 600
aroA reverse 13303 RU80         ............G....................................................................................... 600
aroA reverse 13304 RU80         ......A.....G..........................A............................................................ 600
aroA reverse 13308 RU80         ......A.....G..........................A............................................................ 600
K-12                            .................................................................................................... 600
aroA reverse 13298 RU80         ............G....................................................................................... 600
aroA 12596 GLY 1.25             .................................................................................................... 600

                                ----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|
1 aroA 13233 reverse GLY10 RU40 ATCGACATCACACTCAATCTGATGAAGACGTTTGGTGTTGAAATTGAAAATCAGCACTATCAACAATTTGTCGTAAAAGGCGGGCAGTCTTATCAGTCTC 700
aroA 12642 RU80                 .....T.....C....................................................................G...........C....... 700
aroA 12628 RU80 (reversed)      ..........................................................................T......................... 700
aroA 12657 reverse RU80         ...........C.....C..............................................................G................... 700
aroA 12663 RU80                 .....T.....C....................................................................G...........C....... 700
aroA 12685 reverse RU80         .................................................................................................... 700
aroA 13257 RU80                 .................................................................................................... 700
aroA 13281 GLY40                ..........................................................................T......................... 700
aroA 13282 GLY40                .................................................................................................... 700
aroA reverse 13296 RU80         ...........C....................................................................G................... 700
aroA reverse 13303 RU80         ...........C.....C..............................................................G................... 700
aroA reverse 13304 RU80         ................................................................................G...........C....... 700
aroA reverse 13308 RU80         ...........C....................................................................G................... 700
K-12                            .................................................................................................... 700
aroA reverse 13298 RU80         .....T.....C....................................................................G...........C....... 700
aroA 12596 GLY 1.25             .................................................................................................... 700

                                ----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|
1 aroA 13233 reverse GLY10 RU40 CGGGTACTTATTTGGTCGAAGGCGATGCATCTTCGGCTTCTTACTTTCTGGCAGCAGCAGCAATCAAAGGCGGCACTGTAAAAGTGACCGGTATTGGACG 800
aroA 12642 RU80                 ....C...........T..........................T..C...................G........................C........ 800
aroA 12628 RU80 (reversed)      ..............................................C............................................C........ 800
aroA 12657 reverse RU80         ...........................................T..C...................G........................C........ 800
aroA 12663 RU80                 ....C...........T..........................T..C...................G........................C........ 800
aroA 12685 reverse RU80         ..............................................C............................................C........ 800
aroA 13257 RU80                 .................................................................................................... 800
aroA 13281 GLY40                ..................................C...........C..........................T.................C........ 800
aroA 13282 GLY40                .................................................................................................... 800
aroA reverse 13296 RU80         ...............................C...........T..C...................G........................C........ 800
aroA reverse 13303 RU80         ...........................................T..C...................G........................C........ 800
aroA reverse 13304 RU80         ....C...........T..........................T..C...................G........................C........ 800
aroA reverse 13308 RU80         ...............................C...........T..C...................G........................C........ 800
K-12                            .................................................................................................... 800
aroA reverse 13298 RU80         ....C...........T..........................T..C...................G........................C........ 800
aroA 12596 GLY 1.25             .................................................................................................... 800
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                                ----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|
1 aroA 13233 reverse GLY10 RU40 TAACAGTATGCAGGGTGATATTCGCTTTGCTGATGTGCTGGAAAAAATGGGCGCGACCATTTGCTGGGGCGATGATTATATTTCCTGCACGCGTGGTGAA  900
aroA 12642 RU80                 C...................................T...............................................................  900
aroA 12628 RU80 (reversed)      C..................................................T................................................  900
aroA 12657 reverse RU80         C..................................................T.........C......................................  900
aroA 12663 RU80                 C...................................T...............................................................  900
aroA 12685 reverse RU80         C............................................................................................G......  900
aroA 13257 RU80                 ....................................................................................................  900
aroA 13281 GLY40                C............................................................................................G......  900
aroA 13282 GLY40                ....................................................................................................  900
aroA reverse 13296 RU80         C..................................................T................................................  900
aroA reverse 13303 RU80         C..................................................T.........C......................................  900
aroA reverse 13304 RU80         C...................................T...............................................................  900
aroA reverse 13308 RU80         C..................................................T................................................  900
K-12                            ....................................................................................................  900
aroA reverse 13298 RU80         C...................................T...............................................................  900
aroA 12596 GLY 1.25             ....................................................................................................  900

                                ----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|
1 aroA 13233 reverse GLY10 RU40 CTGAACGCTATTGATATGGATATGAACCATATTCCTGATGCGGCGATGACCATTGCCACGGCGGCGTTATTTGCAAAAGGCACCACCACGCTGCGCAATA  1000
aroA 12642 RU80                 ........C..........................C.......................C........................................  1000
aroA 12628 RU80 (reversed)      ........C..........................C.......................C......................A.................  1000
aroA 12657 reverse RU80         ........C..........................C.......................C........................................  1000
aroA 12663 RU80                 ........C..........................C.......................C........................................  1000
aroA 12685 reverse RU80         ....................................................................................................  1000
aroA 13257 RU80                 ....................................................................................................  1000
aroA 13281 GLY40                ....................................................................................................  1000
aroA 13282 GLY40                ...................................C................................................................  1000
aroA reverse 13296 RU80         ........C...........................................................................................  1000
aroA reverse 13303 RU80         ........C..........................C.......................C........................................  1000
aroA reverse 13304 RU80         ........C..........................C.......................C........................................  1000
aroA reverse 13308 RU80         ........C...........................................................................................  1000
K-12                            ....................................................................................................  1000
aroA reverse 13298 RU80         ........C..........................C.......................C........................................  1000
aroA 12596 GLY 1.25             ...................................C................................................................  1000

                                ----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|
1 aroA 13233 reverse GLY10 RU40 TCTATAACTGGCGTGTTAAAGAGACCGATCGCCTGTTTGCGATGGCAACAGAACTGCGTAAAGTCGGTGCGGAAGTAGAAGAGGGGCACGATTACATTCG  1100
aroA 12642 RU80                 ............................................................................C...........T....T......  1100
aroA 12628 RU80 (reversed)      ............................................................................C................TT.....  1100
aroA 12657 reverse RU80         ............................................................................C................TT.....  1100
aroA 12663 RU80                 ............................................................................C...........T....T......  1100
aroA 12685 reverse RU80         ................................................................T...................................  1100
aroA 13257 RU80                 ...................................................................C........G.......................  1100
aroA 13281 GLY40                ................................................................T...................................  1100
aroA 13282 GLY40                ......................A.............................................................................  1100
aroA reverse 13296 RU80         ................................................................T...........C.......................  1100
aroA reverse 13303 RU80         ............................................................................C................TT.....  1100
aroA reverse 13304 RU80         ............................................................................C...........T....T......  1100
aroA reverse 13308 RU80         ................................................................T...........C.......................  1100
K-12                            ...................................................................C........G.......................  1100
aroA reverse 13298 RU80         ............................................................................C...........T....T......  1100
aroA 12596 GLY 1.25             ......................A.............................................................................  1100

                                ----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|
1 aroA 13233 reverse GLY10 RU40 TATCACTCCACCGGAAAAACTGAACTTTGCCGAGATCGCGACATACAATGATCACCGGATGGCGATGTGTTTCTCGCTGGTGGCGTTGTCAGATACACCA  1200
aroA 12642 RU80                 C..T..G.................A.....T........C..T..T...................................C..A..A........G..T  1200
aroA 12628 RU80 (reversed)      C..T..G.................A.....T........C..T..T...................................C..A..A........GG..  1200
aroA 12657 reverse RU80         C..T..G.................A.....T........C..T..T...................................C..A..A........GG.T  1200
aroA 12663 RU80                 C..T..G.................A.....T........C..T..T...................................C..A..A........G..T  1200
aroA 12685 reverse RU80         .......................................A............................................................  1200
aroA 13257 RU80                 .........T..........................................................................................  1200
aroA 13281 GLY40                .......................................A.........................................C..A..A........GG.T  1200
aroA 13282 GLY40                ....................................................................................................  1200
aroA reverse 13296 RU80         .........T.............................C..T..T................................A.....................  1200
aroA reverse 13303 RU80         C..T..G.................A.....T........C..T..T...................................C..A..A........GG.T  1200
aroA reverse 13304 RU80         C..T..G.................A.....T........C..T..T...................................C..A..A........G..T  1200
aroA reverse 13308 RU80         .........T.............................C..T..T................................A.....................  1200
K-12                            .........T..........................................................................................  1200
aroA reverse 13298 RU80         ...T..G.................A.....T........C..T..T...................................C..A..A........G..T  1200
aroA 12596 GLY 1.25             ....................................................................................................  1200

                                ----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|----
1 aroA 13233 reverse GLY10 RU40 GTGACGATTCTTGATCCCAAATGTACGGCCAAAACATTTCCGGATTATTTCGAGCAGTTGGCGCGGATTAGCCAGGCAGCCTGA                  1284
aroA 12642 RU80                 .................A........C..T.....T.....A..C...........A...........----------------                  1268
aroA 12628 RU80 (reversed)      .......................C.................................C..........................                  1284
aroA 12657 reverse RU80         .................A........C..T.....T.....A..C...........A...........A..T..AC.C.G....                  1284
aroA 12663 RU80                 .................A........C..T.....T.....A..C...........A...........A..T..AC.C.G....                  1284
aroA 12685 reverse RU80         .......................C.................................C..........................                  1284
aroA 13257 RU80                 .......................C.................................C..........................                  1284
aroA 13281 GLY40                .................A........C..T.....T.....A..C...........A...........A..T..AC.C.G....                  1284
aroA 13282 GLY40                ....................................................................................                  1284
aroA reverse 13296 RU80         .......................C.................................C..........................                  1284
aroA reverse 13303 RU80         .................A........C..T.....T.....A..C...........A...........A..T..AC.C.G....                  1284
aroA reverse 13304 RU80         .................A........C..T.....T.....A..C...........A...........A..T..AC.C.G....                  1284
aroA reverse 13308 RU80         .......................C.................................C..........................                  1284
K-12                            .......................C.................................C..........................                  1284
aroA reverse 13298 RU80         .................A........C..T.....T.....A..C...........A...........A..T..AC.C.G....                  1284
aroA 12596 GLY 1.25             .......................C.................................C..........................                  1284
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LIST OF PUBLICATIONS

Journal Articles

Bote, K.; Pöppe, J.; Merle, R.; Makarova, O.; Roesler, U. (2019):

Minimum Inhibitory Concentration of Glyphosate and of a Glyphosate-Containing Herbicide Formulation for *Escherichia coli* Isolates - Differences Between Pathogenicand Non-pathogenic Isolates and Between Host Species.

Frontiers in microbiology; 10, p. 932

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European journal of microbiology & immunology, 9(3), p. 94–99.

https://doi.org/10.1556/1886.2019.00010

Pöppe, J.; **Bote, K**.; Merle, R.; Makarova, O.; Roesler, U. (2019):

Minimum Inhibitory Concentration of Glyphosate and a Glyphosate-Containing Herbicide in *Salmonella* enterica Isolates Originating from Different Time Periods, Hosts, and Serovars.

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Does selection for resistance to a popular glyphosate-based herbicide lead to increased tolerance and cross-resistance to antibiotics in *Salmonella enterica*?

Applied and Environmental Microbiology, vol. 86, no. 24

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The glyphosate formulation Roundup influences the global metabolome of pig gut microbiota *in vitro.*

Science of The Total Environment, 745, p.140932

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Oral presentations

Bote, K.; Pöppe, J.; Makarova, O.; Merle, R.; Roesler, U. (2019):

Minimum Inhibitory Concentrations for glyphosate and glyphosate-containing formulation in livestock-associated Enterobacteriaceae.

XIX International Congress of ISAH 2019

08.09.-12.09.2019.

Wrocław, Poland

Bote, K.; Pöppe, J.; Makarova, O.; Merle, R.; Roesler, U. (2017):

MIC Distributions for Glyphosate in Farm Animal-Associated Enterobacteriaceae

DRS-Doktorandensymposium

22.09.2017

Berlin, Germany

2nd prize: Best oral presentation

Bote, K.; Pöppe, J.; Makarova, O.; Rösler, U. (2017):

MIC distributions for glyphosate in farm animal-associated Enterobacteriaceae determined by the broth microdilution method.

National Symposium on Zoonoses Research 2017

12.10. - 13.10.2017

Berlin, Germany

Posters

Bote, K.; Pöppe, J.; Johnston, P.; Rösler, U.; Makarova, O (2019):

More than just a weed killer: exposure to Roundup selects for resistance with low fitness cost in pathogenic enteric bacteria *Escherichia coli* and *Salmonella enterica* from farm animals

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16.10.– 18.10.2019

Berlin, Germany

Bote, K.; Pöppe, J.; Riede, S.; Breves, G; Makarova, O.; Roesler, U. (2018):

Impact of Roundup, a glyphosate-containing herbicide, on pathogenic Enterobacteriaceae in a ‚Rumen Simulation Technique‘-fermenter model

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18.10. - 19.10.2018

Berlin, Germany

Poeppe, J.; **Bote, K**.; Roesler, U.; Makarova, O. (2018):

Induction of Resistance to Roundup, a Glyphosate-Containing herbicide, in Enterobacteriaceae *In Vitro*

National Symposium on Zoonoses Research 2018 – German Research Platform for Zoonoses

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27.07.2018

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Effects of Glyphosate, a Common Herbicide, on Farm Animal-associated Enterobacteriaceae.

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07.06.2018

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Makarova, O.; **Bote, K.;** Poeppe, J.; Roesler, U. (2018):

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19.03.2018

Paris - France

Pöppe, J.; **Bote, K.;** Rösler, U.; Makarova, O. (2017):

Induction of Resistance to Glyphosate, a Common Herbicide, in *E.coli In Vitro*.

National Symposium on Zoonoses Research 2017

12.10.-13.10.2017

Berlin, Germany

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Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen Anspruch genommen habe.

Berlin, den 07.01.2021 Katrin Bote