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Effects of glyphosate on the intestinal microbiota

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Content

Preface

This report is written as an extended summary of the scientific work conducted during the PestiGut project “Tarmmikrobiota som følsom indikator for biologisk relevante restkoncentrationer af kemiske pesticider i fødevarer eksemplificeret ved glyphosat (Roundup®)”. The report includes an introduction to the area, overview of methods, main results and conclusions. For a more detailed presentation of the work please refer to the accompanying scientific manuscript entitled: “Glyphosate has limited short-term effects on commensal bacterial community composition in the gut environment due to sufficient aromatic amino acid levels” [1].

1. Introduction

1.1 Glyphosate

Glyphosate-based products represent the most widely used herbicide group in the world. The herbicide is used on feed and food crops during cultivation, to desiccate the crop before harvest, and more intensively during the cultivation of the genetically modified glyphosate-resistant crop varieties that are engineered to tolerate glyphosate [2,3]. Globally, glyphosate use has risen almost 15-fold since the genetically engineered glyphosate-tolerant "Roundup Ready" crops were introduced in 1996. The total volume applied by farmers world-wide rose from 51 million kg in 1996 to 747 million kg in 2014 [4]. In this same period, glyphosate sales in Denmark rose from 514.000 kg to 627.000 kg with a peak in 2011 where 1.941.000 kg was sold (Figure 1) [5,6]. Global non-agricultural uses have increased fivefold since the introduction of genetically engineered crops from 16 million kg in 1995 to 79 million kg in 2014 [7]. Total worldwide glyphosate use (agricultural plus non-agricultural) rose more than 12-fold from about 67 million kg in 1995 to 826 million kg in 2014 and over the last decade 6100 million kg of glyphosate have been applied [4].

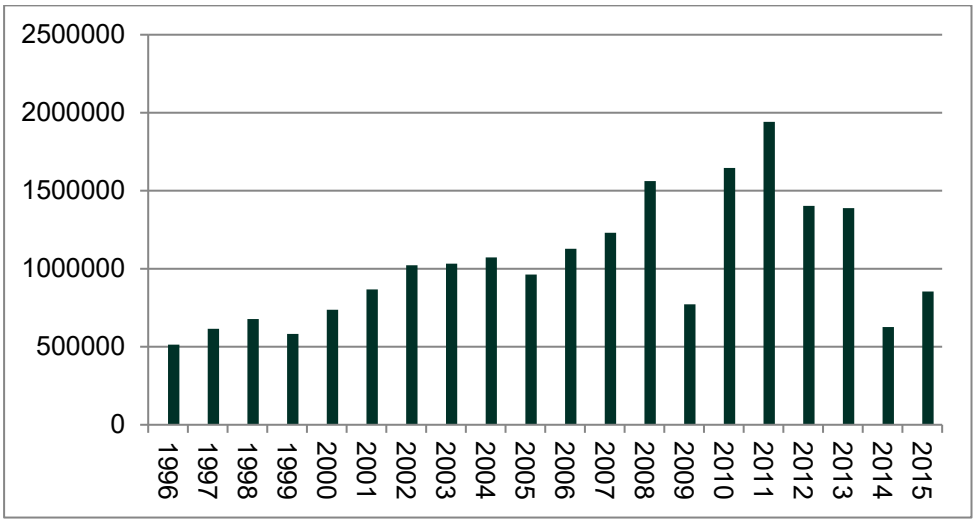

FIGURE 1. Kilo gram glyphosate sold in Denmark from 1996 to 2015 [8].

Glyphosate has been detected in air during spraying, in water, in food, and additionally in the urine of agricultural workers, as well as the general population, indicating both exposure and absorption[9–11]. The acceptable daily intake (ADI) of glyphosate is currently set to 0.5 mg/kg body-weight (bw.) pr. day within EU, based on the maternal and developmental No-observed-adverse-effect level (NOAEL) of 50 mg/kg bw. pr. day from development toxicity studies in rabbits and applying a standard uncertainty factor of 100 [12]. The maximum residue level (MRL) in food commodities varies dependent on product type and is thus defined for each product separately e.g. for barley and oats it is 30 mg/kg.

Glyphosate has for many years been believed to be a relatively safe compound, however during the last decades, an increasing number of studies and data have indicated putatively

toxic effects of glyphosate towards mammals including humans [13,14]. In March, 2015, 17 experts from 11 countries met and decided to classify glyphosate as “probably carcinogenic to humans” based on available data at the International Agency for research on Cancer (IARC; Lyon, France) (Group 2A) [2,9]. From case-control studies of occupational exposures, mostly agricultural, in USA, Canada, and Sweden limited evidence suggested carcinogenicity in humans for non-Hodgkin lymphoma [15–17]. Some evidence additionally suggested that glyphosate may cause cancer in laboratory animals, however this is still debated [18–20].One study reported increases in blood markers of chromosomal damage (micronuclei) in humans after glyphosate formulations were sprayed nearby [9,21]. In Europe, glyphosate as an active ingredient is assessed by the European Food Safety Authority (EFSA) together with member states. However the herbicide formulation and the distribution of it is regulated by the individual member states as for example in Denmark [22]. Following a second mandate from the European Commission to consider the findings from IARC, EFSA concluded that glyphosate is unlikely to pose a carcinogenic hazard to humans and that the evidence does not support classification with regard to its carcinogenic potential according to Regulation (EC) No 1272/2008 [12]. A similar conclusion was recently reached by the European Chemicals agency (ECHA). The main difference between the IARC and EFSA evaluation is that IARC considers both the active compound glyphosate itself and glyphosate-based formulations regardless of their composition, while EFSA considers only the active compound glyphosate. This is an important point because it is likely that the observed genotoxic effects are related to other ingredients or co-formulants [12]. However, the major difference was in the database that was available for the evaluations by IARC and EU.

It is generally accepted that the toxicity of commercially formulated glyphosate herbicides exceeds the toxicity of the active compound glyphosate, which has been demonstrated in several studies both *in vitro* and *in vivo* [23–27]. Although the toxicity of pure glyphosate towards mammals is reported to be very low, the exposure to high doses of formulated products has been shown to cause serious poisonings in human subjects [28–30]. One of the most commonly used herbicide formulations world-wide is Roundup®, which contains an aquatic solution of glyphosate in the form of its isopropylamine salt, together with a number of co-formulants. The composition of co-formulants is often confidential, but in some cases, these have comprised polyethoxylated tallow amine (POEA). There are numerous studies demonstrating that the toxicity of POEA towards mammals clearly exceeds the toxicity of glyphosate [26,31,32]. In June 2016 the EU commission decided to extend the approval of glyphosate for a limited period pending a final decision once the ECHA had concluded its review. The extension was however subject to certain precautions including a ban of POEA containing products for sale in EU [33].

1.1.1 Mode-of-action of glyphosate

The chemical name of glyphosate is N-(phosphonomethyl) glycine, as defined by the International Union of Pure and Applied Chemistry (IUPAC) (Figure 3). In its pure form it is an odorless white powder with a molecular weight of 169.1 g/mol and a solubility of 10.5 g/L at 20 °C (pH 1.90 – 1.98) in water [34]. Glyphosate specifically inhibits the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), which is a central enzymatic step of the shikimate pathway of aromatic acid biosynthesis in plants as well as some bacteria, algae, fungi and parasites.

Glyphosate thus effectively suppresses the synthesis of aromatic amino acids (tyrosine, tryptophan and phenylalanine) (Figure 2) and consequently also reduces downstream secondary metabolite synthesis [35].

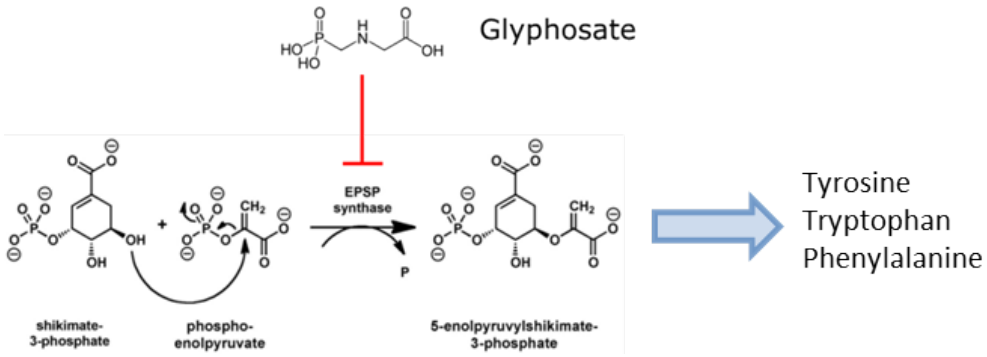

FIGURE 2 Mode-of-action of glyphosate. Glyphosate inhibits the 5-enolpyruvylshikimate-3-phosphate synthase enzyme (EPSPS) and thus suppresses the synthesis of downstream aromatic amino acids tyrosine, tryptophan and phenylalanine).

Glyphosate is stable in air, and practically insoluble in most organic solvents (e.g. acetone, ethanol and benzene) because of its high polarity, but is somewhat soluble in water [36]. In the soil environment glyphosate has a high affinity to soil particles and may be metabolized into plant nutrients by soil microorganisms [37]. It can be metabolized through two pathways; the C-P lyase pathway or the primary aminomethylphosphonic acid (AMPA) pathway [38] (Figure 3). Microorganisms reported to have the capacity to degrade glyphosate include *Pseudomonas* sp., *Arthrobacter* atrocyaneus and *Flavobacterium* sp. [35]. Detection of the primary metabolite AMPA in the blood of humans following oral intoxication further suggests intestinal microbial metabolism [39].

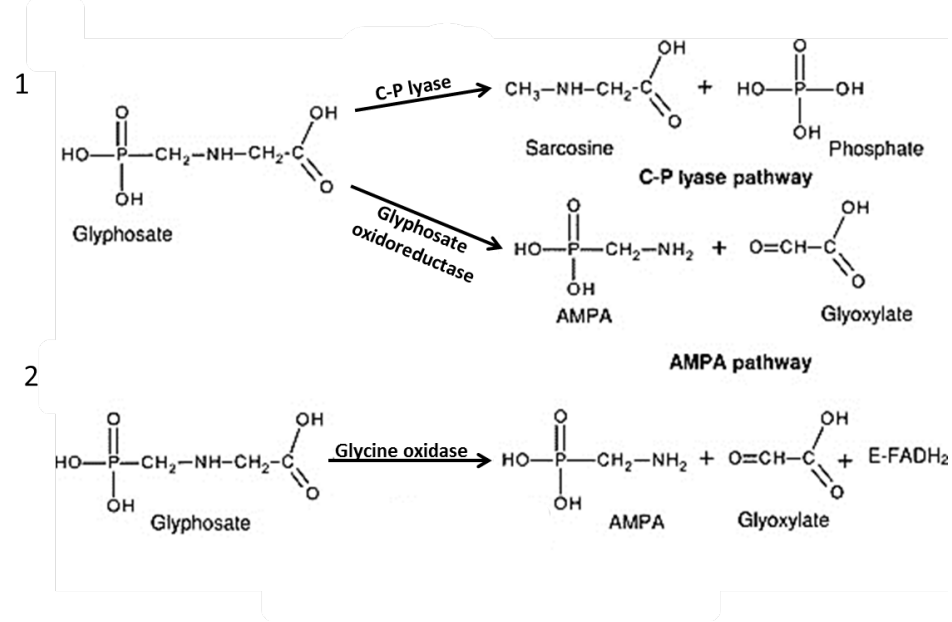

FIGURE 3. Microbial mechanisms of glyphosate degradation with the two principal pathways; 1) the C-P lyase pathway and 2) the AMPA pathway (Figure modified from Pollegioni *et el.* (2011) [38]).

In addition to inhibiting the shikimate pathway glyphosate can form chelates or complexes with micronutrient metal ions in solution [40] and may thus reduce their bioavailability. Both the carboxyl and the phosphonate groups can thus bind to cations such as Ca^{2+}, Mg^{2+}, Mn^{2+} and Fe^{2+} forming poorly soluble and stable complexes [41].

1.2 The intestinal microbiota

The human intestine is colonized by an extremely complex and dense community of microbes, collectively referred to as the gut microbiota. In recent years numerous studies have demonstrated and revealed important links between these commensal microbes and human health [42–44]. The bacterial load (i.e. concentration of bacterial cells) and diversity of the microbiota increases throughout the gut and is most dense and diverse within the colon, where more than 10^{11} bacteria per gram of intestinal content are found [45]. The microbiota contains bacteria which may be classified as commensals, symbionts or pathobionts. Commensals are permanent residents of the microbiota that do not cause damage to the host organism, while symbionts are generally associated with known health promoting functions. Pathobionts are like commensals permanent in the microbiota, but they are opportunistic pathogens, which can potentially induce infection or other pathologies and thus cause damage to the host [42,46]. The microbiota of infants is normally dominated by facultative anaerobes such as *Escherichia coli* and other *Enterobacteriaceae* species. As the infant grows, the oxygen level within the gut is quickly lowered due to the metabolism of the microbiota resulting in successional colonization by strict anaerobes such as *Clostridium*, *Bacteriodes, and Ruminococcus* species[47]. In adults, the microbiota is typically dominated by the phyla *Firmicutes* and *Bacteriodetes* and relatively few *Proteobacteria, Actinobacteria, Fusobacteria* and *Verrucomicrobiota* are present. Some bacteria are considered important for human health, including species belonging to the *Bifidobacterium* and *Lactobacillus* genera. These are also applied as probiotics, defined as live microbial food supplements, which benefit the host through improving the gastrointestinal microbial equilibrium [48,49]. The bifidobacteria belong to the phylum *Actinobacteria* and are Gram-Positive anaerobic bacteria [50]. They protect e.g. against enteropathogenic infection by producing the short-chain fatty acid acetate. Acetate induces anti-inflammatory and/or anti-apoptotic effects on the colonic epithelium, e.g. by preventing the translocation of the enterohaemorrhagic *E. coli* O157:H7 shiga toxin, which causes diarrhoea, haemorrhagic colitis and haemolytic uraemic syndrome [48]. *Lactobacillus* species are facultative anaerobic, Gram-positive bacteria belonging to the phylum *Firmicutes* and some members of the *Lactobacillus* genus have been shown to influence intestinal physiology, regulate the immune system and balance the intestinal ecology of the host [51]. The finely tuned balance within the gut microbiota is very sensitive towards external influences such as diet and oral antibiotic treatment, the latter of which causes dramatic alteration to the community structure [45,52]. Changing the balance can lead to undesirable effects, i.e. shifting the composition of the microbiota by reducing the numbers of symbionts and/or increasing the number of pathobionts, which can influence human health [42]. Disturbance of a healthy microbiota has been associated with a variety of disorders including metabolic as well as inflammatory diseases [53–59].

1.2.1 Gut microbiota and glyphosate

At present relatively little is known about potential effects of glyphosate on the gut microbial community composition and function, despite the fact that it is well established that similar to plants, microorganisms also harbor the Shikimate pathway, which is the target of the pesticide as described in the patent of glyphosate [60,61]. Considering the established ADI of 0.5 mg/kg within EU and the previously published minimal inhibitory concentration of 0.075 mg/mL for e.g. bifidobacteria [62] found in the intestinal environment, it seems probable that some inhibitory effect may be possible if exposure occurs at the ADI threshold concentration. For an average person weighing 70 kg, the established ADI allows ingestion of 35 mg glyphosate per day, which equates 0.22 mg/mL fecal content under the assumption that 80% is excreted in feces and a median of fecal wet mass of 128 g/day is produced [63,64]. The strong chelating ability of glyphosate may also reduce the bioavailability of important cations, which may potentially affect bacterial growth (Duke et al., 2012). Lastly, the various adjuvants used in commercial formulation may further increase potentially toxic effects in this environment [66]. Recently the effect of glyphosate on bacterial growth has been investigated in several *in vitro* studies. In one of these studies, performed on bacteria isolated from poultry, it was reported that bacteria generally regarded as beneficial were more susceptible to the effect of glyphosate than potentially pathogenic bacteria including *Salmonella* Typhmurium, *Clostridium perfringens* and *Clostridium botulinum*, which appeared more resilient [62]. Despite the fact that this study was performed only on bacteria in pure culture, indicates that the ecological fine-tuned balance of the gut bacterial community may be affected by glyphosate. Indeed another study from the same research group suggests that enterococci isolated from cattle are particularly susceptible to glyphosate. The authors speculated that this effect may in part drive the observed increase in *Clostridium botulinum* mediated botulinum disease in German cattle, since a reduction of enterococci may lead to a reduced intrinsic production of bacteriocins, which are known to inhibit growth of specific pathogens [67]. From a scientific standpoint it is clear that more studies, particularly well-controlled *in vivo* studies in laboratory- or production animals, are required in order to confirm these findings. Interestingly, some studies published in scientific journals appear rather speculative concerning the effects of glyphosate on health. This includes a recent review article linking glyphosate to a long list of different life-style diseases including diabetes [68]. Lately, concerns related to potential negative effects of glyphosate in feed for production animals on the gut microbiota have also been raised in Denmark following a report from Aarhus University [69]. However, a clear gap exists in our current knowledge of effects of glyphosate and its formulations on the gut microbiota *in vivo,* and of biologically relevant residue concentrations.

1.2.2 Gut microbiota and other pesticides

A number of studies have investigated effects of other pesticides on the gut microbiota. In one such study, chronic exposure to the insecticide chlorpyrifos, which is an organo-phosphate known to inhibit acetylcholine esterase, was shown to induce microbial dysbiosis in both an *in vitro* model of the human intestine (SHIME) and in a rodent model [70]. The relative abundance of lactobacilli and bifidobacteria were reduced significantly in the rodent model, which may affect intestinal integrity [71]. In addition to this, it has been shown that chlorpyrifos may directly affect the tight-junction protein structures connecting the endothelial cells and thus reduce intestinal

integrity [72]. Another example of a group of pesticides, which has been shown to affect the bacteria in the gut environment, is the organochlorine-based pesticides. In one study a positive association was found between the serum/feces concentration of pesticide and the number of pesticide-degrading *Methanobacteriales* in the gut microbiota in a group of Korean women [73]. The same authors further demonstrated a positive correlation between pesticide concentration and obesity, which they suggested may be related to the gut microbiota [74]. Results from these studies collectively advocate that cumulative effects of different classes of pesticides may potentiate the effect previously described as a cocktail-effect [75]. Apart from the above mentioned specific examples of interactions between pesticides and the gut microbiota, it is becoming evident that the microbiota itself may also potentiate the effect of different pesticides by modifying absorption, distribution, metabolism, and excretion (ADME) characteristics [76] [77].

2. Methods

In the present study we aimed to clarify the effects of glyphosate and its formulations on specific bacteria relevant to the intestinal environment. We used Sprague-Dawley rats as model animals to explore the effects of pure glyphosate and a commercial formulation on the intestinal bacterial community *in vivo*, which to our knowledge has not been studied previously. The overall study design for the project is shown in Figure 4.

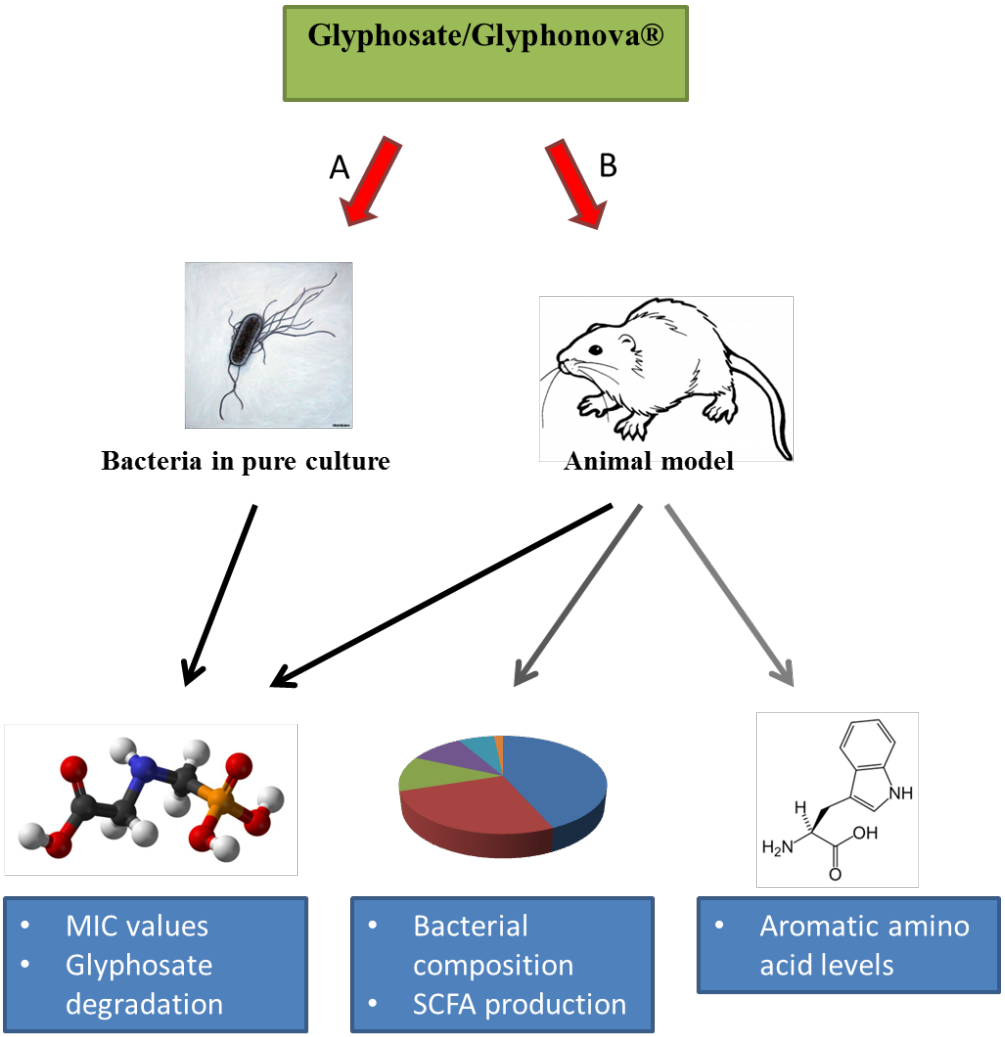

FIGURE 4. Overall design of the study in two parts; A) assessment of bacteria in pure culture related to MIC values and glyphosate degradation and B) rat model to study effects of glyphosates on the bacterial composition, short chain fatty acids and aromatic amino acid levels in the animals.

2.1 Bacteria in pure culture

To study how bacteria in pure culture respond to glyphosate and formulations hereof, we determined the minimal inhibitory concentration (MIC) in different growth media and measured bacterial growth. In total we tested 22 different bacterial strains relevant for the human gut microbiota representing 5 bacterial phyla (Table 1). To determine whether the composition of

growth media influenced MIC, we used two different rich growth media; Brain Heart Infusion broth (BHI) and Reinforced Clostridial Medium (RCM).

TABLE 1. Bacterial strains tested for MIC to glyphosate in BHI and RCM medium respectively.

		Strains	MIC (mg/ml) BHI	MIC (mg/ml) RCM
Gram	+	*Bifidobacterium adolensis* DSM 20083	10	20
	+	*Bifidobacterium bifidum* DSM 20456	10	20
	+	*Bifidobacterium breve* DSM 20091	10	20
	+	*Bifidobacterium longum subsp. infantitis* DSM 20088	10	20
	+	*Bifidobacterium animalis* DSM 10140	10	20
	+	*Bifidobacterium animalis* lactis BL-04	10	20
	+	*Clostridium perfringens* CCUG 1795	10	20
	+	*Clostridium leptum* DSM 753	10	20
	+	*Clostridium nexile* DSM 1787	10	20
	+	*Enterococcus faecalis* ATCC 29212	80	40
	+	*Enterococcus faecalis* DSM 2570	80	40
	+	*Lactobacillus johnsonii* DSM 10533	20	20
	+	*Lactobacillus planetarum* DSM 20174	40	40
	+	*Lactobacillus reuteri* DSM 20016	40	40
	+	*Lactobacillus rhamnosus* ATCC 53103	40	40
	-	*Bacteroides uniformis* DSM 6597	5	10
	-	*Bacteroides vulgatus* DSM 1447	5	20
	-	*Bacteroides ovatus* DSM 1896	10	20
	-	*Bacteroides fragiles* DSM 2151	5	40
	-	*Escherichia coli* ATCC 25922	80	20
	-	*Escherichia coli* DSM 18039	80	20
	-	*Akkermansia muciniphila* DSM 22959	20	20

The MIC is defined as the lowest concentration of an antimicrobial agent that under specified test conditions inhibits the visible growth of the bacterium being examined based on the guidelines of the Clinical and Laboratory Standards Institute and the European Committee on Antimicrobial Susceptibility Testing and according to the method previously described [78]. The method was also used to examine *E. coli* in minimal growth medium supplemented with aromatic amino acids. Bacteria for this study were chosen to represent common members of the human gut and included commensals, symbionts and pathobionts that in previous studies have shown responses to glyphosate. In this assay we used the commercial formulation Glyfonova® because of the low solubility of glyphosate *N*-(phosphonomethyl)glycine (acid).

MIC assays are used to give an estimate of the susceptibility to a specific compound, however because of the standard two-fold dilution steps of the compound tested, some uncertainties of the results are expected. For example if one bacterial strain is just able to grow at 2 mg/ml and another is just not, this will result in a two-fold difference in MIC value (4 mg/mL and 2 mg/mL, respectively). To study effects of glyphosate on growth at higher resolution we therefore also included 24-hour growth experiments and chose *E. coli* as a model organism, due to the prototrophic nature of this bacterium, which is able to synthesize all of its amino acids, nucleic acids and vitamins from inorganic nutrients. The applied *E. coli* strain is thus able to synthesize amino acids *de novo* via the Shikimate pathway, and therefore capable of growth in minimal medium without amino acid supplementation. This provided us with the possibility to study whether the absence or presence of free aromatic amino acids in the medium affected bacterial

growth in the presence of glyphosate in its pure form *N*-(phosphonomethyl)glycine, as well as of different formulations including glyphosate isopropylamine salt, Glyfonova® (450 g/L) and Roundup® (120 g/L).

TABLE 2. The different types of glyphosate formulations used in this study.

Common name	Ingredients	Reference
Glyphosate	Phosphonomethyl)glycine	79
Glyphosate salt	*N*-(Phosphonomethyl)glycine, monoisopropylamine salt solution (40 wt. % in H_2O)	80
Glyfonova®	The product contains 607 g/L glyphosate as monoisopropylamine salt that corresponds to 450 g/L glyphosate acid (37 %)	81
Roundup®	The product contains 120 g/L glyphosate acid (11.3 %)	82

2.2 Animal model

The *in vivo* study involved a total of 80 Sprague-Dawley adult male rats aged 4 weeks at arrival, and purchased from Taconic [83]. The animal study was performed at the National Food Institute DTU, adhered to regulations set out by the Danish Animal Experiments Inspectorate, took place with ethical approval and were overseen by the National Food Institutes in-house Animal Welfare Committee for animal care and use. At arrival animals were caged randomly in pairs and following an acclimatization period of 1 week, the cages were divided evenly into four separate treatment groups, taking animal weight into consideration (Figure 5). The four treatment groups were: 1) control (CON) group receiving only water, 2) 5xADI glyphosate group (GLY5), 3) 50xADI glyphosate group (GLY50), and finally 4) 50xADI Glyfonova® group (NOVA), which received the formulated commercial product.

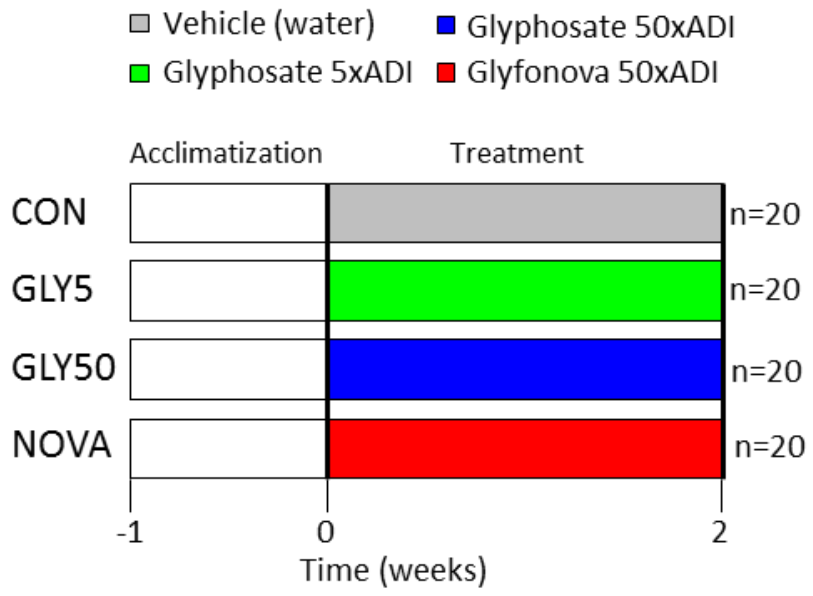

FIGURE 5: Study design.

The rats were dosed daily by oral gavage with 5xADI pure glyphosate, 50xADI pure glyphosate and 50xADI glyphosate formulation (Glyphonova®450 PLUS). The control group was dosed in the same way with water. The dosages were decided based on previous studies showing that 0.075 mg/ml is the lowest MIC value (for bifidobacteria) [62]. From the literature it is reported that Sprague-Dawley rats on average produce 8 g feces per day, which approximately corresponds to 10 ml [63]. The bio-absorption of glyphosate passing through the intestinal tract is approximately 20%, leaving 80% to be excreted with the feces [64]. To obtain a concentration in the colon corresponding to at least the MIC of 0.075 mg/ml we calculated a theoretical minimum dosage of 0.075 mg/ml * 10 ml/day = 0.75 mg/day and with the 20% bio-adsorption, the theoretical minimum is 0.9 mg/day glyphosate in the animals. The ADI for glyphosate is 0.5 mg/kg/day which correspond to 0.1 mg/day if the rat weighs 200g. With the applied strategy we expected to reach a concentration above the MIC value for the 50xADI groups. It is however important to note that below the MIC, we expected that the bacterial growth could still be partly inhibited, and that this could result in changes in the composition of the bacterial community in the gut.

After the treatment period of two weeks we measured the concentration of glyphosate by LC-MS in three intestinal compartments (ileum, cecum and colon) to test and confirm that we reached the theoretical calculated levels of glyphosate. We also used the method to quantify levels of AMPA, the primary metabolite resulting from the degradation of glyphosate, in the three compartments, in order to assess whether the gut microbiota was able to degrade glyphosate. We also tested 7 bacterial strains from our strain collection (*E. coli, E. faecalis, L. reuteri, C. nexile, Bact. uniformis, Bif. adolensis* and *A. mucinophilia*) (Table 1) and two human fecal samples for fermentation of glyphosate to AMPA.

Because of the significant importance of intestinal aromatic amino acids for this study, we additionally applied LC-MS to quantify phenylalanine, tyrosine and tryptophan in ileum, cecum and colon. We suspected that inhibition of the Shikimate acid pathway could also affect downstream metabolite production, and therefore quantification of these was also included in the study (Fig. 6).

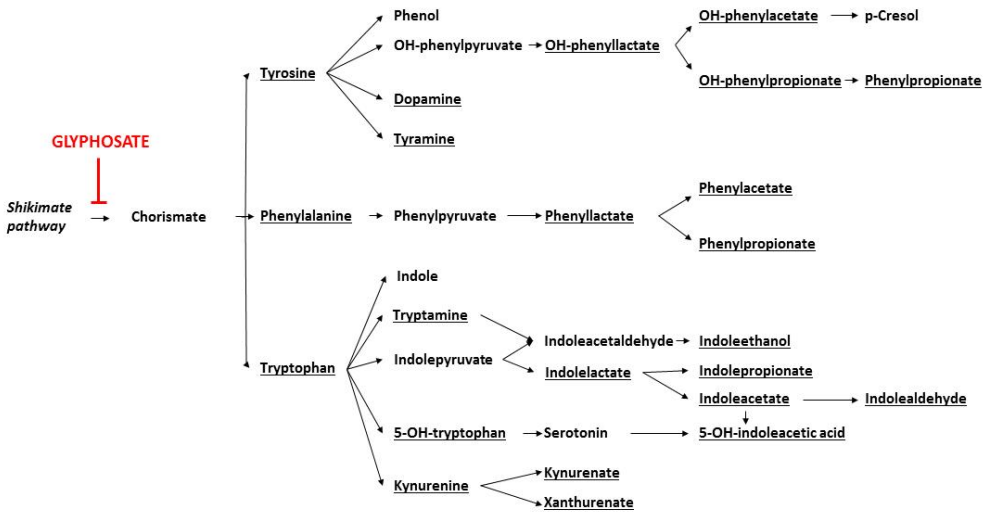

FIGURE 6. Outline of how glyphosate may affect the catabolism of aromatic amino acids in the intestine. Underlined metabolites are those targeted by chemical analysis in the present study.

The gut microbial composition was determined by sequencing the hypervariable V3-region of the 16S ribosomal RNA gene (16S rRNA gene), which is forms part of the 30S small subunit of a prokaryotic ribosome that binds to the Shine-Dalgarno sequence of mRNA [84]. We further measured SCFAs in the cecum compartment, which are important degradation products from bacterial fermentation of dietary fibers. Likewise, pH in fecal samples from the last day of treatment was determined. Blood samples were collected at termination and serum levels of the acute phase protein haptoglobin as well as IL-6 were determined. Tight junction proteins (claudin-1 and ZO-1) of ileum were analyzed by Western blotting and normalized to the housekeeping β-actin protein.

3. Results and discussion

The main result of our study was that at genus level, no major structural changes occurred in the gut microbial communities of the rats treated with glyphosate in relatively high concentrations, as compared to the control group. However, slight differences in alpha diversity were observed between treatment groups. We showed by *in vitro* assays that absence of free aromatic amino acids is necessary for glyphosate to affect bacterial growth. If high aromatic amino acid concentrations are available, blocking of the Shikimate pathways thus has only minor effects on the proliferation of microbes. In rich growth medium, containing sufficient amounts of aromatic amino acids, we detected some differences in susceptibility between the studied bacteria, but in general a very high tolerance to glyphosate was found. Strains of *E. coli* and *Enterococcus faecalis* had the highest tolerance with an MIC of 80 mg/ml and *Bacteriodes* had the lowest tolerance with a MIC of 5 mg/ml. We suspected the rich medium with a high concentration of free available aromatic amino acids to be responsible for the high MICs and therefore we included a study in minimal medium that did not contain any amino acids. The minimal growth medium supports growth only of prototrophic bacteria and we therefore chose *E. coli* as a model for testing. Importantly, when grown in minimal medium the MIC of the *E. coli* strain was 100-fold lower (0.08 mg/ml) than in rich medium. By adding increasing concentrations of aromatic amino acids we were able to decrease the susceptibility of *E. coli towards glyphosate,* thereby demonstrating that free aromatic amino acids reduce the inhibitory effect of glyphosate on this bacterium. This phenomenon has indeed previously been reported for *E. coli* [85] as well as for carrot and tobacco cells [86].

We further compared the growth of *E. coli* in different formulations of glyphosate (Table 2); glyphosate in its pure form *N*-(phosphonomethyl)glycine, glyphosate isopropylamine salt, Glyfonova® and Roundup®. Our findings support that different formulations can affect bacteria differently and we noted for *E. coli* that glyphosate in the pure form has a lower inhibitory effect on growth compared to both the glyphosate isopropylamine salt and the formulations Glyfonova® and Roundup®. A similar observation was made in a study where Roundup® had an inhibitory effect on microbial growth, but glyphosate at the same level did not result in any effect on the three food microorganisms *Geotrichum candidum*, *Lactococcus lactis* subsp. *cremoris* and *Lactobacillus delbrueckii* subsp. *bulgaricus* [87]. Other studies including trials with eukaryotic cells have suggested that the impact of glyphosate is not proportional to its concentration in formulations, confirming that adjuvants are not inert [27,88], and similar effects on microorganisms have been reported. The protozoan *Ichthyophtirius multifiliis* and the bacteria *T. thermophile* tolerate glyphosate but not Roundup®, and the commercial formulation was found to be 100 times more toxic than the active ingredient [89]. Amongst these adjuvants, POEA which promotes xenobiotic penetration into cells has been shown to be more toxic than glyphosate itself [66]. In the studies to which we compare our data, formulations including POEA have primarily been used. However POEA was recently banned in glyphosate-formulated products in EU and in our study we therefore chose to use the Glyfonova® product not containing POEA.

In vitro studies previously performed on gut bacteria in pure culture or as communities found inhibitory effects of glyphosate and even suggested that beneficial bacteria are more sensitive to glyphosate compared to pathogenic bacteria [62,67,87]. In this context, it is important to notice that different forms of glyphosate, different formulations, and different media and growth conditions were applied, which makes direct comparison between the studies difficult. In the *in vivo* study with Sprague-Dawley rats treated with either water, glyphosate or the formulation Glyfonova®, we found that levels of the three aromatic amino acids tyrosine, tryptophan and phenylalanine in the gut environment were relatively high and probably sufficient for the bacteria to grow by uptake of these aromatic amino acids, and they were therefore not inhibited by blocking of the Shikimate pathway by glyphosate. A similar observation was made for *Klebsiella pneumoniae* in pure culture where a mixture of the 3 aromatic amino acids reversed the growth inhibition caused by glyphosate and lowered bacterial sensitivity towards this pesticide [87,88]. However, the results from our study, together with studies where no effects of glyphosate were observed [91,92], indicate that the mode of action of glyphosate on bacterial communities is complex and highly dependent on the surrounding environment. A genomic study supports this, as most free-living soil bacteria appear to contain a complete and functioning Shikimate pathway, while for host-associated bacteria in the gut environment, more than one-quarter have incomplete pathways, indicating that they have access to sufficient amounts of aromatic amino acids by sequestering from the host as a shared metabolic evolutionary adaptation [93]. In environments with low concentrations of aromatic amino acids e.g. soil or gut distal compartments such as the colon, where we measured the lowest concentration of aromatic amino acids, we would expect the highest effect of glyphosate on the microbial gut community.

Even though the effect of glyphosate can be largely alleviated by aromatic amino acids present in the intestinal environment, it is still possible that certain bacteria are affected by glyphosate in terms of slower growth rates or decreased activity. We measured slight, but significantly lower concentrations of acetic acid and increased pH that could indicate changes in activity of acetic acid producing bacteria. Acetic acid is produced by most anaerobes, including acetogens that are able to perform reductive acetogenesis from formate or hydrogen plus CO_2 and it is usually fully ionized to acetate [94]. Exogenous acetate formed by colonic bacterial fermentation enters the blood compartment and is mixed with endogenous acetate released by tissues and organs [95,96]. Up to 70% of the acetate is taken up by the liver [97], where it is not only used as an energy source, but also as a substrate for the synthesis of cholesterol and long-chain fatty acids and as a co-substrate for glutamine and glutamate synthesis. Other tissues including the heart, adipose tissue, kidney, and muscle metabolize the remainder of acetate [95].

Interestingly, we found a significantly higher number of bacterial species (richness) in cecum and colon in the group of rats treated with Glyfonova® than in the control group. Additionally, positive correlations were identified between the measured concentration of glyphosate and the alpha diversity parameters in all three intestinal compartments. Previous studies have found a stimulation of bacterial growth, biomass and enhanced bioactivity under certain conditions after application of glyphosate [98,99] indicating that some bacteria can utilize glyphosate as a source of carbon, nitrogen or phosphorus. Our study included quantification of the primary metabolite AMPA from degradation of glyphosate (Figure 4) and we found that the AMPA to glyphosate

ratio increased through the gut, indicating that there are bacteria present in the gut, which are capable of degradation of glyphosate to AMPA. We further measured AMPA in samples from seven individually cultured bacteria (pure culture) and two human fecal samples in the presence of glyphosate, but did not detect AMPA. We found very low concentrations of both glyphosate and AMPA in the intestinal content of animals in the control group, which was attributed to very low residues of both compounds in their feed (un-supplemented). We find it unlikely that these low levels of glyphosate (approximately 10-fold lower than measured in the GLY5 group and thus equating to exposure to 0.5*ADI) would affect the microbiota of the animals in the control group, however this cannot be ruled out, and any effect could even be augmented following long-term exposure.

In the treated groups of animals we did not observe any physiological abnormalities of organs, changes in proinflammatory IL-6 levels or changes in expression of tight junction proteins. However, we found significantly higher serum levels of the acute phase protein haptoglobin in the group treated with Glyfonova®. An increase of haptoglobin indicates ongoing (potentially low-grade) inflammatory responses [100]. Glyfonova® includes non-declared additives besides glyphosate and it is possible that one or more of these compounds are also involved in the observed increase in haptoglobin.

4. Conclusion and perspectives

The possible impact of glyphosate on human health is currently highly debated and very relevant in light of the extension period for approval of glyphosate and the ban of the co-formulant POEA from glyphosate based products. We studied the inhibitory effect of glyphosate on bacterial growth in both pure cultures and *in vivo* within the complex bacterial communities present in the intestinal tract of rats. We found that absence of free aromatic amino acids is essential for bacterial inhibition. Sufficient amounts of bioavailable aromatic amino acids thus almost completely alleviated the effect of glyphosate on bacteria. In rats, we measured relatively high concentrations of aromatic amino acids in the small intestine compared to the cecum and colon segments where most of the aromatic amino acids have been absorbed. Although the relatively low aromatic amino acid concentrations in the cecum and colon, and very high bacterial load, could provide an environment where glyphosate impacts bacterial growth, we found only very limited changes in the bacterial community structure within any of the treatment groups. We did however note an increase in pH and a slight decrease in the concentration of the short chain fatty acid acetate, which could indicate an effect on bacterial activity. It is however important to note that concentrations of glyphosate tested in this study were between 5 and 50 times higher than the established ADI for humans and in most cases much higher than normally achievable based on actual residue levels in food commodities in Denmark. It is possible that low-protein diets, states of general malnutrition or even generally lower levels of bioavailable aromatic amino acids in the intestines of humans compared to laboratory rats could provide conditions where glyphosate causes a more pronounced effect on the bacterial community. Also conditions in production animals could be different from those determined in the rodents in the present study. Collectively, further studies on microbial inhibition should include determination of aromatic amino acid levels in different segments of the intestinal tract. Despite the relatively low impact of glyphosate on the gut microbiota reported in the present study, we see a general need to consider the intestinal microbiota as an important end-point during risk assessment of xenobiotic compounds including pesticides. The gut microbiota has proven to be very important for human health, and several pesticides have already been shown to affect the natural balance of this complex ecosystem. The present study emphasizes the need to conduct this kind of studies on complex bacterial systems where environmental nutrients and different growth rates are weighted and not to rely solely on effects on isolated bacterial species such as determination of minimum inhibitory concentrations. To further develop exposure tests, it would be highly relevant to seek standardization in the form of well-defined bacterial communities housed in laboratory animals, such as rodents. Potentially also more simple animal forms, such as nematodes and zebrafish, could be developed as high-throughput models. In conclusion we find very limited effects of glyphosate on the intestinal microbiota in a rodent model, but suggest that similar testing should be considered during risk assessment of pesticides and other xenobiotics in the future.

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Appendix 1. Scientific paper

Scientific paper

Nielsen, L.N., Roager, H.M., Casas, M.E., Frandsen, H.L., Gosewinkel, U., Bester, K., Licht, T.R., Hendriksen, N.B., Bahl, M.I., 2018. Glyphosate has limited short-term effects on commensal bacterial community composition in the gut environment due to sufficient aromatic amino acid levels. Environ. Pollut. 233, 364–376.

Link to journal paper: https://authors.elsevier.com/sd/article/S0269749117328099

Effects of glyphosate on the intestinal microbiota

The intestinal bacterial community is now recognized as an important factor for health and implicated in numerous states of disease. Despite the fairly extensive regulatory demands for risk assessment of pesticides in relation to human exposure, there is currently very little knowledge related to potential effects on the gut microbiota. It has however recently been speculated that glyphosate based herbicides may affect the gut microbiota of humans and animal husbandry due to inhibition of the Shikimate pathway in bacteria causing loss of aromatic amino acid synthesis and thus growth inhibition. In this study Sprague Dawley rats were exposed to glyphosate at 5x and 50x the acceptable daily intake (ADI) for humans. Profiling of the bacterial community and aromatic amino acids and their downstream metabolites was performed on intestinal samples obtained after two weeks of oral dossing. We found that glyphosate had very limited effects on bacterial community composition even at the highest exposure concentration. Also we measured relatively high concentrations of aromatic amino acids in the intestine of the animals. Our data show that glyphosate inhibits bacterial growth in minimal medium but this inhibitory effect is relieved in the presence of aromatic amino acids in the growth medium. Results from the animal trial therefor suggest that sufficient levels of aromatic amino acids are present in the rat intestine to alleviate the need for bacterial synthesis and thus prevent an antimicrobial effect of glyphosate in vivo.

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