

BOARD INVITED REVIEW

Glyphosate in livestock: feed residues and animal health¹

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Abstract

Glyphosate is a nonselective systemic herbicide used in agriculture since 1974. It inhibits 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, an enzyme in the shikimate pathway present in cells of plants and some microorganisms but not human or other animal cells. Glyphosate-tolerant crops have been commercialized for more than 20 yr using a transgene from a resistant bacterial EPSP synthase that renders the crops insensitive to glyphosate. Much of the forage or grain from these crops are consumed by farm animals. Glyphosate protects crop yields, lowers the cost of feed production, and reduces CO₂ emissions attributable to agriculture by reducing tillage and fuel usage. Despite these benefits and even though global regulatory agencies continue to reaffirm its safety, the public hears conflicting information about glyphosate's safety. The U.S. Environmental Protection Agency determines for every agricultural chemical a maximum daily allowable human exposure (called the reference dose, RfD). The RfD is based on amounts that are 1/100th (for sensitive populations) to 1/1,000th (for children) the no observed adverse effects level (NOAEL) identified through a comprehensive battery of animal toxicology studies. Recent surveys for residues have indicated that amounts of glyphosate in food/feed are at or below established tolerances and actual intakes for humans or livestock are much lower than these conservative exposure limits. While the EPSP synthase of some bacteria is sensitive to glyphosate, *in vivo* or *in vitro* dynamic culture systems with mixed bacteria and media that resembles rumen digesta have not demonstrated an impact on microbial function from adding glyphosate. Moreover, one chemical characteristic of glyphosate cited as a reason for concern is that it is a tridentate chelating ligand for divalent and trivalent metals; however, other more potent chelators are ubiquitous in livestock diets, such as certain amino acids. Regulatory testing identifies potential hazards, but risks of these hazards need to be evaluated in the context of realistic exposures and conditions. Conclusions about safety should be based on empirical results within the limitations of model systems or experimental design. This review summarizes how pesticide residues, particularly glyphosate, in food and feed are quantified, and how their safety is determined by regulatory agencies to establish safe use levels.

Key words: feed safety, glyphosate, pesticide residues, rumen microbes

Introduction

Glyphosate is a nonselective herbicide that inhibits 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, an enzyme in the shikimate pathway found in the chloroplasts of plants (Franz et al., 1997). It was marketed under the Roundup brand beginning in 1974 after human and environmental safety data were reviewed and approved by global regulatory agencies, including for its application to control weeds with crops earmarked for animal feed. Since going off patent in 1991 several companies have performed their own regulatory studies, obtained approvals and commercialized their own formulations under multiple names (GTF, 2019). Biotic and abiotic threats reduce crop yields and the greatest source of loss is due to weeds, which accounts for approximately 34% of potential crop losses (Oerke, 2006). Reducing pre- and postharvest food/feed waste is considered an attainable and critical way to reduce the environmental impact of agriculture while maintaining food security for a growing population (Dou et al., 2016). Glyphosate-based weed control is a critical tool to growers and the impact of yield losses is also important to animal agriculture because feed costs are the greatest expense to animal production systems. For instance, in the United States for 2017, feed costs as a percentage of total reported costs were 46% and 40% for dairy and hog production, respectively (USDA ERS, 2018). Additional characteristics of glyphosate-based weed control include a low acute toxicity to animals, a relatively short half-life in soil and limited movement from soil to groundwater (Rueppel et al., 1977; Giesy et al., 2000). An under-recognized value of glyphosate is that it enables the adoption of no-till farming, which reduces CO₂ emissions from soil (Lal, 2004; Fernandez-Cornejo et al., 2014; Brookes et al., 2017). Considering that crops are a significant source of the greenhouse gasses attributed to animal agriculture life-cycle analyses (Pitesky et al., 2009; Asem-Hiablue et al., 2019), no-till agriculture is a crop management method that can reduce the total CO₂ emissions from crop production earmarked for animal feed (Vicini, 2017).

A significant percentage of crops (>90% of hectares) grown in the United States are glyphosate tolerant and livestock consume the majority of these crops (Van Eenennaam and Young, 2014). Studies show that today's genetically engineered (GE) crops are compositionally equivalent to their conventional comparators (Herman and Price, 2013). Moreover, residues of recombinant DNA or novel proteins are not detectable in meat, milk, or eggs (Flachowsky et al., 2005) as these components are digested normally in the intestinal tract. An expert panel of the U.S. National Academies of Sciences conducted a comprehensive review and concluded "that there was no evidence of a risk to human health from GM crops compared to conventional crops" (NAS, 2016). Nevertheless, some concerns have been expressed by the public regarding the safety of glyphosate because of: 1) increased use of glyphosate following the introduction of GE crops, 2) detection of glyphosate residues in GE crops earmarked for feedstuffs, and 3) allegations pertaining to the safety of glyphosate.

This paper reviews studies related to the effect of glyphosate on animal health and, more specifically, on gut microbes by inhibition of EPSP synthase. Emphasis will be given to ruminants, due to their reliance on rumen microbes for the efficient digestion of fibrous feedstuffs and their conversion of fibrous feedstuffs to nutritious meat or milk.

Mechanism of Action and Development of Glyphosate Tolerant Crops

Glyphosate is a broad-spectrum herbicide because it inhibits plant EPSP synthase, an enzyme in the shikimate pathway

responsible for the de novo synthesis of aromatic amino acids (Phe, Trp, and Tyr). This pathway is critical in plants, not only for the amino acids required for protein synthesis, but also for the synthesis of other abundant plant compounds such as lignin (Tzin and Galili, 2010). Because human and other animal cells do not have this pathway, these amino acids must be obtained from the diet and this enzyme is not a target for these species (Giesy et al., 2000).

EPSP synthase catalyzes the conversion of phosphoenolpyruvic acid (PEP) and 3-phosphoshikimic acid to 5-enolpyruvyl-3-phosphoshikimic acid. Glyphosate blocks this step by competing with PEP for binding to the enzyme's active site. Glyphosate is the only known inhibitor of this reaction. It was first used as a nonselective herbicide in row crops, orchards, aquatic systems, along highways, and to control invasive and noxious weeds. Crop scientists screened for an EPSP synthase that was resistant to glyphosate to enable the use of a nonselective herbicide with crops that would be resistant to the herbicide. This allows the use of this broad-spectrum herbicide to control weeds in these crops. No EPSP synthase was found in plants; however, through microbial screens several enzymes were identified. These enzymes are classified as Class I, which are enzymes sensitive to glyphosate, and Class II, which are enzymes that are not affected. As stated, all known plant EPSP synthase enzymes are Class I. Within the Class II category are enzymes from several microbial species that are insensitive to glyphosate. There are 2 requirements for a Class II EPSP synthase to be a suitable enzyme to transform into plants for commercial application. First, the enzyme should enable the plant to resist the inhibitory effects of glyphosate. Second, PEP binding for plants not sprayed with glyphosate needs to be maintained. A suitable candidate was discovered from the CP4 strain of *Agrobacterium tumefaciens* that was isolated from wastewater at a glyphosate manufacturing facility (Barry et al., 1997). Crops with this transgene have been tested using a rigorous, internationally agreed on system and have been approved for cultivation in many countries and import globally.

The major soil degradation pathway for glyphosate results in the formation of aminomethylphosphonic acid (AMPA) and CO₂ (Rueppel et al., 1977). Von Soosten (2016) detected AMPA in feed but glyphosate is not the only source of AMPA (Nowack, 2003). AMPA does not compete with PEP for enzyme binding (Reddy et al., 2004; Duke et al., 2012). The lack of inhibition of EPSP synthase can also be demonstrated by a second mechanism of action for another glyphosate tolerant crop, which greatly slows down development of resistance to the herbicide. Insertion of the gene for glyphosate oxidoreductase detoxifies glyphosate in the plant by metabolizing it to AMPA (Pline-Srnac, 2017).

Residues, Exposures, and Risk Assessment

Many pesticides, whether those allowed in organic production systems or synthetically produced chemicals, leave residues. In fact, plants naturally produce many pesticidal chemicals (Ames et al., 1990). The presence of a pesticide residue is not indicative of a health concern and EPA relies on a well-defined process to determine safe exposure levels and establish allowable residues in food and feed, which has been reviewed by Reeves et al. (2019). It includes a series of chronic toxicological tests used to establish a no observed adverse effect level (NOAEL). The NOAEL is the highest dose in collective toxicological studies that does not produce any adverse effect in the most sensitive species of test animals. Toxicology studies use numerous endpoints to assess health, including clinical signs, blood analytes, and gross and microscopic pathology of tissues that includes the

gastrointestinal tract (US EPA, 2015). A reference dose (RfD), expressed as daily pesticide exposure per body weight (BW) (mg/kg/d), is the maximum allowable exposure intended to provide a “reasonable certainty of no harm” to humans. EPA derives the RfD by dividing the NOAEL by a factor of 10 to account for animal to human extrapolation and by a second factor of 10 to account for sensitive human populations. An additional factor of up to 10 to account for effects specific to children can be used resulting in the RfD being 1/100th to 1/1,000th of the NOAEL. Europe and other regions use a similar value referred to as the acceptable daily intake (ADI). EPA proposed a chronic RfD for glyphosate of 1.0 mg glyphosate per kg of BW/d (EPA, 2019), which includes two 10-fold safety factors. The sum of the most conservative or greatest possible exposures resulting from all uses cannot exceed the RfD.

Based on empirical data, tolerances (in some countries referred to as maximum residue limits), are then determined for each crop or animal products. Tolerances establish the legal limits for pesticide residues in a food or feed when the pesticide is applied according to the maximum rates on the product label. These data are the result of multiple field trials in which residues are measured and the tolerances are selected from the upper portion of the statistical range. This process ensures that the tolerances will be based on highly conservative assumptions. Tolerances by definition are not safety standards. Instead, they are the highest level of residues allowed for legal use of a pesticide regardless of whether even greater residue amounts might be acceptable from a safety perspective (Winter and Jara, 2015). Therefore, a food/feed that has a detectable residue, whether less than or greater than the established tolerance does not indicate a safety concern. Rather it can be used as an indicator of proper application practices, a means to track potential human dietary exposure, and is used as a standard for commerce.

Recently, The European Food Safety Agency (EFSA) and the U.S. Food and Drug Administration (FDA) published results of surveys of foods and feeds in which glyphosate residues were measured using highly sensitive methods (EFSA, 2018b; FDA, 2018). As an example, the limit of detection (LOD) provided for all compounds in the FDA report ranged from 0.1 to 50 mg/kg and the default limit of quantification (LOQ) was 0.01 mg/kg. EFSA detected glyphosate residues in 3.6% of food samples. Of these foods, glyphosate was detected in commodities that are commonly used in feeds such as soybean (16%), barley (19%), and wheat (13%) and none of these amounts exceeded established tolerances for the European Union. Likewise, FDA-tested animal feeds and nonviable residues of glyphosate were found in 63% of corn samples and 67% of soybean samples.

It is important to understand the analytical methods available to test for glyphosate in foods/feed. The most sensitive and selective method that has been validated for multiple feeds and other matrices is liquid chromatography tandem mass spectrometry (LC-MS/MS) (Jensen et al., 2016). The former Monsanto Company developed a cheaper, antibody-based ELISA method to qualitatively test glyphosate in water—a simple matrix. The kit was sold by the Abraxis Co. (Warminster, PA) and the instructions state that the “kit provides screening results. As with any analytical technique (GC, HPLC, etc.) positive results requiring some action should be confirmed by an alternative method.” Use of the ELISA test in complex matrices, however, has generated some questionable results. An example is a report of glyphosate detected in human milk that was posted on a website (summarized by Bus (2015)). The ELISA assay used to produce these results and

validation information in a milk matrix was not provided. In contrast, glyphosate was not detected in milk from humans or cows for studies using validated LC-MS/MS methods with selectivity for glyphosate (NZ Ministry for Primary Industries, 2012; Ehling and Reddy, 2015; McGuire et al., 2016; Steinborn et al., 2016; von Soosten et al., 2016; EFSA, 2018b; FDA, 2018; Zoller et al., 2018). Another questionable result from use of the ELISA was the alleged detection of glyphosate in deformed piglets (Krüger et al., 2014). Validation for this matrix was not reported in detail and there was no control group consisting of normal piglets tested for the presence of glyphosate. In contrast, metabolism studies with high doses of glyphosate in feed (up to 400 mg/kg) were used to establish the tolerance for glyphosate in meat (muscle) and it was set as 0.05 mg/kg, which was the LOD of the assay, an indication that glyphosate in meat was not detectable (FAO, 2005). These data combined with other properties of glyphosate, such as its ionized state, its low octanol-water partition coefficient and its rapid excretion from the body via the urine and feces suggest that glyphosate should not accumulate in the body (Bus, 2015) or be detectable in meat, milk or eggs (Van Eenennaam and Young, 2017). FDA recently monitored food for residues of glyphosate and, as in previously mentioned studies, glyphosate was not detectable in milk nor was it detected in eggs (FDA, 2018). Zoller et al. (2018) tested meat ($n = 12$) and fish ($n = 1$; salmon with no indication if farm raised) and samples that appear to be only meat ($n = 13$) or fish ($n = 1$) had no quantifiable glyphosate. Three samples had glyphosate residues that were slightly above the LOQ, but these 3 were sausages or meat loaf that do not describe if they had nonmeat ingredients derived from grain.

The theoretical maximum exposure is based on the most conservative assumptions such as a person consuming all possible food items containing the highest level of permissible pesticide residue, being exposed to a product through maximum allowable home uses, and consuming drinking water containing the pesticide in question at the maximum allowable level. The theoretical maximum exposure must be less than or equal to the RfD.

Because glyphosate is not metabolized and is eliminated rapidly from the body, urine values can be used to get a realistic estimate of actual consumption. Using the highest glyphosate concentration from a urine sample collected in the previously mentioned study of lactating women, it was estimated that this person consumed approximately 0.0002 times EPA’s RfD (McGuire et al., 2016). At the time this value was published, the EPA RfD was 1.75 mg/kg. Based on a revised RfD of 1.00 mg/kg (EPA, 2019), this calculated value would change from 0.0002 to 0.0004. Despite the increased use of glyphosate due to the widespread adoption of GE crops, there still exists at least 4 orders of magnitude of a margin of safety, based on empirical data (Solomon, 2016).

A formal risk assessment based on information available about possible routes of exposure is typically not conducted for livestock as it is for humans. Given the at least 100-fold safety factor on allowable human exposures, and the fact that most allowable residues are well below levels of safety concern, the sum of tolerances is usually protective of animal health. EFSA conducted a risk assessment for glyphosate residues in animal feed and calculated that the maximum dietary burdens for cattle and swine are 13.2 and 2.85 mg/kg BW/d, respectively (EFSA, 2018a). Even at the maximum dietary burden based on tolerances, cattle or swine could consume 4 or 18 times as much glyphosate before their intakes would be at the level of the NOAEL, which incorporates a 10-fold uncertainty factor.

As stated previously, the issue of exposure is further complicated in that tolerances of individual feed ingredients are all based on the application regimens that result in the greatest amounts of herbicide residue. This exposure value will therefore over predict the average or typical herbicide residue for the feedstuff. For example, according to EFSA, the major contributor of glyphosate exposure for ruminants is grass (non-GE). Pastures can be treated with glyphosate for weed control at either preplant, pre-emergence, or postemergence for renovation or spot treatment. Depending on application, cattle can graze on treated pastures with no restriction on time (depending on dose) or up to a 7-d restriction. Yet, because glyphosate kills growing grass, most pastures are rarely, if ever, treated. Therefore, the majority of grass fed to cattle would have no residue since it is untreated. This exposure difference between the most extreme pasture herbicide applications and actual applications also exists for Roundup Ready Alfalfa. According to label instructions, Roundup Ready Alfalfa can be sprayed several times in a season, yet often is sprayed only once in the establishment year. The World Health Organization (WHO) has suggested that the mean or median residue level in pasture grass may be more appropriate for estimating long-term dietary intake, although the median residue levels are not appropriate for short-term exposures (GEMS, 1997).

Understanding the possible residues in a crop is of practical significance when designing studies and selecting appropriate doses. An alternative would be to calculate the maximum reasonably balanced diet (OECD, 2009). And still another approach is to use empirical data, such as urine values, for a specific population of animals. Validated assay data on urine values for livestock are not as extensively available as values for human exposure (Niemann et al., 2015). Examples of these measurements of toxicity and resulting calculated glyphosate exposures are listed in Table 1. While there might not be a single best method for calculating intake, it is important that the dose be put into the proper context when interpreting results of a study. The glyphosate concentration sprayed onto a crop, as some have used, is not a correct or accurate estimate of exposure to livestock. When calculating the exposure of ingested glyphosate residues on the ruminant's gut microbes, the intake, metabolism and absorption, and volume/turnover of the rumen or other compartments of the digestive tract all must be taken into consideration.

Glyphosate and Gut Microbiota

Glyphosate tolerant crops have been commercialized since 1996 and are widely adopted in the United States with no apparent effects on animal productivity (Van Eenennaam and Young,

2014). Moreover, experimentally determined NOAELs reflect the lack of adverse finding, including within the full length of the gastrointestinal tract. Notwithstanding these conclusions, some have speculated whether gut microbes could be affected by the inhibition of microbial Class I EPSP synthase from glyphosate residues in the digesta. Microbial fermentation and digestive physiology are complex processes and are intricately interwoven. Therefore, conclusions about animal health, or even gut health, are not just a matter of the presence of Class 1 or Class 2 EPSP synthase. For instance, some or many strains of bacteria may not need the shikimate pathway to synthesize amino acids de novo when amino acids are present in their environment. The impact of glyphosate on microorganisms would be dependent on several factors, such as 1) the concentration of glyphosate within the gastrointestinal tract to allow for competitive inhibition with PEP; 2) the need for, or flux through, the shikimate pathway; and 3) the availability of Trp (usually the least abundant of the aromatic amino acids). NH₃ provides the nitrogen for de novo synthesis of amino acids, although the addition of amino acids has been shown to be beneficial (Russell et al. 1992). In general, a complete mixture of amino acids is needed for the maximum microbial growth response but supplementation with only aromatic amino acids results in improved fermentation (Argyle and Baldwin, 1989). In one study with ¹³C-labeled amino acids in rumen fluid, 90% of the Phe-C in microbial protein was derived from the C-skeleton of soluble amino acids (Atasoglu et al., 2004), suggesting direct use, or uptake of carbon skeletons (Leibholz, 1969). *Bacillus subtilis* which is sensitive to glyphosate when grown in minimal media with glucose and succinate as carbon sources, is not affected when Phe and Tyr are added to the media (Wicke et al., 2019). Walker et al. (2005) suggest that although the chorismate pathway exists, other pathways are available such as reductive carboxylation of phenylacetate (Allison 1969; Sauer et al., 1975). Taken together, these studies show that there are multiple means to amino acid incorporation/synthesis and the importance of the interaction (cross-feeding) among mixed populations of intestinal microorganisms. So, rather than base possible glyphosate toxicity to bacteria on a single mechanism, it is critical that in vivo studies or model systems accurately replicate the conditions of particular sections of the gastrointestinal tract.

Batch culture studies

The simplest study design for testing effects of glyphosate or other compounds on gut microbes is to test individual strains for growth on media using batch culture systems. These in vitro tests are commonly used for quantifying the bactericidal effects of antibiotics against a single strain. The antibiotic is titrated in

Table 1. Measurements of toxicity and range of glyphosate exposures that can be estimated for dairy cows

Measurements of toxicity or exposure	Value	
Acute toxicity (LD 50)	>5,000 mg/kg BW	
Chronic EPA NOAEL (EPA, 2019)	100 mg/kg BW	
Chronic EFSA NOAEL (EFSA, 2018a)	50 mg/kg BW	
Calculated exposures for dairy cow:	Value	Assumptions
Most conservative estimate (100% grass hay with highest tolerance)	20 mg/kg	
Maximum Reasonably Balanced Diet	11 mg/kg	1) 24 kg DMI and 600 kg cow; 2) diet with highest tolerances of a roughage source, a carbohydrate grain and a protein concentrate.
Based on urine data (von Soosten et al., 2016)	0.007 mg/kg	1) 600 kg cows; 2) all AMPA from glyphosate; and 3) uses highest urinary values reported.

concentrations intended to exceed that at which the microbe will grow. Results yield an estimated concentration above which growth is inhibited. The single strain exposed to a compound inside a glass tube is cultured in conditions that are quite unlike *in vivo* conditions. Inside the rumen, metabolism is carried out by a complex interaction of thousands of types of microbes. Yet, these single-strain systems have been used to test the effects of adding glyphosate to media, the same as is used to determine the minimum inhibitory concentration (MIC) value of an antibiotic, but at much higher concentrations than antibiotics. It is important that these *in vitro* tests be done in appropriate media and growth conditions. Due to the conditions of some studies (Krüger et al., 2013; Shehata et al., 2013) they are difficult to extrapolate to actual conditions in the gastrointestinal tract. Many intestinal bacteria only grow anaerobically; yet these *in vitro* tests were conducted aerobically. Claims that certain bacteria were killed therefore cannot be made as the cells simply did not grow adequately due to inappropriate media and growth conditions for the test bacteria (little change was observed between beginning and ending cell numbers). Furthermore, these studies used formulated glyphosate that contained surfactant. Surfactants (soap-like substances) are known for their bactericidal properties when applied in large amounts to unprotected cells in *in vitro* systems because they disrupt membranes, which do not mimic *in vivo* exposures (Levine et al., 2007). Therefore, these *in vitro*, batch culture studies do little to demonstrate that gut microbes are affected by glyphosate via EPSP synthase.

Bacteria in batch culture techniques as described in the MIC studies above were grown for 24 to 48 h. During this time, microorganisms typically go through a lag phase and an exponential growth phase. Then cells enter a stationary phase that is dependent on either exhaustion of nutrient supply, accumulation of inhibitory compounds or space constraints, or a combination of these conditions. Stationary phase is when cells are no longer actively growing. Measuring growth during the stationary phase is difficult to interpret. The batch culture studies cited above used incubation times of 24 to 48 h, but growth curves were not presented to indicate the growth phase of these cultures. Culture conditions are critical as diet changes are known to result in adaptation of not only species of bacteria, but also in adaptive changes within a bacterial species (Saluzzi et al., 2001). For instance, growth rates of batch vs. continuous cultures affect susceptibility to antimicrobial agents (Brown et al., 1990).

Nielsen et al. (2018) measured the MIC value for glyphosate added to anaerobic cultures grown on minimal media. Overall, they found MICs to be “very high” (5 to 80 mg/mL), but more important, they found that supplementation of their minimal media with aromatic amino acids increased the MIC in a dose-dependent manner. When measuring growth of *Escherichia coli* over a 24-h period, they detected a lag phase even with aromatic amino acid supplementation, but maximum growth rates were unaffected. This might suggest that in a couple of hours, *E. coli* adapted to utilizing aromatic amino acids from the substrate rather than depend on *de novo* synthesis.

In vivo studies

In 2015, 29% and 75% of the global total hectares planted to corn and soybean were GE, respectively (Brookes and Barfoot, 2017). In the United States, herbicide tolerant corn accounted for 89% of the corn crop, and 94% of the soybean crop, and presumably most of it was sprayed with glyphosate. GE crops are significant sources of the feedstuffs fed to livestock. Van Eenennaam and

Young (2014) estimated that 85% of soybean and 57% of corn grain are used in global livestock diets annually. Studies have been conducted to examine the feeding of glyphosate-tolerant GE crops with various farm animals. These studies involved dairy cows (Grant et al., 2003; Ipharraguerre et al., 2003; Castillo et al., 2004; Combs and Hartnell, 2008), beef cattle (Erickson et al., 2003), sheep (Hartnell et al., 2005), and broilers (Taylor et al., 2003; Kan and Hartnell, 2004; Taylor et al., 2005; Taylor et al., 2007a, 2007b; McNaughton et al., 2011). None of these studies found that feeding crops sprayed during cultivation with glyphosate had an impact on animal productivity.

Not only are ruminants a significant user of GE crops, but they are models for studying the effects of pesticide residues on gut microbes since end products of microbial fermentation and bacterial protein make up a large portion of their metabolizable nutrients. Likewise, bacterial fermentation in the hindguts of monogastric animals is important for some nutrients, although microbial proteins are not utilized (Walker et al., 2005). In an *in vitro* setting, glyphosate can affect EPSP synthase of some bacteria, and thus has the potential to impact gut microbes, but the critical question is whether the normal use of glyphosate *in vivo* results in changes in digestive function, altered performance or impaired animal health. The rumen ecosystem is complex and highly adaptable (McSweeney and Mackie, 2012); therefore, other studies were conducted to more specifically examine microbial populations using either a more dynamic *in vitro* system or animal models. These systems allow for longer incubation times and use of mixed populations of rumen microbes. In contrast, testing single strains of bacteria in batch culture does not replicate the gut environment or growth rate, which is somewhat determined by gut turnover, or the interactions of the numerous strains and species present in the gut ecosystem. Riede et al. (2016) tested a glyphosate formulation (Plantaclean 360 (Plantan GmbH, Buchholz, Germany) added to a semicontinuous culture system (Czerkawski and Breckenridge, 1977), which uses a mixed population of microbes from rumen fluid and provides for semicontinuous addition of nutrients and removal of waste products to more closely resemble ruminal conditions. Their incubations consisted of a 6-d adaptation period, a 5-d control period (no glyphosate formulation), and a 5-d experimental period (added glyphosate formulation) and no deleterious effects were detected with a low and high dose (Table 2). In a second experiment Riede et al. (2016) specifically looked for effects on *Clostridium botulinum* and the addition of formulated glyphosate had no effect. This experiment was conducted to examine claims that farms in northern Germany were suspected of having a rare form of visceral botulism, which had been hypothesized as being caused by glyphosate application (Krüger et al., 2012; Rodloff and Krüger, 2012). The hypothesis was proven false. Moreover, a team of university veterinarians investigated this claim by screening dairy cattle at 92 “affected” farms, which were herds meeting criteria that suggested chronic health issues based on recent health and productivity records. Fecal samples from cows in these farms and from 47 control farms were sampled and tested for *C. botulinum* neurotoxins using a mouse bioassay. Testing was performed on 1,388 animals. Again, there was no evidence of *C. botulinum* neurotoxins in these cows from the targeted farms (Seyboldt et al., 2015).

In addition to livestock studies, some rodent models have also been used to study impact of glyphosate on gut microbes. Nielsen et al. (2018) also conducted an *in vivo* mouse study using daily oral administration. They calculated that at the amounts administered, the mice received 5 and 50 times the

Table 2. Experimental conditions of studies that tested effects of glyphosate or formulated glyphosate on growth of rumen microbes or ruminal function

Citation	Product tested ¹	Culture system	Source of microbes	Concentrations in culture ² (range of non-zero amounts in mg/mL)	Reported findings ³
Shehata et al. (2013)	Formulated (Roundup Ultra Max)	Batch culture	Single strain bacteria	0.075–5 ⁴	MIC values ranged from 0.15 to 5 mg/mL
Krüger et al. (2013)	Formulated (Roundup Ultra Max)	Batch culture	Single strain bacteria	0.1–10	Results are artifact of confounding incubation times and glyphosate amounts.
Krüger et al. (2013)	N-(Phosphono-methyl) glycine	Batch culture	Single strain bacteria	0.1–10	Glyphosate or glyphosate formulations more toxic at large doses and extended incubations for <i>E. faecalis</i> and <i>C. botulinum</i>
Ackermann et al. (2015)	Glyphosate	Batch culture with rumen fluid	Rumen fluid	0.001–0.1	Differences in ciliate protozoa are presented but they do not appear meaningful. No differences were observed for any ciliates with high vs. low grain diets.
Hüther et al. (2005)	Formulated (Roundup Ultra)	Fistulated sheep rumen	Rumen fluid	0.77 g/d a.i. Avg conc. = 0.07 mg/mL	None
Riede et al. (2016)	Formulated (Plantaclean 360)	Semicontinuous culture	Rumen fluid	0.42–2.92 mg/d added. 0.0006 - 0.004 and 0.0003 - 0.003 mg/mL, initial and average.	No adverse findings.
Nielsen et al. (2018)	Formulated (Glyphonova® 450 PLUS)	Batch culture	Batch culture	5 - 80	All bacteria had high MIC values.
Nielsen et al. (2018)	Glyphosate, glyphosate salt, Glyphonova®, Roundup®	Batch culture	Batch culture	0.04–0.16 of the active compound	Dose-dependent alleviation of the inhibitory effect of formulated glyphosate with aromatic amino acids.
Nielsen et al. (2018)	water (CTR), glyphosate 2.5 mg/kg/day (GLY5), glyphosate 25 mg/kg/day (GLY50) or Glyphonova 25 mg/kg/day glyphosate acid equivalent (NOVA) by oral gavage	In vivo		0.006–0.03 (in colon)	Glyphosate or formulated glyphosate administered at up to 50× the European ADI had limited effects on bacterial community composition in Sprague Dawley rats.
Wicke et al. (2019)	Glyphosate	Batch culture	<i>B. subtilis</i> or <i>E. coli</i>	0.1–1	Glyphosate effects were reduced by amino acids in media and microbes adapt to grow in presence of glyphosate.

¹Roundup Ultra Max (Monsanto, St. Louis, MO); Plantaclean 360 (Plantan GmbH, Buchholz, Germany); Glyphonova 450 Plus (FMC Corporation).

²Values presented are targeted amounts added to media, unless otherwise noted, and all values are expressed as concentrations on an acid equivalent (a.e.) basis.

³Paper states that formulated glyphosate was used and presumably the amounts added to tubes are calculated based on glyphosate concentrations.

⁴Conclusions are those of the cited authors and not necessarily those of the authors of this manuscript.

RfD. There were no deleterious effects on the gut microbes. Glyphosate concentrations were measured in the ileum, cecum and colon, and were greatest in the colon. The colonic values were approximately 1, 10, 50, and 50 $\mu\text{g/g}$ for mice given control, glyphosate (5 \times RfD), glyphosate (50 \times RfD) and glyfonova (formulated; 50 \times RfD), respectively. The gut concentrations from mice receiving the relatively high doses were in the $\mu\text{g/g}$ range. These values are considered low compared to MIC values from their in vitro study as well as the in vitro study from Shehata et al. (2013) that were in the mg/mL range (described previously). For reference, antibiotic MIC values are usually in the $\mu\text{g/mL}$ range.

Hüther et al. (2005) added formulated glyphosate directly into the rumen of fistulated sheep and found no effects on endpoints that evaluated rumen function. They hypothesized a priori that supplemental aromatic amino acid would ameliorate any potential effects of glyphosate, but due to the lack of observed changes in rumen function, this study is not relevant for testing that hypothesis. Amino acids are present in large amounts in the rumen from feed proteins as well as in free form (Leibholz, 1969).

Glyphosate in the rumen

Theoretical concentrations of glyphosate in the rumens of cattle can be calculated to put the experimental conditions of the above cited studies into context. von Soosten (2016) measured glyphosate intake from the feed for dairy cattle and found a range of glyphosate intakes from 0.08 to 6.67 mg/d. Assuming an average BW of 680 kg, intakes would have ranged from 0.0001 to 0.01 mg/kg and the no effect level from EFSA is 50 mg/kg (EFSA, 2018a). Estimates for rumen volume and turnover (Stokes et al., 1985) can be used to determine daily liquid flow through the rumen. Based on the amount of glyphosate found in the dairy cow feed above, the concentrations of glyphosate ranged from 0.0000004 to 0.00004 mg/mL. Concentrations of glyphosate or other culture conditions from the studies cited above are provided in Table 2. In the risk assessment done by EFSA (2018a), the calculated maximum dietary burden for glyphosate consumed by dairy cattle using the most conservative assumptions of the highest possible intakes for legal application of glyphosate (predominantly based on glyphosate applied to grass) is 13.17 mg/kg. Using the same assumptions for ruminal kinetics, calculations result in a glyphosate concentration of 0.05 mg/mL. These data suggest that in vitro studies that use glyphosate at concentrations greater than 0.05 $\mu\text{g/mL}$ use concentrations greater than ruminal bacteria would be exposed.

Chelating Properties of Glyphosate

Glyphosate is a zwitterion with 3 acidic protons that make it a tridentate chelating agent of divalent and trivalent metals, forming either 1:1 or 1:2 complexes. Many publications suggest that glyphosate was patented originally as a chelator in a patent issued to the Stauffer Chemical Company in 1964 and critics cite knowledge of this characteristic as an example of corporate malfeasance. However, glyphosate was discovered and patented as a herbicide in 1969 and was never even part of a claim in the Stauffer patent (Swarthout et al., 2018). This chemical property is often overstated as a mechanism whereby glyphosate application to plants limits mineral availability either by limiting uptake from the soil or limiting mineral transport in the phloem. This claim is not corroborated by the commercial viability of herbicide tolerant crops, since the EPSP synthase transgene does not provide any protection from chelation of minerals and yet these transgenic varieties have not shown yield losses when sprayed with glyphosate (Duke et al., 2012).

Likewise, some have claimed that absorption of minerals in the digestive tract is perturbed by chelation from ingested residues of glyphosate. Most dietary minerals are fed at levels that greatly exceed the amounts of glyphosate residue that would be consumed. For ruminants, cobalt is required for microbial synthesis of vitamin B₁₂ and the recommended daily amount in the diet is the least of all the minerals, thus Co would be at the lowest concentrations of the minerals in the rumen. Other ubiquitous anionic chemicals, such as amino acids and phytic acid can form complexes with cationic minerals in the rumen (Durand and Kawashima, 1980) and some amino acids are more prevalent and more potent chelators than glyphosate (Harris et al., 2012). Formation constants measure the strength of complexes between ions and ligands. By comparing these values for complexes of Co²⁺ with glyphosate (Motekaitis and Martell, 1985) and Co²⁺ with amino acids it is apparent that Co²⁺ is more likely to be bound to amino acids than glyphosate. Furthermore, glyphosate is more likely to bind Fe than Co²⁺ because the formation constant for glyphosate and Fe²⁺ is similar to that of Co²⁺, and that with Fe³⁺ is significantly higher.

Conclusion

The mode of action for the herbicidal effect of glyphosate is through EPSP synthase. This enzyme does not exist in the cells of humans and other mammals, which is why aromatic amino acids are considered essential nutrients that must be supplied in the diet. Although some microbes have an EPSP synthase that is susceptible to glyphosate, it does not mean that glyphosate alters their ability to compete or function in the gut. Important factors when designing or interpreting model systems of gut microbes are to consider the impacts of single vs. multiple strains, batch vs. semicontinuous or continuous systems, turnover rates (i.e., growth rate), the concentration of glyphosate in the digesta, aerobic vs. anaerobic, the duration of culture and the relevance of endpoints to function. As with any model system, conclusions should be based on empirical results within the limitations of the model system. Likewise, the ability to form complexes with certain metal ions is a property of glyphosate, but it has not been found to impact animal nutrition due to the concentrations and interplay among competing ligands and ions, and the relative stabilities of alternative chelators to form complexes. The weight of the evidence suggests that glyphosate use in crops fed to poultry and livestock has not affected animal health, rumen/gut microbes or production without affecting the safety of consuming meat, milk, and eggs.

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