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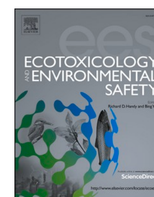

From dysbiosis to neuropathologies: Toxic effects of glyphosate in zebrafish

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ABSTRACT

Glyphosate, a globally prevalent herbicide known for its selective inhibition of the shikimate pathway in plants, is now implicated in physiological effects on humans and animals, probably due to its impacts in their gut microbiomes which possess the shikimate pathway. In this study, we investigate the effects of environmentally relevant concentrations of glyphosate on the gut microbiota, neurotransmitter levels, and anxiety in zebrafish. Our findings demonstrate that glyphosate exposure leads to dysbiosis in the zebrafish gut, alterations in central and peripheral serotonin levels, increased dopamine levels in the brain, and notable changes in anxiety and social behavior. While the dysbiosis can be attributed to glyphosate's antimicrobial properties, the observed effects on neurotransmitter levels leading to the reported induction of oxidative stress in the brain indicate a novel and significant mode of action for glyphosate, namely the impairment of the microbiome-gut-axis. While further investigations are necessary to determine the relevance of this mechanism in humans, our findings shed light on the potential explanation for the contradictory reports on the safety of glyphosate for consumers.

1. Introduction

The microbiome-gut-brain (MGB) axis connects the gut microbiome to the central nervous system (Cussotto et al., 2018; Nagpal and Cryan, 2021; Sherwin et al., 2019). As summarized in Supplementary Fig. S1, the effect of microbiome on the brain function is mainly mediated by some signaling molecules, such as short-chain fatty acids (SCFAs), tryptophan metabolites and some neurotransmitters, produced by several bacterial taxa in the gut (Sherwin et al., 2019). These exogenous effectors are recognized by intestinal enteroendocrine cells (EECs), electrically excitable polymodal chemosensors that excrete the neurotransmitter serotonin and some peptide hormones (Bellono et al., 2017). Serotonin released by EECs can then propagate the action potential along the vagal afferent fibers to the hindbrain (Bonaz et al., 2018), finally modulating different neurotransmitter systems in the brain and resulting in changes in anxiety and social behavior. The effects of the stimulation of vagal afferent fibers from the intestinal tract on the dopaminergic system through the mesocorticolimbic circuit in the brain are also well established (González-Arancibia et al., 2019; Hamamah et al., 2022). Whereas the MGT axis is a bidirectional channel essential for exchanging information between the intestinal lumen and the brain, some recent studies have proposed that some xenobiotics may produce adverse effects on the central nervous system acting indirectly through this axis (Balaguer-Trias et al., 2022; Bertotto et al., 2020), although further efforts are needed to obtain evidence of this new mode of action (MoA).

Glyphosate [N-(phosphonomethyl)glycine] is the most widely used herbicide in the world, due to its broad spectrum and to the development of resistant crop varieties (Annett et al., 2014). Glyphosate concentrations in the environment range from less than 1 $\mu g\ l^{-1}$ in surface water to more than 10 mg kg^{-1} in some foods (Annett et al., 2014; Faria et al., 2021; Vicini et al., 2021). There is a considerable controversy about the toxicity of glyphosate for animals in general and for humans in particular. Glyphosate is a competitive inhibitor of

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5-enolpyruvylsikimate-3-phosphate synthase (EPSPS), an enzyme of the shikimate pathway essential for the synthesis of aromatic amino acids in plants and some microorganisms. As animals lack the shikimate pathway, glyphosate was initially considered a very safe pesticide. However, increasing evidences have suggested that glyphosate might indirectly lead to adverse effects in animals, including humans, although the interpretation of these findings is complicated by the co-exposure to several adjuvants that are present in most commercial formulations (Costas-Ferreira et al., 2022).

An indirect effect of glyphosate exposure can be alteration of microbial populations. Not all EPSPS enzymes have similar sensitivity to glyphosate, with class I, expressed in plants and some bacterial groups, being very sensitive to glyphosate, and class II, expressed only in bacteria, being quite tolerant to this chemical (Mesnage and Antoniou, 2020). This selective inhibitory activity of glyphosate constitutes a plausible MoA for the adverse effects of glyphosate on animals could be the disruption of the gut microbiome, leading to intestinal dysbiosis (Motta et al., 2018; Shehata et al., 2013). Many bacterial groups, including some present in gut microbiome, also use the shikimate pathway to produce antibiotic and other metabolites, and it has been proposed that its suppression may alter microbiomes both in the environment and in the guts (Mesnage and Antoniou, 2020). In fact, glyphosate has been shown to induce intestinal dysbiosis in different animal species, reducing the abundance of beneficial gut bacteria, such as *Lactobacillus* and *Bifidobacterium*, while increasing the abundance of pathogenic bacteria such as *Clostridia* and *Salmonella* (Motta et al., 2018; Shehata et al., 2013).

Zebrafish is a vertebrate model increasingly used in different areas of the biomedical research, including neurobiology (Berg et al., 2023; Kunst et al., 2019), and studies related to the effects of microbiota manipulations on brain and behavior (Cornuault et al., 2022; Nagpal and Cryan, 2021; Soares et al., 2019). In a previous study (Faria et al., 2021), we described a distinct neurotoxic effect of glyphosate in adult zebrafish, characterized by an anxiety-like behavior and tightly packed shoals, increased serotonin levels, dopaminergic activation and changes in expression of genes involved in the dopaminergic system, and altered antioxidant systems and significant lipid peroxidation in the brain (Faria et al., 2021).

Considering the lack of direct molecular targets of glyphosate in animals, and the reported effects of this herbicide on the intestinal microbiota, in this study we have tested the hypothesis that the observed adverse effects of glyphosate on the central nervous system of adult zebrafish were mediated through the MGT axis. With this aim, we analyzed the effects of glyphosate in zebrafish gut microbiomes in parallel with a comprehensive targeted metabolomic analysis on feces, gut and brain samples from control and treated animals. Finally, anxiety and social behavior of the exposed animals were determined and compared with the corresponding controls.

2. Material and methods

2.1. Animals and housing

Adult wild-type zebrafish (standard length: 3.1–3.5 cm) were obtained from Exopet (Madrid, Spain) and maintained into a recirculating zebrafish system (Aquaneering Inc., San Diego, United States) at the Research and Development Center (CID-CSIC) Aquatic Vertebrate Platform for 2 months before starting the exposures (see Supplementary Methods for additional housing details). All procedures were approved by the Institutional Animal Care and Use Committees at the CID-CSIC (OH 1032/2020) and conducted in accordance with the institutional guidelines under a license from the local government (agreement number 9027).

2.2. Experimental procedure

Glyphosate (CAS # 1071–83-6, 98% purity, HPLC) was purchased from ChemCruz (sc-211568; Santa Cruz Biotechnology, Dallas, TX). The day of the experiment a 3 mg/L stock solution was freshly prepared in fish water, fish water, out of which test solutions of 0.3 and 3.0 μg/L glyphosate (pH: 6.8) were diluted. These glyphosate concentrations were previously identified as having specific effects in the zebrafish monoaminergic systems (Faria et al., 2021). Stability of these glyphosate aqueous solutions (90% stability in 48 h) was also tested in this previous work, as well as the good agreement (>95%) between nominal and actual concentrations (Faria et al., 2021) Adult zebrafish (50:50 male: female ratio) were randomly selected and exposed for 2 weeks at 28.5 °C and 12 L:12D photoperiod. Experiments were conducted in duplicate or triplicate. Experimental solutions were renewed every 48 h, 30 min after the first feeding of the day. Tanks were kept in an incubation chamber set to 28.5 °C and 12 L:12D photoperiod (POL-EKO APARATURA Climatic chamber KK350, Poland). For brain sample collection, fish were euthanized by inducing hypothermic shock in ice-chilled water (2–4 °C). Brains and gut were immediately dissected and individually stored at − 80 °C for further analysis. For intestinal histopathological assessment, fish were also euthanized by inducing hypothermic shock in ice-chilled water, the whole digestive system was immediately dissected and fixed in 10% buffered formalin, and individually stored at room temperature for further analysis.

2.3. Neurobehavioral assessment

Anxiety-like behavior and social behaviors were assessed using the Novel Tank Test (NTT) and Social Preference Test (SPT), respectively. The protocols for these behavioral paradigms were based in video-tracking technologies, and have been reported elsewhere (Bedrossiantz et al., 2021; Faria et al., 2021). More details are available at Supplementary Methods.

2.4. Respiration rate determination

Respiration rate of each fish was assessed by transferring one fish inside a 50 mL gastight syringe (Hamilton, USA) and measuring the oxygen after 15 min at 28ºC (Fig. S6). Oxygen consumption of adult zebrafish inside was determined at 28ºC using an oxygen meter (Model 782, Strathkelvin Instruments, Glasgow, Scotland) following the protocols described elsewhere (Zhou et al., 2018). More details are available at Supplementary Methods.

2.5. Inflammation assessment

2.5.1. Histological analysis

Previously fixed samples of the digestive system were processed by routine paraffin histology. Each sample was sectioned at 4–5 μm and stained with Haematoxylin & Eosin. All the stained slides were thoroughly screened and examined under a Leica DM500B microscope for the detection and identification of potential disorders. Images were taken with a Leica camera model CTR5000 connected to the microscope.

2.5.2. Gene expression analysis

Real Time PCR was performed in LightCycler ® 480 Real-Time PCR System using SYBR Green PCR Master Mix (Roche Diagnostics, Mannheim, Germany). Cycling parameters were 95ºC for 15 min followed by 45 cycles of 95ºC for 10 s and 60ºC for 30 s. Three technical replicates were run for each sample. Primer sequences for the selected genes (Supplementary Table ST1) were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA) and synthesized by Sigma-Aldrich. Primers were checked for their efficiency and specificity previous to analyses. Results were normalized using the housekeeping *ppia2* as reference gene and the relative abundance of mRNA was

calculated from the second derivative maximum of their respective amplification curves (ΔΔCt method) (Livak and Schmittgen, 2001), and deriving fold-change ratios from these values.

2.6. Microbiome analysis

2.6.1. Acid Nucleic extraction

Fecal pellets from three fish per replicate (5 replicates per treatment) were collected for microbiome analysis. Extraction of DNA from zebrafish was performed using an adapted Phenol:Chloroform method, as previously described (Cerro-Gálvez et al., 2020) with few modifications, which included the homogenization of fish feces with DNA lysis buffer using a TissueLyser® (Qiagen, Germantown, MA, USA) and the adjustment of the final DNA solution in RNAse free water to 25 µl. The quality and quantity of total DNA was determined in a NanoDrop Spectrophotometer 8000 (Thermo Fisher Scientific, Inc).

2.6.2. 16 S amplicon sequencing

The V3 and V4 hypervariable region of 16 S rRNA genes were amplified by PCR using barcoded primers with Illumina adapters (341 F CCTAYGGGRBGCASCAG, 806 R GGACTACNNGGGTATCTAAT). All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). Quality-checked PCR products were purified by Qiagen Gel Extraction Kit (Qiagen, Germany). Sequencing libraries were generated using NEBNext Ultra DNA Library Pre ®Kit for Illumina, following manufacturer's recommendations, and index codes were added. Sequencing was carried out on an Illumina platform and 250 bp paired-end reads were generated. Quality control, trimming of the reads, processing, inference of amplicon sequence variants (ASV) and taxonomical classification was done using the R package DADA2 (Callahan et al., 2016) and the SILVA database (v.138) through the Classify-sklearn moduler in QIIME2 software. ASV counts were rarefied using the package *vegan* (software R) prior to data analysis. Relative abundances were calculated, when necessary, as the abundance of a given taxa divided by the sum of frequencies of all the taxa in a sample. Analysis of β-diversity was performed using calculated Bray-Curtis distances and represented in a nonmetric multidimensional scaling (NMDS), and significant differences among samples were assessed using the PERmutational Multivariate ANOVA (PERMANOVA) test (vegan package). As for α-diversity, Chao and Shannon diversity indexes were calculated (vegan package) and significant differences were assessed using ANOVA followed by Tukey post-hoc test ($p < 0.05$). All statistical analyses were performed using the R software (version 4.2.0). Lastly, partial least square discriminant analysis (PLS-DA) was performed with the mixomics R package (Rohart et al., 2017) and Variable in Importance of Projection (VIPs) were calculated. All 16 s DNA sequence data can be downloaded from the European Nucleotide Archive (ENA) under the project accession PRJEB64277.

2.7. Metabolomic analysis

2.7.1. Sample preparation

First, frozen samples from brain and whole gut (individual animals), and fecal pellets (from three animals of the same treatment group) were accurately weighed on a Sartorious CPA225D semi-micro electronic balance (Mississauga, ON, CA). Tissue samples were homogenized and shaken to achieve metabolite's extraction, centrifuged, and supernatants were kept at − 20 ºC for further steps. From the resultant extraction, samples were split into two aliquots for two different methodologies: Phenyl isothiocyanate (PITC) derivatization (targeting amine-containing compounds) and 3-Nitrophenylhydrazine (3-NPH) derivatization (targeting keto- and carboxyl- containing compounds). These two metabolites' derivatization procedures were previously optimized for human urine samples (Zheng et al., 2020). In this study, the protocol was modified for the three different matrices. All derivatization details are described in the Supplementary Information.

2.7.2. LC-MS/MS analysis

The LC-MS methodology employed in this study is an extension of a previously established method (Zheng et al., 2020), using the same chromatographic conditions and mass spectrometric parameters for the analysis of target metabolites. All LC/ MS/MS analyses were conducted using an Agilent 1260 series UHPLC system (Palo Alto, CA, U.S.A) coupled to AB Sciex QTRAP 4000 mass spectrometer (Concord, ON, CA). The data management was processed using Analyst 1.6.2 software. All chromatographic and MS detector conditions for both methodologies are described in the Supplementary Information.

2.7.3. Quality assurance

Concerning the quality of the methodology, an extensive validation of the method was performed in previous studies and the quality parameters are described in previously published articles (Zheng et al., 2020). The method has been demonstrated to be accurate, precise, and the limits of detection and quantification indicated that the optimized methodology is very sensitive. Moreover, it has been proved to be a highly reproducible method. However, since the matrix of study is quite different comparing to the sample used in previous studies (Zheng et al., 2020), several samples of the three matrices were spiked to assess recoveries by comparing the calculated spiked concentration with the fortified amount. Samples were spiked at different concentration levels depending on the analyte and the matrix, considering the intrinsic concentration of each analyte in the sample. Recoveries obtained for all metabolites in the three matrices (brain, gut and feces) are reported in the Supplementary Information (Supplementary Table ST2 and ST3). Metabolomic data is publicly accessible at Metabolights (EMBL-EBI), access number MTBLS8165.

3. Results

3.1. Environmental levels of glyphosate modify gut microbiota composition

Adult zebrafish exposed to 0.3 µg/L and 3 µg/L glyphosate for 14 days showed no differences at any experimental group (Supplementary Table ST4). Microbiome composition of fecal pellets was analyzed by 16 S rDNA sequencing (64896 to 91042 reads per library; one sample was excluded due to low read counts, 30564) and taxonomic annotation using DADA2. The number of unique ASV per sample ranged from 558 to 60 (Fig. S2). Fusobacteriota and Proteobacteria dominated microbial communities with maximum and minimum relative contributions of 79.5–27% and 67.6–19.7% respectively (Fig. 1). *Aeromonas* and, as minor contributers, *Plesiomonas* and *Chitinibacter*, dominated the Proteobacteria, whereas *Cetobacterium* (Fusobacteriota) was the most abundant Genus. No differences in α-diversity indexes Chao and Shannon were observed among samples (Fig. S3). ASV distribution analysis by non-metric multidimensional scaling (NMDS) separated control and 0.3 µg/L and 3 µg/L glyphosate-treated samples (Fig. 2). PERMANOVA analysis confirmed a significant treatment-dependent variation of the fecal microbiome composition ($p = 0.007$, Supplementary Table ST5). At the Genus level, these changes corresponded to a substitution of *Aeromonas* and *Undibacterium* by *Cetobacterium* (among others), in a non-linear dependence of the glyphosate concentration, for low-dose samples differed more from control samples than the high-dose ones (Fig. 2). In contrast, the multi-variant test PLS-DA showed a rather monotonic response in the changes in microbial structure, which were stronger in the high-dose samples than in the low-dose ones (Fig. S4). Consistently, some taxa contributing to the Variable Importance in Projection (VIP) scores were more abundant in the 3 µg/L treatment, with *Pseudomonas*, *Bradyrhizobium*, *Bryobacter* and *Vibrio* species contributing to the VIP score (Fig. S4).

The potential metabolic consequences of the observed changes in fecal microbiome were further analyzed by PICRUST Predicted Metagenome Analysis. The analysis identified 368 altered pathways, of which

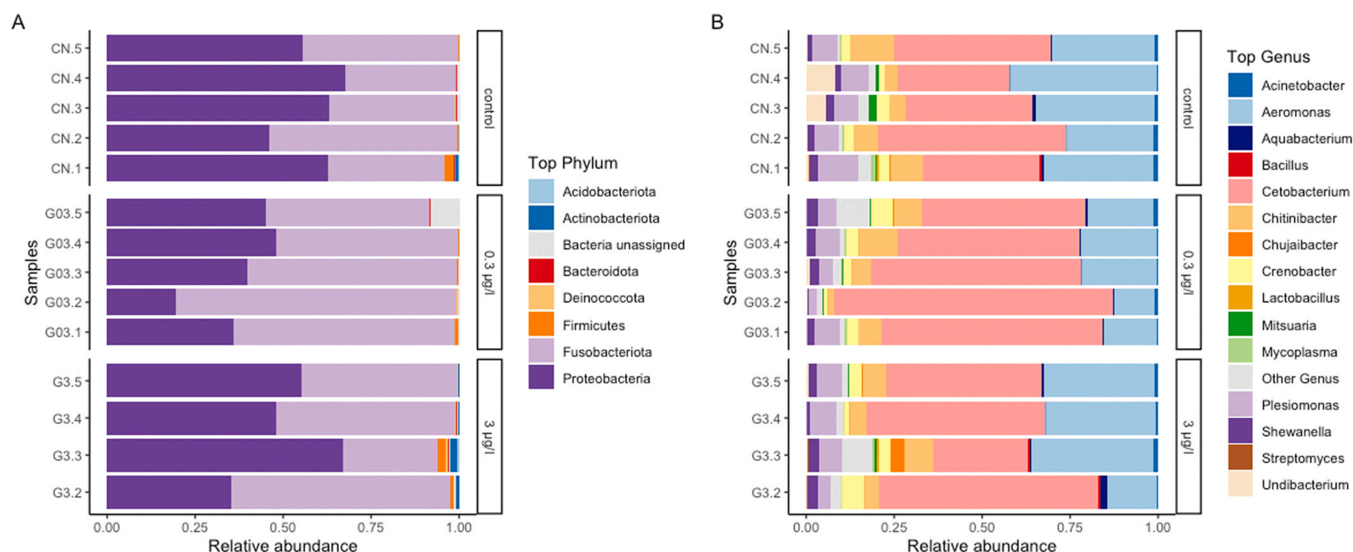


Fig. 1. Microbiome composition in the analyzed fecal pellet samples. The panel shows taxonomic composition of the samples at the Phylum level (panel A) and Genus level (panel B); estimated taxonomy classification is provided in the legend at the bottom. The top 10 classes and top 12 families were plotted and the other reads grouped as “Other" bacteria. "C", "G0.3" and "G3" indicate data from Control, 0.3 μg l^{-1} and 3 μg l^{-1} treatment groups, respectively. Each row corresponds to data from a single animal.

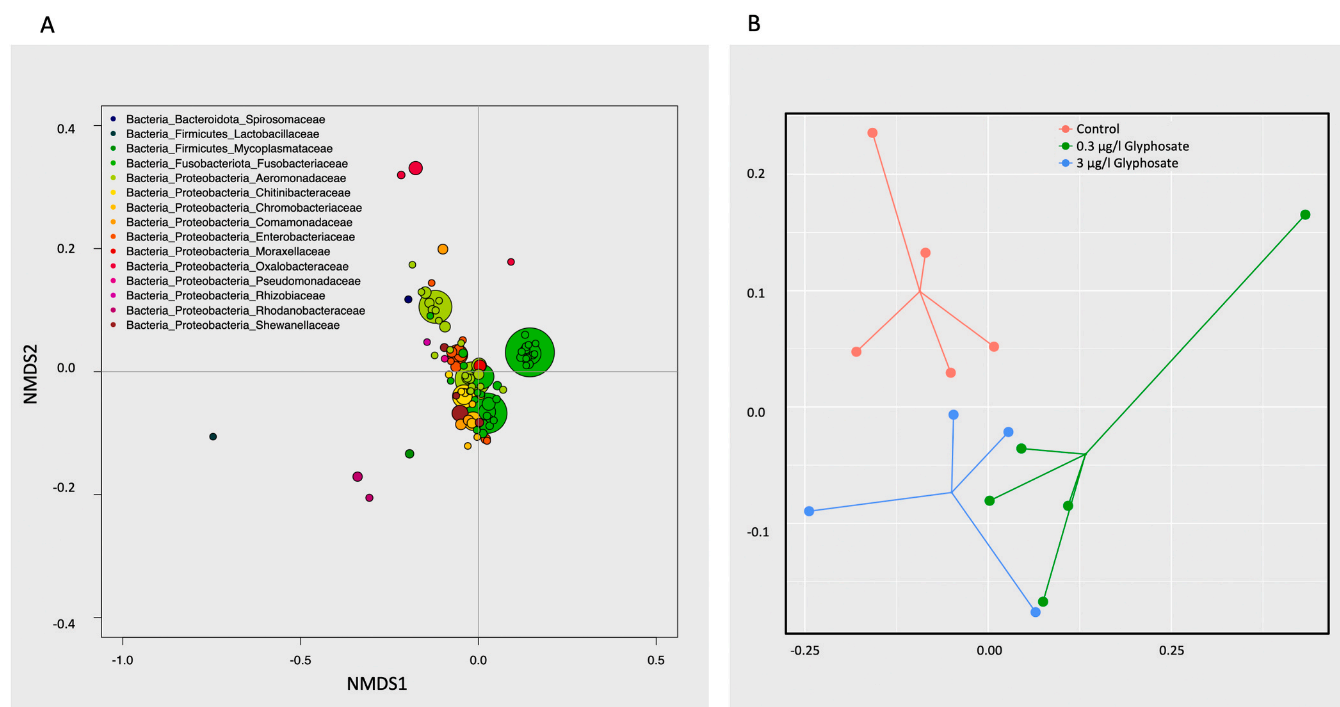
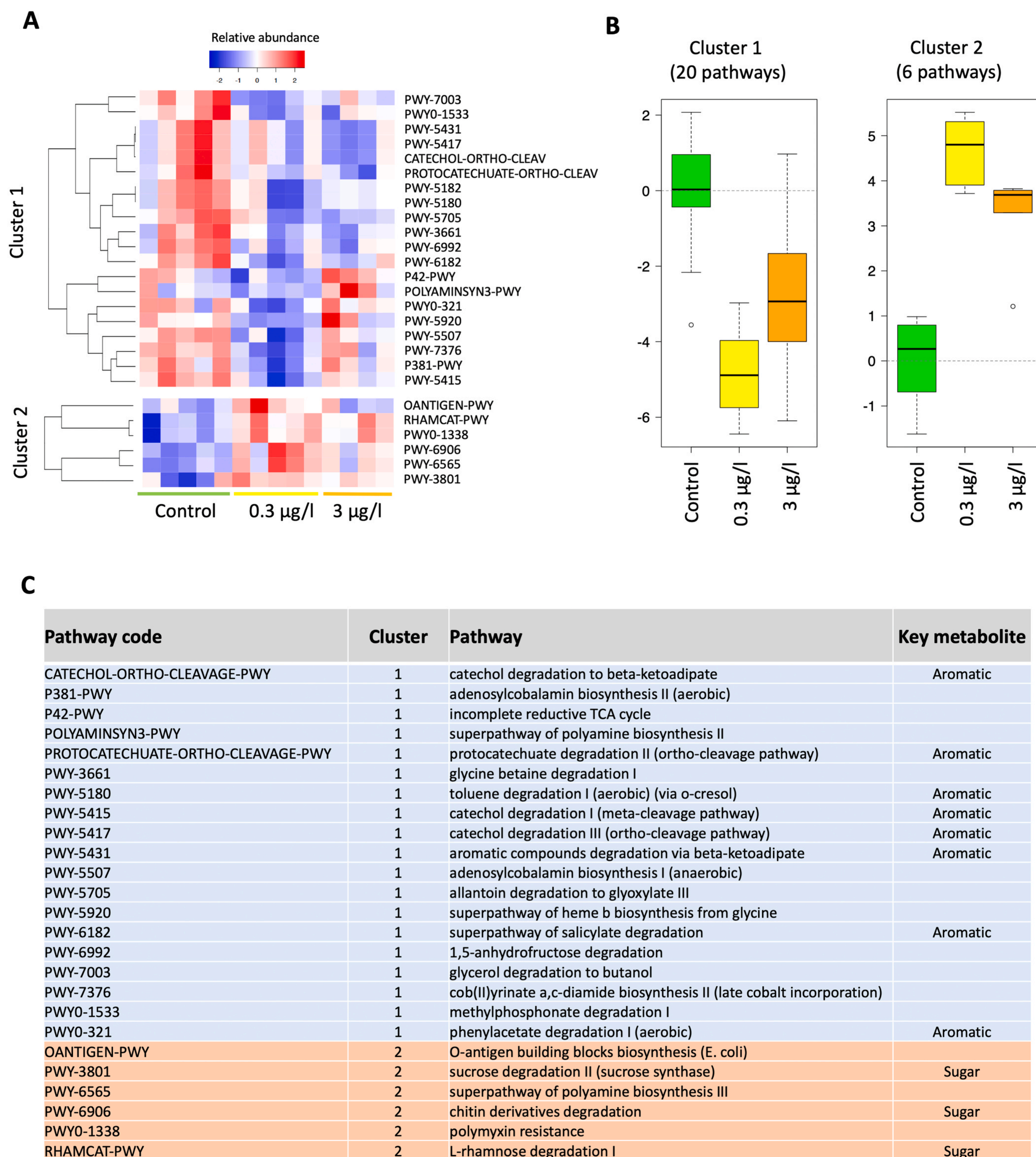


Fig. 2. NMDS plot showing the ASV distribution among the different samples. (A) Species plot: each dot represents an individual ASVs, only those showing, on average, two reads per sample (747 ASVs in total) were included. Colors represent different Families, as indicated in the inset. Dots size represents their absolute abundance in all samples (size scale on the bottom). Only the 100 ASVs with higher relative abundances in the whole dataset are shown;(B) Sample plot. Red, blue and green dots correspond to control, low-dosed and high-dosed samples, respectively.

329 had 10 or more hits in the microbiome per sample as median value. 26 of these pathways showed significant abundance distribution variations among treatment groups (PLS-ANOVA), and they could be classified into two clusters (Fig. 3). Cluster 1 corresponded to 20 pathways more represented in the control microbiome than in the treated ones, whereas Cluster 2 correspond the 6 pathways showing the opposite distribution (Fig. 3A,B). As for the general ASV analysis, low-dosed samples (0.3 μg l^{-1} glyphosate) showed stronger changes than high-dosed ones (0.3 μg l^{-1} glyphosate) relative to controls (Fig. 3B). Many of the pathways that became underrepresented in glyphosate-treated samples (Cluster 1) were related to degradation of different aromatic and/or organic compounds, whereas pathways increased by the treatment were mainly related to metabolism of complex sugars (Fig. 3C), perhaps related to differences in cell wall (OANTIGEN-PWY, PWY0–1338) or carbon source availability (PWY-3801, PWY-6906, RHAMCAT-PWY).



Pathway code	Cluster	Pathway	Key metabolite
CATECHOL-ORTHO-CLEAVAGE-PWY	1	catechol degradation to beta-ketoadipate	Aromatic
P381-PWY	1	adenosylcobalamin biosynthesis II (aerobic)	
P42-PWY	1	incomplete reductive TCA cycle	
POLYAMINSYN3-PWY	1	superpathway of polyamine biosynthesis II	
PROTOCATECHUATE-ORTHO-CLEAVAGE-PWY	1	protocatechuate degradation II (ortho-cleavage pathway)	Aromatic
PWY-3661	1	glycine betaine degradation I	
PWY-5180	1	toluene degradation I (aerobic) (via o-cresol)	Aromatic
PWY-5415	1	catechol degradation I (meta-cleavage pathway)	Aromatic
PWY-5417	1	catechol degradation III (ortho-cleavage pathway)	Aromatic
PWY-5431	1	aromatic compounds degradation via beta-ketoadipate	Aromatic
PWY-5507	1	adenosylcobalamin biosynthesis I (anaerobic)	
PWY-5705	1	allantoin degradation to glyoxylate III	
PWY-5920	1	superpathway of heme b biosynthesis from glycine	
PWY-6182	1	superpathway of salicylate degradation	Aromatic
PWY-6992	1	1,5-anhydrofructose degradation	
PWY-7003	1	glycerol degradation to butanol	
PWY-7376	1	cob(II)yrinate a,c-diamide biosynthesis II (late cobalt incorporation)	
PWY0-1533	1	methylphosphonate degradation I	
PWY0-321	1	phenylacetate degradation I (aerobic)	Aromatic
OANTIGEN-PWY	2	O-antigen building blocks biosynthesis (E. coli)	
PWY-3801	2	sucrose degradation II (sucrose synthase)	Sugar
PWY-6565	2	superpathway of polyamine biosynthesis III	
PWY-6906	2	chitin derivatives degradation	Sugar
PWY0-1338	2	polymyxin resistance	
RHAMCAT-PWY	2	L-rhamnose degradation I	Sugar

Fig. 3. Cluster analysis of metabolic pathway predicted by PICRUST (K-means PAM method) (A) heatmap representing normalized relative abundances for each MetaCyc pathway in log scales. Red and blue sectors represent over- and under-represented pathways in a given sample relative to the rest; (B) Relative abundance of ASVs annotated in each pathway group (log-transformed, normalized and grouped data) in both clusters. Boxes represent the second and third quartile intervals, whiskers cover the rest of the distribution, and outliers are represented by empty dots. The thick line in the boxes indicates the median value. Control data was averaged to 0 (mean the log distribution) for both clusters; (C) Description of the 26 pathways predicted by PICRUST and whose relative relevance changed in treated groups relative to controls. Pathways included in Clusters 1 and 2 are indicated by cyan and orange sectors, respectively.

3.2. Environmental levels of glyphosate do not result in intestinal inflammation

Both the observed dysbiosis of gut microbiome and the reported properties of glyphosate as an irritant agent might lead to intestinal inflammation, with activation of the immune system, cytokine release and activation of the microbiome-gut-brain axis. Therefore, the potential intestinal inflammation in the glyphosate-exposed animals was

evaluated by histological and using the transcriptional markers *il-10* and *tnfα* (Hanyang et al., 2017). However, no evidences of inflammation, such as loss of intestinal fold architecture, infiltration of eosinophils or depletion of Goblet cells were found in the intestine of the glyphosate exposed fish (Fig. S5). Moreover, real-time PCR analysis on total intestinal RNA showed no changes in the relative expression of *il-10* or *tnfα* after glyphosate exposure.

3.3. Exposure to glyphosate leads to changes in the metabolomic profile in feces, intestine and brain

Analysis of metabolite concentrations in brain, gut, and fecal pellets identified 122, 128 and 113 metabolites, respectively (132 metabolites in total, Supplementary Table ST6). 85 of them showed concentrations changes upon exposure to glyphosate in at least one of the tissues (PLS-ANOVA), corresponding the highest number of changes to the gut metabolome (27 metabolites up and 33 down), followed by the fecal pellets (12 up and 25 down) and brain (20 up and 10 down). Analysis of the data revealed a major decrease in amino acid contents in the intestine and, partially, in the brain, and increase in PCs, lyso PCs and some SMs in the intestine, while lyso PCs were reduced in fecal pellets, and an increase of SCFA (beta-hydroxy butyric, butyric, iso-butyric and propionic acids) in fecal pellets. It was also noticeable a strong increase in serotonin in brain (14-fold) and gut (5-fold, Supplementary Table ST6).

3.4. Glyphosate exposure induces anxiety in adult zebrafish

Glyphosate exerted a pronounced anxiogenic effect in zebrafish, as treated animals (3 μg/L) displayed positive geotaxis, spending more time (Student's t test, $P = 0.0124$) and swimming longest distances (Student's t test, $P = 0.0076$) in the bottom of the tank (Fig. S6). No differences were found in the total distance moved between the control and glyphosate-exposed fish (Student's *t* test, $P = 0.138$).

As anxiety often triggers an increase in respiration rate (Krohn et al., 2023), in order to confirm the anxiogenic effect found in the novel tank paradigm, oxygen consumption (MO_2) of the fish was analyzed. Despite that the exposure to 3 μg/L glyphosate had any significant effect on total distance moved in the tank, (Student's t test, $P = 0.138$), there was an increase in MO_2 in the exposed fish (Fig. S7; $F_{2,21} = 6.887$, $P = 0.005$).

At the same time, glyphosate-exposed fish showed a general trend to decrease the time spent near the conspecifics in a social preference test, although this effect was only significant for the high-dose treatment group ($H(2) = 6.442$, $P = 0.040$ for "Time in conspecific zone", Fig. 4). When the frequency in each zone was determined, a significant increase was found for all zones and glyphosate concentrations (Fig. S8B), probably related to the hyperactivity observed for the glyphosate-treated fish in this test (Fig. S8A; $F_{2,44} = 3.974$, $P = 0.0259$).

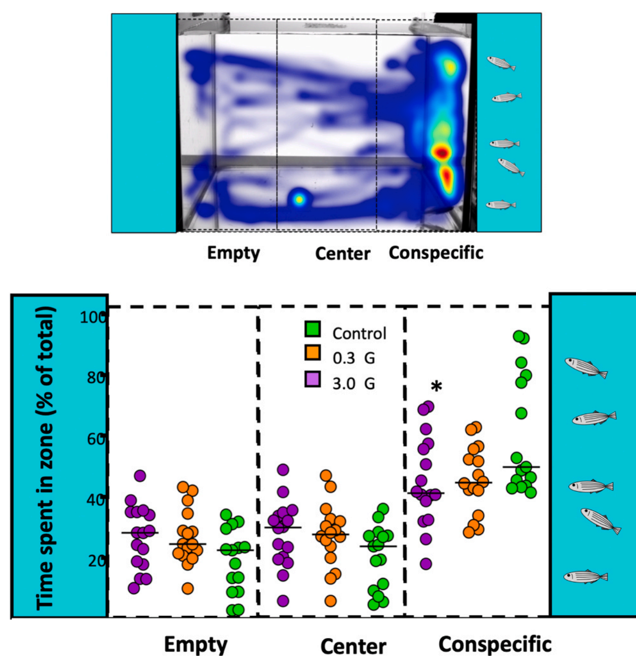


Fig. 4. Social behavior analysis of control and glyphosate-treated fish using the social preference test paradigm. (A) Heat map of a control fish in the social preference test paradigm, showing that it spends most of the time close to its conspecifics; (B) Time of the fish in each of the three virtual zones of the experimental tank: empty, center and conspecific. Data from each experiment were normalized to the corresponding control values. Data are reported as scatter plot with the median (n = 15–16) *p < 0.05; Kruskal Wallis test with Bonferroni correction. Data from two independent experiments.

4. Discussion

The potential neurotoxicity of glyphosate has been a matter of debate, first because most of these studies used unrealistic concentrations, at or surpassing the U.S. EPA reference concentrations (1.75 mg kg b.w.$^{-1}$ day^{-1}), and second because the lack of suitable mode of action (Faria et al., 2021; Hsiao et al., 2023). In other cases, the use of commercial herbicide formulations, instead of the pure substance, prevents the adequate assessment of glyphosate toxic effects (Ait Bali et al., 2022; Aitbali et al., 2018; Hsiao et al., 2023). In this paper we explored one of the proposed mechanisms, namely the differential effects on gut microbiome, at environmentally relevant concentrations of pure glyphosate.

Sequence-based studies of intestinal bacterial communities in adult zebrafish reared in different lab facilities have revealed a core microbiota composed by Fusobacteriota and Proteobacteria (Roeselers et al., 2011; Stephens et al., 2015), the two prevalent phyla in the gut microbiome also in the present study. In the gut of the glyphosate-exposed fish, the predominant groups were still Fusobacteriota and Proteobacteria, although in this case the relative abundance of Fusobacteriota increased whereas the abundance of Proteobacteria decreased. These results are consistent with a recent report on zebrafish exposed for 21d to 3500 μg/L glyphosate in water, in which a similar decrease in Proteobacteria and increase in Fusobacteriota were found compared with the controls (Ding et al., 2021). The fact that glyphosate has a similar effect at 0.3 (present study) and 3500 (Ding et al., 2021) μg/L strongly suggest a high potency of glyphosate. Interestingly, a similar profile has been reported in zebrafish exposed to 0.42 μg/L oxytetracycline, a broad-spectrum antibiotic, for 6 weeks (Zhou et al., 2018). However, animals exposed to oxytetracycline exhibited also some evidences of inflammation of the intestine that were not found in the present study. Differences in the exposure time, 6 weeks vs 2 weeks, could be related with these differences in inflammatory response between both studies.

The overall changes induced by glyphosate in the fecal microbiome lead an increase in SCFAs in the feces, suggesting a higher short fatty acid production from glyphosate-resistant class II EPSPS bacteria or due to the substitution of *Aeromonas* and other taxa by *Cetobacterium,* known for producing high amounts of acetate (Wang et al., 2021). SCFAs produced by the gut microbiome are known to lead to an increase in the biosynthesis and release of systemic serotonin by EECs (Michaudel and Sokol, 2020). These cells express in the basolateral membrane the SCFAs receptors FFA2R (GPR43) and FFA3R (GPR41) (Lu et al., 2018). After activation by SFCAs these receptors induce an increase in cytosolic calcium leading to the release of serotonin and gut hormones such as glucagon-like peptide (GLP-1) and peptide YY (PYY) (De Vadder et al., 2014). Moreover, in their apical membrane they express the transient receptor potential ankyrin A1 (TRPA1), an irritant receptor activated by allyl-isothiocyanate (AITC), lipopolysaccharide (LPS), hydrogen sulfide (H2S), and, interestingly, by tryptophan catabolites such as indole and indole-3-carboxaldehyde (Nozawa et al., 2009). Activation of TRPA1 results in a Ca^{2+} influx and the release of serotonin by intestinal EECs in

human, mice and zebrafish (Ye et al., 2021). In spite to the observed increase in the levels of the SCFAs beta-hydroxy butyric, butyric, iso-butyric, and propionic acids in the feces of the glyphosate treated fish, it is not possible to preclude the precise molecule activating the MGB axis in glyphosate-treated fish. On one hand, all the available information on the signaling molecules of the zebrafish MGT axis has been obtained in 6 days post-fertilization (dpf) larvae (Ye et al., 2021, 2019), and it is not possible to discard differences in the expression patters of fatty acid and TPRA1 receptors between early larvae and adults. Unfortunately, our metabolomic target analysis was unable to identify some relevant signaling molecules, like indole and indole-3-carboxyaldehyde.

In mammals, vagal afferents convey the signals from intestinal EECs to the neurons of the nucleus of the solitary tract (NST), at the brainstem, and then, these neurons project to different monoaminergic nuclei at the mid- and forebrain. Moreover, the fact that long-term vagus nerve stimulation has been used as an adjunctive therapy for treatment of depression as a results of an increase in monoaminergic neurotransmission in the brain (Manta et al., 2013) demonstrate the relevant role of vagal afferents activation on serotonergic system in mammals. Although zebrafish has not NTS, there are molecular evidences that the secondary gustatory nucleus (SGN) is in this species is homologous to the NTS in mammals (Yáñez et al., 2017). Therefore, activation of vagal afferents by intestinal EECs should be able to modulate monoaminergic system in the zebrafish brain in a similar way as it does in mammals. In this study we have demonstrated an increased levels of dopamine and serotonin in the brain of the glyphosate-exposed fish, a result consistent with a previous report (Faria et al., 2021). In different studies performed on rodents, however, authors reported a decrease in these monoaminergic neurotransmitters in the brain after exposure to glyphosate (Hernández-Plata et al., 2015; Martínez et al., 2018). The high concentrations of glyphosate used in these studies, resulting in deleterious effect on dopaminergic and serotonergic neurons, could explain, at least partially, the observed differences between rodents and zebrafish.

Anxiety and social behaviors are modulated by an extremely complex interplay between the dopaminergic and serotonergic systems (Gordon and Hen, 2004; Miller, 2020; Moratalla et al., 2017), so the observed increase in the levels of these neurotransmitters in the brain of glyphosate exposed fish might be directly related with the changes in anxiety and social behaviors. These neurotransmitters systems play also an important role in the modulation anxiety and social behaviors by the microbiota-gut-brain axis (Dinan et al., 2015; Sherwin et al., 2019). The effect of glyphosate on anxiety-like behavior has been determined by analyzing geotaxis and using oxygen consumption as an additional marker of anxiety, as this emotional state often triggers an increase in respiration rate (Krohn et al., 2023). The positive geotaxis found here in the exposed fish, consistent with previous reports (Faria et al., 2021) along with the increase in MO_2 strongly support the anxiogenic effect of glyphosate. We have also found that glyphosate impairs social behavior in the social preference paradigm, as this chemical decreased the preference of the fish to spend more time close to conspecifics. Glyphosate has been previously reported to alter social behavior by increasing social cohesion in a shoal (Faria et al., 2021). Whereas the increase in the social cohesion can be only reflecting the anxiogenic effect of glyphosate, the decrease in the social preference observed in this study strongly suggest that glyphosate specifically targets social behavior.

5. Conclusions

In this work we observed that glyphosate-exposed fish present altered gut microbiomes, which increased their production of SCFAs relative to those from non-exposed animals. High levels of SCFA in the gut may explain their increased peripheral and brain serotonin levels and their increased dopamine levels in the brain (Sherwin et al., 2019), which in turn may relate to their altered anxiety and social behaviors. A similar concatenation of effects may explain similar effects observed in glyphosate-based pesticide-exposed mice (Aitbali et al., 2018). The observed increase in SCFAs in the gut may be a secondary consequence of the substitution of *Aeromonas* and *Undibacterium* species by *Cetobacterium*, among others. Whereas direct evidences on the release of serotonin by EECs or the activation of vagal afferents making synapses on these cells at the intestinal tract have not been provided, it rrepresents the only potential link connecting the increase in the levels of peripheral serotonin and the increase found in dopamine and serotonin levels in the glyphosate treated animals is the MGB axis. Therefore, gut dysbiosis induced by glyphosate is relevant enough to explain most, if not all, symptoms associated to the exposure to this herbicide observed in zebrafish, which may indicate the existence of new neurotoxicological modes of action that have been overlooked up to now.

CRediT authorship contribution statement

Bedrossiantz Juliette: Investigation, Methodology, Writing – original draft. **Zheng Jiamin:** Investigation, Supervision. **Mandal Rupasri:** Investigation, Methodology. **Wishart David S.:** Formal analysis, Supervision. **Gómez-Canela Cristian:** Funding acquisition, Methodology, Supervision, Writing – original draft, Writing – review & editing. **Vila-Costa Maria:** Conceptualization, Methodology, Supervision, Writing – original draft. **Prats Eva:** Methodology. **Pina Benjamin:** Conceptualization, Formal analysis, Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing. **Raldúa Demetrio:** Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Validation, Writing – original draft, Writing – review & editing. **Bellot Marina:** Investigation, Methodology, Writing – original draft. **Carrillo Maria Paula:** Investigation, Methodology, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Metagenomic and metabolic data have been deposited in appropriate public databases, as indicated in teh amterial and Methods section.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2023.115888.

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