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# Glyphosate has limited short-term effects on commensal bacterial community composition in the gut environment due to sufficient aromatic amino acid levels☆

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ABSTRACT

Recently, concerns have been raised that residues of glyphosate-based herbicides may interfere with the homeostasis of the intestinal bacterial community and thereby affect the health of humans or animals. The biochemical pathway for aromatic amino acid synthesis (Shikimate pathway), which is specifically inhibited by glyphosate, is shared by plants and numerous bacterial species. Several *in vitro* studies have shown that various groups of intestinal bacteria may be differently affected by glyphosate. Here, we present results from an animal exposure trial combining deep 16S rRNA gene sequencing of the bacterial community with liquid chromatography mass spectrometry (LC-MS) based metabolic profiling of aromatic amino acids and their downstream metabolites. We found that glyphosate as well as the commercial formulation Glyfonova®450 PLUS administered at up to fifty times the established European Acceptable Daily Intake (ADI = 0.5 mg/kg body weight) had very limited effects on bacterial community composition in Sprague Dawley rats during a two-week exposure trial. The effect of glyphosate on prototrophic bacterial growth was highly dependent on the availability of aromatic amino acids, suggesting that the observed limited effect on bacterial composition was due to the presence of sufficient amounts of aromatic amino acids in the intestinal environment. A strong correlation was observed between intestinal concentrations of glyphosate and intestinal pH, which may partly be explained by an observed reduction in acetic acid produced by the gut bacteria. We conclude that sufficient intestinal levels of aromatic amino acids provided by the diet alleviates the need for bacterial synthesis of aromatic amino acids and thus prevents an antimicrobial effect of glyphosate *in vivo*. It is however possible that the situation is different in cases of human malnutrition or in production animals.



## 1. Introduction

Globally, glyphosate-based herbicides are widely applied in agriculture for the control of weeds and for green burndown (Bøhn et al., 2014). Since the late 1970s, the volume of glyphosate-based herbicides applied world-wide has increased approximately 100-fold, especially after the introduction of genetically modified plants tolerant to these herbicides (Myers et al., 2016). Application of glyphosate-based herbicides on crops will inevitably result in glyphosate residues and potentially its primary metabolite, aminomethyl phosphonic acid (AMPA), in crops at harvest, which may reach the consumers (Myers et al., 2016). In recent years the biologically significant residue level of glyphosate in food commodities has been much debated. Some studies claim possible negative effects following exposure below the regulatory maximum residue (MRL) and accepted daily intake (ADI) levels (Benedetti et al., 2004; Larsen et al., 2014). In Europe the MRL varies for different crops, and is defined for each product type separately. For barley and oats (grains) the MRL is 30 mg/kg, while the ADI is set to 0.5 mg/kg body weight per day (EFSA, 2015). Humans may be exposed to

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glyphosate residues from consuming fruits, vegetables and other agricultural products as well as from drinking-water. This represents a significant public health concern, fueled by the fact that several studies demonstrate the presence of glyphosate in the urine of the general population (Conrad et al., 2016; Krüger et al., 2014).

When assessing oral toxicity of pesticides including glyphosate, the potential impact on the bacterial community within the gut environment has received very little attention until recently (Jin et al., 2017; Liu et al., 2017; Shehata et al., 2013). The significance of this highly complex ecosystem, collectively termed the gut microbiota, has however attracted immense scientific attention in recent years, due to a growing recognition of its importance in human health (Arrieta and Finlay, 2012; Butel, 2014; Jandhyala et al., 2015). It is also well established that the natural homeostasis of the gut microbiota is sensitive towards external influences, such as antibiotic treatments (Dethlefsen et al., 2008), dietary habits (David et al., 2013) and exposure to xenobiotic compounds (Lai et al., 2016; Nasuti et al., 2016). Modulation of this intricate balance has been associated with several disorders such as metabolic diseases, hepatic, coronary and gastrointestinal diseases (e.g. inflammatory bowel disease) (Sheehan et al., 2015). The link between gut microbiota and host health is partly driven by bacterial breakdown of non-digestible dietary fibers, resulting in the generation of short-chain fatty acids (SCFA) (Russell et al., 2013). The SCFA, including acetic acid, propionic acid and butyric acid, play an important role for human health as they serve as nutrition for enterocytes (Brüssow and Parkinson, 2014), and are involved in both appetite regulation (Byrne et al., 2015) and immune homeostasis (Wu and Wu, 2012). Acetic acid, which is the most predominant of the SCFAs in the gut and is produced by many different bacterial taxa, has been shown to induce anti-inflammatory effects in the colonic epithelium, and to prevent *E. coli* infection (Fukuda et al., 2011).

Already around the time glyphosate was patented as a herbicide (Franz, 1974) it was realized that the active compound could have antibacterial capabilities. Glyphosate specifically inhibits the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), which is a central enzymatic step of the Shikimate pathway of aromatic amino acid biosynthesis in plants as well as some bacteria, algae, fungi and parasites (Clair et al., 2012; McLeod et al., 1998; Priestman et al., 2005). Reports of non-target effects of glyphosate on aquatic and soil-living microorganisms are numerous and show that glyphosate does indeed impact environmental microbial communities, for example by selectively enhancing bacteria belonging to the Gammaproteobacteria and inhibiting *Pseudomonas* spp. and *Acidobacteria* (Newman et al., 2016; Zobiole et al., 2011). Therefore, glyphosate could also potentially modify the composition of the microbial community in the gut, and thereby potentially exert a negative effect on the host organism. According to recent *in vitro* studies, bacterial communities associated with the intestinal environment are indeed susceptible to interference by glyphosate. In one study the minimum inhibitory concentration (MIC) for 23 different bacterial species relevant to the intestinal environment was determined for the glyphosate-containing herbicide Roundup UltraMax®. This study indicated that bacteria commonly recognized as beneficial, including bifidobacteria, enterococci and lactobacilli, were more susceptible to the glyphosate formulation than pathogenic bacteria such as *Clostridium perfringens* and *Salmonella* spp. (Shehata et al., 2013). However, an *in vivo* study of rumen fermentation in sheep fed either corn silage alone or mixed with Roundup Ultra® at a high concentration (0.2% wt/wt) revealed no changes in rumen fermentation parameters (pH, ammonia and volatile fatty acids) following 15 days of treatment (Hüther et al., 2005). In agreement with the latter study, an *in vitro* bovine fermentation model did not reveal any significant effects on ruminal metabolism or composition of the bacterial communities (Riede et al., 2016). It is important to note that several studies have shown that the effect of glyphosate depends on the way it is formulated. In one *in vitro* study, effects of pure glyphosate and Roundup® (R400) on three food microorganisms *Geotrichum candidum, Lactococcus lactis* subsp. *cremoris* and *Lactobacillus delbrueckii* subsp. *Bulgaricus* were compared. It was found that Roundup® had an inhibitory effect on microbial growth, but that exposure to glyphosate at the same concentrations had no effect (Clair et al., 2012). Also Braconi et al. (2006) report that pure glyphosate affects cell growth and metabolism in the yeast *Saccharomyces cerevisiae* less than commercial preparations (Braconi et al., 2006).

Overall, the available data from literature is scarce and partly contradictory in terms of the effects of glyphosate on intestinal microbial communities. It remains unclear whether oral exposure to glyphosate residues has the potential to modulate the human gut microbiota at a level of concern for human health. Here, we report on the effects of pure glyphosate and a commercial glyphosate formulation on the intestinal microbial composition, activity and host response during a short-term exposure trial in Sprague Dawley rats. In contrast to previous studies, we address the potentially alleviating effects of endogenous aromatic amino acids in the intestinal environment.

## 2. Materials and methods

### 2.1. Bacterial strains

Bacterial strains used in this study are listed in Table 1. All strains were grown in brain heart infusion broth (BHI) (Oxoid) or reinforced clostridial medium (RCM). *Escherichia coli* ATCC 25922 was additionally grown in AB minimal medium (Clark and Maaløe, 1967) containing 2.5 mg thiamine/mL and 0.5% glucose (ABTG) to allow investigation of the effects of aromatic amino acids in the growth media.

### 2.2. Chemicals

Glyphosate was used in the formulations; Glyphosate (*N*-phosphonomethyl)glycine (Sigma-Aldrich 1071-83-6), Glyphosate salt *N*-(Phosphonomethyl)glycine with monoisopropylamine as counter-ion (Sigma-Aldrich 38641-94-0), Glyfonova® 450 Plus (450 g/L glyphosate acid equivalent) (kind gift from FMC Corporation, previously Cheminova A/S) and Roundup® Garden (120 g/L glyphosate equivalent) (Monsanto). Underlined names are used henceforth.

For the aromatic amino acid analysis, LC-MS grade acetonitrile, methanol, ammonium hydroxide and formic acid were obtained from Merck (Darmstadt, Germany). All aqueous solutions for LC-MS analysis were prepared using ultrapure water obtained from a Milli-Q Gradient A10 system (Millipore, Bedford, MA). Authentic aromatic amino acid compounds (Table S1) including L-Tyrosine, L-Tryptophan and L-Phenylalanine were obtained from Sigma-Aldrich. Aromatic amino acid internal standards (L-Phenylalanine (ring-d5, 98%), L-Tyrosine (ring-d4, 98%), L-Tryptophan (indole-d5, 98%) and indoleacetic acid (2,2-d2, 96%)) of the highest purity grade available were purchased from Cambridge Isotope Laboratories Inc. (Andover, MA).

For the Glyphosate and AMPA analysis: Ammonium acetate, AMPA, ammonia solution 25%, and HPLC-MS grade water were obtained from Sigma-Aldrich (St. Louis, MO). Direct labelled internal standards, glyphosate-2-13C and glyphosate (13C, 99%; 15N, 98%; methylene-D2,98%), were obtained from Sigma Aldrich and Cambridge Isotope Laboratories Inc. (Andover, MA) respectively.