Aus dem Institut für Tier- und Umwelthygiene

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"Impact of glyphosate and glyphosate containing herbicides on *Salmonella enterica* from farm animals"

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"Holzhacken ist deshalb so beliebt,

weil man bei dieser Tätigkeit den Erfolg sofort sieht."

Albert Einstein

Für Henriette.

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AMPA	Aminomethylphosphonic acid
a.i.	After infection
cfu	Colony forming unit
DM	Dry mass
DNA	Desoxyribonuceid acid
GBH	Glyphosate based herbicides
gly	Glyphosate
GM	Genetically modified
LFGB	Lebensmittel- Bedarfsgegenstände und Futtermittelgesetzbuch
MH	Mueller hinton medium
MH I	Mueller hinton I
MH II	Mueller hinton cation adjusted
MIC	Minimum inhibitory concentration
MSRV	Modified semi-solid Rappaport vassiliadis
NOAEL	No-observed-adverse-effect-level
OD	Optical density
POEA	Polyetoxylated tallow amine
rRNA	Ribosomal ribonucleic acid
RU	Roundup LB plus
Rusitec	Rumen Simulation Technique
SCFA	Short chain fatty acids
XLD	Xylose lysine deoxycholate

III. List of genes and proteins

acrB	multidrug efflux pump subunit acrB
ahpC	Alkyl hydroperoxide reductase C gene
ArcAB	ArcAB two-component signal transduction system gene
argF	Ornithine carbamoyltrnasferase subunit F gene
aroA	3-phosphoshikimate 1-carboxyvinyltransferase gene
CydAB	Cytochrome D oxidase gene
deoA	Thymidine phosphorylase gene
E4P	Erythrose 4-phosphate
EPSP	5-Enolpyruvylshikimat-3-phosphate
EPSPS	5-Enolpyruvylshikimat-3-phosphate-synthase
FtsZ	Cell division protein FtsZ gene
fusA	Elongation factor G gene
ivbL	ilv operon leader peptide gene
LolA	Outer-membrane lipoprotein carrier protein gene
Lpp	Lipoma prefered partner gene
mutS	DNA mismatch repair protein MutS
OmpA	Outer membrane protein A gene
osmY	Osmotically-inducible protein Y gene
PEP	Phosphoenolpyruvate
pflB	Formate acetyltransferase 1 gene
rcsB	Transcriptional regulatory protein RcsB
rpoD	RNA polymerase sigma factor RpoD gene
RpoE	RNA poylmerase sigma-E factor gene
RpoS	NA polymerase sigma factor RpoS gene
rrfA-H	5S ribosomal RNA gene
ruvAB	DNA binding protein
sdhA	Succinate dehydrogenase flavoprotein subunit
SlyB	Outer membrane lipoprotein SlyB gene
speA	Exotoxin type A gene
SufB	FeS cluster assemply protein SufB
SufC	SufC protein
SufS	Cysteine desulfurase
TolC	Outer membrane protein TolC
uxaC	Uronate isomerase gene
YehZ	Glycine betaine-binding protein YehZ gene
yhhS	Uncharacterized MFS-type transporter YhhS

1. Introduction

1.1 Glyphosate, a "once in a century" herbicide

Glyphosate, N - (phosphonomethyl) glycine (figure 1), is the most widely-used herbicide in the world (1). The chemical was invented in the 1950s (2) but was first patented for weed control in 1974 (3) by Monsanto, a worldwide operating company producing seeds and pesticides.

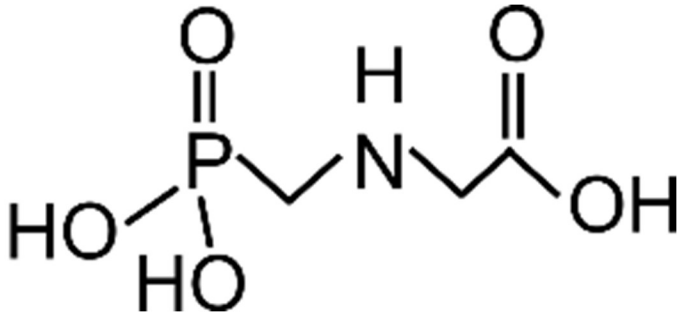

Figure 1: Chemical structure of N - (phosphonomethyl) glycine, glyphosate.

Until the 1990s, it was only possible to use glyphosate to prepare fields in between cultivation of grains and as a pesticide for weed control. The rise of its use as a herbicide began in 1996, when the first glyphosate resistant seeds were available on the market (figure 1). From this point on, the amount of glyphosate use in agriculture substantially increased worldwide (4). Also the usage of glyphosate changed in its function as it is now additionally used for desiccation of crops and selection during growth phase (5). Aside from agricultural use, glyphosate is also common for weed control in urban areas (6) and on the rail network (7).

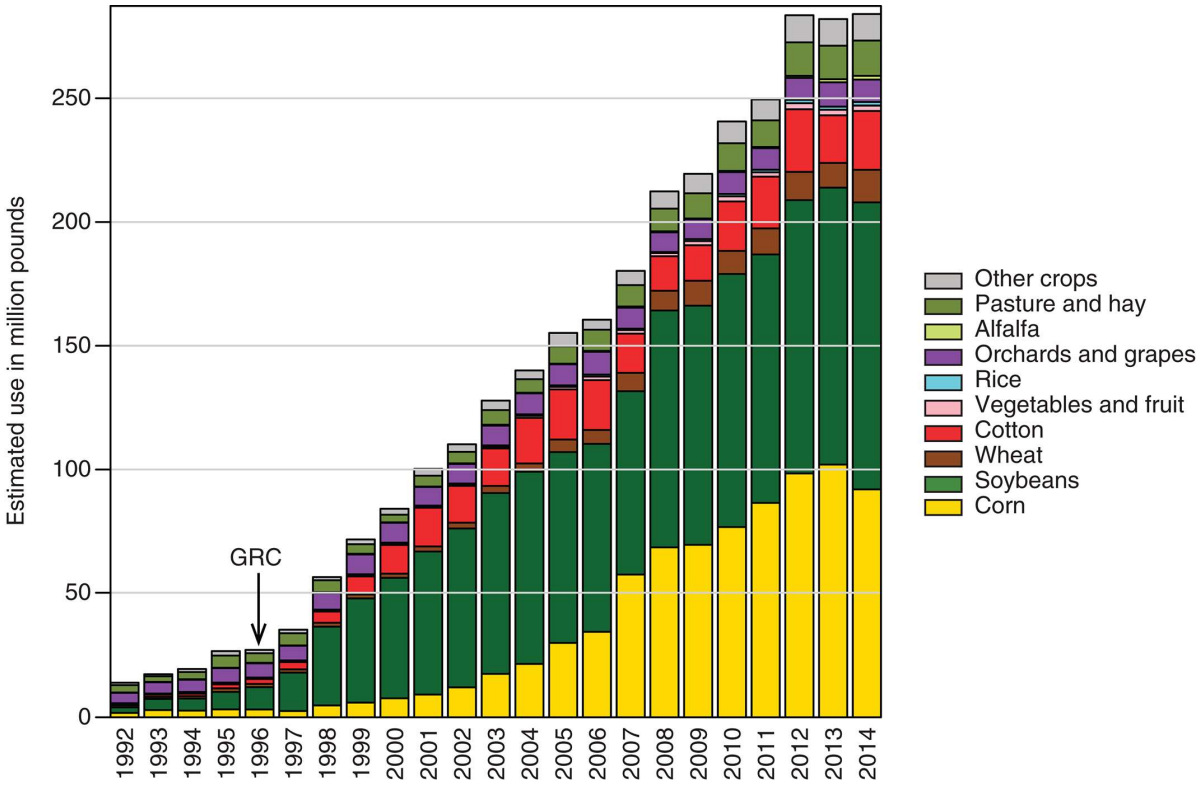

Figure 2: Duke et al 2017. The history and current status of glyphosate. Pest Management Science (4).

Since the amount of glyphosate used in agriculture heavily increased in the mid 1990s (figure 2), critical concerns arose surrounding the pesticide. In European countries, the use of glyphosate during the growth phase is prohibited, due to the ban on genetically modified crops. There is general concern about residues in feed and food and a potential, but limited risk for consumers (8). In other countries glyphosate is used within the whole agricultural cycle and is transferred in large amounts into the food chain (9). First, glyphosate was categorized as `least toxic` for animals by the United States Environmental Protection Agency (EPA)(10), but this acceptance has changed over the recent years. Worldwide there is no consensus about the risk of cancer or other diseases in association with glyphosate so the call for independent research on the topic is also made in science (11, 12). In respect of direct effects on higher organisms, most studies came to the conclusion, that there is no evidence for toxicity, DNA damage or carcinogenicity in glyphosate or its metabolite aminomethylphosphonic acid (AMPA) (10, 13, 14) although the debate is ongoing.

When glyphosate is introduced to fields it is always combined with adjuvants which have the function to allow the glyphosate to enter the plant cells (15). In many cases, these adjuvants consist of polyetoxylated tallow amine (POEA). These chemicals are known to be more toxic to higher organisms than glyphosate itself (16). However, even without POEA, glyphosate based herbicides (GBHs) still contain a number of chemicals as adjuvants, which do not have to be declared. Also these were discussed to be more dangerous than glyphosate, or at least to be dangerous when combined with glyphosate or other substances (17). Therefore, the

debate about investigations of toxicity of the combination of substances came into the focus of public interests. Many researchers supported the need to look into that topic and started to investigate not only the effects of glyphosate as a pure substance but also the effect of ready to use mixtures for agriculture (18–20). In fact, in these studies the GBHs showed different effects, like being more or less harmful, compared to glyphosate as a pure substance and studies started to also focus on glyphosate as formulations. The German Federal Institute for Risk Assessment (BfR) declared GBHs as potentially more dangerous than the pure substance (8). Plants are not able to metabolize glyphosate and so the herbicide accumulates in plant tissues (21). Moderate to high residues of glyphosate and its metabolite AMPA can also be found in soil. In the laddder, degradation of glyphosate is mostly performed by microorganisms (22).

1.2 Glyphosate target structure and mode of action

The fact that glyphosates target structure cannot only be found in plants but also in bacteria, some fungi and unicellular parasites and the resulting idea to use glyphosate as antimicrobial was already part of its patent in the 1970s. Therefore, this aspect has already been investigated. The target structure is the enzyme of the penultimate step in the shikimate pathway (Figure 3), the 5-Enolpyruvylshikimate-3-phosphate-synthase (EPSPS) (23, 24). The biochemical pathway can only be found in plants, bacteria and some fungi and unicellular parasites (25–27). The EPSPS is coded by the gene *aroA*. The final step of shikimate pathway is the production of chorismate (28). It is the intermediate product for the biosynthesis of aromatic amino acids like phenylalanine, tryptophan and tyrosine (29). The interruption of shikimate pathway leads to an interruption of carbon fixation and a complete stop of the metabolism and the death of the plant (5, 30). Since this biochemical pathway does not exist in animals and humans the common opinion has been formed, that glyphosate is not toxic for higher organisms (31).

The EPSPS occurs naturally in different variants in bacteria. The EPSPS I and II have similar genetic backgrounds, but differ in their sensitivity against glyphosate (32). The third variant, EPSPS III, has only recently been described. It was found in *Pseudomaonas putida* isolated from a glyphosate polluted area in china (33) and shows a much higher tolerance against glyphosate than the first described. The variants I and II have less than 30 % amino acid identity in common. Class II enzymes have higher affinity to phopshoenol pyruvat (PEP), which is an intermediate metabolic product of shikimate pathway, and are more tolerant to glyphosate. The variant III seem to be related to class I, but it remained unclear if this variant

occurred in the *P. putida* isolate or if it was taken from another unknown bacterial species via horizontal gene transfer (33). The sensitivity against glyphosate decreases from variant I to variant III.

For the creation of glyphosate resistant plants, in most cases resistance genes from other organisms were transferred, for example from *Escherichia* (*E.*) *coli,* into the agricultural plant species (34). For this purpose, some EPSPS variants less susceptible to glyphosate were created in bacteria, either through direct or random mutagenesis (35, 36) also for *Salmonella* (*S.*)*. enterica* and other Enterobacteriacea (37). Unspecific increase of glyphosate resistance was created via gene shuffling (38) or direct evolution (39). Naturally or directly created non-target resistances against glyphosate were relatively unexplored. One mechanism detected by Staub et al. 2012 (40) is the overexpression of an membrane efflux pump in *E. coli* and *Pseudomonas*, which lead to a high-level resistance to glyphosate. In Actinobacteria *Corynebacterium glutamicum* Liu et al. 2013 (41) found, that a mechanism for maintaining reactive oxygen species also led to resistance against glyphosate and many other chemicals. From an isolate of Enterobacter, resistant to glyphosate via a mutation in EPSPS it has been shown, that resistance to glyphosate can also have effect on non-target genes, like for stress response (42). The potential of bacteria to develop resistance against glyphosate due to exposure to the herbicide has so far not been investigated under controlled laboratory conditions.

Besides the target in plants and bacteria, glyphosate has an additional effect on the environment, the chelating effect. Glyphosate presumably binds to ions of copper, calcium, magnesium, iron and manganese and therefore decreases the availability of these ions for plants and other organisms (43). Interestingly, this chemical reaction was also known even before the discovery of its herbicidal effect and was patented by Toy and Uhing in 1964 (44). Despite the inhibition of EPSPS the chelating effect of glyphosate can have a concerning effect on plants and animals but also on bacteria and microbiological communities in soil, plants and in animal and human hosts. Lacking essential minerals, many biological processes can be influenced or even interrupted.

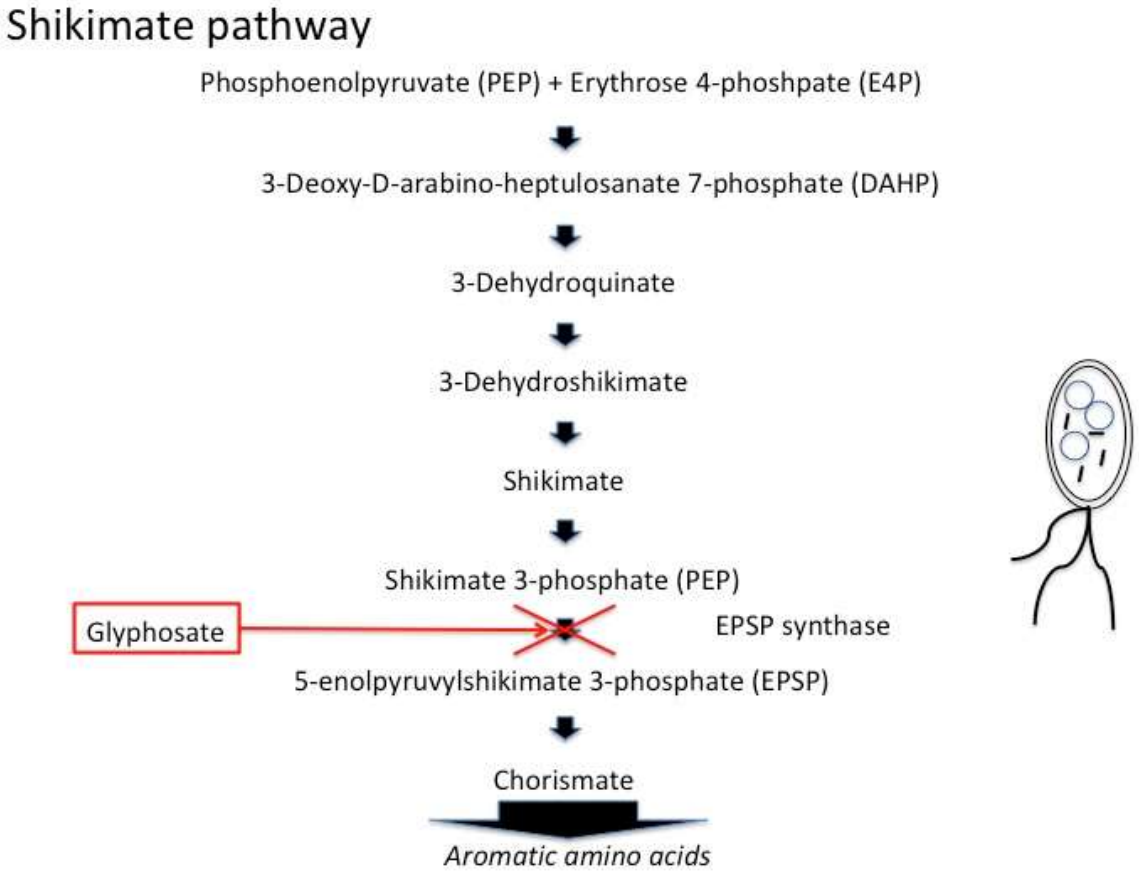

Figure 3: Shikimate pathway in bacteria as simplified scheme with glyphosate effect modified after Helander et al 2012 (45).

1.3 Glyphosate residues in the environment

As glyphosate and its formulations are heavily used on fields, the topic of glyphosate residues in human and animal feed came into public awareness. While the usage of glyphosate during growth period is prohibited in the European Union, in most other countries in the world this is common practice. Therefore, even in the European Union glyphosate residues in animal feed are easy to find, as many components of the feed are imported from non-EU countries. However, glyphosate residues were found especially in soy (9, 46–48), maize (4) and therefore in feed for almost all farm animal species (49–51). An overview of the amount of glyphosate residues in animal feed investigated in Europe in the most recent published official reports can be found in table 1.

Citation	Type of animal feed	Amount of residues
EFSA report 2018 (51)	Cattle	342 mg/kg DM
	Sheep	530 mg/kg DM
	Swine	123 mg/kg DM
	Poultry	33,4 mg/kg DM
Renewal Assessment Report 2015 (8)	Chicken	4,6 mg/kg DM
	Dairy cattle	43,4 mg/kg DM
	Beef cattle	104,9 mg/kg DM
	Pig	5,2 mg/kg DM

Table 1: Amount of glyphosate residues in animal feed published in the two most comprehensive reports commissioned by the European Union. DM = dry matter.

As the feed of many farm animals contains a high amount of soy, many publications focus on the content of glyphosate in soy and soy products. In the United States in 2015, in over 90% of soy samples, glyphosate residues could be found (52). Residues of glyphosate are particularly found when the crop is made up of genetically modified (GM) glyphosate resistant plants and these plants make a very high proportion of the total harvested soy crop, especially in South America (Argentina 100%, Brazil 83%) (46). It has been also shown previously, that the amount of glyphosate residues in plants increases with the intensity of treatment of plants with glyphosate during the crop cycle (53). In the European Union the amounts of residues found in animal feed is dependent on the concentrations of the pesticide in imported grains. However, as farm animal feed does not only consist of soy and imported soy, but of a mixture of different crops from different countries, the particular level of glyphosate contamination varies. For Germany Schnabel et al 2017 (50) showed an amount of glyphosate daily intake for dairy cows of up to 84,5 mg. European food safety authority (EFSA) calculated up to 530 mg/kg dry mass glyphosate for farm animal feed (51). This shows clearly that although glyphosate and glyphosate formulation output on fields in Europe is limited; glyphosate reaches animal feed also in Europe in high amounts. In ground water too, especially in agricultural regions, many investigations were made to show how glyphosate pollution increases. For example, in water used for farm animals, glyphosate and glyphosate formulations could be found (54). Still the impact of those findings for the environment and the animals is not clear. Direct toxic effects of glyphosate and GBHs may not be expected in concentrations like that as the no-observed-adverse-effect-level (NOAEL) for chronic exposure is recommended to be 560 mg/kg body weight and day (13). The amount of residues is very disparate and also the results of research vary a lot.

Because of the residues in animal feed, it is no surprise that residues of glyphosate could also be found in farm animals and their excretions. Krüger et al showed in 2013 (55) for a small sample size of Danish dairy cows, that in urine samples of all sampled cows residues of glyphosate could be found in different concentrations from 10,0 up to over 103,3 ng/ml glyphosate in urine. This shows that glyphosate from feed reaches the organs of the farm animals. Residues of glyphosate were also found in farm animal organs and even in humans (56, 57). This underlines, that glyphosate can be found all along the food production chain through to the consumer (58).

The fact that glyphosate can be found as residues in animal feed and drinking water leads to a further topic in the debate around glyphosate, independent from the question of toxicity. As glyphosate can be found almost everywhere in the environment and also in animals, what kind of impact can glyphosate and formulation have on the bacteria and bacterial communities within this eco-system? As already mentioned before, glyphosate was postulated to be a possible antibiotic when it was patented in 1974. Glyphosate was never established as an antimicrobial, even though some investigation about the shikimate pathway and glyphosate took place for general drug design (59, 60).

1.4 Glyphosate as an antimicrobial

Monsanto`s patent already included a potential antimicrobial effect of the chemical. Side effects of glyphosate and formulation on bacteria and bacterial communities were expected. Also, the varying sensitivity against glyphosate by different variants of *aroA* gene lead to the suggestion, that an impact of glyphosate not only on individual bacterial species is logical, but also an impact of glyphosate on bacterial communities and therefore on microbiota is highly probable. Glyphosate may act as a selection factor for more tolerant bacterial species.

While the discussion about the direct impact of glyphosate on health for humans and animals was triggered by the heavy use of glyphosate in agriculture, the discussion about the influence of glyphosate and glyphosate formulation on organism interaction and species communities is relatively new. However, as the target structure of glyphosate, the shikimate pathway can be found not only in plants but also in bacteria, fungi and some unicellular organisms (e.g. parasites), the effect of the pesticide on bacteria itself came into focus very early. In recent times, the connection between those two aspects came to attract attention and research started to concentrate on the effect glyphosate can have on the microbiota in many different environments due to the different sensitivities of the bacterial species. For host-associated bacteria, pathogenic species, like *Salmonella enterica* seem to have lower

sensitivity towards glyphosate than commensal ones (61). For example, *Clostridium* species showed higher resistance for glyphosate in *in vitro* experiments and lead to higher concentrations of botulinum neurotoxin in the presence of glyphosate (62). These results were supported by the findings of higher sensitivity of commensal species in the same host-related environment, for example for Enterococci species. This change in bacterial composition had an impact on the production of botulinum neurotoxin which is suppressed by the presence of commensal bacteria *in vitro* (63). Other *in vitro* experiments could not confirm these findings. Riede et al 2016 compared differences in the total bacterial composition in an *in vitro* experiment with rumen simulation technique and did not find significant changes within the use of glyphosate and glyphosate formulation (64). In non-host associated environments, different sensitivities for bacterial species against glyphosate were found, like for bacterial species in food production, and not only for glyphosate as a pure substance but also as a formulation (65). Many experiments investigated the potential effect of glyphosate on soil microbiota, as this is one first point of entry of glyphosate into the environment. Some investigations were made about the inhibitory effect of glyphosate on soil microbiota for example in *Bacillus subtilis* (66, 67), a widespread bacterial species. Also for fungi as part of the soil microbiota, it has been shown that glyphosate as an ingredient of formulation inhibit growth from a certain amount and in lower concentration it decreases sporulation and pigmentation. The fungi species also show differences in sensitivity which might result in a shift of species composition (68). This direct effect on soil microbiota can lead to implied effects on other organisms as part of the soil community (69). Also in rhizosphere and soil related bacteria these results were confirmed. Susceptibility to glyphosate and formulation differs for different rhizosphere related bacteria (70–72). Three specific isolates of *Pseudomonas* sp., *Actinobacteria* and *Seratia* sp from waste water and, agricultural soil and plant tissue grew in increased glyphosate concentration and showed resistance to glyphosate. They were also able to degrade glyphosate based herbicides (73).

Investigations for complex ecosystems like soil show controversial results. For example in vineyard soil the total count of bacteria increased by 260 % in presence of glyphosate in comparison to mechanical weeding. Also the abundance of soil bacteria increased by 264 %. Even if both effects were not statistically significant, the trend is not towards a decrease of bacterial count or species variability due to herbicide usage. The authors try to explain this by a domino effect in the complex environment (74). The impact of glyphosate in long term experiments (six month) on soil health, including higher organisms also relevant for soil ecology, showed that the numbers of heterotrophic microbes increased during the experiment compared to untreated soil (75). The impact of the pesticide could particularly be detected in experiments over much longer periods. Some of these soil related experiments also hint, that

the effect of glyphosate on the bacterial diversity can be reduced by application of amino acids, at least for fungi (68).

In host associated environments glyphosate enters via the food chain (9). Many studies were made about toxicity and health effect of glyphosate on higher organisms like rodents (76), mice, frogs (16), fish (77) and humans (78). Results of these studies are highly controversial and debate is still ongoing. Nevertheless, apart from direct health influence of glyphosate the discussion of indirect influence on individuals and ecology increased more and more. The impact of glyphosate on insects, especially on bees, was one of the most common topics. Some studies showed for example, the negative impact of glyphosate on honeybee behavior (79, 80). But also the microbiota of honeybees came into focus and it was shown that glyphosate has effects on the bee microbiota in composition, whereby a negative effect could not be proven (81). A recent study went even further and postulated a negative effect of glyphosate on the microbiota of bees and a direct loss of young working bees (82). They fed glyphosate containing sucrose sirup to adult working bees in two different glyphosate concentrations (5mg/l and 10 mg/l) and compared the microbiota via 16S rRNA to a control group fed with sucrose syrup. In another experiment, they fed glyphosate to young bees in early stage of gut colonization. They found out, that glyphosate had an effect on number of bacteria and diversity of microbiota, but results were not conclusive. For the young bees with developing microbiota they found, that the young bees were more susceptible to the pathogen *Serratia marcescense* (82) At least this last study shows a direct effect of glyphosate on the microbiota, which leads to a direct effect on individuals and ecology and a selection advantage for pathogenic bacterial species.

In other studies investigating the composition of microbiota in hosts relations between the effect of glyphosate on microbiota and behavior for mice (83) and rats (84) were found.

Compared to these results, effects of glyphosate on the microbial community in farm animals are largely unknown. For cattle *in vitro* experiments suggests little effects on microbiota composition (64). Except for this work, most of the authors concluded, that glyphosate can possibly act as a modulator of bacterial communities and may lead to selection for pathogenic bacteria (61–63). For other farm animals like chicken and pigs, the question whether glyphosate intake has an effect on bacterial composition or the excretion of pathogenic bacteria is still not answered.

All these findings underline that the impact of glyphosate and glyphosate containing formulation has been underestimated in the discussion of the risks for glyphosate use in agriculture. As the herbicide can be found in water, soil and every part of the food chain the question of side effects is important to investigate. In addition, predictions of the impact on

complex ecosystems via laboratory experiments are shown to be limited and *in vivo* experiments are critical to understand and prevent nature and environment from lasting damages.

1.5 Interactions of glyphosate with antimicrobials

The induction of tolerance and resistance is a complex topic and has been heavily discussed due to the increase of antibiotic resistances in many bacterial species (85). The development of resistance against herbicides, especially glyphosate has before only been discussed in the context of the finding of new genes for the use of creation of resistant crops (86). In research like this, resistances against glyphosate developed by bacteria themselves are not target of the question but the impact on other resistances. Only after glyphosate came into focus as a potential risk to human end environment a debate started about the impact of glyphosate on bacteria themselves.

The definition of resistance comes from antimicrobial drugs from medical and veterinary field and was defined in a clinical context. The Clinical and Laboratory Standard Institute (CLSI) defines the methods of testing the susceptibility of microbes against antimicrobials and gives cut off values for clinical relevant antimicrobials (87). Recently, differences in definition of clinical and microbiological resistances were established (88). Following this, microbiological resistance has to be lead back to genetically mutations. This disqualifies the term for phenotypically detected reduced susceptibility. This is important especially for biocides and other antimicrobial active substances, which includes some herbicides. For other antimicrobial substances like herbicides and biocides clinical cut offs are not applicable and therefore missing.

Further, the resistance to glyphosate has to be distinguished to effects of cross- and co-resistance against antibiotics. As definitions are always a point of contention, for further discussion, this study considers cross-resistance as one biochemical system, confer resistance to two or more different chemicals and co-resistance as a genetic link between one or more resistances. For example in nearby places in genome or on the same plasmid as described and visualized in Baker-Austin et al. 2006 (89).

As resistance to glyphosate has been described above, cross- and co-resistance is an additional topic. It has been described especially for classical antibiotics. This means specifically, resistance against one antibiotic initiated or modified by another classical antibiotic. Especially for Enterobacteriaceae and here for *S. enterica* (90) but even more for

E. coli , these effects were already shown in numerous publications (for example 91–93). But not only antibiotics can lead to co-resistance, it has also been shown for biocides and heavy metals for foodborne pathogens (94).

Shehata et al. 2013 were the first to discuss whether the presence of glyphosate selects for certain bacterial species, especially for pathogens. In addition to the question of direct effect of glyphosate and formulation on resistance and selection of pathogenic bacteria, Kurenbach et al. showed in 2015, that glyphosate can increase and decrease susceptibility of *E. coli* and *Salmonella enterica* against different antibiotics (95). The same working group postulated in a subsequent publication that parallel use of glyphosate and antibiotics modified susceptibility against antibiotics in *E. coli* and *Salmonella enterica* ser. Typhimurium and that active ingredient of herbicide formulations are accountable for that effect (96). But, the group did not investigate the genetic background of those results.

Also other chemicals, like biocides in general are suspected to increase resistance to antibiotics, as it was shown for benzalkonium chloride and *Pseudomonas aeruginosa*, where co-selection via the biocide lead to increased resistance against polymyxin B through overexpression of multidrug efflux pumps (97). As glyphosate has side effects besides the target structure, for example the chelating effect, and as the impact of glyphosate as a formulation is an important topic, the comparison of to the effects of biocides is evident. As herbicides also biocides were used in high concentrations and are often a mixture of different ingredients. Also in GBH the effect of other undeclared ingredients has to be considered.

Many other substances can increase resistance to antibiotics in Enterobacteriaceae *in vivo,* like here shown for ions of zinc (98), so the anticipation of glyphosate, as an influence of the environment and with many effects in different directions is consistent.

Indirect induction of antibiotic resistance is also a possibility. It is known, that glyphosate is a chelator (99) and therefore reduces available ions, like iron from the environment. Lack of nutrients, especial metal ions, is known to be a driver for the development of antibiotic resistances like magnesium in *S. enterica* (100) or iron in *E.coli* (101).

1.6 Development of tolerance and resistance in bacteria

Tolerance and persistence are, together with cross- and co-resistance, an upcoming topic of bacterial resistance genesis. The evolution of resistance is mostly described as a single step mutation but recently it has been demonstrated, that tolerance can initiate the evolution of resistance (102). This can lead also to multidrug tolerance (103) or collateral sensitivity

(increase of sensitivity against an antibiotic class in context of the resistance against another antibiotic class) to other antimicrobials (104).

The correlation between tolerance and resistance and the impact of other chemicals like biocides and herbicides in the development of resistances against antibiotics is a complex and so far not fully understood topic. Induction of resistance in bacteria has been shown to happen due to many reasons. Excessive use of antibiotics lead to a very fast developing resistance against the used antibiotic in bacterial species (105). Glyphosate was also mentioned as a broad spectrum antibacterial target (106),(59). Still the question is not answered whether glyphosate can induce resistance or at least tolerance and change the susceptibility against antibiotics due to long-term exposure, like in animal gut.

The selection of biocides for antibiotic resistant bacteria has been tested (107). As the comparison of GBH and biocides may be valid, this can lead to the question, if the selection for antibiotic resistant bacteria is happening in an environment polluted with glyphosate. Biocides are shown to have an impact on more than one target gene (108, 109), this can also be possible for GBH. As described earlier, for GBH the comparison with biocides could be helpful, as both sorts of substances have some comparable characteristics.

1.7 Salmonella and its role in livestock and food chain

Salmonella enterica is a worldwide-distributed species of the family *Enterobacteriaceae* that causes gastrointestinal diseases in almost every kind of farm animal, wild animals and in humans. Worldwide *S. enterica* is a major cause for enteric disease and is mostly transmitted via contaminated food. Majowicz et al. 2010 (111) calculated the number of foodborne *S. enterica* infections worldwide with 93.8 million cases a year and around 155.000 deaths. That makes Salmonellosis one of the most important food borne diseases. As there is a reduction of *S. enterica* infections in industrial countries a reduction of *S. enterica* infections worldwide is not predicted as some scientists see a possible increase of foodborne diseases in the context of climate change (112).

Salmonella is a genus belonging to the family of Enterobacteriaceae. It consists only of two species, *Salmonella enterica* and *Salmonella bongori*, which is very rare. Further classification of the species Salmonella has been made phenotypically via differences of surface antigens, which can be distinguished by antibodies. These serovars although separated so, are taxonomically the same species but show differences in many ways, like host range and disease progression, which should not be underestimated (113). Although

the species *Salmonella enterica* show phenotypically a high variability from the point of genetics, *Salmonella* serovars can share more than 96% of sequence identity in genes existing in two serovars (114). The success of Salmonella as a pathogen has more than one component. One is the ability to persist in the host while host does not show symptoms and, for example, *Salmonella* ser. Typhimurium can be found in feces for up to a year (115). Also Salmonella strains are able to colonize in plants, which requires specific variability and adaptation in metabolism (116). In this context Hao et al. 2011 found, that *aroA* mutant of *Salmonella enterica* was less able to colonize roots of alfalfa seed due to reduced replication. In rich media growth of *wild type* and mutant was comparable (117). This underlines the role of *aroA* in bacterial life cycle and a possible impact of glyphosate on *Salmonella enterica*. If findings like these are transferrable and play a role in host-associated environments has to be investigated.

Salmonella is also known for high acid tolerance described as adaptive acid tolerance response in 1990 (118) which plays an important role for Salmonella virulence (119). Also acid induced stress reaction can induce protection of Salmonella against heat, salt and even antibiotics, like polymixin B (120). The relatively low sensitivity against many different agents of *Salmonella enterica* compared to other bacteria of the intestine lead to the assumption, that *Salmonella enterica* may have a selection advantage in the intestine of host animals and therefore may have the potential to prevail in a glyphosate polluted environments (61).

Livestock animals act as reservoir hosts for many *Salmonella* serovars. Often pork and chicken products are a source of the disease for humans (121). Despite the development of acute disease due to Salmonella infections also asymptomatically carriers and intermittent shedders are known (122, 123). The eradication of Salmonella from livestock is not easy and therefore Salmonella can enter the food chain. It can cause infections in humans as important Salmonella serovars like S. Typhimurium have an extended host spectrum (124). Normally, *S. enterica* were transmitted from mothers to their offspring in a very early stage, but vertical transmission is also possible. Infections of pigs with *Salmonella enterica* show that low-shedders and high shedders differ in their microbiota before infection (125) and that gut microbiota of pigs differs to gut microbiota of non-infected pigs (126). Therefore, gut microbiota has a crucial impact on the development of *S. enterica* shedding, but at the same time also the infection with Salmonella has an impact on the microbiota (125). As glyphosate has been shown to have an impact on gut microbiota (82, 83) an interaction is evident.

In livestock, the distribution of *S. enterica* serovars differs depending on the host species. Except for species-specific serovars, in pigs the most common serovar is *S.* Typhimurium whereas in poultry *S.* Enteritidis is more common. From all *S. enterica* infections worldwide, the serovar Typhimurium is among the two most common serovars to cause infections in

humans and animals (110, 127–129). However, these data vary depending on the region and time.

2. Study overview

2.1 General approach of the study

The impact of glyphosate and GBH on bacteria has been known for decades and the pollution of the environment increases year by year. The investigations of the impact of GBH and glyphosate on the bacteria and potential selection for pathogenic bacteria is an important topic and has to be investigated. In this work, we focus on the impact of glyphosate and GBH on *Salmonella enterica*, as one of the most important food borne diseases. As a first step, we focused on the adaptation of pathogenic bacteria to the considerable pollution of the environment with GBH within the last decades.

The potential advantage for selection in a glyphosate contaminated environment is priority of the *in vitro* experiments with artificial animal intestine. Furthermore, in *in vivo* experiments, the shedding of *S. enterica* from pigs modified by glyphosate and GBH will be investigated, as pork is one of the most important sources of food related infections in Germany. As there are high glyphosate residues found in animal feed, in the intestine of food producing animals' microbiota and pathogenic bacteria get in contact with glyphosate. The induction of resistance *in vivo* is conceivable. Some bacteria, especially pathogenic bacteria are able to develop higher glyphosate tolerance than commensal bacteria as a part of a healthy microbiota. While still be in contact with glyphosate residues, this can lead to a selection of pathogenic bacteria and to an increase of shedding into the environment.

Under laboratory conditions, the question of inducible glyphosate tolerance and resistance is addressed. Turner et al 2011 first introduced the term *´evolve and resequence´* (130) and described the method of sequencing and comparing before and after selection experiment. Figuring out the influence of certain compounds like herbicides and the influence on adaptation due to beneficial mutations has not been investigated extensively. The understanding of the molecular changes and impact of environmental factors is still vestigial. In this scientific study, we used an *evolve and resequence* approach to investigate the potential of *Enterobacteriaceae* to adapt to high concentrations of the herbicide glyphosate, which is almost ubiquitously present in the agricultural environment. As the adaption to laboratory conditions also lead to mutations in *Salmonella enterica* (131), we compared

adapted ancestors with selected mutants. Also this study deals with the question if there are any side effects of adaptation to this herbicide, as described in former publications (95). One of these side effects important to environmental hygiene is the question of cross and co-resistance (94) against antibiotics used in human medicine.

To test the glyphosate driven tolerance and resistance induction we wanted to

i) Screen clinical and environmental *Salmonella* strains for their level of glyphosate tolerance
ii) Show that glyphosate resistance can be induced by increasing glyphosate concentrations under laboratory conditions. We want to show side effects which influence pathogenicity of the concerning species, like increase or decrease of antibiotic resistance or environmental fitness. We also want to see the genetically background of this adaptations and investigate the impact of this to the development of co- and cross resistance and co- and cross- tolerance to important clinical relevant antibiotics
iii) Show *in vitro* a selection advantage of pathogenic bacteria *S. enterica* under influence of GBH and
iv) Show *in vivo* that pathogenic bacteria like *S. enterica* have selection advantage, when they get in contact with concentrations of glyphosate and GBH in the complex surrounding of animal intestine, as GBH residues can be found in animal feed, and that this leads to increased shedding of *S. enterica* in pig infection model.

Details on the study design and study conduct for the individual points listed above are described in the respective publications below.

The research took place within a decision support project for the German federal government founded by the German Federal Ministry of Food and Agriculture based on an formal vote of the German parliament.

2.2 Screening of minimum inhibitory concentration

In the screening for minimum inhibitory concentration (MIC), we addressed the question, whether there was a change in glyphosate susceptibility over the last thirty years. For this, we screened isolates of *Salmonella enterica* from different farm animals and from three different serovars. For this purpose, we had access to the archive of the German Federal

Institute for Risk Assessment, which collected *S. enterica* isolates from animals from 1981 until 2016 from different infection outbreaks. Two hundred and twenty five isolates of *Salmonella enterica* from three different kinds of farm animal types (pigs, poultry and cattle) and from the most common zoonotic serovars, Typhimurium, Enteritidis and Infantis, were investigated. We determined the MIC in glyphosate as a pure substance and in the formulation Roundup LB plus, which is one of the most frequently sold formulations with glyphosate as active ingredient in Germany.

2.3 Evolution experiments

The basis for the selection of strains for the evolution experiment were the data from MIC screening. The strains selected were those with already high MIC. To create an environment comparable with real natural habitat of the strains MIC was repeated in pH-adjusted media.

2.3.1 Evolutionary processes under laboratory conditions

Evolution is a process first described and defined by Charles Darwin in his monumental work "On the origins of species by means of natural selection" in 1859. Since then hundreds of scientists started to discover the field of evolution and evolution theory. One of the many ways of investigating the species adaption process to changing environmental conditions is by challenging them under laboratory and therefore measurable conditions. This technique was gained from combination with molecular methods like whole genome sequencing.

Ten isolates of *Salmonella enterica* with already high MIC were selected from the screening experiment. These ten isolates were adapted to experimental conditions for three days. After adaptation, the isolates were challenged with increasing amounts of glyphosate formulation Roundup LB plus. Isolates which were able to grow in the highest concentration of GBH were selected for further characterization via MIC against RU, whole genome sequencing, growth fitness, resistances against antibiotics and differences in protein expression.

2.3.2 Whole genome sequencing

The strains selected for WGS were the ones with visible growth in concentrations over estimated epidemiological MIC (80 mg/ml GLY in RU), which was still stable after stability

passaging. For whole genome sequencing, DNA was extracted and send to BeGenDiv (Berlin Center for Genomics in Biodiversity Research, Berlin, Germany) for further analysis. For every one isolated with increased MIC the isolate after stability passage was sequenced and, for comparison, the adapted ancestor.

2.3.3 Proteomics

For Proteomics investigations, the three isolates with decreased susceptibility and mutations within sequence against RU and their ancestral isolates were investigated for their different protein expression in medium and in medium combined with a sub lethal concentration of RU (1/4 of MIC). As the whole genome sequencing of these strains should show an adjustment of the bacteria in presence of RU on genome level, proteomics data should show also an adaptation of the strains in the expression in proteins applicable in the defense against higher concentrations of the herbicide.

The isolates showing a higher tolerance against RU (mutants) were, together with their ancestral isolates, investigated with label free proteome quantification. Comparisons were made between the isolates in media with isolates challenged with RU in 1/4 of MIC concentration. They were grown until mid-exponential growth phase and subsequently investigated for the composition of expressed proteins.

2.4 Fermenter experiments

Enterobacteria, especially pathogenic species like *S. enterica*, have a permanent impact on farm animals. The use of techniques for simulation of natural habitats, also in animals, is a good way to investigate different scenarios without harming laboratory animals. In cooperation with the Institute of Physiology and Cell Biology of the University of Veterinary Medicine Hannover, Foundation, together with Prof. Gerhard Breves and Dr. Susanne Riede, we investigated via Rumen Simulation Technique (Rusitec) the question whether different concentrations of GBH change the residence time of colony forming units of Enterobacteriacea, especially *Salmonella enterica* ser. Typhimurium, in an artificial rumen. Rumen liquid was transferred into vessels and held under rumen typical conditions. Bacteria were added and numbers were counted over time with and without GBH.

2.5 Animal experiments

The animal experiments were used to compare results found in the *in vitro* studies to results reachable in the complexity of *in vivo* studies. Laboratory experiments can in many cases only partly represent the processes and potential outcome of experiments *in vivo*. In this study, the main focus was in the potential of RU to influence composition of microbiota and as a result shedding of resistant bacteria or pathogens. For the animal experiment we compared three groups of weaning piglets. One group of piglets was fed with glyphosate as a pure substance, a second group was fed with RU and a last served as control. All groups were infected with the same number of *Salmonella enterica* cells in a known infection model (132). Fecal samples were taken for a comparison of shedded *Salmonella enterica* with or without glyphosate/RU. In a final sampling, organs, lymph nodes and intestinal contents were also investigated for the amount of *Salmonella enterica*. Fecal and ceacal samples were frozen at -80 °C for metagenomic analysis and a comparison of sequencing of 16S DNA.

3. Publications

3.1 Publication I: "Minimum inhibitory concentration of glyphosate and a glyphosate-containing herbicide – screening of *Salmonella enterica* isolates originating from different time periods, hosts and serovars"

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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Glyphosate, the active compound of Roundup, is one of the most used pesticides in the world. Its residues are often detected in animal feed, but the impact on the animal gut microbiota and on pathogens of the intestine has not intensively been investigated. In this study, we analyzed the minimum inhibitory concentration (MIC) of glyphosate isopropylamine salt and a common glyphosate-containing herbicide formulation in 225 *Salmonella enterica* isolates by broth microdilution. A bacteriostatic effect of glyphosate on *Salmonella* growth was detected at the concentration range of 10 to 80 mg/mL for both the active ingredient and the ready-to-use formulation. Time/year of isolation, host species, and serovars revealed a statistically significant influence on MIC values. Recently collected *Salmonella* isolates had significantly higher MIC values for glyphosate and the glyphosate-containing product compared with isolates collected between 1981 and 1990. Isolates from pigs showed significantly higher MIC values compared with isolates from poultry, and isolates of the *Salmonella* serovar Typhimurium had significantly higher MIC values than *Salmonella* Enteritidis and Infantis isolates.

Keywords: glyphosate, enterobacteriaceae, *Salmonella*, minimum inhibitory concentration, MIC, historic isolates

Introduction

Glyphosate (*N*-(phosphonomethyl)glycine) is one of the most widely used herbicides in the world [1]. Its usage has increased significantly since the first genetically glyphosate-resistant agricultural crops were introduced to the market in the 1990s [2]. In parallel to the increase of the usage of glyphosate-containing herbicides (GCH), the discussion about its toxicity on higher organisms and other impacts on the environment, e.g., on freshwater communities [3–5] or soil organisms [6, 7], came intensively into the focus of public interest.

The target structure of glyphosate is the penultimate step in the shikimate pathway and well described [8–10]. The herbicide binds to the complex of 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase and its substrate and inhibits the production of aromatic amino acids. This metabolic pathway is present in plants, bacteria, some fungi, and unicellular parasites but not in vertebrates [11]. In bacteria, 3 variants of the targeted enzyme gene have been described so far, accounting for the distinct effect of glyphosate in microbiology. Two of the gene variants show a high similarity, while the third one, which has only recently been found, differs [12]. The resulting variations of the target enzyme provide a conceivable reason for diverging sensitivities to glyphosate, acting as an antimicrobial substance in bacteria.

Nowadays, genetically modified plants are commonly grown and highly polluted with glyphosate in many countries in the world, because the pesticide can be applied throughout the whole growth phase.

Residues of glyphosate were found in soy [13, 14] and maize [15], which both play an important role in animal nutrition. Hence, livestock-associated microbiota can get in contact with glyphosate through residues in animal feed as demonstrated by glyphosate detection in urine and feces of dairy cows [16].

If glyphosate and GCHs cause adverse effects on the environment remains unclear, especially regarding complex bacterial communities.

It was postulated by Krüger et al. [17] and Ackermann et al. [18] that a daily intake of glyphosate with animal feed can lead to dysbiosis with an increased number of pathogenic bacteria surviving in the intestine, depending on the sensitivity to glyphosate. Regarding a ruminal setting, these findings could not be confirmed [19]. Shehata et al. [20] determined differing minimum inhibitory concentrations (MIC) for glyphosate and a formulation of glyphosate for Enterobacteriaceae of farm animal origin. In the case of food microorganisms, results show differences in the susceptibility between glyphosate and glyphosate formulations. While glyphosate itself did not affect microbial growth, an inhibitory effect was determined when using the formulation [21].

In general, chances for survival and reproduction of pathogens in the intestine are higher in an imbalanced microbiological environment. *Salmonella* species are pathogens causing subclinical infections in pigs and leading to chronic carriage in poultry. Its shedding and spread from farm animals into the environment is of major concern in food hygiene, due to its ability to cause foodborne diseases. The World Health Organization ranks non-typhoidal salmonellosis as one of the four key global causes of diarrheal diseases [22]. Majowicz et al. [23] estimated the worldwide number of Salmonella infections to 93.8 million. Following poultry, pork is considered the

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second leading source of Salmonella infections in humans [24]. Non-species specific serovars pose zoonotic potential and occur in host species in different frequencies. The most important livestock-associated serovars are *Salmonella* Typhimurium, *Salmonella* Enteritidis and *Salmonella* Infantis.

For this study, we investigated MICs for glyphosate and a commonly used formulation of glyphosate to quantify potential inhibitory effects on different strains of the major foodborne zoonotic pathogen *Salmonella enterica* isolated from livestock animals.

The study aimed to i) determine the MIC for glyphosate and the glyphosate-containing formulation Roundup LB plus in *Salmonella* isolates originating from farm animals, ii) compare the trend of MIC development within the last three decades comprising the period before and after the immense utilization of glyphosate in agriculture, and iii) reveal the influence of the animal host and the serovar on the MIC of the *Salmonella* isolates.

Materials and Methods

Two hundred twenty-five *Salmonella enterica* isolates were selected (Table 1), belonging to the serovars Typhimurium, Enteritidis, or Infantis. They originated from fecal samples of pigs and poultry. One hundred twenty of the isolates were sampled between 2014 and 2016 (recent isolates). Inclusion criteria for this study consisted of widespread sampling locations representing strains from all over Germany and a variability in antibiotic susceptibility. Sixty isolates originated from pigs and chicken, respectively. Each serovar was represented by 40 isolates. One hundred five isolates were isolated from 1981 until 1990 (historic isolates). Thirty-six of these isolates were assigned to serovar Typhimurium, 37 to serovar Enteritidis, and 32 to serovar Infantis. Forty-eight isolates were isolated from pigs, and 57 from poultry.

MICs were determined in 96-well plates by broth microdilution, described by Wiegand et al. [25]. The concentration of glyphosate was calculated based on the concentration of glyphosate isopropylamine salt in a 40% solution, purchased from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Taufenkirchen, Germany) with a purity of 98% (GLY) or in the herbicide formulation Roundup LB Plus (German license number: 024142–00, Monsanto) (RU). Starting from a concentration of 80 mg/mL, a twofold dilution series was prepared down to a concentration of 1.25 mg/mL. Ready-to-use MIC plates were stored at −80 °C. Nutrient rich Mueller Hinton medium (MH) (Oxoid GmbH, Wesel, CM0405) was used.

Inoculum was prepared as an overnight culture in Mueller Hinton broth and aerobically incubated for 16 h at 37 °C, with shaking. Subsequently, optical density of the overnight cultures was measured, adjusted to a number of 1×10^6 colony forming units (cfu) per milliliter. An inoculum of 5 µL for each well and isolate was transferred into prepared 96-well plates (with conical bottom; Sarstedt GmbH, Nürnberg) resulting in a final number of 5×10^4 cfu per well. All samples were processed in technical triplicates. Every isolate was tested for GLY and RU. The 96-well plates were aerobically incubated over night at a temperature of 37 °C in a humidity chamber [26].

The MIC was defined as the lowest concentration without visible growth in at least 2 out of 3 technical replicates. It was determined visually by using an impinging light and a mirror (SensiTouch by Sensititre).

Statistical analysis was performed with IBM SPSS statistics Version 24. Prior to calculation, data were transformed to an ordinal scale (10 mg/mL = 1; 20 mg/mL = 2; 40 mg/mL = 3; 80 mg/mL = 4). Because data were not normally distributed, comparisons between groups were calculated using the Mann–Whitney *U* test for two groups or the Kruskal–Wallis test for more than two groups, respectively.

Multivariable analysis of variance model was used to investigate the influence of isolation time (historical or recent isolates), serovar (*S*. Typhimurium, *S*. Infantis, or *S.* Enteritidis) and host (poultry or pig) on MIC values. Further, proportional-odds ordinal regression models were run with the MIC values as dependent variable and isolation time, serovar, and hosts as independent variables.

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 considered statistically significant. Model diagnostics included check for normality and homoscedasticity of residuals. Ordinal regression models were additionally tested for the assumption of proportional odds.

Ethics. All isolates were provided by German Federal Institute of Risk Assessment. Non of the samples from which the *Salmonella enterica* were isolated were collected directly from animals from the investigating institute. Therefore no ethical approval was necessary.

Results

MIC for glyphosate isopropylamine salt (GLY) and for glyphosate salt within the formulation Roundup LB plus (RU) was investigated for 225 isolates of *Salmonella enterica* from fecal samples from pigs and poultry. The MICs varied slightly between replicates and between RU and GLY for the particular isolate. Hence, the MIC data comprised a very narrow range and were not normally distributed. The results of the statistical analysis are summarized in Table 2.

GLY vs. RU. The distribution of MICs of all isolates investigated for GLY and RU is shown in Figure 1. The determined values for GLY ranged from 10 mg/mL (lowest MIC) to 80 mg/mL (highest MIC). The median and the mode for the whole dataset were 40 mg/mL. For RU, MICs had a range of 20 mg/mL to 80 mg/mL. The median and the mode were 40 mg/mL. The 95% cutoff for both GLY and RU was located at 80 mg/mL (Figure 1).

Comparison of Recent and Historic Isolates. The distribution of MICs expressed as percentage for the investigated *Salmonella* isolates separated according to historic and recent isolates to GLY and RU is presented in Figure 2. One hundred twenty of the investigated *Salmonella enterica* isolates where collected between 2014 and 2016. One hundred five isolates were collected between 1981 and 1990, prior to the intensive usage of glyphosate in agriculture. MIC values for the isolates collected before 1991 showed a distribution of 20 to 80 mg/mL for both GLY and RU. In general, equal ranges were determined for recent isolates regarding RU. For GLY, recent isolates showed a distribution of MIC ranging from 10 mg/mL to 80 mg/mL. The median was 40 mg/mL for both GLY and RU. In addition, the mode

Table 1. Overview of isolates. Number and distribution of tested isolates for the different collection times, species, and serovars. Historic isolates have been collected between 1981 and 1990; recent isolates have been collected between 2014 and 2016. All isolates were provided by the German Federal Institute of Risk Assessment

Serovar	Poultry		Pig		Total
	Historic	Recent	Historic	Recent	
S. Typhimurium	19	10	17	30	76
S. Enteritidis	25	30	12	10	77
S. Infantis	14	20	18	20	72
Total	58	60	47	60	**225**

Table 2. Statistical comparisons. Statistical comparison between the different groups with univariable Mann–Whitney U^1 rpt. Kruskal–Wallis[2] test as non-parametric test for not normally distributed data and the multivariable models of analysis of variance and ordinal regression. (* = statistically significant). Groups with higher MICs in bold

	Glyphosate isopropylamine salt (GLY)			Roundup LB plus (RU)		
	Mann–Whitney U^1/Kruskal–Wallis[2] univariable	Multivariable analysis of variance	Multivariable ordinal regression	Mann–Whitney U^1/Kruskal–Wallis[2] univariable	Multivariable analysis of variance	Multivariable ordinal regression
Isolation time: **recent** vs. historical	$P = 0.012^1$*	$P = 0.006$*	$P = 0.008$*	$P < 0.001$*	$P < 0.001$*	$P < 0.001$*
Host: **pig** vs. poultry	$P = 0.027^1$*	$P = 0.030$*	$P = 0.031$*	$P = 0.021$*	$P = 0.174$	$P = 0.097$
Serovars	$P < 0.001^2$*	$P < 0.001$*	–	$P < 0.001^2$*	$P < 0.001$*	–
Typhimurium vs Enteritidis	$P = 0.147^1$	$P = 0.238$	$P = 0.381$	$P = 0.002$*	$P < 0.001$*	$P = 0.003$*
Typhimurium vs Infantis	$P < 0.001^1$	$P < 0.001$*	–	$P < 0.001$*	$P < 0.001$*	–
Enteritidis vs. Infantis	$P < 0.001^1$*	$P = 0.001$*	$P < 0.001$*	$P = 0.030$*	$P = 0.030$*	$P = 0.006$*

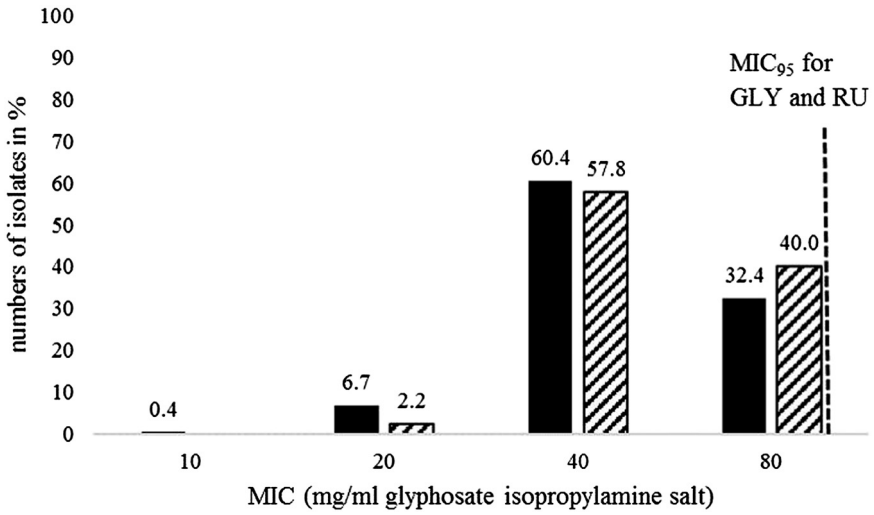

Figure 1. Distribution of the MIC values of the investigated *Salmonella enterica* isolates in percentage for glyphosate isopropylamine salt (GLY) (black) and Roundup LB plus (RU) (cross hatched). Dashed line marks 95% epidemiological cutoff for both GLY and RU

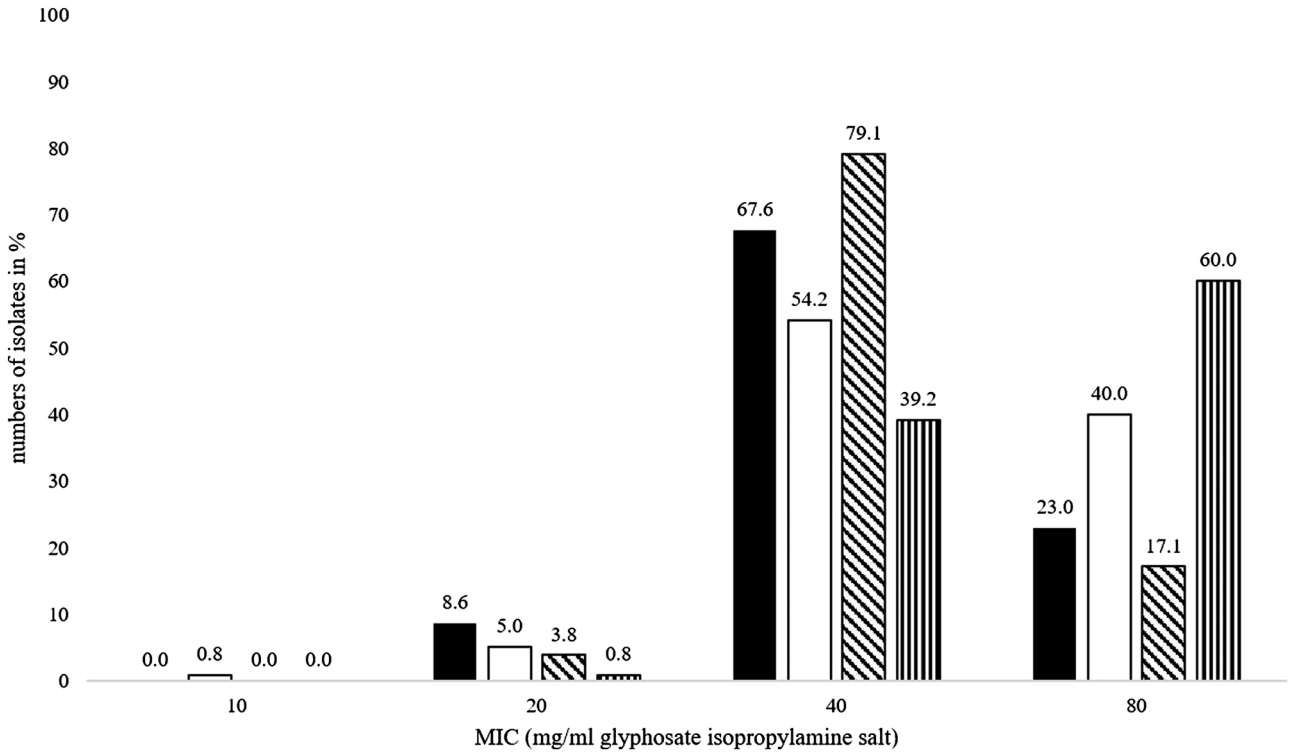

Figure 2. MIC values for the investigated *Salmonella enterica* isolates for glyphosate isopropylamine salt (GLY) and Roundup LB plus (RU) in comparison, differentiated between historic isolates collected before 1991 and recent isolates collected between 2014 and 2016. GLY before 1991 in black, GLY between 2014 and 2016 in white, RU before 1991 oblique crosshatched, and RU between 2014 and 2016 vertical crosshatched

for both datasets was 40 mg/mL as well. The statistical analysis and the two statistical models used showed that the obtained differences of MICs between historical and recent isolates were highly significant (Table 2; Mann–Whitney *U* test $P = 0.012$; univariate analysis of variance $P = 0.006$; ordinal regression $P = 0.008$). Historical isolates had lower MIC against GLY compared with recent isolates ($P = 0.006$). For RU, the statistically significant influence of isolation time was also determined by univariable tests. Historical isolates had lower MIC compared with recent isolates ($P < 0.001$).

Comparison of Host Species. A comparison of the MIC values of GLY and RU expressed as percentage and separated according to host species is shown in Figure 3. Regarding *Salmonella* isolates originating from pigs, a MIC of 80 mg/mL was more frequently determined than for those originating from poultry. This was the case for both GLY and RU. By contrast, the percentage of isolates from poultry showing low MICs of 20 mg/mL or 10 mg/mL for RU, respectively, was higher compared to that from pigs. The univariate analysis of variance confirmed that MICs of pig isolates were significantly higher than those obtained for poultry isolates (Table 2; $P = 0.030$). For RU, statistical significance could also be shown in the non-parametric tests ($P = 0.021$), whereas the analysis of variances and the ordinal regression model did not reveal a statistically significant influence of the host ($P = 0.174$; $P = 0.097$).

Comparison of *Salmonella* Serovars. A comparison of the datasets obtained for the different serovars included in the study revealed that with the exception of the serovar Typhimurium, MICs of 40 mg/mL were most frequently determined. This accounts for both GLY and RU. For GLY, the obtained differences of MICs according to serovars were classified as significant by the Kruskal–Wallis test for more than 2 groups ($P < 0.001$).

In the statistical model for GLY, the comparison of all 3 serovars also revealed significant differences in MIC values (analysis of variance $P < 0.001$). Post-hoc pairwise comparison revealed a significance of the difference obtained for Typhimurium and Infantis ($P < 0.001$; higher values for Typhimurium than for Infantis), whereas that between the serovars Enteritidis and Typhimurium was rated as not significant by the modeling ($P = 0.238$). In the two calculated models, the serovar Typhimurium had highest MICs, followed by the serovar Enteritidis. The lowest MICs were obtained by the serovar Infantis. The differences between Enteritidis and Infantis and Typhimurium and Infantis were statistically significant, whereas between Typhimurium and Enteritidis, they were not (Table 2).

For RU, the Kruskal–Wallis test showed statistically significant differences between all 3 serovars. This correlated with the results of the analysis of variance and the ordinal regression model. MIC values of the serovar Enteritidis were significantly higher than those of the serovar Infantis ($P = 0.030$). Between the serovars Enteritidis and Typhimurium, a statistically significant difference ($P < 0.001$) was detected, where the serovar Typhimurium showed higher values than Enteritidis. This was also reflected by the ordinal regression ($P = 0.003$ Typhimurium vs. Enteritidis; $P = 0.006$ Enteritidis vs. Infantis). In summary, the comparison of the 3 serovars in RU revealed the highest MICs for Typhimurium compared to Enteritidis and Infantis, while the serovar Enteritidis showed higher values than Infantis.

Discussion

For the first time, we aimed to systematically investigate different *Salmonella enterica* isolates from food-producing animals for their susceptibility against the herbicide glyphosate (GLY) and a glyphosate-containing tallow amine free formulation, Roundup LB Plus (RU). Furthermore, we intended to analyze the potential impact of time of isolation, host species, and serovar on susceptibility to glyphosate.

MIC Values. In general, the range of MIC values determined for all isolates included in the study was rather narrow. Nonetheless, a statistical analysis of the generated study data revealed a significant impact of some parameters

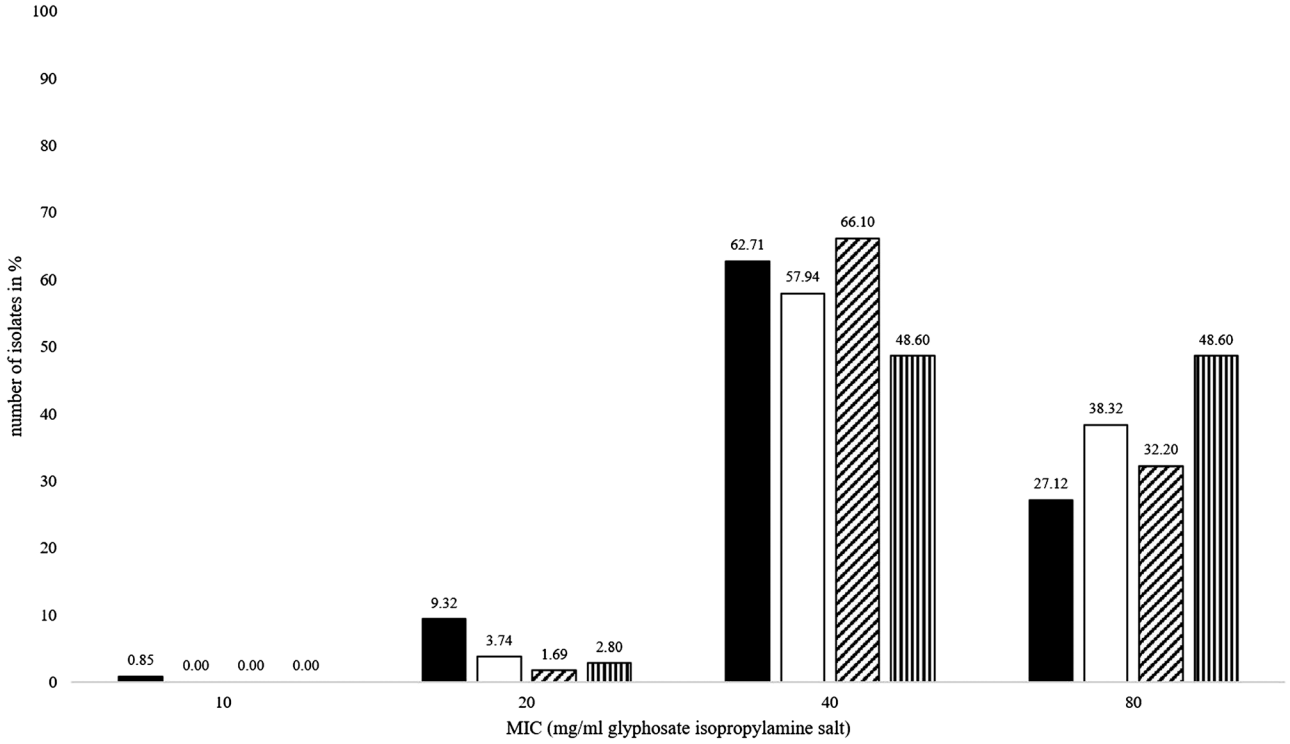

Figure 3. MIC values of *Salmonella* isolates from pig for glyphosate isopropylamine salt (white) and poultry for glyphosate isopropylamine salt (black) and for pig in Roundup LB plus (vertical crosshatched) and poultry in Roundup LB plus (oblique crosshatched)

that were considered as potential factors beforehand and thus investigated.

In comparison with data published before, the mean MIC value of 40 mg/mL isopropylamine glyphosate in both the herbicide and the formulation as determined by our study has to be considered as rather high. Shehata et al. [20] determined MICs of 5.0 mg/mL glyphosate for 3 different *Salmonella* strains using the formulation "Roundup UltraMax". Within the study conducted by Kurenbach et al. [27], MIC was investigated for one *Salmonella* Typhimurium isolate, resulting in a value of 6.19 mg/mL. The group used the formulation "Roundup Weed killer". Only the work of Nielsen et al. [28] revealed comparable high MIC values, for example, a MIC of 80 mg/mL for the formulation "Glyfonova 450 plus" for two *E. coli* isolates. Unfortunately, they had no *Salmonella enterica* isolates in their dataset to compare with.

Different Formulations. The differences between reported MIC values and those determined by our study could be due to the usage of different glyphosate formulations. An impact of the formulation was considered by different authors [21, 29], as well as the usage of different media [28]. Further deviations were occurring because of a lack of standardized methods. For example, the formulation "Roundup UltraMax" used in some studies contained tallow amine as a surfactant, which is known to be more toxic than glyphosate [29, 30]. This remarkable difference underlines the issue of an insufficient comparability of results obtained for variable glyphosate formulations. Generally, little is known about the additional ingredients and their potential interactions with bacteria [31]. Glyphosate is known to reduce the amount of manganese, magnesium, and calcium in plants due to chelation [32]. This renders a possible interaction of glyphosate with the media used for MIC determination.

Different Media. Since bacteria could balance a lack of aromatic amino acids with an uptake of free amino acids from a nutrient-rich media to some extent, differences in media composition could also lead to different results. Also, the usage of different media and different methods in general is a possible factor, generating the big differences in MIC values [28]. Zucko et al. [33] investigated 488 prokaryotes sequences for the completeness of the genes for production of aromatic amino acids and came to the conclusion that host-associated bacteria may not process a complete shikimate pathway but instead gather aromatic amino acids from their host environment. While susceptibility testing in our study was conducted in Mueller Hinton broth, Kurenbach et al. [27] used LB media. Within the publication of Shehata et al. [20], it is not stated which media were used. The most comparable MICs, as mentioned before, were obtained by Nielsen et al. [28], who cultivated bacteria anaerobically and used Brain Heart Infusion broth and Reinforced Clostridial Medium. Overall, comparability of the results has to be considered to be limited if they were produced using different herbicide formulations and according to different protocols.

Time of Isolation. We compared MICs of *Salmonella enterica* isolates before and after the rise of the herbicide glyphosate in the 1990s [1]. Isolates collected before 1981 had significant lower MIC values for GLY and RU than those collected from 2014 to 2016. However, this does not necessarily imply that the decrease in sensitivity against both tested solutions is due to the vast increase of glyphosate usage leading to more resistant recent *Salmonella enterica* strains compared to historical strains.

Antimicrobials and other potential stressors like residues of pesticides [27] and biocides [34] are able to cause an increase in persistence and a decrease in susceptibility in microorganisms against different antibiotic agents [27]. The decrease in susceptibility does not necessarily require a change in the specifically targeted structures. It could be due to general mechanisms against stressors as well. The occurrence of co- and cross-resistance enables a decrease in susceptability against certain agents even when they are absent. Hence, not only an increase in residues of glyphosate itself within the environment could have led to a decrease of susceptibility against the herbicide, but also the increasing burden of residues of further active substances.

For example, MIC increase could be an increased tolerance due to unspecific modifications, like overexpression of multidrug-resistance efflux pumps [35]. This was recently shown for Enterobacteriaceae and biocides [36]. Bailey et al. [37] revealed an increase in tolerance of *Salmonella* Typhimurium after short time exposure to the biocide triclosan, which was due to an overexpression of efflux pumps.

Similar to glyphosate, the use of biocides increased in many environments within the last decades [38]. Especially in food [39] and farm surroundings, low biocides concentrations could be detected. It was stated that the presence of residues provides an environment, in which the selection of isolates with increased tolerance to different agents is potentially favored [40, 41]. As shown by Karatzas et al. [34], *Salmonella* species that survive at low doses of biocides are more likely to be resistant against antibiotics. The group concluded that a high MIC of biocides in *Salmonella* can lead to a selection for antibiotic-resistant isolates. This can for example happen due to co-selection, also in food-borne pathogens [42]. Furthermore, Parikh et al. [43] even detected a linked resistance between biocides and antibiotics. Whitehead et al. [44] showed also that high doses of biocides could lead to the selection of multidrug-resistant *Salmonella enterica* strains.

Another example specific for the livestock sector is the use of heavy metals like copper and zinc as growth promotors [45]. The heavy usage of these animal food components can lead to different modifications in bacteria, for example co-resistances [46] and enrichment of certain bacteria in the animal intestine due to other mechanisms [47].

Differences in MIC for Different Host Species. Our study revealed significantly higher MICs for glyphosate in pigs than in poultry. However, the differences are not highly significant. Moreover, host-dependent differences in MICs were not detected when using the formulation RU. An explanation for higher MIC values in pigs than in poultry could be a greater uptake of glyphosate residues with feed. Compared to poultry, daily feed rations for pigs are much higher, and they additionally have a much longer lifespan. A comparison of the results for RU as a complex formulation is always more complicated than with glyphosate as a pure substance due to the lack of knowledge of the additional ingredients in the formulations. In general, the results obtained for the different host species are very similar. A further investigation, comprising also species-specific *Salmonella* serovars (e.g. *S.* Pullorum for poultry or *S.* Derby for pigs), the analysis of a bigger sample set, or the comparison of the pig isolates with samples from only one poultry species (e.g. chicken) could potentially lead to more pronounced differences in results.

Differences in MIC for the Different Serovars of Salmonella enterica. Classification of *Salmonella enterica* according to serovars is a very important tool that relies on phenotypic properties only. For most serovars, the specific genetic background causing the phenotypic differences is not investigated. Nonetheless, the classification of the serovars is based on the differences in O and H antigens, which results in modified lipopolysaccharides (LPS) on the cell surface or flagella. This can lead to variations in polarization of cell surface [48] or other differences, which change the

susceptibility for chemicals and other active substances [49]. In this study, the serovar Typhimurium provided higher MICs compared with the other two serovars Enteritidis and Infantis. However, based on the data obtained within the study presented here, this assumption cannot sufficiently be investigated. For proving the possible impact of differences of the cell surface, further investigations, like a comparison of MICs of closely related serovars, should be carried out. Also, whole genome sequencing would be required to properly address this issue.

Since the serovar Typhimurium is the predominant serovar in pigs [50] but not in poultry [51], differences in the serovars can be biased by this fact. This explanation would be in concordance with the assumption that glyphosate intake of pigs is higher than that of poultry, due to the longer lifespan and higher daily feed rations. Hence, it is conceivable that Enterobacteriaceae get in contact with glyphosate more frequently and at potentially higher doses, leading to a higher risk for the development of resistance. In addition, this matches our results of MICs being higher in pigs than in poultry. Underlining that our statistical models should exclude the mutual influences within the analyzed variables the dataset could still be too small to show this relationship.

However, the glyphosate residues detected in poultry and pig feed [20, 52] were considerably lower than the MIC.

Conclusion

To the best our knowledge, this is the first large scale study that systematically assessed glyphosate sensitivity in *Salmonella enterica* isolates of animal origin. We demonstrated significant differences between the MICs before and after the massive increase of glyphosate usage in agriculture. Further investigations are needed to show causality between the increase of glyphosate tolerance and the usage of glyphosate or other chemicals, antibiotics, or heavy metals in agriculture. Furthermore, resistance mechanisms behind the increased MIC should be clarified, and whether they are transferable between bacteria. In addition, investigations are needed to determine the impact of an increased resistance against glyphosate on the occurrence of pathogenic, zoonotic, and commensal bacteria, as well as on the composition of bacterial communities, especially for food-producing animals and for the environment.

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Authors' Contribution

JP performed the experiments, collected, analyzed and interpreted the data, drafted the manuscript and figures with critical evaluation, and supported of all other authors. KB 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, as well as critically revised the manuscript. All authors approved the final version to be published.

Conflict of Interest

The authors declare that there is no conflict of interest.

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3.2 Publication II: "Selection for Resistance to a Glyphosate-Containing Herbicide in Salmonella enterica Does Not Result in a Sustained Activation of the Tolerance Response or Increased Cross-Tolerance and Cross-Resistance to Clinically Important Antibiotics."

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Selection for Resistance to a Glyphosate-Containing Herbicide in *Salmonella enterica* Does Not Result in a Sustained Activation of the Tolerance Response or Increased Cross-Tolerance and Cross-Resistance to Clinically Important Antibiotics

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ABSTRACT Evolution of bacterial tolerance to antimicrobials precedes evolution of resistance and may result in cross-tolerance, cross-resistance, or collateral sensitivity to other antibiotics. Transient exposure of gut bacteria to glyphosate, the world's most widely used herbicide, has been linked to the activation of the stress response and changes in susceptibility to antibiotics. In this study, we investigated whether chronic exposure to a glyphosate-based herbicide (GBH) results in resistance, a constitutive activation of the tolerance and stress responses, and cross-tolerance or cross-resistance to antibiotics. Of the 10 farm animal-derived clinical isolates of *Salmonella enterica* subjected to experimental evolution in increasing concentrations of GBH, three isolates showed stable resistance with mutations associated with the glyphosate target gene *aroA* and no fitness costs. Global quantitative proteomics analysis demonstrated activation of the cellular tolerance and stress response during the transient exposure to GBH but not constitutively in the resistant mutants. Resistant mutants displayed no cross-resistance or cross-tolerance to antibiotics. These results suggest that while transient exposure to GBH triggers cellular tolerance response in *Salmonella enterica*, this response does not become genetically fixed after selection for resistance to GBH and does not result in increased cross-tolerance or cross-resistance to clinically important antibiotics under our experimental conditions.

IMPORTANCE Glyphosate-based herbicides (GBH) are among the world's most popular, with traces commonly found in food, feed, and the environment. Such high ubiquity means that the herbicide may come into contact with various microorganisms, on which it acts as an antimicrobial, and it may select for resistance and cross-resistance to clinically important antibiotics. It is therefore important to estimate whether the widespread use of pesticides may be an underappreciated source of antibiotic-resistant microorganisms that may compromise efficiency of antibiotic treatments in humans and animals.

KEYWORDS glyphosate, *Enterobacteriaceae*, resistance, tolerance, fitness costs, experimental evolution

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Antimicrobial resistance (AMR) is a complex problem and a major existential threat as described by the World Economic Forum (https://www.weforum.org/reports/the-global-risks-report-2020). Chronic exposure of bacteria to sublethal concentrations of antimicrobials has long been identified as the major driver of the *de novo* evolution of resistance and cross-resistance to antibiotics (1). Tolerance is a transient phenotypic ability of the bacterial population to tolerate antimicrobials associated with general stress response (2). It has recently been shown that evolution of tolerance in response to subinhibitory concentrations of antibiotics precedes and facilitates emergence of resistance (3), making it an important but underappreciated contributor to AMR.

Glyphosate-based herbicides (GBH) are among the world's most popular herbicides (4). While the potential toxic effects of glyphosate on humans, animals, and the environment are subjects of heated scientific and public debates (5, 6), its effects on gut bacteria have only recently attracted attention, despite its known antimicrobial properties (7) and frequent exposure through food and feed (8). Indeed, we recently found that *Salmonella enterica* sampled after the introduction of GBH into agricultural practice tended to have higher levels of resistance to glyphosate and GBH than the historical isolates from the preglyphosate era (9), while direct exposure to GBH has been shown to enrich for pathogenic bacteria in the gut (10) and change susceptibility to antibiotics in *S. enterica* and *Escherichia coli* through the activation of AcrAB efflux pumps (11), which are known to be involved in drug tolerance and resistance (12–14).

Intrigued by the findings of Kurenbach et al. (11), who found that transient exposure to subinhibitory concentrations of GBH resulted in altered antibiotic susceptibility profiles, and by the pervasive nature of glyphosate contamination, we sought to investigate whether chronic exposure to GBH results in the genetic fixation of this tolerance response and thereby may permanently compromise the efficiency of antibiotics.

RESULTS AND DISCUSSION

First, we attempted to obtain stable mutants resistant to GBH. For this, 10 clinical isolates of *S. enterica* from farm animals were passaged daily at increasing concentrations of the GBH Roundup LB Plus (RU), starting from 1/2× to 1/4× the MIC (20 mg/ml RU, equivalent to isopropylamine salt of glyphosate), depending on the strain, along with nonselected wild-type controls (Fig. 1). The overall dynamics of adaptation was slow and marked by early extinctions. Although all isolates were initially able to grow at 60 mg/ml, only three demonstrated a 2- to 4-fold MIC increase after the "stability of resistance" passage (in the absence of GBH) (Table 1). These data suggest that although evolution of resistance to GBH does not occur easily, it nonetheless has the potential to become fixed in resistant isolates.

To gain insights into the molecular mechanisms of resistance, we sequenced the resulting GBH-resistant mutants and their respective ancestors. All three strains had missense mutations either upstream of (*S. enterica* serovar Typhimurium 12468M and *S. enterica* serovar Enteritidis 12539M) or inside (*S.* Typhimurium 12472M) *aroA*, the gene encoding the molecular target of glyphosate (15, 16). Additionally, mutations in the genes frequently associated with stress response and tolerance (17, 18) were also found (Table 2; Table S1): truncation of *rpoS* in *S.* Typhimurium 12472M, which encodes RNA polymerase sigma factor, a master regulator of the general stress response (19), and a missense mutation in *rcsB* in *S.* Typhimurium 12468M, encoding the transcriptional regulator of a two-component system. These data suggest that while evolution of resistance to GBH converges at the target gene and functional levels (various genes related to stress and tolerance response), individual strains employ different strategies to achieve this adaptation.

To probe more deeply into what cellular processes are affected by resistance to GBH, we performed global label-free quantitative proteomics analysis of the resistant mutants and their ancestors in the presence and absence of subinhibitory (1/4× MIC) concentrations of GBH (Table S2; Fig. S1 and S2). We wondered whether the acute response to the sublethal concentration of GBH in the sensitive ancestor simply became

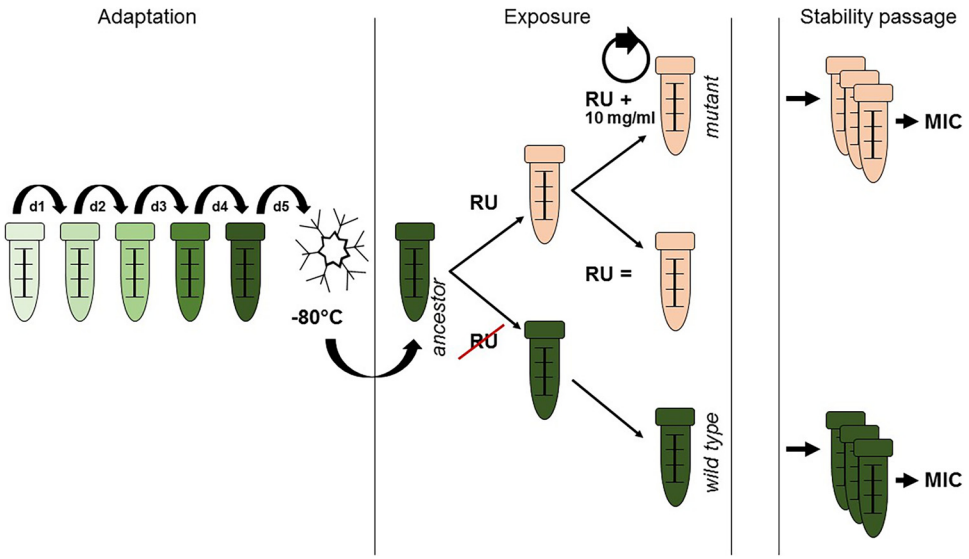

FIG 1 Schematic representation of the evolution experiment. Bacterial cultures were preadapted to the experimental conditions prior to the evolution experiment, where evolving populations were passaged daily 1:100 with the same Roundup LB Plus (RU) concentration and a concentration that was increased by 10 mg/ml, along with the nonselected controls. After the evolution experiments, populations were passaged in the absence of the herbicide and assessed by MIC testing for stability of resistance.

amplified and constitutively fixed in resistant mutants following chronic exposure. While upregulation of proteins involved in oxidative stress response was consistent in both challenged sensitive ancestors and constitutive GBH-resistant mutants, the overall cellular responses were vastly different (Fig. 2A and B), suggesting that evolution of resistance to GBH does not simply result in fixation of the acute response to GBH stress.

When we searched the combined proteome of the four challenged ancestors (three ancestors of the resistant mutants—12468A, 12472A, and 12539A—and one ancestor of the extinct line 12538A used as a control) for proteins involved in the processes known to be affected by glyphosate (production of aromatic amino acids, chelation of iron, and stress response in bacteria) and plotted them together with the 10 most up- and downregulated proteins for each strain using STRING network analysis (20), we found a striking convergence at the tolerance response (Fig. 2A). The acriflavine resistance AcrAB multidrug efflux pump, which is associated with tolerant persister state in nongrowing and nondividing cells, including herbicide paraquat-induced tolerance (21, 22), was upregulated in all challenged ancestral isolates. This is consistent with the findings of Kurenbach et al., who demonstrated that exposure of *Enterobacteriaceae* to subinhibitory concentrations of GBH resulted in activation of efflux pumps and was associated with increased antibiotic tolerance (11), while deletion of *acrA*, *acrB*,

TABLE 1 Dynamics of adaptation in the evolution experiment[a]

Serovar	Isolate no.	MIC before expt (mg/ml)	Highest concn with visible growth (mg/ml)	MIC after expt (mg/ml)	MIC after stability passage (mg/ml)	Day of extinction
***S.* Typhimurium**	**12468**	**40**	**90**	**160**	**160**	**17**
S. Typhimurium	12469	40	70			7
S. Typhimurium	12470	40	70			7
S. Typhimurium	12471	40	80			22
***S.* Typhimurium**	**12472**	**40**	**80**	**80**	**160**	**24**
S. Typhimurium	12473	40	60			5
S. Enteritidis	12538	80	80	80	80	10
***S.* Enteritidis**	**12539**	**80**	**70**	**80**	**160**	**10**
S. Enteritidis	12541	40	80			13
S. Enteritidis	12543	40	60			5

[a]The number of passages equals the number of days of the experiment before extinction. Resistant isolates that were subjected to whole-genome resequencing are in bold.

TABLE 2 Overview of the nonsynonymous mutations detected in GBH-resistant mutants[a]

Isolate	Affected gene	Type and location of mutations in coding and protein sequences	Protein function
S. Typhimurium 12468M	*aroA*	SNP in scaffold_16:G41393A upstream of *aroA*	3-Phosphoshikimate 1-carboxyvinyltransferase
S. Typhimurium 12472M	*rcsB*	Missense variant c.530G>T/651 p.Arg177Leu/216	Two-component system transcriptional regulator RcsB
	rpoS	Stop gained c.361G>T/993 p.Glu121*/330	RNA polymerase sigma factor RpoS
	aroA	Missense variant c.289A>G/1284 p.Thr97Ala/427	3-Phosphoshikimate 1-carboxyvinyltransferase
	Multiple	91 missense mutations in prophage genes	Prophage genes
S. Enteritidis 12539M	*aroA*	SNP in scaffold_0:G272493A upstream of *aroA*	3-Phosphoshikimate 1-carboxyvinyltransferase

[a]Stable resistant mutants (single colonies after the stability passage) were subjected to whole-genome resequencing and compared to the genomes of the nonevolved controls and ancestors. Mutation locations are indicated as follows: type of mutation, followed by the variant of nucleotide or amino acid present in the ancestor, position (relative to the gene or protein start) at which the substitution occurred, substituted nucleotide or amino acid in the mutant relative to the complete nucleotide coding (c) and translated protein (p) sequences (after the slash). Mutations in the *aroA* gene encoding the molecular target of glyphosate are shaded in gray. Note that *S.* Typhimurium 12468 and *S.* Enteritidis 12539 appear to have the same mutation in the intergenic space upstream of *aroA*, while *S.* Typhimurium 12472 has the amino acid substitution in the location previously associated with resistance to glyphosate (47), suggesting a high degree of convergent evolution between these environmental isolates. SNP, single-nucleotide polymorphism.

and *tolC* (but not of *ompF* and *acrD*) caused a reduction in the MIC of GBH (23). Furthermore, TolC, which is frequently associated with tolerance, was also upregulated in all but one strain. Among other upregulated proteins were those involved in uptake and metabolism of iron and other divalent trace metals (siderophores and a number of transporters), consistent with the chelating effects of glyphosate (24). General envelope (RpoE, OmpA, LolA, Lpp, and SlyB), osmotic (osmolarity response proteins and osmoprotectants YehZ and OsmY), and oxidative (SufB, SufC, and SufS) stress response proteins were also upregulated, as well as respiration (CydAB), DNA recombination (RuvAB), and cell division (FtsZ) proteins. No effects directly on the target of glyphosate (3-phosphoshikimate1-carboxyvinyltransferase or 5-enolpyruvylshikimate-3-phosphate synthase [EPSPS]) were found in any of the strains, although other proteins involved in the shikimate pathway (chorismate mutase and synthase) were upregulated in all strains except *S.* Typhimurium 12468.

Recently, a relationship between stress response, iron limitation, and amino acid uptake was demonstrated (25). Banerjee et al. reported that for pathogenic *E. coli*, survival in the urinary tract is linked to the stress response-mediated ability to increase amino acid uptake under iron-limiting conditions (25). It is conceivable that similar processes occur in *Salmonella* exposed to glyphosate, which acts as a potent iron chelator, and these would be consistent with the increased stress response and iron metabolism observed in our experiments. In short, our data strongly suggest that activation of iron limitation and tolerance response precedes activation of expression of the specific glyphosate target following acute GBH stress in a rich medium.

In contrast, similar analysis of the combined proteomes of the three constitutively GBH-resistant mutants (*S.* Enteritidis 12539M, *S.* Typhimurium 12468M, and *S.* Typhimurium 12472M) and their ancestors revealed few similarities between the isolates, with the exception of the molecular target of glyphosate EPSPS, which was upregulated in all three mutants (Fig. 2B; Table S2; Fig. S2). Interestingly, iron metabolism-related proteins were not as strongly affected by the evolution of resistance to GBH, despite a strong activation of these proteins in the challenged sensitive ancestors. Nonetheless, bacterioferritin, which is used for storage of intracellular iron, was upregulated in resistant *S.* Typhimurium 12468M but downregulated in resistant *S.* Typhimurium 12472M. It is important to note that none of the ferritins were upregulated during the GBH exposure of the sensitive ancestors, suggesting that *Salmonella*'s short-term response to iron limitation by glyphosate chelation is to increase transport but not storage of iron. There were fewer proteins involved in tolerance and envelope stress represented in this data set, and those that were present tended to be downregulated, in contrast to the response in challenged ancestors. Altogether, proteomics data suggest that chronic exposure to GBH results in constitutive fixation of the resistance traits associated with the direct effects of the herbicide on bacteria (EPSPS and iron chelation) but not the tolerance response, which is activated by the presence of GBH in both sensitive ancestors and resistant mutants.

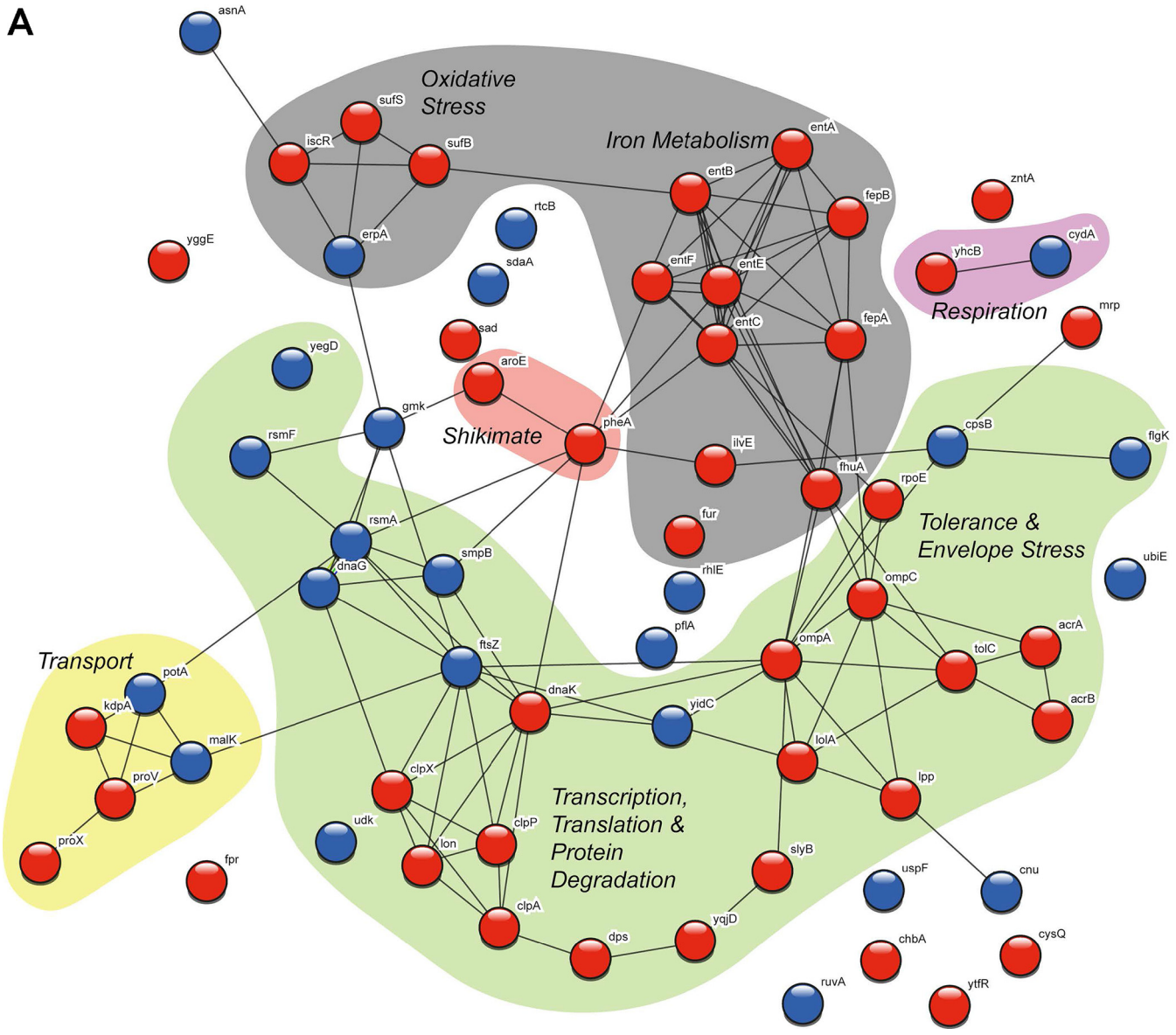

FIG 2 STRING network analysis of the proteome. The combined proteome of four challenged ancestors (A) and that of the three resulting constitutively GBH-resistant mutants (B) are shown, representing proteins involved in the processes known to be affected by glyphosate (production of aromatic amino acids, chelation of iron, and stress response in bacteria) and the 10 most up- and downregulated proteins for each strain. Blue spheres represent downregulated proteins, red spheres represent upregulated proteins, and yellow spheres are proteins which are upregulated in one strain and downregulated in another strain. Proteins highlighted with the same color belong to a functional group.

It was demonstrated previously that evolution of tolerance precedes evolution of resistance (3) and may result in cross-tolerance (26) and collateral sensitivity (27) to other antimicrobials. Indeed, our experimental evolution resulted in resistance to GBH, while proteomics demonstrated activation of the tolerance response upon transient exposure to GBH in both ancestors and mutants. To check whether genetically fixed resistance to GBH affects cross-tolerance and cross-resistance/collateral sensitivity, we subjected the ancestors and the mutants to TDtest assays (28) and MIC testing by Vitek automated susceptibility testing (AST) (in the absence of GBH) against a number of antibiotics relevant to human medicine. We found no tolerant bacteria in TDtest assays with the β-lactam antibiotics ceftazidime (CAZ; third-generation cephalosporin) and cefepime (FEP; 4th-generation cephalosporin) or with rifampin (RIF) or colistin (CT; also known as polymyxin) (Fig. S3), consistent with the lack of tolerance response at the proteome level. Interestingly, we found colonies in tolerance assays with fosfomycin

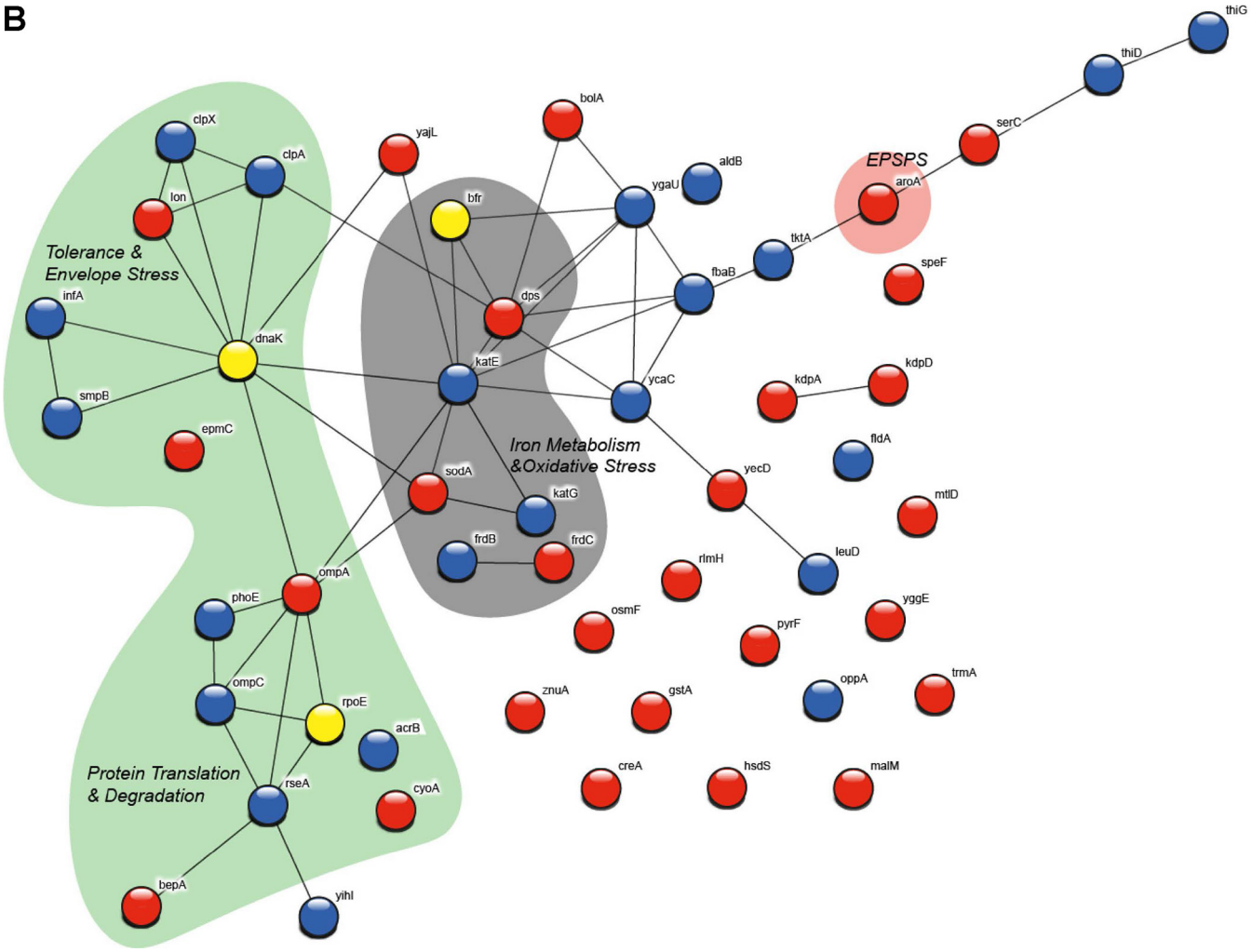

FIG 2 (Continued)

(FOS) which upon retesting by MIC testing displayed an elevated level of resistance and are likely spontaneous mutants (Fig. S4), a phenomenon frequently described for this antibiotic (29). MIC assays by Vitek showed no changes between nonchallenged ancestors and mutants with the exception of the isolate 12472, where the mutant had a decreased MIC of piperacillin (Table 3), likely a sign of collateral sensitivity. In short, GBH resistance had no effect on cross-tolerance and cross-resistance to antibiotics.

Evolution of resistance is often accompanied by fitness costs, meaning that resistant mutants would not survive as well as their sensitive ancestors. While subinhibitory concentrations of antibiotics are usually necessary for AMR selection, they are not always needed for maintenance, once fitness costs become sufficiently reduced by compensatory mechanisms (30). To test whether resistance to GBH is associated with fitness costs or advantages, we compared the growth of resistant mutants and their GBH-sensitive ancestors in the absence and presence of several concentrations of the selective agent. For this, we performed growth curves of ancestors and mutants individually and used the final biomass at 16 h as a proxy for fitness. In the absence of GBH, two strains (*S.* Enteritidis 12539M and *S.* Typhimurium 12468M) demonstrated no fitness costs, and *S.* Typhimurium 12472M displayed a fitness advantage. All three resistant mutants had a higher biomass than the ancestors at 80 mg/ml GBH (the highest level of resistance achieved in the experiment and the highest concentration tested), as well as small fitness advantages across the range of subinhibitory concentrations (0.312 to 20 mg/ml) (Fig. 3). Additionally, we performed a growth rate inhibition

TABLE 3 Susceptibilities to antibiotics in GBH mutants and ancestors

Agent	Antibiotic class	MIC (mg/liter) and category for[a]:					
		S. Typhimurium 12468		*S.* Typhimurium 12472		*S.* Enteritidis 12539	
		Ancestor (40)	Mutant (160)	Ancestor (40)	Mutant (160)	Ancestor (80)	Mutant (160)
Piperacillin	β-Lactam	≥128 R	≥128 R	≥128 R	≥128 R	≤4 S	≤4 S
Piperacillin-tazobactam	β-Lactam–β-lactamase-inhibitor	≤4 S	≤4 S	**8 S**	**≤4 S**	≤4 S	≤4 S
Cefotaxime	3rd-generation cephalosporin	≤1 S	≤1 S	≤1 S	≤1 S	≤1 S	≤1 S
Ceftazidime	3rd-generation cephalosporin	≤1 S	≤1 S	≤1 S	≤1 S	≤1 S	≤1 S
Cefepime	4th-generation cephalosporin	≤1 S	≤1 S	≤1 S	≤1 S	≤1 S	≤1 S
Aztreonam	Monobactam	≤1 S	≤1 S	≤1 S	≤1 S	≤1 S	≤1 S
Imipenem	Carbapenem	≤0.25 S	≤0.25 S	≤0.25 S	≤0.25 S	≤0.25 S	≤0.25 S
Meropenem	Carbapenem	≤0.25 S	≤0.25 S	≤0.25 S	≤0.25 S	≤0.25 S	≤0.25 S
Amikacin	Aminoglycoside	≤2 R	≤2 R	≤2 R	≤2 R	≤2 R	≤2 R
Gentamicin	Aminoglycoside	≤1 R	≤1 R	≤1 R	≤1 R	≤1 R	≤1 R
Tobramycin	Aminoglycoside	≤1 R	≤1 R	≤1 R	≤1 R	≤1 R	≤1 R
Ciprofloxacin	Fluoroquinolone	≤0.25 R	≤0.25 R	≤0.25 R	≤0.25 R	≤0.25 R	≤0.25 R
Tigecycline	Glycylcycline	≤0.5 S	≤0.5 S	≤0.5 S	≤0.5 S	≤0.5 S	≤0.5 S
Fosfomycin	Epoxide	≤16 S	≤16 S	≤16 S	≤16 S	≤16 S	≤16 S
Trimethoprim-sulfamethoxazole	Folate inhibitor-sulfonamide	≤20 S	≤20 S	≤20 S	≤20 S	≤20 S	≤20 S

[a]MICs were determined using the Vitek2 AST N-248 panel of antibiotics. Changes in MIC between ancestors and mutants are in bold. Numbers in parentheses are concentrations of Roundup LB Plus, in milligrams per milliliter. R, resistant; S, susceptible.

analysis of the growth curves in the presence and absence of GBH (Fig. S5). This method is independent of cell division rate and assay duration and is considered more robust than traditional 50% inhibitory concentration (IC_{50}) determination for estimation of cellular responses to drugs (31, 32). Similarly to the biomass analyses, comparison of the growth rate inhibition of ancestral and mutant strains in the presence of nine different concentrations of GBH showed no statistically significant differences. Our data indicate that stable resistance to GBH not only is possible but also is free of fitness costs or even advantageous, in both the presence and absence of GBH. While this suggests that the resistant GBH mutants may persist in the environment even when the selective pressure is not present, a competition assay between the mutant and the ancestor *in vivo* would provide evidence as to whether this is indeed the case, and this is the subject of further investigations.

Our study demonstrated that transient exposure of pathogenic *Salmonella enterica* bacteria to subinhibitory concentrations of the herbicide GBH in a rich growth medium and at a physiological (neutral) pH (a situation resembling *in vivo* conditions) readily elicits a tolerance response at the cellular level and upregulation of the AcrAB-TolC efflux system, while chronic exposure to GBH results in selection for GBH resistance but not cross-tolerance or cross-resistance to clinically relevant antibiotics. Importantly, although our results indicate that GBH resistance does not occur easily and is relatively low level (2 to 4× MIC increase), it is stable and is associated with no fitness costs in the absence of GBH and even a fitness advantage in its presence.

Our results are in line with the findings of Randall et al. (33), who demonstrated that exposure of *Salmonella enterica* serovar Typhimurium to commonly used farm disinfectants resulted in upregulation of the AcrAB-TolC efflux system, while selection for resistance to these biocides largely did not result in resistance to multiple antibiotics. Similar to our results, these biocide-resistant mutants did not show any fitness losses relative to parent strains. Kurenbach et al. demonstrated through the use of efflux pump and stress response regulator reporter assays and efflux pump gene deletion experiments that efflux plays an important role in the response of *Enterobacteriaceae* to GBH (11, 23), which is also in agreement with our data showing upregulation of the AcrAB-TolC efflux pump.

Interestingly, no noticeable upregulation of efflux pump genes was found in a transcriptomic study of *E. coli* in the presence of glyphosate (34), where largely energy- and metabolism-related genes were downregulated, while cell motility and

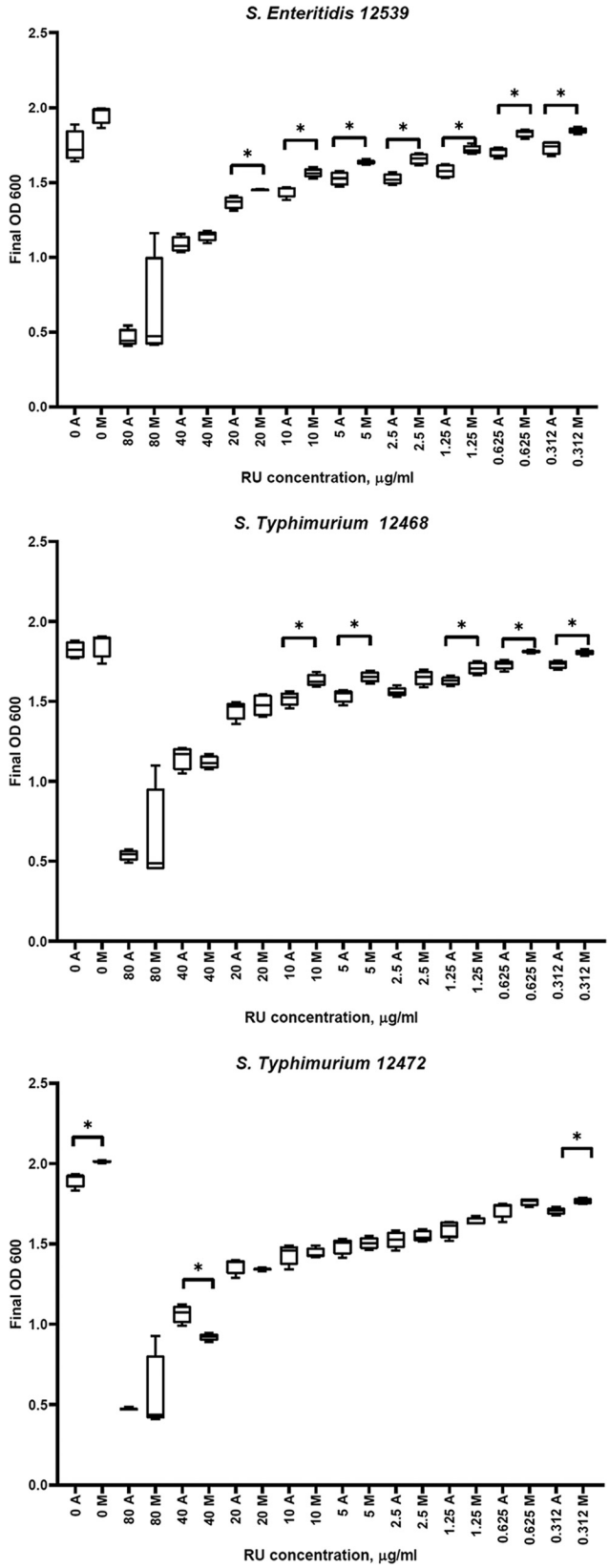

FIG 3 Fitness costs. Final biomass (OD_{600} values at 16 h) was used as a proxy for bacterial fitness in the presence and absence of Roundup LB Plus (RU). M, mutant; A, ancestor. Asterisks indicate statistical significance as determined by the *t* test ($P < 0.05$). Note the absence of fitness costs in the absence of RU and the subtle fitness advantage at certain subinhibitory concentrations of RU.

chemotaxis-related genes were upregulated. This discrepancy may be explained by the fact that different types and concentrations of GBH were used: 200 mM glyphosate (or 33.814 mg/ml) (34) versus 1,250 ppm (or 1.25 mg/ml) complete formulation Roundup weed killer (11) and 10 to 20 mg/ml Roundup LB Plus in the present study, both containing 360 g/liter isopropylamine salt. Indeed, it has been demonstrated that inert wetting agents found in complete formulations may also change bacterial sensitivity to antibiotics (23), highlighting the importance of making a distinction between the effects of the active ingredient and the complete formulation. Additionally, differences in the sensitivity of the methods, culturing conditions, and bacterial strains may also offer an explanation. At the same time, very few changes were found in the transcriptome of *E. coli* during the heterologous expression of the resistant *aroA* variant in the absence of the herbicide (35). This mirrors our observations of the proteomes in the resistant mutants in the absence of GBH, where only the proteins directly involved in resistance to glyphosate were differentially expressed.

Overall, there appears to be a consensus that while acute exposure to GBH triggers activation of efflux pumps and stringent response, no such effects are observed during the constitutive expression of the resistant EPSPS variant in the absence of GBH. It is also noteworthy that although the GBH concentrations used in this study exceed the concentrations typically found in animal feed (8), they are not unlikely and can be found during handling of the undiluted herbicide. Interestingly, it has been reported that exposure to higher GBH concentrations decreases the genome-wide mutation rate in *E. coli*, suggesting that long-term exposure to GBH does not compromise bacterial genome stability (36). This is in agreement with our observations of slow resistance evolution at increasingly higher concentrations of GBH, frequent extinctions, few observed mutations, and no cross-resistance to antibiotics, assuming that increased mutation supply is a prerequisite for evolution, including evolution of antibiotic resistance (37). Nonetheless, while this study provides important hints for extended risk assessment of ubiquitous herbicides such as glyphosate, the findings may be limited to the specific experimental conditions, and therefore, more studies with a broader range of bacterial species are needed to determine the relevance of these findings *in vivo*.

MATERIALS AND METHODS

Bacterial strains and culturing conditions. Ten isolates of *S. enterica* serovars Enteritidis and Typhimurium originally isolated from pig feces were provided by the German Federal Institute for Risk Assessment. All cultures were grown in Mueller-Hinton I (MHI) medium (CM0405; Oxoid GmbH, Wesel, Germany) and incubated at 37°C with moderate shaking, unless stated otherwise.

Experimental evolution. Single colonies were isolated from blood agar plates and passaged daily 1:100 in 5 ml MHI in a 50-ml Falcon tube for 3 days to help bacteria adapt to the experimental conditions (referred to as the preadaptation passage). Roundup LB Plus (German license 024142-00; Monsanto) was used for experiments. After the preadaptation phase, the medium was supplemented with increasing concentrations of Roundup LB Plus, adjusted with NaOH to pH 7. The starting subinhibitory concentration for the challenge was 20 mg/ml glyphosate isopropylamine salt in Roundup LB Plus. Each day, 50 μl of the overnight culture was transferred into two new tubes, one with the same concentration of Roundup at which the visible growth occurred in the last passage and one with a concentration of glyphosate 10 mg/ml higher than that. Nonevolving controls were handled similarly with the exception that Roundup was absent. The experiment ended when no growth was visible after 24 h in both tubes. Roundup-selected isolates were then passaged 1:100 in the absence of the herbicide (referred to as the stability passage), followed by the determination of the MICs (Fig. 1).

DNA preparation and whole-genome resequencing. Isolates which remained Roundup resistant after the stability passage were subjected to whole-genome resequencing along with nonselected controls and ancestors (each as a single colony and a population) (Table S1). For colony sequencing, individual colonies were isolated from Mueller-Hinton agar plates before culturing in liquid medium. For population sequencing, a loopful of the frozen stock was directly cultivated in 3 ml MHI. DNA was extracted with a GeneMatrix bacterial and yeast genomic DNA purification kit (EURx Molecular Biology Products, Gdansk, Poland). DNA concentration was determined using Nanodrop at 260/280 nm. DNA integrity was ensured by gel electrophoresis (1% agarose). Isolated DNA was stored at −20°C until sequencing.

Sequencing libraries were constructed from 2 μg total genomic DNA using a TruSeq DNA PCR-free kit (Illumina) and sequenced for 600 cycles using a MiSeq at the Berlin Center for Genomics in Biodiversity Research.

Reference genomes were constructed by assembling sequencing reads using the a5-miseq pipeline (38) and annotated using prokka (39). The variant calling pipeline Snippy was used to identify mutations

in the selection lines. Snippy uses bwa (40) to align reads to the reference genome and identifies variants in the resulting alignments using FreeBayes (41). Variants were verified using the breseq computational pipeline (42).

Label-free quantitative proteomics analysis. Isolates from ancestral and mutant lines frozen stocks were grown overnight in MHI at 37°C with shaking, diluted 1:100, and subcultured until an optical density of 0.5 was achieved. Each mutant-ancestor pair was allowed to grow for 25 min more in the presence and absence of Roundup LB Plus; each condition consisted of six biological replicates. Treatment samples were challenged with 1/4× MIC Roundup and had their pH adjusted with 5 M NaOH to neutral.

Following incubation, subcultures were centrifuged for 5 min at room temperature at maximum speed, and the supernatant was discarded. The pellet was washed with 1 ml PBS, followed by addition of 300 μl distilled water and 900 μl ethanol (EtOH) (100%). After 1 h of incubation at room temperature, samples were centrifuged for 10 min at room temperature at 10,000 $\times$ *g*. The supernatant was discarded, and the pellet was air dried and stored at −20°C until protein extraction.

Protein extraction was carried out with the ethanol-fixed cells. In brief, the cells were reconstituted with 100 μl each of acetonitrile (100%) and formic acid (75% [vol/vol]). The samples were sonicated on ice for 1 min (duty cycle, 1.0; amplitude, 100%) (UP100H; Hielscher Ultrasound Technology, Teltow, Germany) and centrifuged at 11,290 $\times$ *g* for 5 min at 4°C. The clear supernatant was collected, and the protein content was quantified using the Qubit method (Thermo Fisher Scientific, Germany) following the manufacturer's recommendations.

In-solution trypsin digestion was carried out at room temperature as described elsewhere (43). The resultant trypsin-digested peptide products were first desalted by solid-phase extraction using C_{18} Empore disc cartridges (Supelco/Sigma-Aldrich, Taufkirchen, Germany) and dried under vacuum. Peptides were reconstituted in 10 μl of 0.05% trifluoroacetic acid (TFA)–2% acetonitrile, and 2 μl was analyzed by a reversed-phase nanoscale liquid chromatography system (Ultimate 3000; Thermo Scientific) connected to an Orbitrap Velos mass spectrometer (Thermo Scientific). Samples were injected and concentrated on a trap column (PepMap100 C_{18} [Thermo Scientific]; 3 μm, 100 Å, 75 μm [inside diameter], 2-cm length) equilibrated with 0.05% TFA–2% acetonitrile in water. After switching the trap column inline, liquid chromatography (LC) separations were performed on a capillary column (Acclaim PepMap100 C_{18} [Thermo Scientific]; 2 μm, 100 Å, 75 μm [inside diameter], 25-cm length) at an eluent flow rate of 300 nl/min. Mobile phase A contained 0.1% formic acid in water, and mobile phase B contained 0.1% formic acid in acetonitrile. The column was pre-equilibrated with 3% mobile phase B followed by an increase from 3 to 50% mobile phase B in 80 min. Mass spectra were acquired in a data-dependent mode using a single mass spectrometry (MS) survey scan (*m/z* 350 to 1,500) with a resolution of 60,000 in the Orbitrap and MS/MS scans of the 20 most intense precursor ions in the linear trap quadrupole. The dynamic exclusion time was set to 60 s, and automatic gain control was set to 1×10^6 and 5,000 for Orbitrap-MS and LTQ-MS/MS scans, respectively. The acquired raw data files from mass spectrometry were processed using the MaxQuant-Andromeda software suite (version 1.6.0.16; Max Planck Institute of Biochemistry, Martinsried, Germany) (44).

Protein identification was carried out by searching MS and MS/MS data against FASTA files of protein sequences produced by translating annotated DNA sequences. The parametric settings were set for protein identification, as follows: mass tolerance, 7 ppm; MS and MS/MS ion tolerance, 0.5 Da; enzymes LysC and trypsin, both with two missed cleavage sites allowed for the database search; variable modification including oxidation of methionine and protein N-terminal acetylation; fixed modification including carbamidomethylation; target-decoy-based false discovery rate (FDR) for peptide and protein identification of 1% for peptides and proteins; and minimum peptide length, 7 amino acids. The software Perseus (version 1.6.1.1; Max Planck Institute of Biochemistry, Martinsried, Germany) (45) was used for identification of differentially expressed proteins. The MaxQuant result file (protein groups.txt) was imported into the Perseus software, and a reduction matrix was applied to remove proteins identified only by site and reverse and potential contamination. The intensity values were transformed to $\log_2$ values, and a reduction matrix based on signal detection in three of six replicates in any one of the group was applied. The two-way Student *t* test, error correction ($P < 0.05$), and FDR correction of the alpha error were applied through the Benjamini-Hochberg procedure for identification of differentially expressed proteins among the compared groups. The STRING online tool (v.11; https://string-db.org/) (20) was used to visualize proteins affected by Roundup in protein networks. For this, significantly differentially expressed proteins from the processes known to be affected by glyphosate and the 10 most up- and downregulated proteins for each strain were used.

Antimicrobial susceptibility testing. The MIC of Roundup LB Plus (German license 024142-00; Monsanto) was determined by the broth microdilution method described in reference 9 in MHI. Because GBH acidifies medium, the pH was adjusted to neutral with NaOH. The concentration of glyphosate was calculated based on the concentration of glyphosate isopropylamine salt in the herbicide formulation. Cross-resistance to a panel of antibiotics relevant to human medicine was determined via antibiotic susceptibility testing with the Vitek system (bioMérieux Deutschland GmbH, Nürtingen, Germany) using the test card Vitek 2 AST N-248. The tested antimicrobials were piperacillin, piperacillin-tazobactam, cefotaxime, ceftazidime, cefepime, aztreonam, imipenem, meropenem, amikacin, gentamicin, tobramycin, ciprofloxacin, moxifloxacin, tigecycline, fosfomycin, and trimethoprim-sulfamethoxazole.

Tolerance detection test. Tolerant colonies were detected by the tolerance detection (TD) test based on the semiquantitative method described in reference 28. The overnight culture for each isolate was prepared with three biological replicates, each of which was individually inoculated from a single cryopreserved stock. An appropriate quantity of cells was resuspended in 0.85% NaCl, and the optical density was adjusted to 0.5. The bacterial suspension was plated on dried Mueller-Hinton I agar plates

containing 0.5% glucose. Ready-to-use discs for CAZ (30 μg per disc), FEP (30 μg per disc), and CT (10 μg per disc) were purchased from Oxoid (Thermo Fisher Scientific, Germany). Fosfomycin (200 μg per disc) and rifampin (10 μg per disc) diffusion discs were prepared by soaking blank discs with 20 μl of antibiotic stock solution per disc. A disc diffusion assay was performed by placing the discs on the lawn of bacteria and incubating them overnight at 37°C. After the overnight incubation, the antibiotic discs were replaced by discs containing 40% glucose and incubated overnight. The isolated colonies found in the zone of inhibition after the incubation with glucose discs represent the tolerant colonies.

Fitness costs. Growth curves were performed in a plate reader (Synergy HTX; BioTech Instruments, Germany). Ancestral and mutant lines were grown in tubes with 3 ml of MHI overnight with shaking. Subsequently, the overnight cultures were diluted 1:100 in MHI and grown for approximately 2 h until an optical density at 600 nm (OD_{600}) of 0.5 was reached. An aliquot of 100 μl was transferred into the wells of a 96-well plate containing 100 μl MHI. Measurements were taken at 10-min intervals after a short period of shaking and incubation at 37°C for 16 h in the plate reader. The assays were performed in the absence and presence of Roundup LB Plus (2-fold dilutions from 0.3125 to 80 mg/ml). Four biological and technical replicates were used for each sample-treatment combination. As a proxy for fitness, mean values of the final biomass (OD_{600} at 16 h) were used. A *t* test was used to calculate statistical significance ($P < 0.05$) of the difference between ancestors and mutants using GraphPad Prism 8. Growth rate (GR) inhibition analysis was performed using GRcalculator (31). For this, three time points (2, 9, and 16 h) and 9 concentrations (0.3125, 0.625, 1.25, 2.5, 5.0, 10.0, 20.0, 40.0, and 80.0 mg/ml glyphosate in RU LB Plus) were used to calculate GR_{50}, GR_{max}, and GR_{inf} values (Fig. S5).

Data availability. Sequence data are available from the NCBI SRA under BioProject accession no. PRJNA485244. The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium via the PRIDE (46) partner repository with the data set identifier PXD019463.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.8 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.

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4. In vitro and in vivo studies on the impact of glyphosate on *Salmonella enterica*

4.1 *In vitro* study for the impact of glyphosate and GBH on the survival of *Salmonella enterica* in rumen simulation

4.1.1 Materials and Methods

The experiment was conducted after Riede et al 2016 (64) in cooperation with the Institute of Physiology and Cell Biology of the University of Veterinary Medicine Hannover, Foundation, together with Katrin Bote, who investigated the effects for *E. coli.*

A pooled sample of rumen liquid from three Holstein Frisian non-lactating cows was taken. Cows were fed with 1/4 grass silage, 1/4 maize silage and 1/2 concentrate.

Rumen liquid was filled into 6 vessels of 700 ml volume. After an equilibration time of 7 days, vessels were inoculated with 1 ml of *Salmonella enterica* ser. Typhimurium strain (DT104 BB440; laboratory number 11386), each (figure 4). The *Salmonella enterica* ser. Typhimurium DT 104 strain was provided by the German Federal Institute of Risk Assessment and originated from pigs. Before the experiment, it showed an initial MIC to RU of 80 mg/ml. For the inoculum, the *S. enterica* strain was taken from frozen stock and incubated overnight in MH broth (CM0405 Oxoid Ltd., Hampshire) at 37 °C, shaking. The culture was incubated until it reached a concentration of 10^9 cfu/ml. This lead to a concentration of 10^6 cfu/ml in every vessel.

Tab. 2: Overview over the infection strain and minimum inhibitory concentration for glyphosate and RU and in pH adjusted media with glyphosate or RU. The infection strain was used in both, *in vitro* and *in vivo* study (Fermenter and animal experiment).

Species	lab number	MIC GLY in mg/ml	MIC GLY pH7 in mg/ml	MIC RU in mg/ml	MIC RU pH7 in mg/ml	Origin	Resistances
Salmonella ser. Typhimurium DT104	13339	40	80	80	80	pig	Nalidixic acid

For isolation of *Salmonella enterica* from the vessels, selective XLD media was used (Oxoid GmbH, Wesel, Germany).

Every day, pH, redox potential (mV) and effluent volume was measured. Concentrations of NH_3 and short chain fatty acids (SCFA) were determined by the Institute of Physiology and Cell Biology of the University of Veterinary Medicine Hannover, Foundation at the end of the equilibration period on day 6.

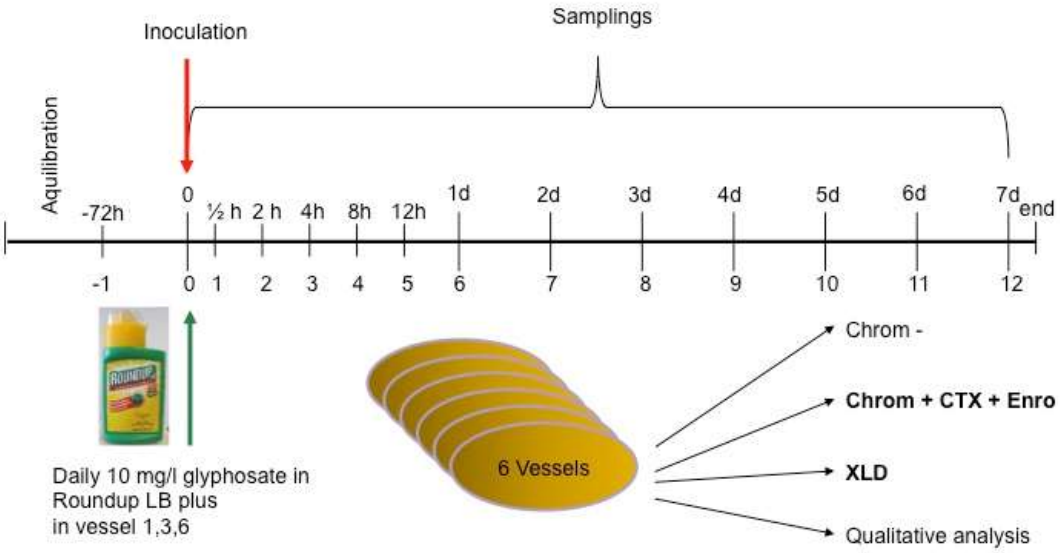


Figure 4: Study design of the RUSITEC experiment.

In each vessel, two nylon bags were inserted, filled with 15 g of fresh substrate. Every nylon bag was exchanged every second day alternately. The bags were filled with grass silage (49,5%), maize silage (39,7 %), wheat meat (5 %), soy cake (5 %) and mineral feed (0,8 %), according to a feed ration of a non-lactating cow.

Right after inoculation of the *S.* Typhimurium strain, challenging of the vessels with RU in a concentration of 10 mg/l started. The vessels were challenged every day at the same time, directly after sampling and changing of the nylon bag to avoid unnecessary opening and interrupting anaerobe conditions. Challenged vessels were the numbers 2, 4 and 6 (RU). Vessels 1, 3 and 5 were used as controls (contr).

Sampling took place at time points 0, 0.5, 2, 4, 8, 12, 24, 48, 72, 96, 120, 144, and 168 h.

Collected samples were plated in a standard dilution row on selective agar plates for quantitative analysis. For qualitative analysis, samples were enriched in overnight cultures in buffered peptone water (DM494D Mast Group Ltd., Merseyside) and plated on selective media, if quantitative plating was unsuccessful.

For investigation of change in susceptibility against glyphosate formulation Roundup LB plus, MIC was determined for *Salmonella enterica* ser. Typhimurium before the beginning of the experiment as comparison and for every three isolates of every vessel at every collection time point. In the end of the experiment, the last possible point of re-isolating during experiment was also determined for its MIC against RU. The determination of MIC was done following the general protocol used in the screening experiment, except for a pH adjusted of RU MH broth. PH 7 was obtained by addition of NaOH.

The initial inoculated *Salmonella enterica* ser. Typhimurium strain and one resulting *S.* Typhimurium isolate from every vessel from the last sampling point of successful recovering was additionally tested for susceptibility against most common antibiotics for human medicine via VITEK system (bioMérieux Deutschland GmbH, Nürtingen, Germany). As test card, the VITEK W2 AST N-248 was chosen. Tested antibiotics from this card are piperacillin, piperacillin–tazobactam, cefotaxime, ceftazidime, cefepime, aztreonam, imipenem, meropenem, amikacin, gentamicin, tobramycin, ciprofloxacin, moxifloxacin, tigecycline, fosfomycin, and tri-methoprim/sulfomethoxazole.

4.1.2 Statistical Analysis

All statistical analyses were performed using IBM SPSS Statistics Version 24. All data of the vessels were compared individually at every time point with t-test. The median of bacterial counts in each fermenter group was compared with Wilcoxon test or t-test.

For quantitative analysis, the median of bacterial counts of every group was compared with Wilcoxon test or t-test. For qualitative analysis a chi-squared test was performed. For results see the publication of Bote et al. 2019 (133) which describes all details.

4.2 *In vivo* study for the impact of glyphosate and glyphosate containing herbicide on shedding of *Salmonella enterica* in pigs

4.2.1 Materials and Methods

Animals. The animal study was permitted by the State Office of Health and Social Affairs Berlin, Germany (Landesamt für Gesundheit und Soziales, G 0318/17). A total of 42 weaning piglets divided into three groups were investigated using the following study design. All animals came from the Institute of Animal Nutrition, Freie Universität Berlin. They were littered by sows which were proven to be free of *Salmonella enterica* via whole genome sequencing of feces. The animals of each group were littered from at least three different sows and selected for comparable weights and health status. Two weeks before weaning, piglets were started to be fed with solid organic piglet feed (Ferkelkorn), purchased from the company Meika Ökologische Tiernahrung. Right after weaning, piglets were transported to the Institute of Animal Hygiene and Environmental health. Animals were held in groups of 14 piglets each in the animal house of the Robert-von-Ostertag House of Freie Universität Berlin. After an adaptation phase of 6 days' animals were infected with a dose of between 1 -3 x 10^8 (Table 2) of *Salmonella enterica* serovar Typhimurium DT104 (DT 104 BB440, laboratory number 11386; same strain was used in RUSITEC experiment; for MIC-data see table 2) with an implemented nalidixic resistance. The infection was done via intragastric application trough a stomach tube. Fecal samples were taken for repeated control for signs of *Salmonella enterica* from the sows five days after littering and from the piglets one day before weaning. For qualitative and quantitative *S. enterica* investigation fecal samples were taken from the piglets after infection at day 1 (s1), day 2 (s2), day 5 (s3), day 7 (s4), day 13 (s5), day 19 (s6) and day 21 (s7) during the last necropsy. Before every fecal sampling rectal temperature was measured. Blood sampling and weighing was performed weekly. In every necropsy, seven piglets were sacrificed (figure 5).

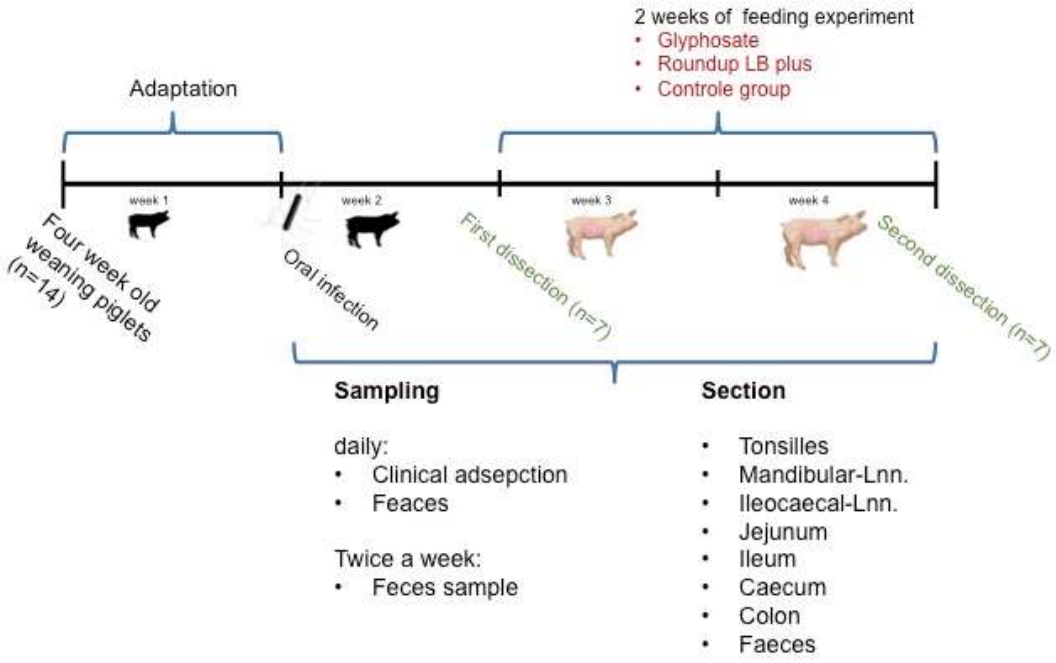


Figure 5: Experimental design of animal trial.

For necropsy piglets were first sedated with azaperone (2 mg/kg BW; Stresnil; Janssen-Cilag GmbH), afterwards with ketamine (15 mg/kg bw; “Ursotamin”; Serumwerk Bernburg, Bernburg, Germany) and xylacine (2 mg/kg BW; 20 mg/ml; Serumwerk Bernburg, Bernburg, Germany) and diazepam (0.5 mg/kg bw; ratiopharm GmbH, Germany). After sedation piglets were sacrificed by intracardial injection of tetracaine hydrochloride, mebezoniume iodide and embutramide (0.12 ml/kg bw T61; Intervet; Germany) and samples were taken in the following order: after opening of the abdomen spleen and ileoceacal lymph nodes. After extraction of complete intestine following parts were sampled or removed: complete ileum, complete caecum, middle part of jejunum, middle part of colon and a fecal sample was taken from rectum. Finally, complete mandibular lymph nodes, the tip of the tongue and complete tonsils were sampled. All collected samples were held sterile and separated until they were prepared in laboratory process.

Glyphosate and Roundup LB plus treatment. The three groups of 14 piglets each were treated with glyphosate (Sigma-Aldrich), Roundup LB plus (RU) or without any treatment as a control. Treatment started after seven days after infection with *Salmonella enterica* Typhimurium 104 and therefore immediately after the necropsy of the first seven piglets of each group as internal control. The piglets were orally treated with glyphosate 2,85 mg/kg/day *per os* either in glyphosate as isopropylamine salt, or in RU as a worst-case scenario (51).

Laboratory investigation. All samples were processed after the same protocol. One gram of each sample was flamed (except feces and samples of intestine), cut into small pieces and mixed with buffered peptone water (DM494D; Mast Group Ltd, Bootle, United Kingdom). Afterwards, samples were homogenized for 2 minutes at 200 rpm (Stomacher 400 circulator; Seward Limited, West Sussex, United Kingdom). For quantitative analysis, the 10^{-1} samples were diluted step by step until a concentration of 10^{-4} and plated in duplets on XLD agar (1.05282.0500; Merck KgaA, Darmstadt, Germany). The agar plates were supplemented with nalidixic acid (CN32.2; Carl Roth GmbH + Co, Karlsruhe, Germany). Incubation took place at 37 °C for 48 hours with a first check after 24 hours. In addition, a qualitative analysis of *S. enterica* was done. Protocol was used after ASU methods for food of §64 LFGB. The solution for the quantitative analysis was prepared as 1 g of organ or feces in 10 ml buffered peptone water. After an incubation of 24 hours, 100 µl of that pre-incubated media was dropped on a supplemented MSRV medium (MSRV; CM1112; Oxoid, Hampshire, United Kingdom) and incubated for another 24 hours. After incubation, a small part of the semisolid media was taken from the very outside of the swarm zone and was transfer on XLD plates. Plates were again incubated at 37 °C for 48 hours. Plates were checked first time for typical growth after 24 hours.

4.2.2 Minimum inhibitory concentration

MIC was performed as described in Pöppe et al. (134) and Bote et al.(135) for RU, glyphosate and, corresponding, with pH-adjusted solution.

4.2.3 Statistical Analysis

All data were analyzed statistically with GraphPad Prism8 and Excel. For the comparison of quantitative Data from feces and organs, t-test was examined for comparison of groups. For qualitative data from feces and organs chi square test was performed. Significance level was set to a maximum of 5% ($P < 0.5$).

4.2.4 Results of the animal experiment

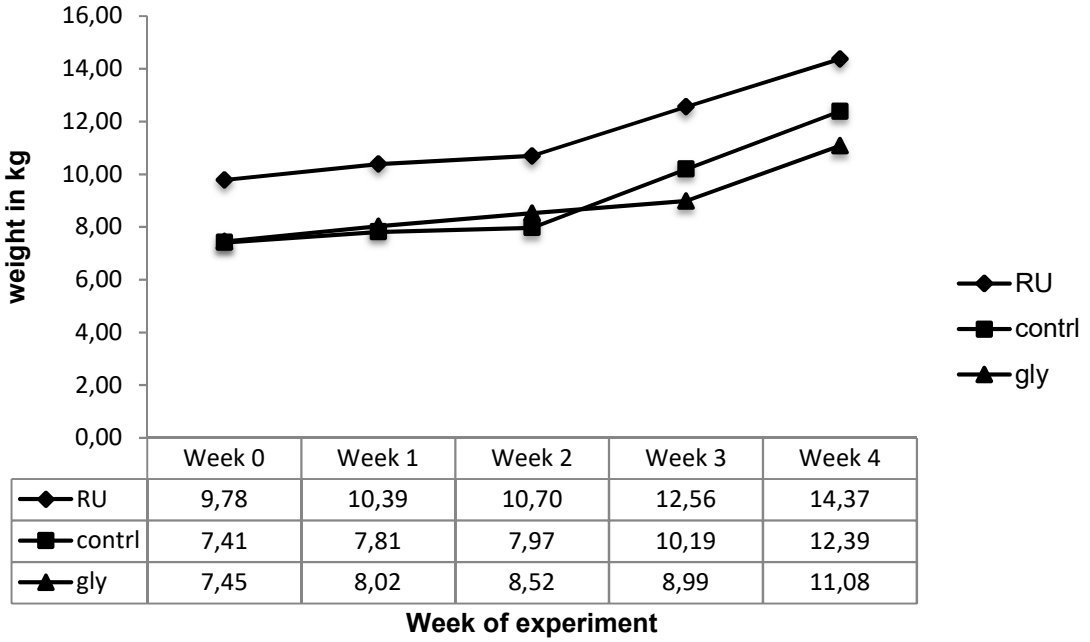
Infection dose

In all three groups, the requested amount of infective cells of *Salmonella enterica* was achieved. Before the experiment, OD was used as reference for number of cells for infection. After preparation, infection dose was controlled on non-selective MH agar plates and number of cells were counted after 24 hours.

Group	OD	cfu
Contr	0.750	1.79E+08
RU	0.759	2.63E+08
gly	0.740	1.59E+08

Table 3: Numbers of cfu of *Salmonella enterica* ser. Typhimurium for the three groups of the animal experiment.

Comparison of animal weight



	Week 0	Week 1	Week 2	Week 3	Week 4
RU	9,78	10,39	10,70	12,56	14,37
contrl	7,41	7,81	7,97	10,19	12,39
gly	7,45	8,02	8,52	8,99	11,08

Week of experiment

Figure 6: Growth of animal weight during the experiment.

In all three groups, Roundup LB plus (RU), glyphosate (gly) and control (contrl) the increase of weight was comparable. The figure 6 shows that the three groups start at different initial weight. This is due to different mother sows in different ages and different litter size.

Qualitative analysis feces

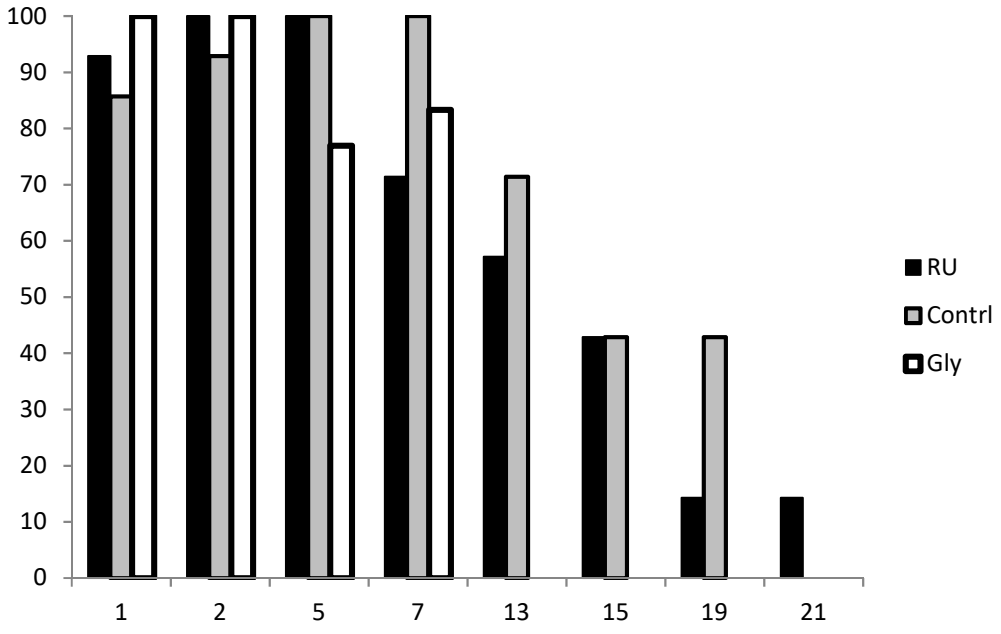


Figure 7: Numbers of qualitative *S. enterica* positive individuals from feces after infection. Numbers are the sampling days after infection. Treatment of RU or glyphosate started at day 5.

Figure 7 shows, that at sampling day 1 to day 5 a.i. in all three groups 100% of individuals were qualitatively *S. enterica* positive. The percentage of qualitatively *S. enterica* positive individuals decreases after day 5 a.i.. This was used as a control for the successful infection of all animals.

Figures 8 and 9 show qualitative comparison of infection in animals of all three groups for section one (figure 8) and section two (figure 9). Section one took place 7 days after infection. Except for bile and spleen for the RU and Gly group, *S. enterica* could be detected in every organ/tissue.

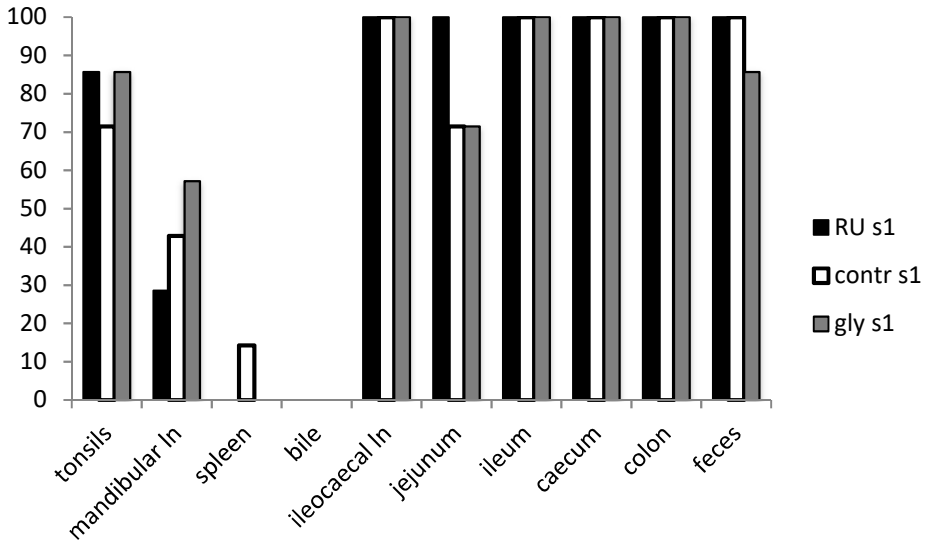


Figure 8: Shown is the qualitative comparison of *S. enterica* for the organs from section 1.

In the organs analyzed after section two, only in ileocaecal lymph nodes, *S. enterica* cells were detectable in every animal. In spleen and bile, no *S. enterica* cells were detectable.

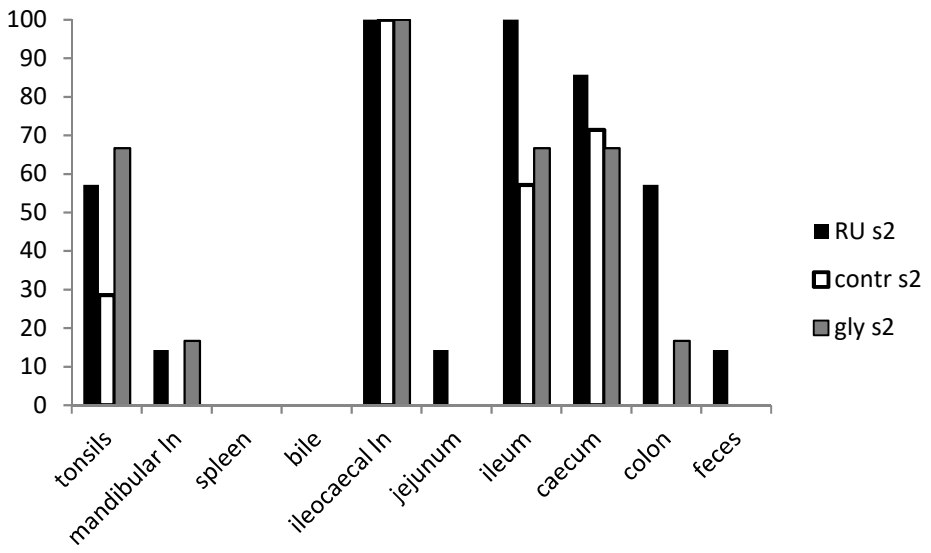


Figure 9: Shown is the qualitative comparison for *Salmonella enterica* for the organs from section 2, after two weeks of feeding of RU or glyphosate and the control group.

Quantitative analysis of *Salmonella enterica* shedding

For feces, the data of the three groups (gly, RU and control) were compared as mean. *S. enterica* was countable from Day 1 till day 5 a. i. in the groups. As the error bar shows, the variability between individuals was high (figure 10). In the beginning of glyphosate or RU feeding after the first section 1 at day 7, in all groups except for the control, number of *S. enterica* was under the detection limit for the method. Only in the control group, did the number of *S. enterica* decreased until day 13. After day 7 a.i., the beginning of the feeding experiment, the numbers already decreased to zero and this did not change until the end of the experiment. The level of *S. enterica* shedding directly after infection at first sampling day 1 is different for all three groups.

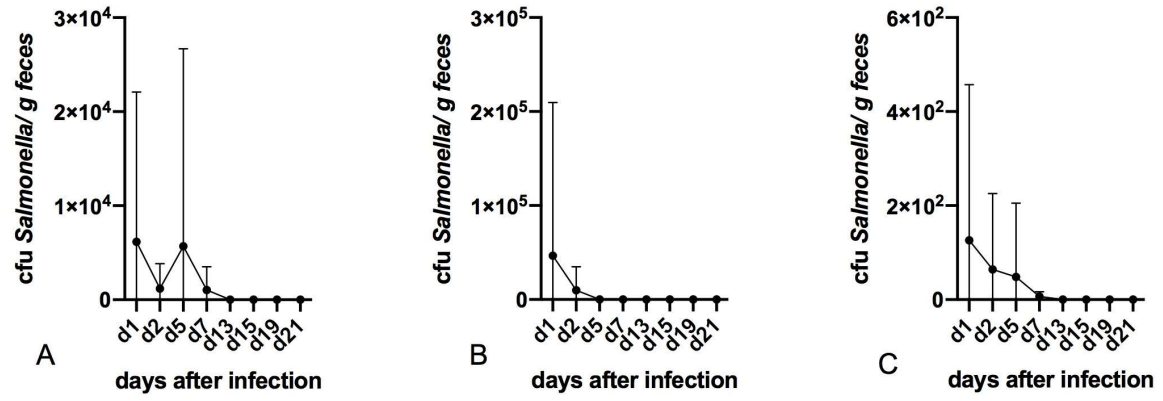


Figure 10: Number of colony forming units of *Salmonella enterica* counted on XLD plates from feces after 48 hours of incubation. A= control group, B = Roundup LB plus group, C= glyphosate group. Sampling days correspond to days after infection. Feeding experiment started at d 7.

In organs after section one, *S. enterica* was not detected quantitatively in the bile and spleen. High numbers of cells were counted in the ileum for the control group. In all investigated organs, numbers of *Salmonella enterica* were very low (figure 11). Colonization of the piglets was successful, as *S. enterica* could be found in most animals in jejunum and feces and in all animals in the other parts of the gut. Although groups are already named RU and glyphosate, a feeding of herbicide started after this section.

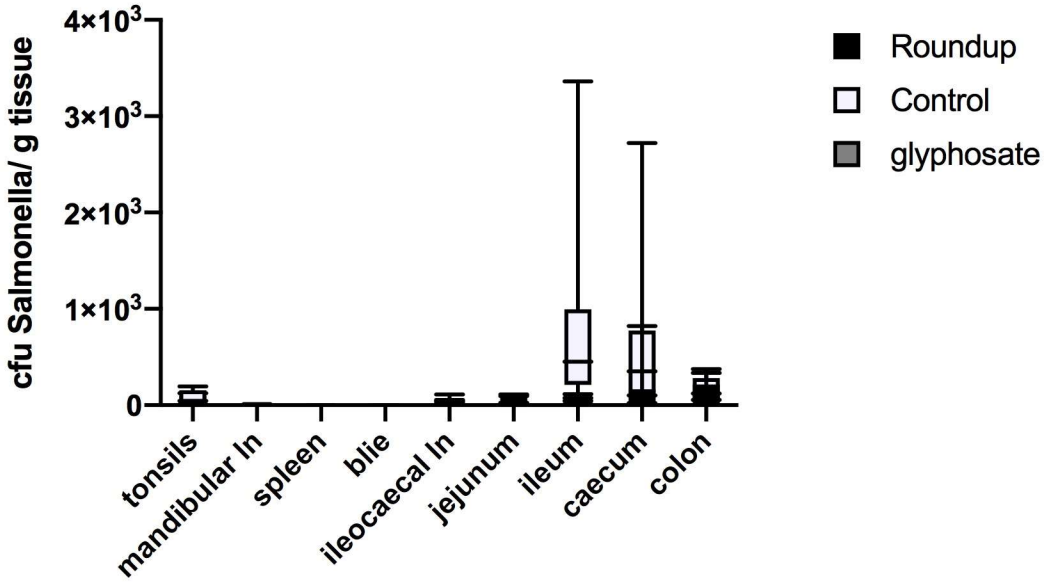


Figure 11: Numbers of *Salmonella enterica* counted in organs from section 1 before the beginning of glyphosate or RU feeding.

Figure 12 shows the quantitative analysis of *S. enterica* in organs after section two. Here, countable amounts of *S. enterica* cells were only found in the tonsils.

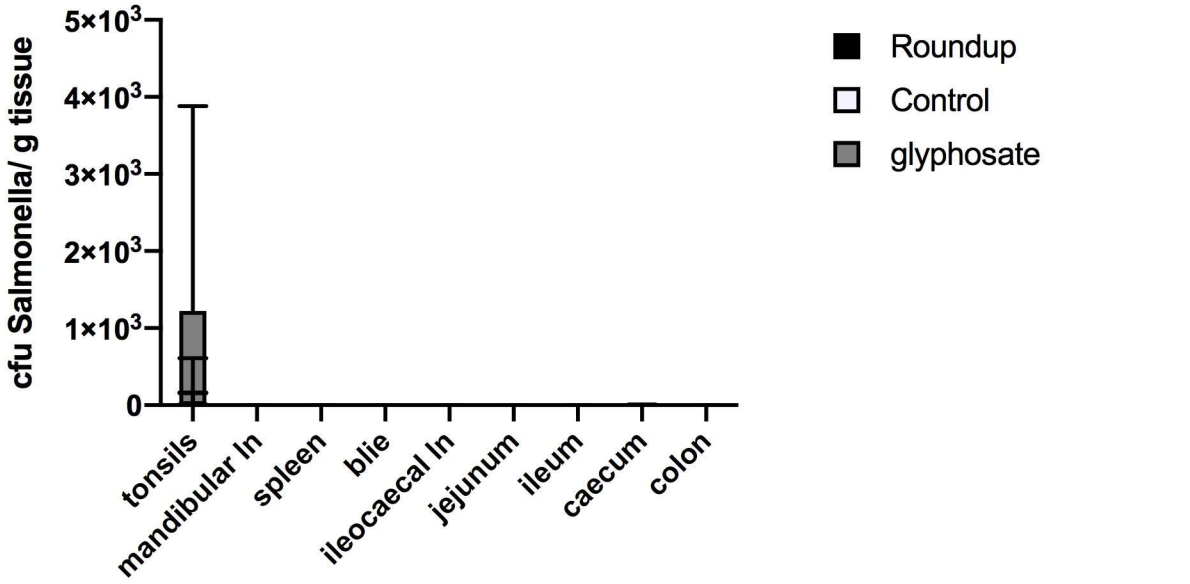


Figure 12: Quantitative analysis of *S. enterica* from section 2 for the three compared groups fed with Roundup, glyphosate and the control.

Minimum Inhibitory Concentration

Minimum inhibitory concentration has been determined for picked *S.* Typhimurium isolates from caecum samples at the end of experiment. No differences in MIC could be detected.

Statistical analysis

The statistical analysis showed a statistically significant difference in the qualitative comparison of *S. enterica* in feces between the glyphosate and the control group at day 13. Numbers in the control group were higher compared to the glyphosate group. This is due to the fact that in the glyphosate group from day 13 a.i. on there was no quantitative evidence of *Salmonella enterica* in the individuals. In the control group no quantitative evidence of *S. enterica* was found since day 15 a.i. and in the Roundup group since day 19 (due to a single individual).

Table 4: Qualitative analysis of *Salmonella enterica* in feces. Chi square test analysis was performed. Significant values are in bold. * no p-value due to no comparable data. a.i.= after infection.

days a.i.	RU vs contr	gly vs contr
1	0,541	0,157
2	0,309	0,326
5	*	0,057
7	0,127	0,261
13	0,577	**0,008**
15	1,000	0,067
19	0,237	0,067
21	0,299	*

Statistical analysis of quantitative analysis of *S. enterica* shedding in feces showed no statistically significant difference (table 5).

Method	day a.i.	RU vs. contr	gly vs. contr	RU vs. gly
Mixed analysis quantitative[1]		0,0549		
multiple t-test	1	0,365	0,185	0,315
	2	0,215	0,139	0,176
	5	0,336	0,342	0,146
	7	0,292	0,327	0,458
	13	0,350	0,377	0,263
	15	0,337	*	0,377
	19		*	*
	21	*	*	*

Table 5: Overview of P-values of analyzed groups and test for quantitative comparison of shedded *S. enterica* in feces. [1] Due to missing data (deletion of piglet number 46 from experiment, mixed model was performed instead of ANOVA analysis). * no analysis due to no comparable data. *P*-values in bold are significant.

The statistical analysis of quantitative results for section one and two showed only significant differences in section one, before the beginning of glyphosate feeding. This is due to individual reactions to the Salmonella infection. In section two, there were no significant differences detectable (table 6). The analysis of qualitative results for section one and two showed only one significant difference for colon in section two for the comparison of RU and control. In the RU group, there are more animals positive for *S. enterica* in colon than in the control group (Table 7).

Multiple t-test quantitative

compared group	organ	section 1	section 2
RU vs contr	tonsils	0,308411	0,398755
	mandibular ln	*	*
	spleen	*	*
	bile	*	*
	ileocaecal ln	**0,030123**	*
	jejunum	0,361304	*
	ileum	0,084078	*
	caecum	0,165713	0,337049
	colon	0,302723	*
	feces	0,259971	*
gly vs contr	tonsils	0,610357	0,229164
	mandibular ln	**0,045118**	*
	spleen	*	*
	bile	*	*
	ileocaecal ln	0,018191	*
	jejunum	0,334961	*
	ileum	0,078689	*
	caecum	0,075329	*
	colon	**0,005625**	*
	feces	0,348542	*
gly vs RU	tonsils	0,454051	0,284033
	mandibular ln	0,045118	*
	spleen	*	*
	bile	*	*
	ileocaecal ln	0,642677	*
	jejunum	0,039233	*
	ileum	0,225494	*
	caecum	0,25262	0,377374
	colon	0,133458	*
	feces	0,83812	*

Table 6: Overview of P-values of analyzed groups and test for quantitative comparison of cfu of *S. enterica* in organs. * no analysis since there were no comparable data (0 or same) . *P*-values in bold are significant.

	section 1		section 2	
	RU vs contr	gly vs contr	RU vs contr	gly vs contr
tonsils	0,906	0,906	0,280	0,170
Mandibular ln	0,714	0,447	0,299	0,261
spleen	0,299	0,299	*	*
bile	*	*	*	*
ileocaecal	*	*	*	*
jejunum	0,127	1,000	*	*
ileum	*	*	0,299	*
caecum	*	*	0,515	0,853
colon	*	*	**0,018**	0,261

Table 7: Statistical analysis of qualitative data from section 1 and 2 for *S. enterica* in organs. Analyzed with chi square test. Significant values in bold. *= no analysis due to no comparable data.

5. Discussion

Glyphosate has been used now for more than 50 years in agriculture as a nonspecific herbicide. Its impact on the environment and its toxicity was always seen as very low (78) so that the amount of usage of the herbicide increased more and more, at least since the mid 90s, were glyphosate resistant crops became common (4). However, in recent years the discussion about the safety of glyphosate started again, even more since the International Agency for Research on Cancer reclassified glyphosate as possibly carcinogenic in 2015 (136). In this comprehensive study we discuss whether glyphosate has an impact aside from classical toxicity or health effects but rather on the composition and perseverance of pathogenic bacteria. From screening isolates sampled in the last 30 years, over evolution experiments and *in vitro* studies in rumen simulation technic towards *in vivo* studies in the pig infection model, the adaptation, implementation and assertion of pathogenic *Salmonella enterica* was investigated in the presence and absence of glyphosate and glyphosate formulation.

5.1 Minimum inhibitory concentration

The value of minimum inhibitory concentration (MIC) showed a small range. In comparison with other MIC determination studies, values of this screening were considerably higher. In studies of other working groups, MIC varied between 5.0 mg/ml (61) and 6.19 mg/ml (95) for *Salmonella enterica*. Comparable results have only been detected by Nielsen et al. of 80 mg/ml glyphosate in formulation (137). The main challenge in comparing these data lies in the different formulations used. Herbicide formulations with glyphosate as active ingredient have many other ingredients, which can have effects on microbiota themselves. This is known for the tallow amines, which are more toxic than glyphosate (52, 138), but similar effects with other undeclared ingredients of formulations are conceivable. Results for *E.coli* with the same MIC determining technique also showed comparatively high MICs as for *S. enterica* (135). In this study *E. coli* has MIC values between <1,25 up to 40 mg/ml glyphosate and glyphosate in formulation. Here, the same formulation, Roundup LB plus, was used. The comparison of the MIC data from literature shows lower MICs for *E.coli*, as was found in the publication of Shehata et al. 2013 (61) 1,2 mg/ml or 7400 ppm in Kurenbach et al. 2015 (95). Likewise for *S. enterica*, in the case for *E. coli* the publication from Nielsen et al 2018 (137) is the publication with comparable results for MICs. Nielsen et al. also use tallow amine free

glyphosate formulation and nutrient rich media. In a small side experiment within this study about MIC values for other Enterobacteriaceae, lower MIC values were examined for *Enterobacter cloacae* and *Enterobacter faecalis*, for glyphosate as a pure substance, in our dilution row (see supplemented material). Nielsen et al. 2018 showed this in their study for Bacteroides and Bifidobacterium for glyphosate formulation. They found MICs for these species between 5 mg/ml and 40 mg/ml. This is again much higher than in literature (1,5 mg/ml for glyphosate formulation Roundup Ultra max (61)) but still lower than MICs found in the same study for pathogenic bacteria. Compared to other species, not part of the Enterobacteriaceae, the MIC values are lower. In the publication of Clair et al. 2012 Lactobacilla species show MIC values between 100 ppm for *Geotrichum candidum* and 1000 ppm for *Lactobacillus delbrueckii* also for a glyphosate containing formulation (65). MIC values are hard to compare, due to different evaluation, different glyphosate containing formulations and different media. Species seem to have the tendency to show higher MIC values in rich media compared with literature. This corresponds with the findings of Hao et al. 2012, that the effect of a defective EPSPS by mutation in *aroA* gene in *Salmonella enterica* can be suspended by the use of rich media (117). Nonetheless, the data of our screening demonstrate continuous higher MIC values for *Salmonella enterica* than for other members of the Enterobacteria family, especially commensals, for glyphosate and also for glyphosate formulation Roundup LB plus. Bote et al. 2019 can also show this result for pathogenic *E. coli* compared to commensals *E.coli* in the same experimental design for glyphosate (135). Likewise, Shehata et al. 2013 showed higher MICs for pathogenic bacteria than for commensal ones (61).

The results in screening of MIC suggest that an increase of glyphosate tolerance in the last decades has taken place. In the work of Bote et al. 2019, the comparison of *E. coli* collected before 1983 and today show also the tendency for an increase of MIC even though these results are not statistically significant. Comparable studies of this range of time are rare. In a study on *Salmonella enterica* and comparison over time, the development of antimicrobial resistances could be linked to the introduction of different antibiotics and to the increased usage (139). So effects like this are verifiable, also for other agents. Nevertheless, it should be noted, that the authors of the previously named study used only phenotypical characteristics for their conclusion. Our sample set and time line was selected to see and compare differences before and after introduction of GM glyphosate resistant crops and the increase of glyphosate usage. However, many other reasons for the increase of MIC have to be considered. In such a way comparable studies show that stressors like heavy metals can lead to a decrease in susceptibility against other agents and to antibiotics (98). Especially in *S. enterica* it has been shown, that stress can lead to higher tolerance against many other environmental factors (120). This takes place outside of cross- or co-resistance or genetically

fixed mutations. A hypothetical reason can be a higher intrinsic stress resistance, but this has to be further investigated. It cannot be excluded that in addition rising pollution of the environment lead to selection of more stress resistant isolates, but not solely due to the increased usage of glyphosate.

It should be recommended that clear evidence of the impact of glyphosate must be demonstrated, for example by genetic comparison based on WGS, to prove this relation. Comparisons are also limited by the use of rich media and of different media and experimental designs, although in this work we based the design of the experiment on the guidelines of The European Committee on Antimicrobial Susceptibility Testing (EUCAST). The rich media was selected due to the fact that this is given for the most MIC testings of antibiotics and it is more closely related to the natural surroundings. We also did no pH-adjustment, as glyphosate shows a low pH. This could also lead to differences in MIC compared to other studies.

5.2 Fermenter

To prove the assumption that pathogenic bacteria have a higher chance to survive in a glyphosate-contaminated host associated environment, we inoculated *Salmonella enterica* ser. Typhimurium in artificial rumen model named Rusitec (Rumen simulation technique). In this *in vitro* experiment, *S. enterica* could not survive better in rumen fluid without RU than with RU contamination. Although *S. enterica* showed a little longer persistence in fermenter vessels compared to *E.coli*, there was no measurable or statistically significant difference in numbers or recovering day between vessels with or without RU. None of the re-isolated *S. enterica* colonies showed changes in MIC against glyphosate or susceptibility against antibiotics. This was also found for *E.coli*. In a comparable experiment, where the working group investigated the effect of specific bacteriophages in an *in vitro* rumen simulation technique, decrease of numbers of *E.coli* were comparable in the control group to the results of our experiments either in control vessels without RU or in RU vessels. Numbers of cfu decreased in comparable time. This underlines the validity of our experiment. Another publication from 1966 showed a continuous decrease of *S. enterica* in rumen of cattle and a disappearance after 40 h. This was influenced by constant feeding (140). As in our Rusitec experiment, also, constant feeding was simulated; *S. enterica* seems to have no chance to survive for a longer period, even under the influence of high concentrations of RU. Of course, even still higher concentrations of RU than were used in this experiment could show other results, but are not realistic to be found in animal feed, as we calculated a worst-case scenario

of 100 mg/d for a dairy cow after Schnabel et al. 2017 (50). To sum up, RU seems to have no effect on the survival of *S. enterica* in Rusitec. The decrease of numbers of *Salmonella enterica* is comparable to the decrease in other experiments, which show a correlation between time of decrease of numbers of pathogens and feeding (140). This suggests that the impact of glyphosate and formulation in the investigated concentration on the survival of pathogens in rumen is low. In addition, it should be considered, that *Salmonella enterica* normally passes the rumen to establish and infect the intestine. For *E. coli* the results are comparable. The decrease of the number of cells is continuously and no significant difference between challenged and unchallenged vessels could be shown. The results for *E.coli* are comparable to results of other experiments without any challenge (141). The time of residence in rumen tends to be short and it is no natural habitat. In addition, MIC of isolates applied in the experiment did not show a transition. Other effects on microbiota of the rumen were not be part of this experiment but were shown to be undetectable by Riede et al. 2016 (64).

5.4 Animal experiment

Studies on the effect of intestinal dysbiosis in humans came to the conclusion that imbalanced microbiota in intestine are a precursor to chronic inflammation and are linked to establishment of pathogens (142). So the relation between shedding of pathogens and stability of intestinal microbiota is widely known. The importance of stability and composition of gut microbiota for farm animals, especially for chickens and pigs is in the interest of agricultural industry as well as for food production and hygiene. For chicken, the gut microbiota plays an important role for health, including protection from pathogens (143, 144). For pigs it is also known, that gut microbiota dysbiosis promotes enteric infections and is highly relevant to health in general (145, 146). This influences successful meat production. In the context of massive pollution of glyphosate in the environment and therefore high amount of residues in animal feed, the question which role glyphosate can play in the complexity in gut microbiota must be raised. *In vivo* experiments were necessary, to prove the hypothesis, that the higher MIC of pathogenic bacteria, especially of Enterobacteria, result in an increased shedding of pathogenic bacteria into the environment under the influence of glyphosate and GBH. To investigate this question, three different groups of weaning piglets were infected with a low dose of *Salmonella enterica* ser. Typhimurium 104, one control group, one group was fed with glyphosate and one group fed with Roundup LB plus. The amount of glyphosate fed was calculated as a worst-case scenario according to EFSA. After two weeks of glyphosate feeding, only in qualitative comparison of RU and the

control in section two, there were a statistically significant higher number of *S. enterica* positive piglets in the control group in colon at day 13 after infection. All other analysis revealed no statistically significant differences in shedding of *S. enterica*. Whether the feeding of glyphosate as pure substance nor the feeding of RU lead to a shedding of higher numbers of *Salmonella enterica,* whether qualitatively nor quantitatively. Additionally, in 16S microbiota analysis no DNA fragment of *Salmonella enterica* has been found in feces, in all three groups at the end of the experiment (Data not shown). This leads to the conclusion, that glyphosate or GBH does not increase the shedding of pathogens, e.g. *Salmonella enterica.*

The infection dosage in our animal experiment was low and there was a comparatively long time between infection and the beginning of glyphosate feeding. At that time, the number of shedded *S. enterica* already slipped under the detection limit of our method in many animals. In comparison to other *S. enterica* infection experiments, the number of shedded *S. enterica* was low. However, in our animal experiment, the infectious dose was also lower. Scherer et al. 2007 used an infectious dose of 4.4 x 10^9 cfu (147). They used the same infection strain like in our experiment and observed a continuous shedding of *Salmonella* Typhimurium in high numbers during the first 14 days. In our experiment, the shedding of *S. enterica* decreased from the first day to only sporadically shedding until day seven. Nevertheless, as mentioned before, the infectious dose was one log level lower. In addition, they found a correlation between fecal shedding of *S. enterica* and bacteremia associated shedding. In our experiment, the piglets show increased body temperature only in the first two days. Szabo et al 2009 also used a higher infectious dose of 3 x 10^9 cfu and the same infection strain of *Salmonella enterica* ser. Typhimurium 104 (132). The piglets were infected 28 days after littering, two weeks earlier than in our experiment. The piglets did not show any symptoms of infection, comparable to our experiment. This can also be found in natural infections with *S. enterica* in pigs, as they mostly show no clinical symptoms (148).

In natural environments, interactions between species and individuals are far more complex and predictions are difficult to make. At least these results do not show if there is no effect of glyphosate on microbiological community in the intestine. Nevertheless, some investigations already made, support the assumption, that the impact of glyphosate on the microbiota is low, at least in short time experiments. Nielsen et al. suggest that the environment in the intestine, with its neutral pH and high nutrient content balance the influence of glyphosate on the microbiota. They fed two different concentrations of glyphosate and the glyphosate formulation Glyfonova to four-week-old rats and analyzed gut microbiota via sequencing of hypervariable V3 region of 16S rRNA. After two weeks of feeding, they found no statistically

significant differences in the composition of microbiota in feces, ileum, colon and caecum (137).

The shikimate pathway is the target structure for glyphosate. The shikimate pathway exists not only in plants but also in bacteria and some fungi. However, in contrast to bacteria, plants depend on the self-production of aromatic amino acids whereas bacteria are able to take up aromatic amino acids from their habitat. As the shikimate pathway exists in bacteria one can conclude that it is necessary for bacteria alimentation, at least in environments of malnutrition as in the soil (149, 150). This is also possible in host-related environments like in the urinary tract (151). For bacteria, it is very useful to have the possibility to switch between uptake of amino acids from the environment and self-production, because many of them, also Enterobacteriaceae, have changes in their surroundings within life cycle and are not reduced to nutrient-rich environments. However, within these nutrient-rich environments, where aromatic amino acids are present, the impact of glyphosate seems to be little. Zucko et al. 2010 show, that host-associated bacteria have greater loss of genes linked to shikimate pathway than non-host related bacteria (152). Thirty percent of host associated bacteria, pathogenic as well as commensal ones, lost part of their shikimate pathway. In an experiment of Hao et al. 2012 a defect *aroA* gene lead to difficulties for *Salmonella enterica* to enter plant root cells. Adding aromatic amino acids neutralized that effect (117). This can predict that the effect of inhibition of EPSPS of bacteria by glyphosate is neutralized in nutrient rich environments, which is the case for the intestine.

Contrary findings are presented also for mice and rats. Aitbali et al. 2018 found that long-term exposure to glyphosate formulation decreased important microbe genera like Corynebacterium, Firmicutes, Bacteroides and Lactobacillus. Especially the ratio of Firmicutes and Bacterioides species seem to have an impact on animal physiology(153). In sum, glyphosate had an impact on phylogenetic diversity (83). The authors used a very high amount of glyphosate of 250 or 500 mg/kg/d in a formulation. These amounts of glyphosate had a different basis for calculation so that they do not correspond to realistic residues in animal feed which were used as basis for calculation in our experiment (2,85 mg/kg/d) (see table 1 one for summary). Differences in the amount of applied glyphosate can be a reason for differences in results. Also, the long-term effect of glyphosate was not considered in our experiment.

Another study with rats described an effect of glyphosate and formulation in a decrease of certain Lactobacillaceae and other commensal genera. In this study, also a high glyphosate resistant strain of *E. coli* was found at the end of the study (84). Unlike the study of Aitbali et al 2018, Lozano et al. 2018 used besides high amounts of glyphosate also moderate quantities. In our animal trial, we could not find any changes of MIC in the end of the

experiment. However, a comparison to our study is difficult, as the sampling in this experiment began after 673 days of supplementation. A long-term effect of the herbicide, also on the shedding of *S. enterica*, for example, in older fattening pigs cannot be excluded by our results. To see whether sub-lethal doses of glyphosate and GBH have an effect on MIC in *S. enterica*, we performed an in vitro evolution experiment (see next chapter).

The effect of glyphosate on the microbiota of honeybees was investigated in 2018 by Motta et al.. In young working bees, glyphosate lead to a positive selection of the pathogenic bacterial species *Serratia marcesces* (82). The bees were exposed to glyphosate and to the opportunistic bacterial species. The mortality rate for young working bees increased. The young bees were in a life stage where the gut microbiota was establishing. This is comparable to our weaning piglets as they are also in a phase of establishing their microbiota. Still our results point into another direction, as the shedding of *S. enterica* was not higher than in the control group. Motta et al. could also show that there was no direct effect of glyphosate on the bees, only in a group challenged with the pathogenic bacteria. A decrease of the genera of Lactobacillus and Bifidobacteria was also found. Interestingly, these effects were only visible in the group fed with moderate amounts of glyphosate and not in the groups fed with high amounts. As explanation the group mentioned, that bees treated with high amounts of glyphosate might not have returned back to the hive and therefore missed for investigation.

In the study of Krause et al. 2020, intestinal contents originating from slaughtered piglet was transfered into bioreactors, as model of pig colonic microbiota intestine. The microbial community in these bioreactors was then challenged with RU in a concentration of a latent dietary burden of 2.85 mg/kg body weight. After equilibration time, the microbial communities were challenged for 3 days and samples were taken and analyzed with 16 S rRNA gene profiling and metaproteomics. The study did not show any differences in the composition of community taxonomy or the enzymatic repertoire (154).

In our experiment, we wanted to see the effect of glyphosate feeding on the number of *S. enterica* shedded, so the beginning of feeding glyphosate and RU after a time of "colonization" of the piglets with *S. enterica* could have increased the visibility of that effect. Also the infectious dose was chosen to be lower than in comparable experiments, because the burden of infection for the piglets should be as low as possible. As we used a worst-case concentration of RU, even a slight effect of GBH should have been visible. Our results cannot exclude an effect after a higher infectious dose, earlier feeding of GBH or feeding a higher amount of GBH. Still, the experimental design is close to realistic conditions and the conclusion of a very low effect of GBH is reasonable. Under usual husbandry conditions one would expect that already the first animal feed is contaminated with the herbicide and the animals start to pick up glyphosate from the very first day they eat solid food. This was also

not addressed with our experimental design. The uptake of glyphosate with mother's milk can be excluded, as this has been shown to be under detection limit at least for cows (155) and breast milk (156).

As an outlook, the impact of glyphosate on microbial communities should be investigated in nutrient-poor environments, also for its impact on bacteria. For example in the urinary tract glyphosate can be found (55) and pathogens which can survive in less nutrient-rich environments have advantages in these habitats (151). Also in combination with the hypothesis, that limitation of amino acids can lead to transient tolerance against antibiotics (157) so that one can predict, that in amino acid limited environment the impact of glyphosate may be much more visible. In complex non-host related environments, relationships are complex and the investigation of glyphosate impact is much more complicated and prognoses are difficult to make.

The collected samples of caecum-content were currently investigated via 16S microbiom analysis in the Helmholtz-Centre for Environmental Research in Leipzig.

5.3 Evolution experiment

Experimental evolution in combination with molecular methods like whole genome sequencing has become an important tool to investigate development of resistances, especially against antibiotics (158), biocides (159) and antimicrobial peptides (160). Here, we used the tool of *evolve and re-sequence* to investigate the adaptation of *Salmonella enterica* to increasing amounts of GBH. In laboratory experiments, systematic increase of glyphosate and/or formulation concentration could not show a fast and easy adaptation or increase of minimum inhibitory concentration. The dynamics of evolution experiment was slow and extinctions appeared early at most of the strains. Even as glyphosate has a clear target enzyme in plants, the effects of the herbicide and maybe of other ingredients in formulation seem not to focus on one metabolic target but on several different ones. That is underlined by *whole genome sequencing*, which reveals mutations in a few strains, being related to the glyphosate target gene *aroA* but also some different genes which were able to increase minimum inhibitory concentration (MIC) to glyphosate formulation.

In this study, we investigated the effects of slowly increasing amounts of Roundup LB plus on 10 different strains of *Salmonella enterica* from animal origin. We started with the investigation of the MIC in pH adjusted MH broth. The MICs of the ancestral isolates before the experiment were comparable to others found in the literature (135, 137). An increase of

one dilution step could be explained due to the pH-adjustment, which has been done within this study but not in the other studies. All other MICs described in the literature are hardly comparable due to a different experimental design and especially the use of different formulation of glyphosate and media (see chapter 5.1).

As the MIC values in the beginning of the evolution experiment were already high, it is not surprising that an increase was only achievable within one to two dilution steps. This is still a measurable increase, as seen in the dynamics of the evolution experiment, due to the fact that extinctions in the most strains happened relatively early.

Three of 10 isolates involved in the evolution experiment showed a higher MIC against RU after the experiment and therefore were whole genome sequenced. In all *S. enterica* strains, we found mutations that refer to glyphosate, the active ingredient in RU. For *E. coli* it has been shown that usually, first-step mutations are limited to a smaller variability (161, 162) The variability increases during ongoing evolution (163). This is also visible in our experiment, as all adapted isolates show mutations in or related to *aroA*. One of our isolates had a missense mutation within *aroA* gene directly; two had mutations upstream the *aroA* gene region, which can have indirect effects on the transcription of *aroA*. In other studies, glyphosate resistance was created by a double mutation also in the *aroA* gene, in *E. coli* in T97I/P101S, which made the EPSPS highly resistant to glyphosate but also still keeps the affinity for PEP (164). Very early it was shown by Comai et al. 1983, that mutations in *aroA* can change EPSPS into an glyphosate insensitive variant (165). Not much later, Stalker et al could show a single substitution of aromatic amino acid Prolin to Serin in a mutagenized *Salmonella enterica* strain and a comparison of *wild type* strain and mutagenized strain, lead to glyphosate resistance. Resistance was investigated via purification of EPSPS of the *Salmonella enterica* mutant and comparing the inhibition (166). The results of these experiments are not easy to compare, as the methods used are very different. Especially the investigation of resistance was done differently to our definition of increased MIC in combination with genetically background. In addition, the authors used glyphosate as a pure substance, whereas a more stringent reaction in the isolates could be expected.

In the literature, not many mutations were identified as most authors were looking only for variants in *aroA* to transfer in plants to therefore increase resistance in crops. One working group identified a single non-target gene in *E. coli*, the gene *yhhS*, which is integrated in drug efflux transport (40). Another group found in a glyphosate polluted soil sample a strain of *Enterobacter* sp. with an increase of glyphosate resistance (42). They could identify 42 genes, which were expressed differently and could be brought into a context with glyphosate. These genes were *argF, sdhA, ivbL, rrfA-H*, which were down regulated, the transcripts of *speA, osmY, pflB, ahpC, fusA, deoA, uxaC, rpoD* genes were upregulated. Other mutations

in context of *aroA* were related to stress response, like truncation of *rpoS,* encoding polymerase sigma factor and *rcsB*, responsible for transcription of the regulator of two-component systems. This shows the different variants in adaptation to stress due to GBH. In the way of non-target adaptation and mutation, the development of resistance against RU seem to be more comparable to resistance against biocides. As biocides, like herbicide formulation, are mostly a mixture of several ingredients, like a GBH, it influences more than the targeted gene. That makes the development of resistance more complicated and single step mutations less probable (167, 168).

Compared to the induction of resistance against antibiotics the dynamics of the induction of resistance against RU are completely different. In antibiotics, in many cases resistance is achievable within a few days, as the stress induced by the antibiotics leads to a massive increase of mutation rate (169). Tincher et al. 2017 found that a glyphosate containing herbicide Roundup concentrate plus even decreased the mutation rate within two (one *wild type* and one delta *mutS* strain) *E. coli* strains (170). This is in contrast, as it was shown that stress leads to increased evolvability in gram negative bacteria (171) and that mutation rate increases (169). Our results are not inconsistent with the findings that RU is not elevating the mutation rate. More comparable is the development of resistance against biocides in the literature. There seem to be more than one target gene when bacteria were exposed to low concentrations of antimicrobials, for example biocides (109, 172). This may be transferable to complex herbicide formulations. Karatzas et al. 2007 used a comparable experimental design for 7 days and were also not able to identify decrease of sensitivity against biocides used, except for triclosan (173). This is comparable to our results, as we also needed much more than seven days to get stable decreased sensitivity. In contrast to Karatzas we could also not find decrease of phenotypical sensitivity for antibiotics, which again lead to the findings of Tincher et al (2017), where glyphosate did not increase mutation rate. This suggests that, although glyphosate has one target structure, the impact and therefore the development of resistance against RU is taking place not only in that direction.

Regarding the fact that we have focused only on the potential changes within strains with increased resistance against RU we have to say that we don’t know anything about potential changes within the genome of the isolates which showed no changes in the sensitivity against glyphosate and we cannot compare the mutations in general. As the mutations, which were found are related to *aroA* in combination with increased phenotypic tolerance to glyphosate it is possible to talk about glyphosate resistance in this case as defined by EUCAST (88). Although clinical cutoffs in this context are not useful, the term resistance is valid.

Cross-resistance is defined as one mechanism, which provides resistance to more than one compound. This has to be distinguished from co-resistance, which means the linkage

between two different resistance genes (174). Cross-resistance can for example occur through an upregulated efflux pump. In our study, we investigated MICs for antibiotics important in human medicine. These antibiotics belong to ten different common antibiotic classes. In contrast to findings in Kurenbach et al. 2015 (95), in this experiment the challenge of *Salmonella enterica* isolates with glyphosate containing herbicide Roundup LB plus had no impact on the investigated antibiotic resistance profile. In the work of Kurenbach et al. strains of *E. coli* and *S. enterica* were challenged with sub inhibitory concentrations of a GBH that lead to increase and decrease of susceptibility against different antibiotics, e.g. ampicillin, ciprofloxacin, chloramphenicol, kanamycin and tetracyline (95). Although in the present study testing was for different antibiotics, comparisons are still possible, because the antibiotics belong to the same classes. The phenomenon of cross-resistance is also known for biocides (175) and heavy metals (176). Biocides have, in contrast to antibiotics and also in contrast to glyphosate, commonly more than a single target structure and show a more general effect on microorganisms (167, 177). However, in some points our experiments of glyphosate formulation show effects more comparable to biocides. For biocides, it has been shown that exposure can lead to induced expression of efflux pumps. This increases tolerance to biocide and changed susceptibility to antibiotics. As in the experiment only a single exposure to biocide has taken place, this did not lead to antibiotic resistances. But it was clearly shown, that *arcB* and *tolC* inactivated strains of *Salmonella enterica* were more susceptible to biocide (178). But still, comparable cross-resistances, like in biocides (90, 107), antibiotics (169) and heavy metals (98), in our experiment they could not be demonstrated.

Concerning the opposite; the development of resistance against biocides in multi drug resistant *Salmonella enterica* could not be found (179). In this work, Humayoun et. al tested 88 multi drug resistant *S. enterica* isolates for their MIC against 17 biocides. A relation of antibiotic resistance and biocides could not be found. So the question of how far these links between resistances against antibiotics and other chemicals go is also questionable. Even if there is an effect of biocides on resistance against antibiotics, results may also lower the transferability of these predictions for glyphosate and GBH in a natural environment. Normally, biocides are used to decrease microbes in specific situations so that concentrations of biocides should normally be very high. In contrast, glyphosate is used on fields in agriculture and has a long way to get into contact of gut microbiota. Therefore, the concentrations are comparably low. This additionally decreases the changes of co- and cross-resistance against antibiotics in the natural habitat of bacteria.

To investigate eventually occurring fitness costs we performed growth experiments in rich media and compared final biomass of the adapted ancestor with mutant isolate. None of the mutants showed a reduced fitness compared to their ancestral strain. One strain had at least

fitness advantage. This is contrary to other findings in which in selection experiments, the adapted strains often show fitness reduction in many environments (180).

Proteomic investigations were made for four strains for comparison of challenged and non-challenged pre-adapted ancestor and for three strains, ancestor and mutant, were compared without RU addition.

Comparison of up- and down-regulated proteins between challenged ancestors and ancestors show a consistent picture. Most up-regulated proteins can be brought into context of stress response, shikimate pathway, iron metabolism and tolerance. Most down-regulated proteins can be brought into context of transcription and translation. This shows an undirected reaction to stress factors, and in case of shikimate pathway and iron metabolism, directed to the presence of glyphosate. In one isolate, in comparison of ancestor and challenged ancestor, there was almost no difference in protein expression.

The comparison of ancestor and RU challenged ancestor revealed that adaptation to glyphosate formulation was not exclusively due to change in EPSPS and related Proteins. Surprisingly, EPSPS was not up regulated, but chorismate mutase and chorismate synthase, both also part of shikimate pathway.

Up-regulated proteins were acriflavine resistance ArcAB, a multidrug efflux pump, which leads to tolerant persisters of non-growing and non-dividing cells. The pump was up-regulated in all four challenged ancestral strains. The efflux pump is known to transport many xenobiotic substances out of the cells and therefore lead to resistance against many antibiotics and also other toxic compounds (Du et al. 2014). This can also include herbicide tolerance (182–184). The finding is consistence with Kurenbach et al 2015, who also found increase of activity of multi drug efflux pumps in *E. coli* and *S. enterica* challenges with sub-lethal concentrations of GBH (95). In this study, the working group used sub-lethal concentrations of GBH Roundup. Different to our study design, they compared efficacy of plating (EOP) on plates only with antibiotics to plates with a combination of antibiotics and herbicides. In contrast to our study, they defined MIC as no growth on an agar plate and did not use a microdilution method (185). In addition, efficacy of plating and calculated killing curves were used as a tool to calculated differences in susceptibility. They found out, that sub-lethal concentrations of herbicides increased and decreased tolerance to different antibiotics. In this method culturing changes from liquid media to solid media. As bacteria may be adapted to laboratory growth condition, this can be an additional stress factor and lead to different results than in our study. Increase of expression of efflux pump was investigated with an enzyme inhibition essay. As the experimental design is greatly different,

the impact of efflux pump in the context of herbicide induced stress and changes in antibiotic susceptibility is visible and comparable.

Many of the up-regulated proteins were proteins associated with tolerance, like TolC, which is a part of the acrAB-TolC three component efflux pump (186). The increase of persistence and tolerance is recently discussed as first steps to resistance against antibiotics (187). This fits with the development of glyphosate resistance, as tolerance in our experiment is also visible. It has been shown, that induction of tolerance can be the first step to resistance (102, 188, 189) but it has only recently been shown that tolerance is a driver for that process (102). Other mutations known for tolerance are within proteins of general stress response, like RpoS (190). Also known to be linked to bacterial persistence and tolerance are proteins of oxidative stress response (191). Here, up-regulated are stress response Proteins OsmY, SufB, SufC and SufS. Therefore, the challenge with RU lead to an increase of tolerance response.

Other up-regulated proteins were RpoE, OmpA, LolA, Lpp, SlyB, all known for bacterial envelope in general, osmolarity response proteins and osmoprotectants YehZ and CydAB as a protein involved in cell respiration, ruvAB, a protein involved in DNA recombination and FtsZ, involved in cell division. One explanation can be the relationship between stress response, iron limitation and amino acid limitation like shown in Banerjee et al (151). As already mentioned glyphosate is a chelator of metal ions and has therefore the potential to create a lack of essential nutrition. Activation of iron limitation and tolerance can be a reaction of naive cells to challenge with RU.

Especially RU does not only influence response in the direct targeted biochemical pathway, as it is suggested by the results of sequencing experiment, but also influences iron metabolism, tolerance, stress response and reproduction. As glyphosate is known to be a chelator for iron and other ions (99) an adaptation in iron metabolism could be expected. So, in most of the strains a difference in regulation of enzymes for iron metabolism could be detected. Nevertheless, solution for the problem of iron availability is solved differently by the strains.

The comparison of unchallenged ancestor and mutant shows an equivocal picture. This is shown best in the fact, that some proteins are up-regulated in one strain and down regulated in one or more other strains. Other proteins responsible for tolerance and stress response, iron metabolism and oxidative stress are up or down regulated in the strains without a recognisable pattern. The exception is the EPSPS, which is up-regulated in all three strains. Down regulated were proteins involved in translation and flagella proteins. This can be a hint for reduced mobility and reproduction.

One explanation could be that adaptation to RU stress lead to many different solutions of the individual isolates. In general, first step mutations promoted by a substance, lead to similar ways of solution (161, 162), on-going evolution than lead to more complex differences (163). Our findings are not a discrepancy to these results, as our evolution experiment took place over several days. The adaptation in protein expression also does not have to be genetically manifested.

The sequencing and comparison of whole genomes show, that the exposure to high amounts of glyphosate has an effect on *aroA* gene. The target gene of glyphosate is affected. However, in proteomics it became clear, that this is not the only effect on bacterial metabolism. It may be the combination of targeting the production of aromatic amino acids, chelating essential metal ions and decrease of mutation rate, which leads to the combination of effects visible in our analysis. The comparison of differences in protein expression of the constitutive ancestor and challenged ancestors with the ancestor and mutant show, that the differences in protein expression after adaptation to high concentrations to GBH are not stringent. Most adaptations seem to be due to a general stress response, which enables microorganisms, especially pathogens from gastrointestinal tract, to respond to the many pressures they are exposed in their habitat. So far, tolerance has been investigated mostly in the presence of antibiotics. It can result from mutations, but in contrast to resistance, it can also derive from environmental stressors, which decrease growth rate (192). Also tolerance and resistance mutations can interact and support each other (193). As Proteomics revealed a high number of tolerance proteins and there was no cross-resistance detectable in MIC for antibiotics, the question arose whether there may be a cross-tolerance to antibiotics in the RU resistant mutants. For this, an experiment was performed as Tolerance Disk (TD) test in comparison of mutant und ancestral strains with several antibiotics. Tolerance is known to be a driver for the development of resistance against antibiotics(194). We could not find any increase of tolerance against the tested antibiotics in the TD test.

To sum up, the impact of glyphosate and the glyphosate based herbicide Round upon *S. enterica* is that over decades the susceptibility against glyphosate decreased, which can be explained by many mechanisms due to the increased pollution of the environment with many stressors and due to the amounts of supplements in animal feed (e.g. metal ions or antibiotics). Under laboratory conditions, an increase of resistance against glyphosate and RU was inducible and sequencing and proteomic data showed the connection to RU use. However, *in vitro* in Rusitec and *in vivo* that increase of minimum inhibitory concentration of glyphosate, as first indication of increased resistance could not be shown. In addition, a higher rate of survival due to a decreased susceptibility against glyphosate or RU was not detectable.

6. Conclusion

We can observe changes in sensitivity against Roundup and glyphosate in *Salmonella enterica* in the last three decades. However, whether glyphosate use is the reason for that has to be further investigated. In an evolutionary experiment, the induction of resistance is not easy to generate, but at least in a few strains, it was successful. This shows that it is, at least under laboratory conditions and with high amounts of GBH, possible to create glyphosate resistant bacterial isolates of *Salmonella enterica*. In an *in vitro* experiment with rumen simulation technique, we could not see an increase of MIC of the infectious strains re-isolated from rumen liquid. This was also not detectable in the animal experiment from pig feces and organs. This may rest upon the high amounts of Roundup needed in a laboratory evolution experiment. These high amounts were not found in animal feed residues and were therefore also not applied in RUSITEC or in the animal experiment. In addition, cross- or co-resistance to antibiotics in the presence of Roundup was not detectable, in contrast to findings of other working groups. Neither was this the case after evolving and re-sequencing of *Salmonella enterica*, nor after re-isolation from *in vitro* or in *in vivo* experiments. As glyphosate is known to reduce mutation rate, maybe even after the development of direct glyphosate resistance, the development of other resistances is unlikely. The question if in a long-term *in vivo* experiment with even higher daily intake of glyphosate resistance against RU *in vivo* can be induced we cannot answer in this study. However, daily intakes of such high amounts of glyphosate represent an unrealistic level, which is unlikely to be found in the environment or food chain. This makes the question whether under unlikely artificial laboratory conditions the glyphosate resistance strains created have a higher possibility of survival in animal intestine or environment or if there is any pressure on a possible selection for these strains – a question not relevant for natural surroundings, at least based on the results of this work in terms of specific serovars in *Salmonella enterica*.

The results of the proteomics displayed the resistance to RU in the mutant *Salmonella enterica* strains and a stress response of ancestral isolates, which goes into direction of tolerance. Whether this can maybe in after long-term exposure lead to co- or cross-resistance with other antimicrobials like antibiotics or biocides, we cannot conclude for our results.

In summary, *Salmonella enterica* isolates collected in the last three decades tend to have a higher tolerance against glyphosate and RU. In experiments, the impact of glyphosate and RU on *Salmonella enterica* can be proven, but a very high amount of herbicide is necessary to induce that effect. In concentrations found in the environment and in the food chain, an impact of glyphosate and RU was not detectable in our experimental setup.

7. Summary

In this study, we extensively investigated the question of the impact of glyphosate and glyphosate formulation on *Salmonella enterica* isolates in context of animal health and environmental hygiene. Within a screening of 225 *S. enterica* isolates from farm animals, a change in susceptibility within the last three decades became visible. Also, different susceptibility against glyphosate was found between different serovars and host animals. In general, the screening revealed higher MIC in isolates that were isolated in the last years. Also, higher MICs were found in pigs compared to poultry and cattle and the serovar Typhimurium had a higher MIC compared to Enteritidis and Infantis. This was the case not only for glyphosate as a pure substance but also for the formulation Roundup LB plus. Further investigations *in vitro* and *in vivo* should show if the generally higher MICs of the *S. enterica* isolates lead to a selection advantage within the microbiota.

In an *in vitro* fermenter experiment, we investigated whether this generally higher glyphosate tolerance in *S. enterica* lead to a better survival within cattle intestine e.g. rumen. Therefore, we performed an experiment with rumen simulation technic and infected fermenter vessels with *S. enterica*. Afterwards we challenged half of the vessels with RU, re-isolated *S. enterica*, counted them and determined the MIC to RU of the re-isolates. It turned out, that RU had no impact on survival time of *S. enterica* in fermenter, that no change of MIC within experiment took place and that no cross-resistance to antibiotics occurred.

In laboratory experiments we finally investigated if it is possible to generate glyphosate resistance in *S. enterica* via a long-term evolution experiment. We challenged the pathogen with sub-lethal concentrations of RU. It emerged that it was not easy to decrease sensitivity in *S. enterica* isolates and many days were needed. In the end, three isolates were selected due to a two to four times higher MIC after the experiment. Of these isolates, the whole genome was sequenced. Comparisons with sequenced ancestral strains revealed mutations in the *aroA* gene and close regions, but also revealed mutations in genes for stress response. In a fitness essay, no fitness costs were measurable. Also, no cross-resistance or cross-tolerance to antibiotics could be detected. In comparison of the proteome of ancestor and mutant not only the increase of EPSPS translation was visible, but also a variety of different proteins were up- and down-regulated, linked with stress response, iron metabolism and reproductions.

In an *in vivo* animal trial, we finally wanted to investigate the impact of glyphosate and GBH on the shedding of *S. enterica*. Therefore, three groups of weaning piglets were fed with RU

or glyphosate or functioned as control group. In qualitative analysis a significantly higher number of *S. enterica* was only found in colon samples of the control group compared to the RU group. All other comparisons, qualitatively and quantitatively, showed no differences between the control group, the group fed with glyphosate or the group fed with RU.

Altogether, our findings show a relatively low impact of glyphosate and glyphosate based herbicides in complex environments and with worst-case but still realistic concentrations on the survival, selection and shedding of *S. enterica*. In laboratory surroundings, it was not easy but possible to generate glyphosate resistant *S. enterica* isolates. These isolates showed mutations in the aroA gene. Furthermore, there was an inconsistent picture of the changes in adaptation to RU which can be brought back to other effects of glyphosate, apart from target enzyme EPSPS and the effects of the undeclared ingredients of glyphosate formulation.

8. Zusammenfassung

Einfluss von Glyphosat und Glyphosat-haltigen Herbiziden auf *Salmonella enterica* von Nutztieren

Ziel dieser Arbeit war die Untersuchung des Einflusses von Glyphosat und Glyphosat haltigen Pestiziden auf den Zoonoseerreger *Salmonella enterica*. In einem initialen Screening wurden hierfür 225 *Salmonella enterica*-Isolate dreier verschiedener Serovarietäten auf ihre minimale Hemmkonzentration hin untersucht. Die untersuchten Isolate wurden zwischen 1981 (und damit vor der Markteinführung von Glyphosat) und 2016 isoliert. Zudem stammten die Isolate von verschiedenen Wirtstierarten. Es zeigte sich, dass die erst jüngst asservierten Isolate eine signifikant höhere MHK aufwiesen, als die in den 1980er und 1990er Jahren asservierten Salmonellen-Isolate. Die Stämme vom Schwein wiesen eine höhere MHK als die vom Huhn und das Serovar *Salmonella enterica* ser. Typhimurium eine höhere MHK als die beiden anderen Serovare Infantis und Enteritidis auf. Dies galt sowohl für die Bestimmung der MHK für Glyphosat als Reinsubstanz als auch als für Roundup LB plus. Weitere Experimente sollten zeigen, ob die generell höheren MHKs der Salmonellen, auch im Vergleich zu anderen Bakterienspezies, insbesondere kommensalen Bakterien, einen Selektionsvorteil *in vivo* und *in vitro* haben. Dazu wurden in einem Fermenterexperiment mehrere Pansenfermenter mit Salmonellen (*S*. Typhimurium DT104) infiziert und einer *worst case* Konzentration von RU ausgesetzt. Anschließend wurden die Fermenter beprobt und die Salmonellen quantifiziert. Hier zeigte sich kein Einfluss der glyphosathaltigen Formulierung auf die Lebensdauer und die Anzahl der pathogenen Salmonellen im künstlichen Pansen.

In einem anschließenden Evolutionsversuch wurden 10 *S. enterica* Isolate mit bereits im Vorfeld hoher MHK für Glyphosat täglich höheren Konzentrationen des Herbizids Roundup LB plus ausgesetzt. Die Versuche zeigten, dass die Induktion einer Glyphosatresistenz langwierig ist, oft ein frühes Aussterben der Isolate-Linien zur Folge hat und nur wenige Isolate am Schluss eine tatsächlich erhöhte MHK aufweisen. Diese Isolate wurden sequenziert und das Genom mit dem Ursprungsisolat verglichen. Mutationen im Bereich der *aroA* zeigten die Resistenz gegen Glyphosat an. In Fitnessvergleichen zeigte sich außerdem bei diesen Isolaten kein Fitnessverlust. Eine veränderte Resistenz gegen Antibiotika, wie in anderen wissenschaftlichen Arbeiten gezeigt, konnten bei diesen Salmonellen-Isolaten jedoch genau so wenig gezeigt werden wie eine erhöhte Toleranz.

In der Proteomanalyse der Ursprungsisolate mit und ohne Exposition zur Glyphosatformulierung zeigte sich ein einheitliches Bild mit erhöhten Expressionen von Proteinen für Toleranzreaktionen. Stressreaktionen und auch des Shikimate Stoffwechselweges. Der Vergleich des Ursprungsisolats mit den mutierten Isolaten zeigte insgesamt jedoch ein wenig einheitliches Bild.

In einem abschließenden *in vivo* Fütterungsexperiment wurden drei Gruppen von abgesetzten Ferkeln mit *Salmonella enterica* ser. Typhimurium DT104 infiziert, um die Auswirkung von Glyphosat und dem glyphosathaltigem Herbizid Roundup LB plus auf die Ausscheidungsrate von *S. enterica* zu untersuchen. Der Vergleich zeigte dabei nur qualitativ im Colon eine signifikant höhere Ausscheidung von *S. enterica*, alle anderen Daten zeigten keinerlei diesbezüglichen Effekt.

Insgesamt zeigen die Experimente einen geringen Einfluss von Glyphosat und glyphosathaltigen Herbiziden auf die Selektion von *Salmonella enterica*-Isolaten, sowohl *in vitro* als auch *in vivo*. Das Erzeugen einer Glyphosatresistenz durch Erhöhung der Glyphosatkonzentration im Medium erwies sich als schwierig, jedoch prinzipiell möglich. Die so erzeugten Mutanten zeigten Mutationen im Bereich der *aroA*. Weiter zeigte sich ein uneinheitliches Bild, das vermutlich auf die weiteren Inhaltsstoffe in der untersuchten Glyphosatformulierung zurück zu führen ist sowie auf weitere Effekte von Glyphosat jenseits der Hemmung des Zielenzyms EPSPS.

9. References

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10. Supplementary material

Table s1: Minimum inhibitory concentration of four isolates from the family of Enterobacteriaceae as a comparison to *Salmonella enterica* and *Escherichia coli*. All MIC were examined after the method in Pöppe et al. 2019.

Species	**Laboratory number**	**MIC Glyphosate mg/ml**	**MIC Roundup mg/ml**
Enterobacter cloacae	LP3.1 KT2 EK	20	40
Enterococcus faecalis	DSM6134	<1,25	10
Enterococcus faecalis	DSM20478	<1,25	20
Enterococcus faecalis	DSM6134	<1,25	5

List of publications

Pöppe J, Bote K, Merle R, Makarova O, Roesler U. Minimum Inhibitory Concentration of Glyphosate and a Glyphosate-Containing Herbicide in *Salmonella enterica* Isolates Originating from Different Time Periods, Hosts, and Serovars. Eur J Microbiol Immunol (Bp). 2019 May 20;9(2):35-41. https://doi.org/10.1556/1886.2019.00005

Bote K, Pöppe J, Merle R, Makarova O, Roesler U. 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. Front Microbiol. 2019 May 3;10:932. doi: 10.3389/fmicb.2019.00932

Bote K, Pöppe J, Riede S, Breves G, Roesler U. Effect of a Glyphosate-Containing Herbicide on *Escherichia coli* and *Salmonella* Ser. Typhimurium in an In Vitro Rumen Simulation System. Eur J Microbiol Immunol (Bp). 2019 Jun 27;9(3):94-99. doi: 10.1556/1886.2019.00010.

Pöppe J, Bote K, Ramesh A, Murugaiyan J, Kuropka B, Kühl M, Johnston P R, Roesler U, Makarova O. Selection for Resistance to a Glyphosate-Containing Herbicide in *Salmonella enterica* Does Not Result in a Sustained Activation of the Tolerance Response or Increased Cross-Tolerance and Cross-Resistance to Clinically Important Antibiotics. Applied and Environmental Microbiology. 2020 10.1128/AEM.01204-20: e01204-20. https://doi.org/10.1128/AEM.01204-20

Fritz-Wallace K, Engelmann B, Krause JL, Schäpe SS, Pöppe J, Herberth G, Rösler U, Jehmlich N, von Bergen M, Rolle-Kampczyk U. Quantification of glyphosate and aminomethylphosphonic acid from microbiome reactor fluids. Rapid Commun Mass Spectrom. 2020 Apr 15;34(7):e8668. doi: 10.1002/rcm.8668.

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Selbstständigkeitserklärung

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

Berlin, 13.07.2021 Judith Pöppe

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