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Investigating the effects of glyphosate on the bumblebee proteome and microbiota

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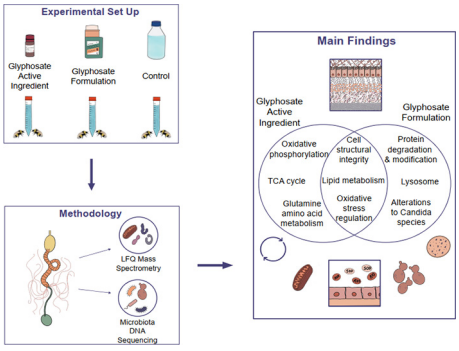
HIGHLIGHTS

- Little is known about glyphosate's impact on the bumblebee digestive tract.
- Mass spectrometry-based proteomics and DNA sequencing were utilized.
- Glyphosate impacts structural, metabolic, and oxidative stress proteins.
- Differences were observed between technical grade glyphosate and RoundUp Optima+®.
- RoundUp Optima+® altered the digestive tract fungal microbiota.

GRAPHICAL ABSTRACT



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ABSTRACT

Glyphosate is one of the most widely used herbicides globally. It acts by inhibiting an enzyme in an aromatic amino acid synthesis pathway specific to plants and microbes, leading to the view that it poses no risk to other organisms. However, there is growing concern that glyphosate is associated with health effects in humans and an ever-increasing body of evidence that suggests potential deleterious effects on other animals including pollinating insects such as bees. Although pesticides have long been considered a factor in the decline of wild bee populations, most research on bees has focussed on demonstrating and understanding the effects of insecticides. To assess whether glyphosate poses a risk to bees, we characterised changes in survival, behaviour, sucrose solution consumption, the digestive tract proteome, and the microbiota in the bumblebee *Bombus terrestris* after chronic exposure to field relevant doses of technical grade glyphosate or the glyphosate-based formulation, RoundUp Optima+®. Regardless of source, there were changes in response to glyphosate exposure in important cellular and physiological processes in the digestive tract of *B. terrestris*, with proteins associated with oxidative stress regulation, metabolism, cellular adhesion, the extracellular matrix, and various signalling pathways altered. Interestingly, proteins associated with endocytosis, oxidative phosphorylation, the TCA cycle, and carbohydrate, lipid, and amino acid metabolism were differentially altered depending on whether the exposure source was glyphosate alone or RoundUp Optima+®. In addition, there were alterations to the digestive tract microbiota of bees depending on the glyphosate source No impacts on survival, behaviour, or food consumption were observed. Our research provides insights into the potential mode of action and consequences of glyphosate exposure at the molecular, cellular and organismal level in bumblebees and highlights issues with the current honeybee-centric risk assessment of pesticides and their formulations, where the impact of co-formulants on non-target organisms are generally overlooked.

Abbreviations: AI, active ingredient; AOP, Adverse outcome pathway; LFQ, label-free quantitative; CF, commercial formulation; ROS, reactive oxygen species; RFC, relative fold change.

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1. Introduction

One of the most widely used pesticides in agricultural and non-agricultural landscapes is the non-selective, systemic herbicide glyphosate ((*N*-phosphonomethyl) glycine) (Benbrook, 2016; Maggi et al., 2019). When applied to plants, glyphosate acts by inhibiting an enzyme, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), specific to the shikimate pathway involved in plant aromatic amino acid metabolism (Steinrücken and Amrhein, 1980; Duke and Powles, 2008). The shikimate pathway is absent in animals, giving rise to the assumption that glyphosate exposure has little to no effect on non-target organisms. However, a growing body of evidence suggests that glyphosate can detrimentally affect animals. Numerous studies have now determined the impacts of glyphosate on learning and memory, reproduction, cell viability, the digestive tract microbiota and organ and mitochondrial function in a wide range of animals including mammals (George et al., 2010; De Liz Oliveira Cavalli et al., 2013; Ford et al., 2017; Bali et al., 2019; Tang et al., 2020; Mesnage et al., 2021), fish (Bridi et al., 2017; A. G. Pereira et al., 2018; Gaur and Bhargava, 2019; Panetto et al., 2019), and invertebrates (Herbert et al., 2014; Motta et al., 2018; Farina et al., 2019; Delkash-Roudsari et al., 2020; Gao et al., 2021).

Insect pollinators contribute an estimated $235–577 billion to the global economy each year through pollination services to crops (Klein et al., 2018; Gallai et al., 2009; Potts et al., 2017). They are also crucial for ecosystem functioning, as they are required for the reproduction of most flowering plants (Ollerton, 2017; Klein et al., 2018). Pollinator diversity and their associated ecosystem services, however, are threatened, as insect pollinator populations and diversity are widely reported to be in decline globally (Nieto et al., 2014; IPBES, 2019; Zattara and Aizen, 2021). Although insecticides have been implicated as one of the principal drivers of this decline, less is known about the impacts of non-insecticidal pesticides (Cullen et al., 2019). Given that glyphosate is one of the most widely applied pesticides worldwide (Benbrook, 2016; Maggi et al., 2019), which bees could be exposed to when foraging (Thompson et al., 2022), determining whether it has impacts on pollinators is essential.

The impact of non-insecticidal pesticides such as herbicides and fungicides on important pollinator groups including bees is beginning to receive attention (see review Cullen et al., 2019). Under certain conditions, glyphosate itself has been shown to impact bee survival (Dai et al., 2018; Almasri et al., 2020; Motta and Moran, 2020), learning and memory (Herbert et al., 2014; Mengoni Goñalons and Farina, 2018; Farina et al., 2019), brood development (Farina et al., 2019; Odemer et al., 2020), and immunity (Vazquez et al., 2018; Zhao et al., 2020; Almasri et al., 2021; Castelli et al., 2021; Motta et al., 2022). Additionally, glyphosate is known to affect the digestive tract microbiota of honeybees at some concentrations (Dai et al., 2018; Motta et al., 2018; Blot et al., 2019; Motta and Moran, 2020; Motta et al., 2020) which may have consequences for development, nutrition, and pathogen protection (Kwong et al., 2017; Zheng et al., 2017; Stefanini, 2018; Miller et al., 2021). Given that the shikimate pathway is present in microbes but not bees (Herrmann and Weaver, 1999) and the impacts of glyphosate on the digestive tract microbiota in honeybees, glyphosate may also result in alterations to the digestive tract microbiota of bumblebees. However, it is unknown whether these effects are typical for all bees as very little research exists that investigates the consequences of glyphosate exposure for bumblebees or solitary bees (Cullen et al., 2019). It is known that different species can display varying sensitivities to pesticides, and differences in lifecycle, habitat, and nutrition can impact risk severity, resulting in a need for pesticide risk assessments on a wider range of bee species (Arena and Sgolastra, 2014; Manjon et al., 2018).

One of the difficulties associated with assessing the potential risks of pesticides to insects relates to the presence of co-formulants in commercial formulations used in both agricultural and non-agricultural settings. Most testing conducted by regulatory bodies for negative impacts of pesticides on bees, for example, focuses on the active ingredient (European Commission, 2009; EFSA, 2012). However, most commercially available products include the active ingredient (AI) and co-formulants, such as surfactants, antifoaming agents, solvents, and dyes that improve the efficacy of the pesticide. In the European Union, risk assessment for regulatory approval of the pesticide active ingredient is assessed according to regulation (EC) No. 1107/2009. Individual co-formulants are not required to undergo the same risk assessment regimes for impacts on bees and risk assessment of formulations is only guaranteed if the formulation contains more than one active ingredient or has toxicity ≥ the active ingredient (The European Commission, 2013, 2009). In addition, formulations may not require testing for toxicity to honeybees if previously tested formulations contain co-formulants which are 'equivalent' – what qualifies as equivalent is not defined (The European Commission, 2009).

Although the effects of glyphosate-based commercial formulations on various nontarget organisms is now being increasingly investigated in academic research (Mesnage et al., 2013; Mesnage et al., 2015; J. L. Pereira et al., 2018; Lopes et al., 2018; Fantón et al., 2020; Pochron et al., 2020; Ruuskanen et al., 2020; Tóth et al., 2020; Zhao et al., 2020), whether these effects are attributable to the active ingredient or co-formulants is seldom determined. This represents a major knowledge gap in our attempts to mitigate the risks posed to pollinators by herbicides. Co-formulants are generally not subjected to the same testing rigour as the AI and where they have been tested, the focus has been on mortality, ignoring possible sublethal effects (Mesnage and Antoniou, 2018; Novotny, 2022; Straw et al., 2022). In addition, many co-formulants are considered proprietary information, making it difficult and sometimes impossible to assess co-formulant impact on the organism being studied (Mesnage and Antoniou, 2018).

We report here, for the first time, an assessment of the impacts of technical grade glyphosate and the glyphosate-based formulation, RoundUp Optima+®, at a field realistic concentration, on the bumblebee *Bombus terrestris*. We follow the adverse outcome pathway (AOP) framework developed by Ankley et al. (2010) which aims to understand and collate ecotoxicological effects of a chemical across different biological levels. We chose to focus on molecular, cellular, and organism level effects of glyphosate exposure on the bumblebee digestive tract by investigating alterations to survival, sucrose solution consumption, behaviour, the digestive tract proteome, and the digestive tract microbiota. We investigated the digestive tract because i) it represents one of the most important organs in the bee for nutrition and pathogen resistance, ii) it is the first point of contact between ingested pesticides and host cells, and iii) it houses an important and diverse microbiota. Using a framework such as the AOP when investigating the impacts of pesticides can provide a clear understanding of how and where a chemical affects an organism and identifies knowledge gaps and research priorities going forward. Molecular level effects of glyphosate were determined using label-free quantitative (LFQ) proteomics (Cox and Mann, 2007; Walther and Mann, 2010) on total protein extracts derived from the digestive tracts of bees after glyphosate or RoundUp Optima+® exposure. This approach permits the identification and quantification of thousands of proteins from a single biological sample, which enables the assessment of physiological changes at the molecular and cellular phenotype level and provides insight at post-transcriptional and translational levels. To further assess the impact of glyphosate (either alone or in formulation) on *B. terrestris*, we conducted survival, behavioural, and consumption assays in addition to 16S and ITS DNA sequencing of DNA extracted from the digestive tract to determine whether glyphosate impacts the *B. terrestris* digestive tract microbiota. Combined, it is anticipated that this multi-level analysis will provide insight into the impacts of glyphosate exposure and the mechanisms behind them (if any) on an ecologically important organism, one that is missing the primary target pathway of this herbicide. Such an approach will also determine whether differences are observed among bees exposed to glyphosate alone or a glyphosate-based formulation, and therefore highlight the potential risks posed by co-formulants.

2. Materials and methods

2.1. Exposure assays for survival analysis

Exposure of bumblebees to glyphosate active ingredient and formulation were carried out following modified OECD 245 guidelines (OECD,

2017) in 2020. Seventy-two bees were chosen randomly from each of four origin commercial colonies (Biobest, Agralan Ltd) and evenly allocated to group isolation chambers resulting in 12 bees/chamber. Each group isolation chamber corresponded to one of six treatments or control: 40 % (w/v) sucrose solution, 1 ppm, 10 ppm or 100 ppm technical grade glyphosate (PESTANAL®, analytical standard, Sigma-Aldrich International GmbH, ≤100 % (phosphonomethyl)glycine) dissolved in 40 % (w/v) sucrose solution, or 1 ppm, 10 ppm or 100 ppm Roundup Optima + ® (18.5 % potassium salt of N-(phosphonomethyl)glycine, <5 % alkylpolyglycoside, <1 % nitroryl, water and >75.5 % water and minor formulating ingredients) dissolved in 40 % (w/v) sucrose solution. The concentrations of RoundUp Optima + ® were calculated based on glyphosate acid content in the formulation so that both the technical grade glyphosate and RoundUp Optima + ® treatments contained the same concentration of glyphosate. Concentrations were chosen based on a conservative field-realistic concentration of 1 ppm (Thompson et al., 2014; Cebotari et al., 2018) with increments by a factor of 10 to determine if higher concentrations could lead to significant mortality. Roundup Optima + ®, a commonly used glyphosate formulation for domestic garden use, was purchased from a local retail outlet in Ireland. All stock solutions were made up in 40 % (w/v) sucrose solution. Group isolation chambers consisted of plastic chambers (11 cm × 7.5 cm × 17.5 cm) with a top-facing lid and a mesh lining for waste to fall through to a plastic bin. Bees had ad libitum access to feeding tubes (15 ml falcon tubes with 2 mm feeding holes) filled with 40 % (w/v) sucrose solution. Group isolation chambers were acclimatised and maintained at 23 ± 3 °C and 58 ± 6 % relative humidity for the duration of the experiment and were continuously kept in the dark. After overnight acclimatisation, each group had ad libitum access to their respective treatments in feeding tubes as described above and were given a fresh suspension every day for the duration of the experiment (ten days). Bee mortality and behaviour were recorded every 24 ± 2 h from the first exposure time for the duration of the exposure (See behaviour descriptions in Table S1). This experiment was carried out three separate times over the course of four weeks and observations from each experiment were used to determine any statistically significant differences in mortality or behaviour.

Statistical analyses were performed using Minitab® 20.3. Normal distribution was confirmed using an Anderson-Darling test. Survival data was analysed using a Kaplan-Meier analysis followed by Log-Rank and Wilcoxon tests to determine if there were statistically significant differences in survival rates between treatments. Behavioural observations were analysed using a generalized linear model with treatment as the only factor.

2.2. Exposure assays for proteomic analysis

2.2.1. Exposure assay

Exposures of bumblebees to glyphosate active ingredient and formulation for digestive tract proteome analysis were carried out following modified OECD 245 guidelines (OECD, 2017) in 2019. Nine bees were randomly chosen from each of four origin commercial colonies (Biobest, Agralan Ltd), with three bees per origin colony allocated to one of three group isolation chambers, in a similar way to Section 2.1. Each group isolation chamber comprised 12 bees in a plastic chamber (17 cm × 14.7 cm × 8.5 cm) with a top facing lid lined with autoclaved sand. Each isolation chamber corresponded to one of the three treatments: 40 % (w/v) sucrose solution, 1 ppm glyphosate (hereafter referred to as active ingredient, AI) or 1 ppm Roundup Optima + ® (hereafter referred to as commercial formulation, CF) dissolved in 40 % (w/v) sucrose solution in feeding tubes as described above.

A conservative and field realistic dose of 1 ppm was selected for this experiment, based on the lowest concentration found in the crops of honeybees after consumption of treated forage, and concentrations found in untreated tree flowers, reported in Thompson et al. (2014) and Cebotari et al. (2018), respectively. Bees had ad libitum access to feeding tubes filled with 40 % (w/v) sucrose. All bees were acclimatised and kept at 20 ± 2 °C and 58 ± 5 % relative humidity for the duration of the experiment and were continuously kept in the dark. After overnight acclimatisation, each group had ad libitum access to their respective treatments in feeding tubes as described in 2.1 and were given a fresh suspension every day for the duration of the experiment (five days). We deviated from the OECD exposure length of 10 days to an exposure length of 5 days as bees can realistically be exposed to glyphosate via pollen and nectar of treated plants for this period (Thompson et al., 2022; Thompson et al., 2014). Bees may also be exposed to glyphosate for longer periods from non-target plants containing glyphosate in pollen or nectar (Cebotari et al., 2018), but the prevalence of this phenomenon is not well documented. Bees were briefly observed every day and mortality was recorded every 24 ± 2 h from the first exposure time for the duration of the experiment.

2.2.2. Proteomic sample preparation and mass spectrometry

Eight bees were randomly selected from each group isolation chamber after the five-day exposure. Bees were administered CO_2 and their digestive tracts were dissected directly below the crop to include the proventriculus to the end of the colon. The dissected digestive tracts were lifted from the abdomen with sterilized tweezers and placed into ice-cold lysis buffer comprising 6 M urea, 2 M thiourea and 1 tablet of Complete™, Mini Protease Inhibitor Cocktail (Roche Diagnostics), snap frozen in liquid nitrogen and kept at −20 °C. Once all tissue samples were dissected, samples were thawed on ice and homogenized for 30 s each. Samples were subsequently sonicated twice for 15 s and centrifuged at 9000 rpm for five minutes at 4 °C to pellet any remaining cellular debris. The supernatants were aliquoted in to clean 1.5 ml tubes and stored at −70 °C.

Protein quantification was conducted using Qubit® Quant-IT™ protein assay kit on a Qubit® fluorometer version 2.0 following manufacturer guidelines. 100 μg of protein was removed from each sample and processed with a 2-D clean up kit (GE HealthCare), following manufacturer guidelines, to remove biological impurities. The resulting pellet was resuspended in 50 μl resuspension buffer (6 M urea, 0.1 M Tris HCl, pH 8.0), of which 20 μl was used for protein digestion. 115 μl of 50 mM ammonium bicarbonate was added to each sample. Proteins were reduced and alkylated by adding 0.5 M dithiothreitol (DTT) at 56 °C for 20 min followed by 0.5 M iodoacetamide (IAA) and incubating at room temperature for 15 min in the dark. 1 μl of 1 % (w/v) Protease Max (Promega) and 1 μl sequence grade trypsin (Promega) were added to each sample and incubated at 37 °C for 16 h. Subsequently, 1 μl of 100 % trifluoroacetic acid (TFA) was added to each sample to terminate digestion and the samples were incubated at room temperature for five minutes and centrifuged at 10,800 rpm for 10 min. The resulting supernatants were purified using Pierce C18 spin columns (Thermo Scientific) following manufacturer guidelines and the eluted purified peptides were dried down using a speedy vacuum concentrator (Thermo Scientific Savant DNA 120) and stored at 4 °C. Peptides were resuspended in a volume of loading buffer (2 % (v/v) acetonitrile and 0.05 % (v/v) TFA) to yield a concentration of 0.5 μg/μl, sonicated for two minutes and centrifuged at 13,400 rpm for five minutes. The supernatant was used for LC MS/MS.

1 μg of peptide mix for each sample was eluted onto the Q-Exactive (Thermofisher Scientific, USA) high resolution accurate mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system. An increasing acetonitrile gradient was used to separate peptides on a Biobasic C18 Picofrit™ column (200 mm length, 75 mm ID), using a 120-min reverse phase gradient at a flow rate of 250 nl/min. All data were acquired with the mass spectrometer operating in automatic data dependent switching mode. A high-resolution MS scan (300–2000 Da) was carried out using the Orbitrap to select the 15 most intense ions prior to MS/MS. MaxQuant version 1.6.17.0 (www.maxquant.org) was used for protein identification and LFQ normalisation of all MS/MS data. The Andromeda search algorithm in MaxQuant was used to correlate all MS/MS data against protein reference sequences obtained from the National Centre for Biotechnology to correlate the data against the protein reference sequences derived from the *B. terrestris* genome (Sadd et al., 2015) obtained from the National Centre for Biotechnology Information (NCBI) repository (17,508 entries, downloaded September 2021). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium

via the PRIDE partner repository with the dataset identifier PXD036578 (Perez-Riverol et al., 2021).

2.2.3. Proteomic data analysis

Perseus version 1.6.1.1 was used for data visualization and statistical analysis. Normalized LFQ intensity values were used as a measure of protein abundance. The data was filtered for the removal of contaminants and peptides identified by site. LFQ intensity values were $\log_2$ transformed and samples were allocated to groups corresponding to treatment. Proteins absent from any samples in at least one group were not used for further analysis. A data imputation step was conducted to replace missing values with values that simulate signals of low abundant proteins chosen randomly from a distribution specified by a downshift of 2.1 times the mean standard deviation (SD) of all measured values and a width of 0.1 times this SD. Normalized intensity values were used for principal component analysis. A two-sample *t*-test was performed using a cut-off value of $p \leq 0.05$ to identify statistically significant differentially abundant (SSDA) proteins. Volcano plots were produced by plotting –Log *p*-values on the y-axis and Log_2 fold-change values on the x-axis to visualize differences in protein abundance between treatment groups. Hierarchical clustering of SSDA proteins was performed using z-score normalized intensity values to produce a heat map of protein abundance.

2.2.4. Functional annotation

The Search Tool for the Retrieval of Interacting Genes/proteins (STRING) version 11 (Szklarczyk et al., 2019) (www.string-db.org) was used to map protein-protein interaction networks. Protein sequences were inputted into the STRING database and protein-protein interactions were analysed using the homologous *Apis mellifera* and *Drosophila melanogaster* match for each identified *B. terrestris* protein. KEGG (https://www.genome.jp/kegg/) IDs were used for GO enrichment to identify enriched biological pathways. Uniprot and QuickGO (www.uniprot.org; www.ebi.ac.uk/QuickGO) were used to identify the associated biological processes, molecular functions, and cellular compartments for SSDA proteins. Interpro (v.67.0) (www.ebi.ac.uk/interpro) was used to characterise protein superfamily and domain homologies of uncharacterised proteins to determine potential protein identity and function.

2.3. Exposure assay for microbiota and sucrose solution consumption analysis

2.3.1. Exposure assay

Exposures of bumblebees to glyphosate active ingredient or formulation for consumption and microbiota analysis were carried out following modified OECD 245 guidelines (OECD, 2017) in 2021. Six bees were randomly selected from each of five origin colonies (Biobest, Agralan Ltd) with bees evenly spread across three group isolation chambers resulting in 10 bees per isolation chamber. Isolation chambers consisted of plastic chambers (11 cm × 7.5 cm × 17.5 cm) with a top-facing lid and a mesh lining for waste to fall through to a plastic bin. Treatment exposures were carried out as outlined in Section 2.2.1. A further three isolation chambers were assembled without bees with the control 40 % (w/v) sucrose solution to measure evaporation. All bees were acclimatised to 25 ± 3 °C and 77 ± 10 % relative humidity for the duration of the exposure and were continuously kept in the dark. After overnight acclimatisation, each group had ad libitum access to their respective treatments in feeding tubes as described above and were given a fresh suspension every day for the duration of the experiment (five days). Three empty control chambers each contained the control solvent, 40 % sucrose solution, in the same feeding tubes administered to bees to determine evaporation. All feeding tubes were weighed at each observation point and directly after fresh serving of treatment each day. Bee mortality and consumption were recorded every 24 ± 2.5 h from the first treatment exposure time for the duration of the exposure. At the end of exposure, bees were individually placed into Eppendorf tubes lined with tissue and placed at −70 °C until DNA extraction. Consumption was analysed as the difference in feeding tube weight at each observation. The average evaporation each day was deducted from the weight of feeding tubes accessed by bees. A two-sample *t*-test was performed to determine any statistical significance in consumption between treatments using Minitab® 20.3.

2.3.2. DNA extraction

DNA was extracted from individual digestive tracts using the DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's instructions except for the following changes. Digestive tracts were homogenized in 200 μl buffer ATL followed by the addition of 50 μl 10 mg/ml lysozyme. Samples were incubated for 30 min at 37 °C and vortexed every five minutes at 800 rpm. 300 mg glass beads and 200 μl buffer AL were added to each sample and samples were bead-beat for five minutes at 30 Hz. Samples were incubated at 56 °C and vortexed at 0 and 30 min at 800 rpm following the addition of 50 μl proteinase K. Extracted DNA was stored at −20 °C before submission to NovoGene for 16S and ITS amplicon sequencing to determine the bacterial and fungal species present. Seven samples per pesticide treatment, and eight control samples, were sequenced.

2.3.3. PCR amplification, library preparation, sequencing, and analysis

PCR amplification, library preparation, and sequencing were carried out at Novogene Europe (UK) Ltd. PCR amplification was carried out using primers specific for 16S (V4) and ITS (ITS1-5F) regions connecting with barcodes. 250 bp paired-end raw reads were generated from libraries sequenced on a paired-end Illumina platform NovaSeq 6000. Raw reads were filtered to obtain high-quality clean reads according to the Qiime (version 1.7.0) (Caporaso et al., 2010). Sequencing data are available from NCBI BioProject PRJNA878707. The SILVA138 database (http://www.arb-silva.de/) was used as a reference database for tag comparison using UCHIME algorithm (Edgar et al., 2011) to detect and remove chimera sequences. On the remaining tags, Uparse software (Uparse version 7.0.1090) was used to determine sequences with >97 % similarity and these were clustered into operational taxonomic units (OTUs). A representative sequence for each OTU was screened using Qiime (version 1.7.0) in Mothur method against SSUrRNA database from the SILVA138 database for species annotation. MUSCLE (version 3.8.31) was used to determine phylogenetic relationships of all OTU representative species. The data was normalized to obtain OTU abundance information and subsequent alpha and beta diversity analysis were carried out using this data. Wilcox and Tukey tests were performed to analyse the significance of differences between treatment groups.

Taxonomy trees were created using independent RandD software showing the top 10 genera in high relative abundance (DeSantis et al., 2006). The relative abundances of the top 10 genera of each group were displayed to demonstrate how they differ between treatment groups. Various indices were used to determine differences in alpha diversity between treatment groups and weighted unifrac distances were used to determine beta diversity between treatment groups using the square matrix of distance between samples. Alpha and beta diversity were calculated using QIIME (version 1.7.0) and displayed with R software (version 2.15.3). Analysis of similarity (Anosim) and multi-response permutation procedure (MRPP) analysis were carried out to determine whether community structure was significantly different between or within treatment groups. A *t*-test was used to determine significant species variation between groups ($p < 0.05$) at various taxon ranks.

3. Results

3.1. Survival assays

No significant differences were observed in survival between any of the pesticide treatments in comparison to the control treatments (Log-rank $p = 0.416$, Wilcoxon $p = 0.436$, Fig. S1, Table S2). Generalized linear model analysis determined there were no differences in the frequency of behavioural observations between bees exposed to any treatment (Table S4).

3.2. The effects of glyphosate and Roundup optima + ® on sucrose solution consumption

A two-sample t-test did not determine any statistically significant differences in consumption of the AI ($p = 0.124$) or CF ($p = 0.623$) in comparison to the control group. In addition, there were no statistically significant differences in consumption between either glyphosate treatment ($p = 0.27$) (Table S3; Table S4).

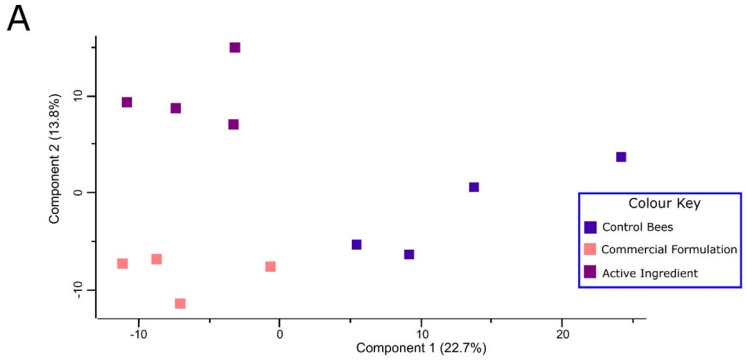
3.3. LFQ analysis of glyphosate exposure on the bumblebee digestive tract

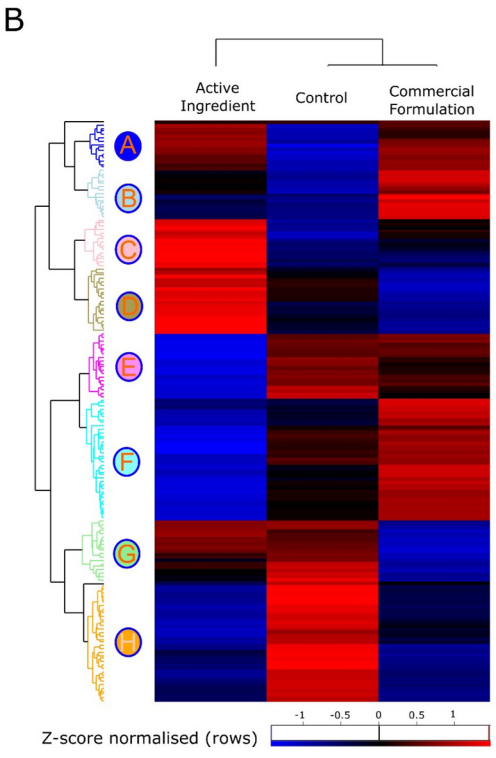
3.3.1. Identified and quantified proteins

In total, 2613 proteins were originally identified from proteins extracted from the *B. terrestris* digestive tract, of which 1365 proteins remained after filtering (Table S5). Principal component analysis (PCA) on all proteins resolved a clear difference between the three treatment groups (Fig. 1A). Variance of both glyphosate-based treatments AI and CF compared to the control sample indicate a clear difference between glyphosate-treated and control samples. There is also a distinct variance between the proteomes of AI and CF bee digestive tracts, demonstrated by the separate clustering of AI samples and CF samples in the principal component analysis (Fig. 1-A).

3.3.2. Hierarchical clustering and gene ontology enrichments

Hierarchical clustering was performed on mean z-scored, normalized LFQ values for 178 statistically significant proteins (ANOVA, Ben-Ho FDR <0.05), which resolved eight clusters (A-H). Each cluster represents a group of proteins with similar expression profiles in the AI, CF, or control-treated digestive tract (Fig. 1-B, Table S6). Proteins in each cluster were analysed using the STRING database using the equivalent *D. melanogaster* Uniprot identifiers to resolve enriched (FDR < 0.05) processes and pathways affected within or across treatments (Fig. 1-C; Table S7). Cluster A comprised 14 proteins with an increased abundance in both AI and CF-treated groups compared to the control group, with enrichment for hydrolases (five proteins). Cluster B included 15 proteins, four and seven of which were associated with nucleotide metabolic processes and hydrolase activity, respectively. Cluster D comprised 20 proteins with an increased abundance in the AI-treated group, a decreased abundance in the control-treated group, and a further decreased abundance in the CF-treated group. These proteins were associated with glycolysis/gluconeogenesis and co-factor metabolic processes (two proteins each). Cluster E contained 20 proteins with a decreased abundance in the AI-treated group compared to CF and control groups, nine of which were associated with the mitochondrion and several proteins were associated with glutamine metabolism. Cluster F contained 37 proteins with a low abundance in the AI-treated group, a slightly higher abundance in the control-treated group, and an increased abundance in the CF-treated group. Enriched processes in this group included fatty acid beta-oxidation (three proteins), oxidation-reduction processes (two proteins), mitochondrial transport (two proteins) and the TCA cycle/respiratory electron transport (three proteins). In total 19 proteins in cluster F were associated with the mitochondrion and when combined with cluster E (both clusters comprising proteins with reduced abundances in the AI-treated group), highlight the dramatic effect of the AI on mitochondrial processes. Cluster G comprised 19 proteins with a low abundance in the CF-treated group compared to AI and control-treated groups, with significant enrichment in proteins associated with the fusome (two proteins). Cluster H consisted of 36 proteins with a low abundance in both glyphosate-based treatment groups in comparison to the control-treated group, with proteins associated with endocytosis (three proteins), regulation of intracellular pH (two proteins) and the plasma membrane (six proteins). There were no significant enrichments in cluster C, which included 15 proteins with an increased abundance in the AI-treated group compared to CF and control-treated groups.





C

Cluster	Term description	Database	Term ID	FDR
A	Hydrolase	Uniprot	KW-0378	0.0232
B	Nucleotide metabolic process	Biological Process	GO:0009117	0.0495
	Hydrolase activity	Molecular Function	GO:0016787	1.91E-05
C	No significant enrichments			
D	Glycolysis / Gluconeogenesis	KEGG	dme00010	0.0042
E	Glutamine amino acid metabolism	Biological process	GO:0009064	0.00074
	Pyruvate metabolism	KEGG	dme00620	0.00021
	Aldehyde dehydrogenase family	PFAM	PF00171	0.0027
F	Mitochondrion	Cellular component	GO:0005739	1.66E-17
	Fatty acid beta-oxidation	Biological process	GO:0006635	2.34E-09
G	Lysosome	KEGG	dme04142	0.034
	Endocytosis	KEGG	dme04144	0.034
H	Endocytosis	Biological process	GO:0006897	0.0066
	Plasma membrane	Cellular component	GO:0005886	0.0171

Fig. 1. Label free quantitative mass spectrometry-based proteomic analysis of the digestive tracts of *Bombus terrestris* exposed to 1 ppm glyphosate active ingredient, 1 ppm glyphosate-based commercial formulation RoundUp Optima + ® or 40 % (w/v) sucrose solution control for five days. (A) Principal component analysis (PCA) of digestive tracts from bees exposed to both glyphosate-based treatments and the control. Each treatment is distinguished from one another, grouping with samples from the same treatment. (B) Hierarchical clustering of z-score normalized values cluster the median protein expression values of SSDA proteins with a similar expression pattern in each treatment. (C) A table displaying GO terms associated with each cluster from hierarchical clustering of protein expression values in B.c.

3.3.3. Two-sample t-tests

Two sample t-tests were performed among treatment groups to determine statistically significant differentially abundant (SSDA) proteins (FDR = 0.05, S0 = 0.1) and their relative fold differences (Table S8). STRING analysis was conducted on SSDA lists to identify pathways, processes, GO, KEGG terms and protein networks that were enriched in one protein set over another.

3.3.4. AI versus control

A total of 152 SSDA proteins (relative fold change (RFC) range: −222.68 to +18.07) were identified from the digestive tracts of AI-exposed bees in comparison with control-treated bees, with 56 and 96 proteins having an increased and decreased abundance respectively. The top 10 proteins with the highest increased abundance included the hydrolase glucosylceramidase (RFC: +18.07), an oxidase; laccase-1 (RFC: +9.56), a venom acid phosphatase; venom acid phosphatase Acph-1 (RFC: +2.78), the lysosomal protein; beta-hexosaminidase subunit (RFC: +2.02), the digestive serine protease; chymotrypsin (RFC: +1.50), a protein with carboxylic ester hydrolase activity; venom carboxylesterase-6 (RFC: +1.36), and two uncharacterized proteins: uncharacterized protein LOC100646617 (RFC: +2.82) and uncharacterized protein LOC100646009 (RFC: + 1.32) which has glutathione transferase and peroxidase activity. There were also proteins associated with 60S ribosomal subunit biogenesis (nucleolar protein 58, RFC: +5.28) and calcium homeostasis (regucalcin, RFC: +2.23). The top 10 proteins with decreased abundance included the transmembrane protein CD151 antigen (RFC: −10.43) and an uncharacterized protein; uncharacterized protein LOC105666313 (RFC: - 68.82). There were also proteins associated with microfibril formation (fibrillin-2, RFC: - 222.68), the basement membrane (collagen alpha-5(IV) chain, RFC: - 9.16 and collagen alpha-1(IV) chain, RFC: - 5.23), proteolysis and ubiquitin-mediated protein catabolism (uncharacterized protein LOC100644923, RFC: - 8.62, ubiquitin carboxyl-terminal hydrolase, RFC: - 10.42 and S-phase kinase-associated protein 1, RFC: - 5.67), cell adhesion (cartilage oligomeric matrix protein, RFC: 6.80) and transmembrane transport (sodium-coupled monocarboxylate transporter 2, RFC: - 8.37).

3.3.5. CF versus control

A total of 119 SSDA proteins (RFC range: −56.83 to +9.69) were identified from the digestive tracts of CF-exposed bees in comparison with control-treated bees, with 47 and 72 proteins having increased or decreased abundance, respectively. The top 10 proteins with the highest abundance in comparison to the control included: a hydrolase involved in sphingolipid metabolism, glucosylceramidase (RFC: +9.69), an oxidase; laccase-1 (RFC: +8.31), a serine-type peptidase; venom dipeptidyl peptidase 4 (RFC: +5.64) and an uncharacterized protein containing a chitin-binding type 4 domain; uncharacterized protein LOC100648508 (RFC: +7.30). There were also proteins associated with carboxylic ester hydrolase activity (venom carboxylesterase-6, RFC: +7.37), cellular protein modification (ubiquitin protein 5, RFC: +3.68), integral component of membrane (transmembrane protein 177, RFC: +3.27), mitochondrial respiratory chain complex I assembly (FAD-dependent oxidoreductase domain-containing protein 1, RFC: +3.15), trehalose metabolic process (trehalase, RFC: +2.33), and calcium homeostasis (regucalcin, RFC: +2.33). Of the top 10 proteins with the lowest abundance in comparison to the control there were the transmembrane protein CD151 antigen (RFC: −9.68) and an uncharacterized protein; uncharacterized protein LOC105666313 (RFC: −25.35). Other proteins were associated with microfibril formation (fibrillin-2, RFC: −56.83), lipid catabolic process (pancreatic lipase-related protein 2-like protein, RFC: −21.15), translation (eukaryotic translation initiation factor 2 subunit 1, RFC: −14.61), proteolysis (uncharacterized protein LOC100644923, RFC: −10.83), Arp2/3 complex-mediated actin nucleation (actin-related protein 2/3 complex subunit 3, RFC: −8.66), cell adhesion (cartilage oligomeric matrix protein, RFC: −6.79), signalling (protein slit-like isoform X1, RFC: −6.71) and, the basement membrane (collagen alpha-5(IV) chain protein, RFC: -5.52).

3.3.6. CF versus AI

A total of 149 proteins (RFC range: −92.29 to +14.63) were identified from the digestive tracts of CF-exposed bees in comparison with AI-exposed bees, with 83 and 66 proteins having an increased and decreased abundance, respectively (Fig. 2). Of the top 10 proteins with the highest

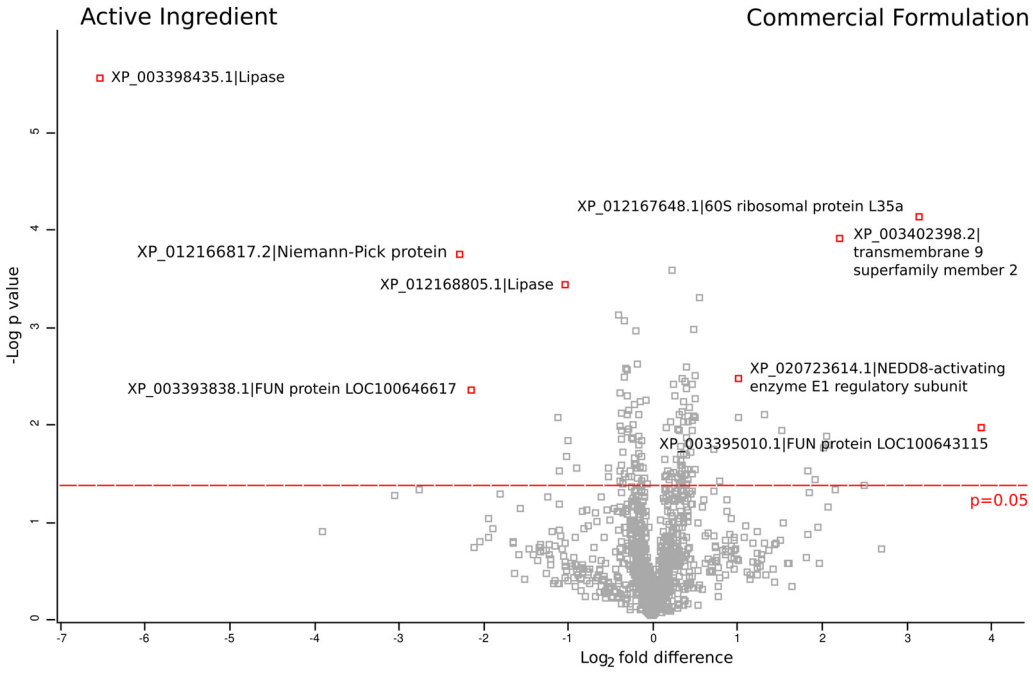


Fig. 2. A two-sample *t*-test was performed to resolve and compare statistically significant differentially abundant (SSDA) proteins in the digestive tract of *Bombus terrestris* exposed to either glyphosate commercial formulation (RoundUp Optima+®) or glyphosate active ingredient (FDR = 0.05, S0 = 0.01). Both treatments were compared based on their -log p value (p = 0.05) and Log2 fold differences. Proteins with p ≥ 0.05 were determined to be SSDA. Compared to the active ingredient, the digestive tract proteome of bees exposed to the commercial formulation had its largest abundance increases in proteins associated with the ribosome and protein degradation/modification (right) and its lowest decreases in proteins associated with lipid metabolism and transport (left).

abundance in the digestive tracts of CF-exposed bees compared to the AI-exposed bees, there were: a transmembrane helix protein (transmembrane protein 256, RFC: + 3.55), protein NipSnap (RFC:+ 4.17), beta-lactamase (RFC: + 4.03) and two uncharacterized proteins; uncharacterized protein LOC100643115 (RFC: +14.63) and uncharacterized protein LOC100648508 (RFC: + 2.86), which has a chitin-binding type-4 domain. There were also proteins associated with translation (60S ribosomal protein L35a, RFC: +8.81), ubiquitin-dependant protein catabolic process (ubiquitin carboxyl-terminal hydrolase, RFC: + 5.65), protein localization to the membrane and negative regulation of peptidoglycan recognition protein signalling pathway (transmembrane 9 superfamily member 2, RFC: + 4.60), carbohydrate metabolic process (myrosinase 1, RFC: + 4.17) and mitochondrial respiratory chain complex I assembly, with oxidoreductase activity (FAD-dependent oxidoreductase domain-containing protein 1, RFC: + 3.78). Of the top 10 most decreased proteins in CF-exposed compared to AI-exposed bees, there were: two uncharacterized proteins (uncharacterized protein LOC100651249, RFC: −2.17 and uncharacterized protein LOC100646617 (RFC: - 4.46) and putative cysteine proteinase CG12163 (RFC: −1.44) which has cysteine-type peptidase and endopeptidase inhibitor activity. There were also proteins associated with sphingolipid metabolic process (glucosylceramidase, RFC: −1.86), ubiquitin-dependent protein catabolic process (E3 ubiquitin-protein ligase UBR4 isoform X1, RFC: - 2.01), carbohydrate metabolic process (L-lactate dehydrogenase-like isoform X2, RFC: - 2.04), lipid transporter activity (Niemann-Pick type protein homolog 1B, RFC: - 4.88) and three pancreatic lipases which are associated with lipid catabolic processes (RFC: - 2.05, − 2.15 and − 92.29).

3.3.7. Proteins with common abundance profiles across both glyphosate-based treatments

To investigate whether a consistent effect of glyphosate exposure could be identified, SSDA protein sets from both glyphosate treatments versus the controls were compared to find common proteins (Table 1). In total eight and 29 proteins were found in common in the increased and decreased glyphosate-treated digestive tracts, respectively. One of the major categories affected by both glyphosate-based treatments was cell structural integrity, comprising 11 proteins with lower abundance, some of which had the greatest fold change differences identified in the experiment. These included fibrillin, CD151 antigen, two collagen proteins and a cartilage matrix protein (combined fold change range of 3.7 to 222.7 across both treatments). 6 proteins associated with metabolism (4 amino acid/carbohydrate metabolism and 2 lipid metabolism) and 5 proteins associated with transport displayed similar fold change values and directions. Categories such as cell signalling, calcium transporting/signalling, gene regulation and protein modification/degradation all had multiple proteins with lower abundances in glyphosate-exposed bees whereas proteins associated with oxidative stress regulation had higher abundances. Individual proteins associated with immunity/detoxification, protein modification and cell migration guidance were identified as having a conserved response and one function-unknown protein (XP_012169860.2) had considerable relative fold change differences of 68.8 and 25.4 in the AI and CF-exposed digestive tracts, respectively, highlighting a potentially novel association with xenobiotic exposure.

Table 1
Conserved response to glyphosate. Functional categories, relative fold changes, MS measurements and characteristics for all statistically significant differentially abundant proteins with similar expression profiles in both active ingredient and commercial formulation – based glyphosate treated bees. Relative fold changes and directions are determined against the procedural control.

Functional annotation	Protein ID	Annotation	RFC AI	RFC CF	FC Direction	No. of peptides	Mol. wt [kDa]	MS/MS count
Cell structural integrity	XP_003399227.2	Fibrillin-2	222.7	56.8	Down	90	314.2	329
	XP_003393881.1	CD151 antigen	10.4	9.7	Down	4	26.5	16
	XP_003399666.1	Collagen alpha-5(IV) chain	9.2	5.5	Down	8	185.2	68
	XP_003399665.1	Collagen alpha-1(IV) chain	5.2	3.7	Down	15	193	197
	XP_003397666.1	Cartilage oligomeric matrix protein	6.8	6.8	Down	8	130.7	15
	XP_003392951.1	Protein BCCIP homolog	2.9	3.2	Down	4	34.4	22
	XP_012174910.1	Basement membrane-proteoglycan	2.5	2.1	Down	158	486	1519
	XP_012164850.1	Protein mesh	1.2	1.2	Down	50	153.9	495
	XP_003394511.1	23 kDa integral membrane protein	1.4	1.3	Down	3	27.2	27
	XP_012172164.1	DE-cadherin	1.3	1.2	Down	19	166.6	104
	XP_003397680.1	Innexin inx7	1.6	1.3	Down	9	45.9	55
Transport	XP_003402688.1	Facilitated trehalose transporter Tret1	4.7	5.2	Down	7	51	29
	XP_003399193.1	Na-independent sulfate anion transporter	4.4	3.6	Down	8	72.6	27
	XP_003396344.1	Organic cation transporter protein	3.4	2.7	Down	4	63	15
	XP_003398230.1	Anion transporter family member 2A1	2.9	2.1	Down	11	79.9	83
	XP_003398587.1	Choline transporter 1	1.5	1.5	Down	5	67.7	41
Metabolism	XP_003397815.1	Beta-hexosaminidase subunit beta	2	2.2	Up	23	61.9	218
	XP_003398461.1	Succinate-semialdehyde dehydrogenase	1.4	1.2	Down	30	56.2	239
	XP_020718677.1	Amino acid transport protein rBAT	1.2	1.3	Down	10	69	64
	XP_003401829.1	ENT diphosphohydrolase 5	1.1	1.2	Up	16	52.6	130
Lipid metabolism	XP_003402892.1	Glucosylceramidase	18.1	9.7	Up	13	58.1	42
	XP_003402390.3	FGGY carbohydrate kinase	1.3	1.1	Up	10	31.1	90
Protein modification/degradation	XP_003394859.1	Uncharacterized protein LOC100644923	8.6	10.8	Down	4	263.2	15
	XP_012174330.1	S-phase kinase-associated protein 1	5.7	4.7	Down	3	18.5	15
Gene regulation	XP_012164476.1	Regulator of chromosome condensation	4.7	3.3	Down	7	46.2	19
	XP_012175550.1	Histone H2B	1.1	1.1	Down	7	13.7	88
Cell signalling	XP_003401488.1	Uncharacterized protein LOC100644037	2.9	3.7	Down	7	75.7	19
	XP_003394892.1	Tubulointerstitial nephritis antigen	1.7	1.5	Down	26	51	266
Calcium transport/signalling	XP_003401730.1	Regucalcin[a]	2.2	2.3	Up	19	36.9	227
	XP_020722918.1	Calcyphosin	1.6	1.7	Down	9	25.1	36
	XP_012175018.1	Plasma membrane Ca-transporting ATPase	1.5	1.4	Down	21	139.1	144
Oxidative stress regulation	XP_003399739.1	Venom carboxylesterase-6	1.4	1.4	Up	14	65.6	116
	XP_003397315.1	Superoxide dismutase [Cu-Zn]	1.1	1.2	Up	13	15.6	218
Function Unknown	XP_012169860.2	Uncharacterized protein LOC105666313	68.8	25.4	Down	7	30.7	53
Immunity/Detoxification	XP_003394143.1	Laccase-1	9.6	8.3	Up	6	76	27
Protein modification	XP_003393428.1	Protein phosphatase methylesterase 1	3	3.1	Down	8	41.8	20
Guidance	XP_003403082.1	Protein slit	4.7	6.7	Down	10	109.1	26

[a] Also involved in lipid metabolism.

3.4. The effects of glyphosate and Roundup optima+® on the digestive tract microbiota

2,128,021 effective tags representing the 16S rRNA V4 gene amplicon region were filtered out from 2,204,903 raw tags. For the ITS (ITS1-5F) gene amplicon region, 1,169,831 effective tags were filtered from 2,320,223 raw tags. The relative abundance of the top ten bacterial genera were not affected by either glyphosate treatment (Fig. 3-A). However, Roundup Optima+® but not glyphosate altered the relative abundance of the top 10 fungal genera across treatment groups, with a reduction in the relative abundance of Ascomycota fungi, particularly *Candida* species (Fig. 3B). In general, this trend did not change when determining the relative abundance of microbial genera in individual samples (Fig. S2). Some samples displayed varying abundance profiles; however, these varying abundance profiles could not be originated back to treatment or origin colony (Table S9). There were no significant differences in species richness (alpha diversity) among bacterial or fungal species (Table S10). Microbial community composition (beta diversity) was analysed and there were no significant differences in beta diversity among bacterial or fungal communities (Fig. S3). Anosim analysis determined that bacterial and fungal variation were not statistically significantly larger between groups than within groups, with all pairwise *P* values >0.05 (Table S11). MRPP analysis did not show any statistically significant differences in microbial community structure among groups with all pairwise P values >0.05. For both Anosim and MRPP analysis, differences between glyphosate and Roundup optima+® exposed groups had the lowest P values at 0.052 and 0.053, respectively, when comparing differences in fungal genera between CF and AI-treated bees (Table S11). *t*-tests determined a statistically significant difference in the abundance of *Candida* species in the digestive tract microbiota between AI and CF-treated bees, with a lower abundance of *Candida* in CF-treated bees ($p = 0.031$) (Fig. 3-C). Additionally, *Trichoderma, Tomentella,* and *Archaeorhizomyces* had in increased abundance in the CF compared to control-exposed bees ($p = 0.035$, 0.040, and 0.045, respectively). *Tomentella* and *Filobasidium* species were found in CF treated digestive tracts, but not the controls ($p = 0.034$ and 0.030, respectively) and the abundance of species from the genus *Archaeorhizomyces* was significantly higher in the CF-treated microbiota compared to control-treated digestive tract microbiota ($p = 0.040$). Whilst the largest impact of any treatment on fungal species was in *Candida* between the CF and AI-treated bees, the AI had some significant differences in comparison to the control, including statistically significant differences in *Rhodotorula* ($p = 0.034$) and *Hannaella* ($p = 0.039$) species. Bees treated with AI had a statistically significant decrease in the bacterial genera *Parabacteroides* in comparison to control-treated bees ($p = 0.022$).

4. Discussion

There is a growing concern that non-insecticide pesticides are having detrimental effects on pollinating insects including bees. As one of the most widely used herbicides in both agricultural and non-agricultural settings, glyphosate can regularly come into contact with bees. The absence of the shikimate pathway in metazoan organisms leads to an expectation for minimal negative consequences for exposed insects. To investigate this further, our study set out to assess glyphosate effects on survival, behaviour, and sucrose solution consumption in the bumblebee *B. terrestris*, and to characterise the molecular and microbiological responses to glyphosate exposure in a key bumblebee organ: the digestive tract. We also set out to compare the molecular level effects of glyphosate when used alone as the AI only or as a CF to determine the potential consequences of co-formulant ingestion in bees.

We found that glyphosate does not have a major direct effect on survival, behaviour, or sucrose consumption at the concentrations used in this study. It does, however, affect the digestive tract proteome of *B. terrestris* and, depending on its consumption as the AI alone or as part of a CF, differential effects were observed. Changes in protein abundance associated with metabolism and the lysosome, were attributable to the co-formulants in the CF. In addition, the AI but not the CF, had a dramatic effect on the abundance of many mitochondrial proteins. However, a

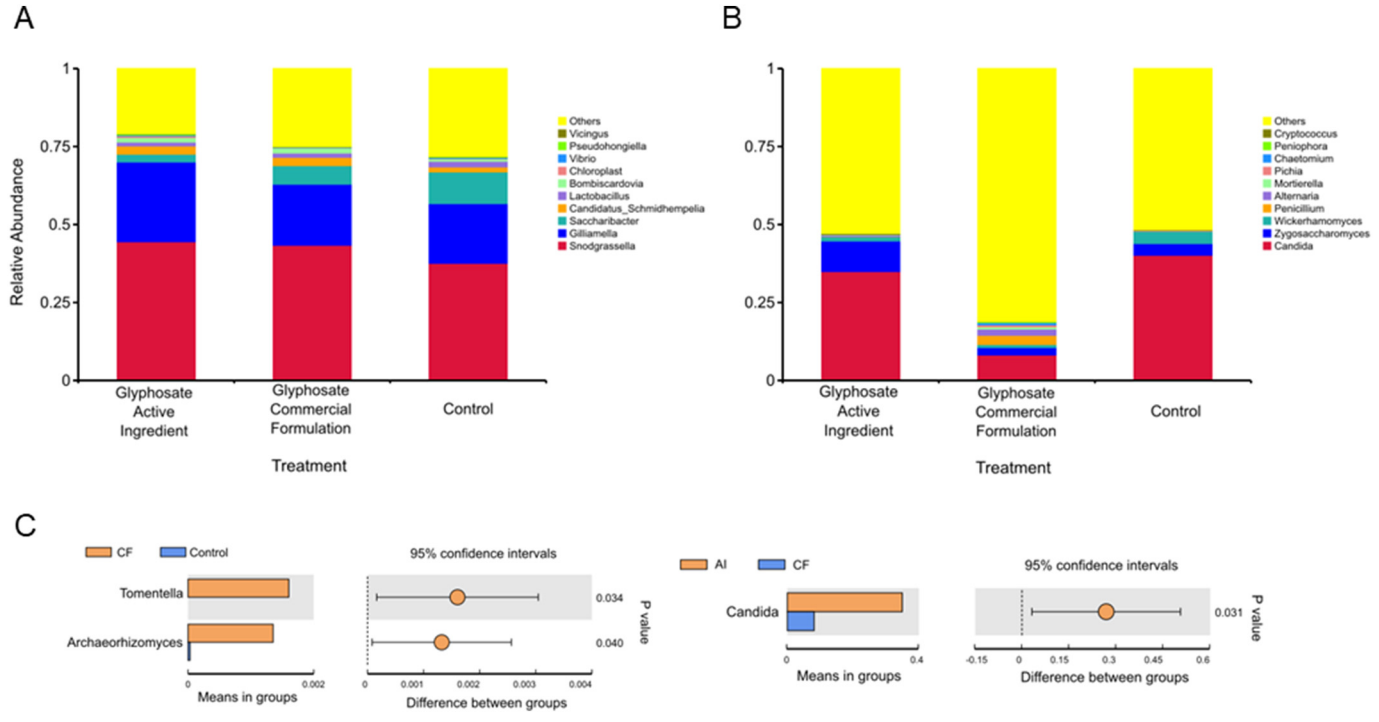


Fig. 3. The relative abundance of (A) bacteria and (B) fungal genera in *B. terrestris* digestive tracts after exposure to 1 ppm glyphosate active ingredient, glyphosate commercial formulation (RoundUp Optima+®) or the 40 % (w/v) sucrose solution control (Control) for 5 days. (C) The mean abundance of species of various genera in the phylum Ascomycota showing a significant difference between fungal species from the digestive tracts of *B. terrestris* exposed to the commercial formulation (CF) in comparison to the control and active ingredient (AI). The confidence interval of between group variation demonstrates the lower and upper confidence limits of the 95 % confidence interval, with the centre (orange circle) representing the difference of the mean value.

common glyphosate signature was also observed through effects on proteins associated with cellular structural integrity and adhesion, the extracellular matrix, oxidative stress regulation and metabolism. Additionally, through ITS and 16S amplicon sequencing, we investigated whether glyphosate AI or CF had impacts on the digestive tract microbiota of *B. terrestris*. Whilst there were some significant impacts on a bacterial species in AI-treated bees, the CF had a significant impact on *Candida* fungi in the *B. terrestris* digestive tract, once again highlighting the potential impacts of co-formulants.

4.1. Glyphosate has no impact on survival, behaviour, or sucrose solution consumption

We determined that glyphosate, regardless of source, had no statistically significant impacts on survival or behavioural measures observed at 1, 10 or 100 ppm. Additionally, 1 ppm glyphosate AI or CF had no impact on the consumption of sucrose solution, demonstrating that bees are neither attracted to nor repelled by glyphosate-contaminated sucrose solution any more than untreated sucrose solution. This result is further evidence that bees are at risk of glyphosate exposure, along with recent research by Thompson et al. (2022), which demonstrated that *B. terrestris* will indiscriminately forage on plants treated with glyphosate. Since glyphosate exposure is plausible in natural settings, it is important to investigate impacts on survival. Whilst we found no impacts on survival at 1, 10 or 100 ppm glyphosate AI or CF after 10 days of exposure, some studies have found impacts at higher levels or over longer periods of exposure. Motta and Moran (2020) found that 16.91 and 169.1 ppm glyphosate exposure decreased honeybee survival, some which were actively obtaining their digestive tract microbiota while other groups had a pre-established microbiota, after 15–40 days of exposure. Interestingly, bees exposed to 1.691 ppm, a concentration closer to the lowest concentration used in our study, had higher survival rates in honeybees. Further, bees without an established digestive tract microbiota still displayed higher mortality than controls after exposure to 169.1 ppm glyphosate. However, from the evidence available, it is unlikely that bees would be orally exposed to such a high concentration for an extended period, but these results give an insight into the possibility that glyphosate may alter bees directly in addition to impacts on their digestive tract microbiota. The proteomic results of our study provide insight into the physiological processes altered by glyphosate in the digestive tract in addition to impacts on the digestive tract microbiota. However, our proteomic study focuses on a single field-realistic dose of 1 ppm glyphosate AI or CF for a shorter period of five days. Additionally, survival impacts observed in Motta and Moran (2020) in microbiota-free bees may be due to impacts on a different tissue. Almasri et al. (2020) determined that glyphosate alters honeybee survival at much lower concentrations over a period of 20 days. In addition to concentration and exposure duration differences, glyphosate may alter honeybee and bumblebee species differently – but this is currently unknown.

4.2. Common proteomic responses to glyphosate regardless of source

4.2.1. Cell adhesion and cellular structural integrity

A major molecular effect of glyphosate exposure, whether ingested as an active ingredient or part of a commercial formulation, involved proteins associated with cell adhesion and cellular structural integrity. Of the twenty-nine proteins with decreased abundance in common to both glyphosate-based treatments, 9 were associated with these processes (Table 1). Several of these proteins represent some of the most differentially abundant proteins in terms of relative fold changes (5 and 4 of the top 10 most decreased proteins in the glyphosate AI and CF-treated bees, respectively). Alterations in these processes would have a marked effect on overall cell integrity, structure, and function. Constituents of the extracellular matrix including fibrillin-2, basement membrane-specific proteoglycan and collagen proteins were significantly reduced in abundance and in fact, fibrillin-2 had the highest fold change decrease in comparison to the control group in both the glyphosate and CF-treated bees by 222.68 and 56.83, respectively. Fibrillin's are necessary for the formation of microfibrils; important components of the extracellular matrix and basement membranes of tissues which act as molecular scaffolds and impart structural integrity, strength, and growth factor regulation to tissues (Kumra and Reinhardt, 2018). A decrease in abundance was also observed for tetraspanin's, CD151 antigen and 23 kDa integral membrane proteins. These plasma membrane-bound proteins have roles in cell adhesion, signalling and immunity via interactions with other proteins e.g. other tetraspanin's and integrins (Maecker et al., 1997; Todres et al., 2000; Zhuang et al., 2007). Collagen IV proteins and heparan sulfate proteoglycans are components of the basement membrane which gives cells structural stability, regulate cell behaviour, and separate epithelial cells from the stroma of the tissue (Lunstrum et al., 1988; Paulson, 1992; Tanzer, 2006; Altincicek et al., 2009). Decreases in collagen proteins have previously been associated with wounding and haemocyte recruitment for cellular repair (Pastor-Pareja et al., 2008; Saleh et al., 2018) highlighting a potential mechanism for glyphosate toxicity (i.e. mechanical damage of digestive tract leading to alterations of the extracellular matrix or basement membrane). Further research into the histology of the *B. terrestris* digestive tract after glyphosate exposure would shed light on this.

Several membrane-associated proteins with roles in cellular signalling were also affected by glyphosate-exposure. These include tubulointerstitial nephritis antigen and the basement membrane-specific heparan sulfate proteoglycan, both of which engage in regulating major signalling pathways such as Wnt, Hedgehog and TGF beta pathways (Theodosiou and Tabin, 2003; Logan and Nusse, 2004; Li et al., 2007; Lin and Perrimon, 2000). Basement membrane proteoglycans are also involved in the regulation of the cytoskeleton itself.

Given that proteins involved in cell adhesion and the extracellular matrix are vital for cell signalling, tissue structural integrity (Gumbiner, 1996; Johansson, 1999), and the proper functioning of the basement membrane (Lunstrum et al., 1988; Paulson, 1992; Tanzer, 2006), alterations in these processes in the insect digestive tract may represent a major detrimental effect of glyphosate exposure on bees.

4.2.2. Lines of defence: oxidative stress regulation, pesticide metabolism and melanisation

Maintaining an equilibrium between free radicals and antioxidants is important to prevent oxidative stress and promote longevity and overall health. Proteins with increased abundance in the digestive tract of bees exposed to both glyphosate-based treatments were associated with oxidative stress regulation and included superoxide dismutase and a venom carboxylesterase. Superoxide dismutase participates in the destruction of free radicals and is expressed in response to oxidative stress in insects (Kim et al., 2005; Choi et al., 2006). Carboxylesterases are involved in lipid metabolism and pesticide detoxification via ester and thioester hydrolysis (Wheelock et al., 2005) and are reported to be involved in insecticide metabolism and oxidative stress regulation in insects (Badiou-Bénéteau et al., 2012; Ma et al., 2018; Mao et al., 2021). The phenoloxidase enzyme, laccase, was also increased in both treatments, having remarkably similar increases in abundance across both treatments (Table 1). Laccase participates in insect immunity and wound healing via melanin production through the catalysis of oxidation-reduction reactions. Laccase also has industrial use for the degradation of various pesticides (Jin et al., 2016; Gangola et al., 2018) which suggests a potential role of laccase in glyphosate metabolism. Multiple studies have identified the role of melanisation in insect immunity and wound healing, as well as the action of glyphosate in altering melanisation in insects (Galko and Krasnow, 2004; González-Santoyo and Córdoba-Aguilar, 2012; Parsons and Foley, 2016; Smith et al., 2021; Motta et al., 2022). Melanisation is an important part of the insect immune response to mechanical injury or infection with pathogens, but if dysregulated, the insect immune response has the capacity to self-inflict major damage to the insect and must remain tightly regulated under normal physiological conditions (Theopold et al., 2004; Dionne et al., 2006; Eleftherianos and Revenis, 2011; Krautz et al., 2014). Recent research suggests that glyphosate exposure inhibits phenoloxidase activity

in the insects *Galleria melonella* and *Anopheles gambiae* in a dose-dependent manner (Smith et al., 2021). The dose used in our experiment was over eighty times lower than the lowest glyphosate dose used in the Smith et al. (2021) study, indicating that higher phenoloxidase abundance may be found at lower field-realistic doses or that glyphosate impacts various insect groups differentially. in the increased abundance of proteins involved in melanisation and oxidative stress regulation found in this study may also highlight a link to the alterations to the extracellular matrix and cellular integrity discussed above, as changes to some extracellular matrix proteins such as a decrease in collagens are associated with mechanical damage and wound healing (Pastor-Pareja et al., 2008; Saleh et al., 2018).

The observed increase in proteins associated with oxidative stress regulation indicates that glyphosate, whether directly or indirectly, either creates or leads to an environment in the digestive tract where there is oxidative stress. Reactive oxygen species (ROS) are important in melanisation, wound repair, and immunity (Nappi and Christensen, 2005; Zug and Hammerstein, 2015) and an estimated 90 % of ROS are produced in the mitochondria, making mitochondrial ROS a likely source of oxidative stress (Balaban et al., 2005). It is also known that exposure to glyphosate CFs result in increased ROS levels and mitochondrial dysfunction in different species and cell lines (Chaufan et al., 2014; Bailey et al., 2018; A. G. Pereira et al., 2018a; Nerozzi et al., 2020; Ravishankar et al., 2020) highlighting an additional mechanism of potential glyphosate-induced impairment.

The increase in the abundance of antioxidant and detoxification proteins demonstrates potential mechanisms to counteract and prevent glyphosate-induced oxidative damage in bees. Considering our other findings, it does seem, however, that this response may be insufficient for protection, particularly when the multiple stressors bumblebees face in the wild are considered.

4.2.3. Lipid metabolism and calcium homeostasis

Six proteins had an increased abundance across both treatments that play key roles in metabolism, four of which are associated with lipid metabolism. These included FGGY carbohydrate, glucosylceramidase and venom carboxylesterase-6 (Acharya and Acharya, 2005; Zhuang et al., 2007; Singh et al., 2017). The glycosyl hydrolase, glucosylceramidase, had the most increased abundance in both glyphosate-based treatments and had similar RFC's indicating a clear and consistent response to glyphosate regardless of the source. Glycosylcerimidase's are involved in sphingolipid metabolism, which are important structural membrane proteins in insects. Carboxylesterases catalyse the breakdown of water-soluble lipids and can be involved in the breakdown of longer, insoluble, fatty acid chains in the presence of surfactants, but at a slower rate than more suitable lipases (Terra and Ferreira, 2012). Other lipid associated proteins: choline transporter protein and phosphatidylethanolamine, had a lower abundance in both glyphosate-treated groups. Choline transporters are found in cellular and mitochondrial membranes and are important for transporting the choline required for phospholipid biosynthesis into the cell. Phosphatidylethanolamine plays a role in ethanolamine transport and ultimately, phosphatidylethanolamine synthesis (Taylor et al., 2021). Both proteins are also important for sphingomyelin production, which plays a structural role in membranes and changes in their abundance could interfere with lipid metabolism (Bridges, 1972; Michel et al., 2006).

Another protein found to be of high abundance in both AI and CF-treated groups was regucalcin (also known as smp-30), a calcium-dependent gluconolactonase involved in regulating intracellular Ca^{2+}, nucleic acid synthesis, proliferation, apoptosis, and intracellular signalling pathways (Yamaguchi and Murata, 2013; Marques et al., 2014). Although regucalcin has also been linked to lipid metabolism in mice, little is known about its involvement in insects (Ishigami et al., 2004; Yamaguchi et al., 2004; Toprak et al., 2020). Interestingly, regucalcin has been identified as an insecticide tolerance-related gene in the grain aphid *Sitobion avenae* after transcriptional increases of regucalcin were observed in response to the neonicotinoid insecticide imidacloprid and the organophosphate insecticide chlorpyrifos (Wei et al., 2019). While an increase in oxidative stress regulation and detoxification proteins could indicate defence against glyphosate toxicity, when this observation is coupled with an increased abundance of regucalcin in response to glyphosate, a conserved xenobiotic response involving these proteins could exist in insects.

Additionally, calcium-transporting proteins were decreased in the digestive tract proteomes of bees exposed to both the AI and CF, including plasma membrane calcium-transporting ATPase 2 and calcyophosin. Calcium is an important mineral for the regulation of a variety of biological functions via its presence as a co-factor or signalling molecule in insects (Taylor, 1987; Teets et al., 2013; Collet et al., 2021). Glyphosate is known to function as a chelating agent which can tightly bind to and sequester calcium from its surroundings (Fon and Uhing, 1964; Mertens et al., 2018). Surprisingly, few studies have assessed the impact of glyphosate on calcium levels within animals, although Gaur and Bhargava (2019) reported calcium signalling alterations in zebrafish embryos after exposure to glyphosate. In insects, calcium homeostasis needs to be maintained within epithelial cells to preserve calcium absorption in the midgut (Taylor, 1985; Taylor, 1987), whereas calcium influxes are involved in the repair of epithelial cells through ROS production, which is important for insect immunity and haemocyte signalling (Davis and Engström, 2012; Krautz et al., 2014; Mikonranta et al., 2014).

4.3. Glyphosate alone is not always comparable to glyphosate-based formulations

Mitochondrial proteins were significantly affected by glyphosate exposure, but to different extents depending on the source of glyphosate. When comparing AI to CF, digestive tract proteomes from AI-exposed bees had a decreased abundance in 48 mitochondria associated proteins in comparison to CF-exposed bees, 22 of which are involved in the TCA cycle and oxidative phosphorylation. Despite this, some proteins associated with ROS production and energy metabolism were also differentially affected by CF exposure. Glyphosate induced damage and dysfunction of the mitochondria has been widely reported in several animals (Peixoto, 2005; Lopes et al., 2018; A. G. Pereira et al., 2018; Neto da Silva et al., 2020) and combined with our findings here, highlight that the mitochondrion and its processes are particularly sensitive to glyphosate-exposure. Given the lack of a known target pathway for glyphosate in metazoans, but the universal presence of mitochondria (a remnant of a symbiosis with a prokaryote), further investigation of the interactions between glyphosate and the mitochondrion at the molecular level is highly warranted.

The AI and CF also differentially affected endocytosis, protein degradation and modification, and plasma membrane proteins which could alter digestive system functioning. Proteins associated with endocytosis and the plasma membrane were decreased in the CF-treated bees, but some proteins associated with endocytosis were low after consumption of both glyphosate-based treatments. When endocytosed molecules are fused with endosomes, maturation into lysosomes relies on hydrolases – enzymes that were increased in both AI and CF-exposed bee digestive tract proteomes- for digestion of endocytosed molecules. Previous studies have found effects of glyphosate on lysosomal integrity (Lopes et al., 2018) with one study by Mottier et al. (2020) demonstrating that a glyphosate-based CF and co-formulant alone can affect phagocytic activity and lysosomal membrane integrity in shellfish haemocytes, with the AI itself resulting in minor alterations. In our study, proteins associated with protein degradation and modification, such as ubiquitin carboxyl-terminal hydrolase, ubiquitin-conjugating enzyme E2 J1, and NEDD8-activating enzyme E1 regulatory subunit, were increased in the CF but not AI-exposed bees. Therefore, it seems that the co-formulants present in the CF can cause alterations to important cellular processes, whether alone or in combination with the AI and/or other co-formulants present. Since we did not assess for the impacts of individual co-formulants, which was partly due to >75.5 % of the formulation listed as 'water and minor ingredients', we cannot determine which scenario is more probable here. Both scenarios have been investigated for various co-formulants found in other pesticide

formulations and some have had impacts on different bee species (Mullin, 2015; Mullin et al., 2015; Straw et al., 2022).

Proteins involved in glutamine amino acid and pyruvate metabolism were of low abundance in the AI-treated bees only, whereas proteins associated with energy production, namely glycolysis and gluconeogenesis had an opposite abundance profile. The effects of glyphosate on nucleic and amino acid metabolic processes have been previously reported for a wider range of animals including fish, frogs, and honeybee species (Rocha et al., 2015; Wang et al., 2019; Zhao et al., 2020). Additionally, various proteins involved in lipid metabolism were decreased in the CF but not the AI-treated bees. Lipases had notable RFC decreases in the CF-exposed bees, with one having an RFC of −92.29 in comparison to the AI, while proteins associated with carbohydrate metabolism such as myrosinase and trehalase were increased in the CF-treated bees. These findings highlight a potential switch from lipid to carbohydrate metabolism attributable to the presence of one or more co-formulants. Various proteins involved in carbohydrate metabolism were increased in abundance in the CF but not AI-treated bees, indicating that the co-formulants may be resulting in alternative responses that could mask the glyphosate specific effects in our data. Shifts in metabolic processes have been previously reported at the transcriptomic and metabolic levels derived from whole-bee samples for two species of honeybees (Zhao et al., 2020) and although our findings are slightly different, they are not unexpected given that different species display varying sensitivities and responses to pesticides (Arena and Sgolastra, 2014). In addition, we focussed on a single organ rather than the whole organism and exposed bees to a lower concentration of glyphosate, which was estimated to be at or below residue concentrations found in glyphosate-treated areas (Thompson et al., 2014; Cebotari et al., 2018).

Our results presented thus far demonstrate that the presence of co-formulants in the CF result in alterations to the proteomic profile of the bumblebee digestive tract after glyphosate exposure compared to the AI. Although we discuss these specific changes in terms of the effects of a formulation on protein function and abundance, it is necessary to consider that co-formulants may be acting as substrates for host enzymes and thus altering the direction of usual metabolic activities in the bee. For instance, the main surfactant used in RoundUp Optima+® used in this study is alkylpolyglycoside which consists of glucose and fatty alcohol, which could account for the shifts in metabolism reported here and elsewhere. What is clear, however, is that current policies on the listing and testing of all co-formulants in commercial formulations are inhibitory to achieving a full understanding of the effects and risks posed by the pesticidal AI and co-formulants alike on bees (Mullin, 2015; Mullin et al., 2015; Novotny, 2022; Straw et al., 2022).

These findings highlight potential issues with the assessment of toxicological risk of commercially available pesticides (Mesnage and Antoniou, 2018; Straw et al., 2022), given that most active ingredients are applied as part of a formulation or are manually mixed with inert ingredients to increase pesticide efficacy (Hazen, 2000). In the EU, formulations are not subject to the same regulatory testing as pesticidal AI's, with the possibility of risk being inferred from other formulations with similar make-ups. In addition, solid formulations are not thoroughly evaluated, as the ingredients used with these formulations in the field are considered 'inert' as they are not known to have direct pesticidal activity (European Commission, 2002; European Commission, 2011; European Food Safety Authority, 2013). Our work highlights the necessity to assess formulations in their entirety if we wish to determine a realistic view of the potential risks on nontarget organisms such as bees. This is a particularly daunting prospect given that there are over 750 glyphosate-containing products available in the US and over 260 in the UK (Henderson et al., 2010; Health and Safety Executive UK, 2022). More recently, a study by the European Food Safety Authority found 182 co-formulants from 82 pesticide products, highlighting the prevalence and diversity of co-formulants in pesticide formulations (European Food Safety Authority, 2022). In addition, not every ingredient in commercial formulations are listed, with >75.5 % of ingredients listed as 'water and other minor ingredients' in the formulation used in this study.

4.4. Alterations to fungal species abundance in the B. terrestris digestive tract

The reported impact of glyphosate on the honeybee microbiota (Dai et al., 2018; Blot et al., 2019; Motta et al., 2020) led us to investigate whether the glyphosate induced changes we observed in the digestive tract at the proteomic level could be explained by an indirect consequence of alterations to the microbiota. In addition, most studies assessing the impacts of glyphosate on the microbiota focussed on honeybees, investigated bacterial alterations only, did not compare glyphosate-based AI and CF's and used relatively high concentrations of glyphosate.

We found little significant effect on bacterial species relative abundance or alpha or beta diversity. However, there were some changes in taxonomic abundance and species present (Fig. S4, Fig. S5). *Lactobacillus bombi* for example, had a higher abundance in control samples compared to those exposed to either glyphosate-based treatment, and *Snodgrasella alvi* was decreased in bees exposed to the glyphosate AI in comparison to the control and CF-treated bees, however, none of these changes were significant. A *t*-test revealed a significantly decreased abundance of bacteria of the genera *Parabacteroides* in the AI compared to the control-treated bees, however, there were no significant differences in bacteria present between the AI and CF, or CF and control-treated bees, indicating that co-formulants may alter how glyphosate impacts bacterial species. We suspect a lack of clear and significant differences may be due to the conservative and low concentration of glyphosate used in our study along with differences in sensitivity between bee species. Castelli et al. (2021), studying honeybees, found that glyphosate AI at concentrations ten times that of ours (in addition to a *Nosema ceranae* infection), resulted in alterations to the relative abundance of *S. alvi* and *Lactobacillus*. In addition, Motta et al. (2018) also used higher concentrations of glyphosate and not only reported changes in the honeybee digestive tract microbiota, but also demonstrated the possession of glyphosate sensitive and insensitive EPSPS (the known target for glyphosate) in different bacterial species and strains. The techniques used here could not determine which strains were present in the digestive tract microbiota or if strains present contained sensitive or insensitive EPSPS classes. This highlights a key difference that may exist between *Apis* and *Bombus* microbiotas which should be further assessed and could explain our failure to identify clear changes here. Interestingly, alterations to the shikimate pathway – the known target pathway of glyphosate - in the digestive tract microbiota may contribute to alterations and physiological changes determined through the characterization of the digestive tract proteome. Mesnage et al. (2021) determined shikimate pathway perturbations in the digestive tract caecum of rats after glyphosate AI and CF (RoundUp MON 52276) exposure, leading to an accumulation of metabolites found upstream of EPSPS. However, this was only significant at higher AI and CF concentrations, which may indicate that physiological changes observed at lower concentrations are consequences of damage to the host organism tissue and not a result of shikimate pathway alterations in the microbiota. In addition, metabolites were expressed in rat serum indicating oxidative stress, a pathway increased in both glyphosate-based treatments in this research, also. Whether this finding in *B. terrestris* is due to a downstream impact of digestive tract microbial alterations or a result of direct impacts on the host organism is currently unknown. Research investigating the metabolome of *the B. terrestris* digestive tract could elucidate whether the shikimate pathway is impacted in the digestive microbiota after glyphosate AI or CF exposure, further piecing together an evidence based AOP for glyphosate and co-formulant impacts on bee health.

The importance of fungi in the bee digestive tract microbiota is understudied in comparison to bacterial communities, with fungi often seen as opportunistic or transient in bees, and therefore unimportant for survival (Batra et al., 1973; Bosmans et al., 2018; Hammer et al., 2021). Despite this, some studies suggest fungi can impact foraging behaviour, nutrition, pathogen protection, longevity, and development in insects, including bees (Cheng and Hou, 2005; Herrera et al., 2013; Stefanini, 2018; Parish et al., 2020; Pozo et al., 2020; Pozo et al., 2021; Cullen et al., 2021) making fungal presence, abundance, and community structure in bees worthy of investigation. In the CF-treated bees, fungal relative abundance and

taxonomic composition were affected, with a statistically significant lower abundance in Ascomycete species observed in comparison to the AI-treated bees. These included the yeasts *Candida apicola* and *Candida bombi*. Less common *Tomentella* and *Filobasidium* species were found in the CF digestive tracts, but not controls. In the wild, this alteration to bee fungal communities in the digestive tract could lead to dysbiosis and infection with opportunistic pathogens (Näpflin and Schmid-Hempel, 2018; Tauber et al., 2019; Pozo et al., 2020). To our knowledge, there is no research available that has assessed the impact of glyphosate on the fungal microbiota in bees. However, Mesnage et al. (2022) found alterations to fungal species in the rat digestive tract caecum induced by two different glyphosate CF's but not the AI. The co-formulants listed in RoundUp Optima + – the CF used in our study – are different to the co-formulants listed in the glyphosate-based herbicides used in Mesnage et al. (2022), however, both studies display a decrease in core ascomycete fungi of the microbiota across different organisms after glyphosate CF exposure. This indicates that negative impacts from a range of co-formulants on the digestive tract microbiota is a possibility, which may be of concern for the health of both mammals and insects likely to encounter glyphosate residues.

Our findings further demonstrate the potential impact of co-formulants on biological systems, with the CF having the most exaggerated effect on the core bumblebee genera *Candida*, implicating one or more of the co-formulant ingredients, in this case, alkylpolyglycoside, nitroryl, or any of the unlisted components of Roundup Optima + ®. Additionally, our results highlight how co-formulants can alter the effects of glyphosate on a core bumblebee organ and its microbiota, as the AI also displayed some significant differences in microbial abundance in comparison to the control, including a decrease in *Parabacteroides* species.

5. Conclusion

Through the application of high-resolution mass spectrometry, new insights into the potential mechanisms and consequences of glyphosate exposure in an organism deficient in the primary target pathway for this herbicide have been elucidated. The identification of proteins affected by glyphosate, and the pathways, cellular processes, and structures they engage in, allow us to populate the molecular, cellular and organ-level categories of an adverse outcome pathway for *Bombus terrestris* and this widely used herbicide (Fig. S6). Coupled with our microbiota analysis, and recent studies such as that of Weidenmüller et al. (2022) who demonstrated the impact of glyphosate on thermoregulation at the colony level, a more complete adverse outcome pathway for *B. terrestris* is becoming clearer. The insights afforded by our proteomic study, when combined with other findings, have also enabled the generation of the components for a putative model for glyphosate effects in bumblebees (Fig. 4). This model involves a series of interrelated steps including i) attempts to metabolise/detoxify glyphosate; ii) major mitochondrial disruption either through the direct or indirect action of glyphosate, presenting a potential mode of action in nontarget organisms; iii) the production of reactive oxygen species and conditions of oxidative stress and mechanisms required for their contention; iv) major effects on cellular integrity through the disruption of extracellular matrix, basement membrane, and cell adhesion proteins; v) alterations to wound repair, melanisation, and signalling mechanisms, vi) alterations to carbohydrate and energy metabolism. An additional component to this hypothetical model is: vii) alterations to the digestive tract microbiota at a low concentration, particularly to fungal species in the presence of co-formulants. Whether the physiological impacts on *B. terrestris* observed in this study are modulated or mediated by microbiota alterations is yet to be determined, however due to a low impact of glyphosate active ingredient on the microbiota at this dose, the former is more likely. Extensive links exist among all components listed above, although the direction and nature of these links under glyphosate exposure has yet to be fully elucidated. The discovery based quantitative proteomic approach adopted here has presented new hypotheses that can now be couched in molecular terms and made available for future testing, bringing us closer to a comprehensive understanding of how glyphosate alters bee health. Further

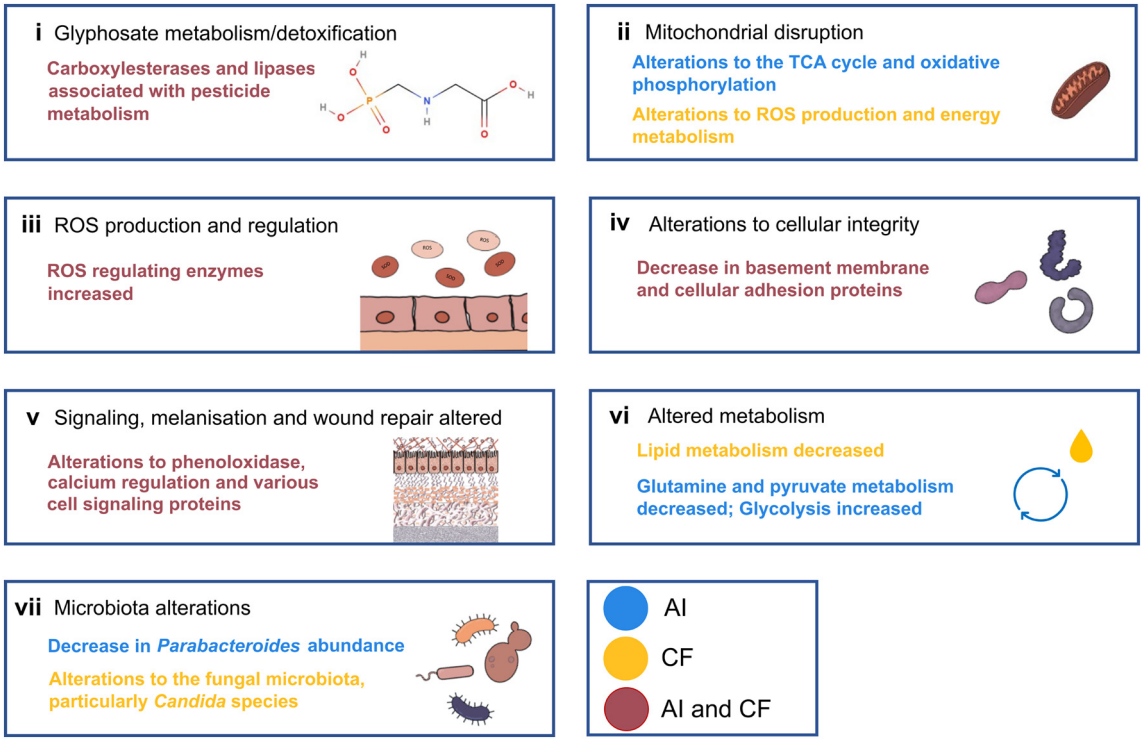


Fig. 4. The components of a hypothetical model for the effects of glyphosate and RoundUp Optima + ® at the same glyphosate concentration on the bumblebee digestive tract and microbiota. From our results, glyphosate exposure over five days at a low dose leads to (i) a detoxification response, (ii) mitochondrial disruption, (iii) reactive oxygen species (ROS) production and regulation responses, (iv) a decrease in cell structural integrity proteins, (v) signalling, melanin and possibly wound repair alterations, (vi) metabolic alterations and (vii) alterations to the digestive tract microbiota, particularly a significant decrease in Candida species after RoundUp Optima + ® exposure. AI = glyphosate active ingredient, CF = glyphosate commercial formulation (RoundUp Optima + ®.

research investigating the impacts of glyphosate and glyphosate-based formulations and co-formulants on non-*Apis* bee species is vital to gauge the risks of this herbicide to wild bee species. The results of this research suggest that glyphosate can impact the digestive tract at various physiological levels – with impacts for the digestive tract proteome and microbiota depending on its ingestion alone or as part of a commercial formulation. Research investigating glyphosate's impact on bumblebee and solitary bee species in other tissues and at various biological levels is currently sparse. Sub-lethal impacts of glyphosate should be further explored at the molecular, physiological, behavioural, and reproductive levels in insect pollinators and other non-target organisms to better inform future policy and risk assessments for clear science-based decision-making and bee decline mitigation strategies regarding glyphosate use.

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CRediT authorship contribution statement

Merissa G Cullen: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, review and editing, Visualization, **Liam Bliss:** Investigation, Writing – review and editing, **Dara A Stanley:** Resources, Writing – review and editing, Supervision, Funding acquisition, **James C Carolan:** Conceptualization, Methodology, Resources, Data curation, Writing – review and editing, Visualization, Supervision, Project administration, Funding acquisition.

Data availability

We have shared links and codes to data in the manuscript materials and methods section, all other data can be found in the supplementary material.

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.

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Appendix A. Supplementary data

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