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Landfill fire impact on bee health: beneficial effect of dietary supplementation with medicinal plants and probiotics in reducing oxidative stress and metal accumulation

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Abstract

The honey bee is an important pollinator insect susceptible to environmental contaminants. We investigated the effects of a waste fire event on elemental content, oxidative stress, and metabolic response in bees fed different nutrients (probiotics, *Quassia amara*, and placebo). The level of the elements was also investigated in honey and beeswax. Our data show a general increase in elemental concentrations in all bee groups after the event; however, the administration of probiotics and *Quassia amara* help fight oxidative stress in bees. Significantly lower concentrations of Ni, S, and U for honey in the probiotic group and a general and significant decrease in elemental concentrations for beeswax in the probiotic group and Li in the *Quassia amara* group were observed after the fire waste event. The comparison of the metabolic profiles through pre- and post-event PCA analyses showed that bees treated with different feeds react differently to the environmental event. The greatest differences in metabolic profiles are observed between the placebo-fed bees compared to the others. This study can help to understand how some stress factors can affect the health of bees and to take measures to protect these precious insects.

Keywords: biomonitoring; ICP-MS; metabolomics; NMR method; pollinators; *Quassia amara*.

1. Introduction

Agricultural resources are a major issue in the oncoming years and decades as the world's population is in constant evolution. Among different related key aspects, such as water supply and agricultural land availability, pollination is as important. Indeed, 76% of the leading agricultural production is pollination dependent (Klein et al. 2007). Moreover, pollination is also one of the most important mechanisms to preserve ecosystems, as pollinators are one of the most important contributors to the conservation of biodiversity through their foraging activity, enabling the maintenance of flowering plant diversity (Ollerton et al. 2011; Thakur 2012; Wei et al. 2021).

Anthropogenic emissions, environmental pollution, climate change, alteration of natural habitats, diseases, and phytosanitary products are a threat to insect pollinators and especially to bees, which are considered among the most important pollinators (Belsky and Joshi 2019; Giannini et al. 2020; Iwasaki and Hogendoorn 2021; Papa et al. 2022). A decline in the number of bee colonies, wild bees, and honey bees (Apis mellifera) has been recorded worldwide but especially in Europe since the 1960s (Potts et al. 2010; Espregueira Themudo et al. 2020; Wood et al. 2020). Bees have since been extensively studied to extend the knowledge to comprehend their decline better, but also because bees can be considered bioindicators. Indeed, thanks to their capability to absorb elements or chemicals from their surroundings, and mostly because their foraging activity gives a realistic and complete screening of the near environment to the bee colony (Conti et al. 2022a, 2022b). During their foraging activity, that is to say, when they collect nectar and pollen from flowers, they also collect pollutants that are present on the flower, in the pollen, and the nectar through the soil, water, and air (Zarić et al. 2022). Their whole body is also covered by hair, and helps capture every pollutant and heavy metal in the environment (Girotti et al. 2020). Knowing that a bee usually covers 7 km^2 on average (up to 100 km^2) (Couvillon and Ratnieks 2015) during its feeding activity, they become representative of the element content of the environment they live in (Kalbande et al. 2008; Lambert et al. 2012; Smith et al. 2019; Zarić et al. 2022). Toxic elements, such as As, Cd, Hg, and Pb, can weaken their immune system but also disrupt their ability to forage through learning and memory capability loss, all leading to less efficient foraging (Sivakoff and Gardiner 2017; Xun et al. 2018; Monchanin et al. 2021a; Astolfi et al. 2022). It has also been observed that a change in the content of Cu, K, P, Na, and Zn can cause a deterioration in the bee's health (Filipiak et al. 2022).

Contaminants such as toxic elements or agrochemicals can also induce oxidative stress in living organisms such as bees (Collin et al. 2010; Koch and Hill 2017; Alburaki et al. 2019). Oxidative stress refers to the uncontrolled production of free reactive oxygen species (ROS), which refers to the superoxide anion radical $\bullet O_2^-$, hydroxyl radical $\bullet OH$, hydrogen peroxide H_2O_2, and others intermediates in the reduction of O_2 to H_2O_2 (Lushchak 2014; Chaitanya et al. 2016). The production of these species occurs naturally in each aerobic organism through diverse reactions, such as the Fenton reaction with Fe^{2+} as a substrate, or through cellular reactions, such as mitochondrial respiration (Winterbourn 1995).

Even though normally regulated and neutralized by antioxidant naturally present, the harmful effects of these highly reactive species, also called oxidative stress, happen when ROS and antioxidant production is no longer in balance (Weirich et al. 2002; Waris and Ahsan 2006). Oxidative stress is characterized by a destructive reaction that is heavily toxic to cells and potentially causes aging, a carcinogenic response and cell death. These reactions are, more precisely, protein oxidation by their sulfhydryl groups, DNA, RNA, and the peroxidation of membrane lipids (He et al. 2021). The effects of oxidative stress can lead to weakened bees and premature death and contribute to their decline.

Toxicity remediation strategies are increasingly studied to avoid contamination of bees. Probiotics as a means of detoxification have already been tested on various living organisms, humans and animals, and they can bind to elements (Astolfi et al. 2019, 2022; Zhai et al. 2019). This comes from the ability of the bacteria composing the probiotic to adsorb elements on the surface of their cell wall or even directly inside the cell. This mechanism comes from an ion exchange, complexation, or nucleation reaction leading to precipitation (Bhakta et al. 2012; Astolfi et al. 2019; Daisley et al. 2019). The detoxification mechanism occurs after binding the bacteria and the element. The latter is eliminated from the organism through excretion (Berenbaum and Johnson 2015; Wang et al. 2015; Zhai et al. 2019). This detoxification mechanism also appears to work with organic substances such as pesticides (Trinder et al. 2015). Medicinal plants have also been used for centuries worldwide as a medicine or pain reliever to treat infections and for other purposes such as gardening. This is the case of the plant named *Quassia amara*, native to the tropics of South America and used as a traditional treatment for a variety of metabolic diseases and as an additive in the food industry (Houël et al. 2009; Husain et al. 2011; Olugbogi et al. 2022). *Q. amara* has also been used to treat stomach and intestinal ailments, for diabetes, as an antimalarial, and as an insecticide (Patel and Patel 2020; Olugbogi et al. 2022). However, this plant is not toxic to bees, larvae and bee broods (EFSA, 2018). One study reports that *Q. amara* prevented Cd-induced oxidative damage in the liver tissue of male Wistar rats (Obembe et al. 2021). *Q. amara* contains many active ingredients, including alkaloids, triterpenes, and bitter ingredients, such as quassinoids (Patel and Patel 2020; Olugbogi et al. 2022).

This study therefore aimed to investigate the protective capacity of probiotics and *Q. amara* against exposure of bees to pollutants. For this purpose, elemental concentrations, oxidative stress and metabolic changes of control groups (placebo-fed) and experimental groups (probiotic or *Q. amara*-fed) were compared.

2. Material and Methods

2.1. Study area and sampling

The sampling site was near the landfill of Malagrotta (Fig. 1), located in Rome province, central Italy (41°51'49.9 N 12°19'46.5 E). Six hives with similar size and number of bees *Apis mellifera ligustica Spinola* were selected for this study: two control hives were each fed only the placebo solution (1 L; Candiplus1; Zucchero and C., Florence, Italy), while one pair of the other four hives (experimental group) was treated with a sugar solution (1 L) containing bee-specific probiotics (10 g; Probee; CHRI.VA, Rome, Italy), and the other pair with a sugar solution (1 L) with 5% *Q. amara* (Bitterholz, Quassiaholz gemahlen, Naturix24, Deutschland). The sugar and probiotic solutions were prepared as described by Astolfi et al. (2022). The elemental concentrations in nutrient solutions used are shown in Table S1.

The treatments started in mid-April 2022 and were given to the bees every two weeks. The first sampling occurred in mid-May, while the second was one week after a major fire broke out on 15 June in the mechanical-biological waste treatment plant about 500 m from the sampling site. The fire caused a very high mushroom-shaped cloud of smoke to rise over the area (Fig. S1). The fire lasted for days and was difficult to put out because combustible material burned. In addition, the fire caused the release of many contaminants.

No smoke was used during sampling. The beekeeper generally uses smoke to calm the bees and work more quickly. However, the smoke inhaled by bees could interfere with the study, as the by-product of burning the wood pellets could contaminate the bees.

For oxidative stress and nuclear magnetic resonance (NMR)-based metabolomic analyses, the bees (about 30) of each hive were sampled separately with 50 mL tubes (Falcon®, Corning Incorporated

Life Sciences, Amsterdam, The Netherlands) and immediately frozen in liquid nitrogen. Once in the laboratory, these samples were stored at -80 °C until treatment.

For elemental analysis, the bees of each hive were sampled as described by Astolfi et al. (2022) and were freeze-dried without being previously washed (Astolfi et al. 2020, 2021, 2022; Conti et al. 2022a, 2022b). Once freeze-dried, the bees are crushed using a mortar and pestle to be homogenized into as fine a powder as possible. Wet bees are only used for oxidative stress and NMR-based metabolomic analyses.

2.2. Elemental analysis

The instrumental conditions (Tables S2-S5) and sample preparations were performed according to previously described methods (Astolfi et al. 2020, 2021, 2022). Mercury was analyzed by cold vapor atomic fluorescence spectrometry (CV-AFS; AFS 8220 Titan; FullTech Instruments, Rome, Italy), while the other elements (Al, As, B, Ba, Be, Bi, Ca, Cd, Ce, Co, Cr, Cs, Cu, Fe, Ga, K, La, Li, Mg, Mn, Mo, Na, Nb, Ni, P, Pb, Rb, Sb, Se, Si, Sn, Sr, Te, Ti, Tl, U, V, W, Zn, and Zr) were quantified by a quadrupole inductively coupled plasma mass spectrometry (ICP-MS; 820-MS; Bruker, Bremen, Germany). For sample treatment, ~0.1 g of bee lyophilized samples were digested at 95 °C for 1 h using a water bath (WB12; Argo Lab, Modena, Italy). A reagent mixture of 0.5 mL HCl (37%, superpure; Carlo Erba Reagents, Milan, Italy), 0.2 mL HNO_3 (69%, superpure; Carlo Erba Reagents, Milan, Italy), and 0.1 mL H_2O_2 (30%, suprapure, Merck KgaA, Darmstadt, Germany) or 1 mL HNO_3 and 0.5 mL H_2O_2 was used for sample digestion and subsequent analysis by CV-AFS or ICP-MS analysis. The digests were diluted to 5 or 20 mL with deionized water (resistivity, 18.2 MΩ cm; obtained by an Arioso Power I RO-UP Scholar UV system, Human Corporation, Songpa-Ku, Seoul, Korea) and filtered using syringe filters (GVS Filter Technology, Indianapolis, IN, USA) before the CV-AFS or ICP-MS analysis. All samples were analyzed in duplicate. Standard solutions for the calibration were prepared from a multielement reference commercial solution (VWR International, Milan, Italy) and a Hg reference solution (SCP Science, Baie D’Urfé, Quebec, Canada). All solvents and gases used were analytical grades.

2.2.1. Element adsorption experiments

For each experiment, an aqueous solution with adsorbent (blank), and a fortified solution with or without adsorbent were considered in triplicate. Fortified solutions at pH 5, containing some toxic or potentially toxic elements (As, Ba, Cd, Ni, Pb, Sb, Sn, Tl, and U) at the concentration of 1 mg/L were prepared by mixing different aliquots of mono-element standard solutions (Merck KgaA, Darmstadt, Germany) into 5 mL of deionized water or aqueous solution of probiotics or *Q. amara*, prepared as described in section 2.1. The pH of the solutions was controlled using a pH meter (Crison MicropH 2002, Crisonb Instruments, Barcelona, Spain) and adjusted using 1% HNO_3 or 5% NaOH (Merck Millipore Ltd, Billerica, MA, USA). The aqueous solutions containing the adsorbents (probiotics or *Q. amara*) and the multi-element solutions with and without the adsorbents were left under mechanical stirring by a rotary shaker (SB2, Cheimika, SA, Italy) at room temperature (21 °C). For the adsorption tests with probiotics, the solutions remained under stirring for 2 h, in agreement with a previous study (Astolfi et al. 2019), while all the other solutions for 24 h. Subsequently, all samples were filtered using syringe filters and diluted 1:40 with 1% HNO_3.

2.3. Spectroscopic characterization of *Q. amara* by FTIR

The *Q. amara* powder was analyzed using a Fourier transform infrared (FTIR) spectrometer (IR Affinity Miracle 10; Shimadzu Scientific Instruments, Columbia, MD, USA) covering a frequency range of 4000–600 cm^{-1} to identify functional groups present on their adsorbent surface.

2.4. Determination of oxidative stress

The methods used to evaluate the oxidative stress of bees were performed according to the procedures described by Alburaki et al. (2019) with minor changes as follows.

2.4.1. Hydrogen peroxide assay

According to the manufacturer's instructions, the physiological stress (PS) of bees induced by exposure to environmental pollutants was estimated using an H_2O_2 assay (Hydrogen Peroxide Assay Kit ab102500, BioVision Kit, Prodotti Gianni, Milan, Italy). The H_2O_2 level was determined in whole bee samples. Bees were crushed individually in 1.5 mL tubes (Eppendorf, Milan, Italy) with 300 µL of deionized H_2O. Subsequently, to separate the supernatant containing the biological liquid from the solid part and eliminate the proteins, the samples were filtered using a microcon-10kDa centrifugal filter unit (Merck KGaA, Darmstadt, Germany). The volume of biological liquid obtained was treated with the kit. The samples obtained were analyzed by UV-Vis spectrophotometer (Varian Cary 50 Bio UV-Vis; Varian Inc., Palo Alto, CA, USA) equipped with 300 µL cuvettes and set at 570 nm.

2.4.2. Protein carbonyl content assay

The potential post-transcriptional damage (PTD) caused to bees by exposure to environmental contaminants was assessed by a protein carbonyl content assay kit (Merck KGaA, Darmstadt, Germany) as described in the manufacturer's protocol. An accurately weighed whole bee was used for each analysis. Bee proteins were solubilized by milling each bee in a 1.5 mL tube with 500 µL of a protein extraction buffer consisting of 20 mM Tris-HCl (pH 8.0; Molecular Biology Grade, Calbiochem, Millipore, Merck KGaA, Darmstadt, Germany), 30 mM NaCl (Merck KGaA, Darmstadt, Germany), and 10% glycerol (Millipore, Merck KGaA, Darmstadt, Germany). To extract the maximum protein content from the bee, the samples were sonicated using an Ulsonix (Germany) proclean 10.0 ultrasonic cleaner (10 L, ultrasonic power 240 W) device for 10 cycles of 30 s at 4 °C. Subsequently, the sample was centrifuged at 5000 g for 12 minutes and the supernatant treated with the kit. The final protein solution was analyzed by spectrophotometry at $\lambda = 375$ nm in a 300 µL cuvette.

2.5. ^{1}H-NMR analysis

Five bees from each hive were extracted following a modified Bligh-Dyer protocol (Tomassini et al. 2016). In brief, each bee was weighted and then ground in a mortar with liquid nitrogen and added to a cold mixture composed of chloroform (3 mL), methanol (3 mL), and distilled water (1.2 mL). The samples were stirred, stored at 4 °C overnight, and then centrifuged for 30 min at 4 °C with a rotation speed of 11,000 rpm. The upper hydrophilic and the lower organic phases were carefully separated and dried under nitrogen flow. The hydrophilic phase was resuspended in 0.7 mL of D_2O containing 3-(trimethylsilyl)-propionic-2,2,3,3-d_4 acid sodium salt (TSP, 2 mM) as an internal chemical shift and concentration standard. The hydrophilic phase was suspended and then analyzed by ^{1}H-NMR. All solvents and chemicals are from Merck KGaA, Darmstadt, Germany.

The NMR experiments were carried out at 298 K on a JNM-ECZ 600R spectrometer operating at the proton frequency of 600 MHz and equipped with a multinuclear z-gradient inverse probe head. The monodimensional ^{1}H NMR experiments were carried out for quantitative analysis, employing a presaturation pulse sequence for water suppression with a time length of 2 s, a spectral width of 9.03KHz, and 64k data points, corresponding to an acquisition time of 5.81 s. The pulse length of the 90° flip angle was set to 8.3 μs, the recycle delay was set to 5.72 s.

Bidimensional ^{1}H-^{1}H Total Correlation Spectroscopy (TOCSY) and ^{1}H-^{13}C Heteronuclear Single Quantum Correlation (HSQC) experiments were acquired according to Spinelli et al. (2022) for the resonance assignment. Quantities were expressed in mmol/mL by comparing of the relative integrals with the reference concentration and normalized to the number of protons (TSP: 9 protons) and the starting milliliter of the sample. The final concentration was expressed as µmol/g.

2.6. Statistical elaboration

Statistical analyses were conducted using IBM SPSS Statistics 27 software (IBM Corp., Armonk, NY, USA), while all descriptive statistics values were calculated using Microsoft Excel. For each element, values below the limit of determination (LOD) were replaced with a value equal to half the LOD (Clarke 1998; Farmaki et al. 2012). When the percentage of values <LOD exceeded 30%, the element was excluded from the statistical elaboration.

The Shapiro-Wilk test was performed on each variable to assess data normality prior to Student's t-test. The differences in the sample concentration were tested by Kruskal-Wallis and pairwise post-hoc tests and Mann–Whitney test. Probability values from multiple pairwise comparisons were adjusted using Bonferroni corrections (Sokal and Rohlf 1981). The results were considered statistically significant with p-values of <0.05.

Regarding the NMR-based metabolomics, multivariate Principal Component Analysis (PCA) and Partial Least Square Discriminant Analysis (PLS) were performed on the data matrix with the Unscrambler ver. 10.5 software (Camo Software AS, Oslo, Norway) for metabolomics data. Data were mean-centered since the variables with the largest response could dominate the models and then autoscaled to equalize the importance of the variation of each variable. To determine which categories were discriminated by metabolites ($p<0.05$), univariate Student's t-test or Mann–Whitney rank sum test were applied according to the normality test. Univariate statistical analysis has been performed with SigmaPlot 14.0 software (Systat Software Inc., San Jose, CA, USA).

Focused PCA was performed accordingly to Falissard (1999) with a MATLAB (R2020b, MathWorks, Portola Valley, CA, USA) own made function, using Spearman's correlation. Data analyses were performed on fused data matrices by combining oxidative stress and elemental or metabolomic matrices.

3. Results and discussion

3.1. Elements

The levels of 42 elements in bees, honey and beeswax are shown in Tables 1, S6-S8. Bismuth, Cr, Nb, Sb, V, and W in bees, Cr, Mo, Nb, Se, Sn, V, and W in honey, and Cr, V, and W in beeswax always resulted <LOD. The most abundant elements in bees were Ca, K, Mg, Na, P, S, and Si in all samples from both periods. Significantly higher concentrations in bees following the fire event (June) were

recorded for As, Ba, Be, Fe, Li, Pb, Se, Sn, Sr, and Ti in control bees, for Ca, Cu, Hg, Mn, Pb, Sr, Ti, and U in bees fed with *Q. amara* and for Ca and Si in bees fed with probiotics.

In agreement with other studies (Dżugan et al. 2018; Borsuk et al. 2021; Astolfi et al. 2022; Conti et al. 2022a, 2022b), higher levels of each element were found in bees (Table 1) than in honey samples (Table S7), confirming the ability of bees to be biofilters to protect honey from toxic or potentially toxic elements. However, some elements significantly increase their concentration in the honey after the fire event, such as Ca, Mn, and Sr from the group fed with probiotics; Al, Ce, Cs, K, La, Mg, Ni, Rb, Si, U, Zn, and Zr in the *Q. amara*-fed group and Sb in the placebo-fed group. Significantly lower levels of honey after the event occurred for Ni, S, Tl and U for the probiotic-fed group and Tl for the placebo-fed group. In the beeswax (Table S8), element levels decreased significantly for most elements (Al, B, Ca, Ce, Co, Cu, Fe, Ga, K, La, Li, Mg, Mn, Mo, P, S, Sb, Si, Sr, Ti, and Zn) in the group fed with probiotics while in the group fed with *Q. amara* only the concentration of Li decreased while that of U and Zr increased. The wax of the group fed with placebo, although showing significantly lower levels after the fire for Al, Cs, Mn, Rb, and Sr, had levels approximately 10 times higher than in the group fed with probiotics for Cu, Mn, Ni, Pb, S, and Sb.

In a previous study (Astolfi et al. 2022), it was highlighted that supplementation with lactic acid bacteria helped honey bee workers to reduce the accumulation of Ba, Be, Cd, Ce, Co, Cu, Pb, Sn, Tl, and U within their bodies. The present study also highlights the lower concentration of Ba, Cd, Co, Pb, Sn and U in bees fed with probiotics compared to those fed with placebo after the fire event, with a relative percentage decrease ranging from 32 (Pb) to 540% (Ba), confirming the protective action of probiotics against the accumulation of toxic or potentially toxic elements. Relative percentage reductions greater than 50% can also be observed for As, B, Fe, Li, Mn, Ni, and Ti in probiotic-fed bees compared to those fed the placebo. Even if to a lesser extent, the bees fed with *Q. amara* also have lower levels than the bees fed with placebo for Ba, Be, Cd, Co, Fe, Li, Mn, Sn, Ti, and U. Similarly to some bacterial strains that can absorb metals (Astolfi et al. 2019; Ali Redha 2020), *Q. amara* could sequester and retain some elements, subsequently favoring their elimination by bees. The preliminary results of some adsorption tests of probiotics and *Q. amara* at a pH ~5, equal to that of bee intestines (Colibar et al. 2010), have shown a good adsorption capacity of *Q. amara* for Ba (75.5%), Cd (77.1%), Pb (98.9%), Ni (45.1%), Sb (77.1%), Sn (87.9%), Tl (16.5%), and U (93.6%) and of probiotics for Pb (16.8%), Sn (84.8%), Tl (6.3%), and U (22.6%). Several factors can affect the adsorption capacity of plants or bacteria, such as biomass dosage, temperature, pH, contact time, initial metal concentration, and the presence of more than one metal ion in the same media (Ali Redha 2020). In fact, it is possible that one metal ion could have a higher affinity to the binding sites of the biosorbent leading to competition on the availability of binding sites (Ali Redha 2020). The adsorption capacity of plants is mainly due to the presence of phenolic and carboxyl functional groups in the cellulose matrix or cellulose-associated components such as lignin and hemicellulose (Abdi and Kazemi 2015). Noli et al. (2019) report that U and Cd can be removed from water using aloe vera wastes, thanks to carboxyl, carbonyl, and hydroxyl groups facilitating metal binding. Also in *Q. amara* these functional groups are present, as shown by Fig. S2 of the IR spectrum. Particularly Fig. S2 shows a broad band around 3400 cm^{-1} due to O-H stretching of various groups like alcohol and phenols; a sharp band around 2900 cm^{-1} assigned to antisymmetric or symmetric CH_2 of lipids; a region between 1720−1420 cm^{-1} assigned to aromatic C=C and asymmetric COO− group vibrations (lignin and other aromatics and aromatic or aliphatic carboxylates), C=O stretch of carbonyl and carboxyl groups (carboxylic acids and aromatic esters), and OH deformations and C=O stretch of phenols or C-H deformation (phenolic and aliphatic structures); and, finally, absorption bands around 1000 cm^{-1} region due to combination of C–O stretching and O–H deformation of polysaccharides (Wongsa et al. 2022).

The adsorption of metals by bacteria takes place through the charges or bonds formed on the surface of their cell wall. Bacteria are divided into gram-positive or gram-negative bacteria depending on the composition and thickness of their cell wall (Zyoud et al. 2019). Notably the cell wall of gram-positive bacteria has thicker peptidoglycan layers connected by amino acid bridges (Abdi and Kazemi 2015; Zyoud et al. 2019). The presence of lipoteichoic acids (polysaccharides) imparts a significant negative charge density to the surface of the cell wall thus allowing a greater removal of heavy metal cations (Tsezos et al. 2006; Abdi and Kazemi 2015). Oxygen-containing functional groups (such as carboxyl, hydroxyl, and amino groups) on the bacterium's surface can form bonds, as in the case of U adsorption with Bacillus amyloliquefaciens (Liu et al. 2019).

Although in different ways, medicinal plants and bacteria decrease the absorption of some toxic or potentially toxic elements in bees. However, it is necessary to continue the studies to deepen the various factors that can affect the adsorption capacity of these bioadsorbents and the excretion routes of bees. Especially if we consider that bees or other pollinating insects can be exposed in the environment to various chemical contaminants and highly variable concentrations of each element with consequent antagonistic, additive, or synergistic effects (Monchanin et al. 2021a,b; Gekière et al. 2023).

3.2. Oxidative stress

Environmental pollution has an important role in the production of oxidative stress in invertebrates due to the possible presence of different pollutants in the air, soil, and water (Chaitanya et al. 2016). In particular, waste fires can release metals (Pb, Cd, Hg, As, Cr, Cu, Ni, Se, and Zn), numerous organic compounds (volatile organic compounds, persistent organic pollutants, ketones, aldehydes), and PM, affecting air quality (Lemieux 1998; EEA 2016; Bihałowicz et al. 2021). Most of these compounds have been shown to induce oxidative stress by generating ROS in non-target species, including invertebrates (Ahmad 1995). Metals, such as Co, Cr, Cu, and Fe, can induce the formation of superoxide and hydroxyl radicals (mainly via the Fenton reaction) and other ROS. Other redox-inactive metals, such as As, Cd, and Pb, can induce toxicity by binding to sulfhydryl groups of proteins and leading to glutathione depletion. Antioxidant or chelating substances can reduce oxidative stress (Chaitanya et al. 2016).

Exposure to different pollutants during the waste burning at the Malagrotta landfill led to increased oxidative stress in honey bees, which was reflected by higher hydrogen peroxide content in control bees (Fig. 2). Elevated levels of hydrogen peroxide occurred as a response to ROS activity induced by exposure to several chemical compounds, which if not diminished by a detoxification process could lead to bee death or protein damage. We have, in fact, identified significantly higher carbonyl protein contents (0.38 nmol/mg) in control bees, which could indicate possible cellular damage in progress (Fig. 2). Physiological stress remains higher in control bees also in the month following the waste fire (July). Instead, bees fed with probiotics or *Q. amara* do not seem to be affected by any oxidative stress induced by the waste fire event. The administration of probiotics and *Q. amara* to bees has shown a protective effect against the oxidative stress caused by an acute environmental pollution event. In particular, probiotics help maintain the balance of the bee microbiota, avoiding dysbiosis with harmful consequences for the bee host (Gekière et al. 2023). Furthermore, the bee microbiota positively influences bee tolerance to chemicals and parasites (Wu et al. 2020; Wang et al. 2021). On the other hand, phenolic compounds present in *Q. amara* (Fig, S2) could contribute to the antioxidant activity of the plant according to other studies (Manach et al. 2004; Zargoosh et al. 2019). In fact, it is known that hydroxyl groups in phenols can scavenge free radicals and reactive oxygen species (Manach et al. 2004). However, future studies should be encouraged to understand better the mechanisms of action of probiotics and medicinal plants against different pollutants, including organic compounds, and the intestinal microbiome of bees.

3.3. NMR-based metabolomic profiling of *Apis Mellifera* bodies

Analyzing the ^{1}H spectra of the hydroalcoholic extracts of bees' bodies allowed the identification of 39 metabolites, classified as amino acids, organic acids, carbohydrates, and miscellaneous molecules, and among these, 36 were quantified. Only quantitative differences were observed when comparing the spectra of different treatments and times. Representative monodimensional ^{1}H and two-dimensional TOCSY and HSQC experiments are reported in Figures S3-S6, and the table of resonance assignment is reported in Table S9.

The different metabolic profiles of probiotic and *Q. amara-fed* bees compared with placebo suggest that these treatments provide protective mechanisms, probably related to improving intestinal microbiota health. Bacteria can directly detoxify xenobiotics and/or stimulate host detoxification, which could be advantageous within environmental stressors, such as food deprivation or exposure to toxins (Jing et al. 2020). A series of PLS was performed to better understand the relationship between the event and the treatments and were analyzed separately before their comparison.

3.3.1. Bees' metabolic profiles related to treatments before the event

Firstly, we performed two different principal component analyses to evaluate if a spontaneous grouping could be observed between placebo, probiotic, and *Q. amara-treated* bees, being equal to the event. In Fig. S7, the PCA among pre-event is shown. The first two components explained 46% of the total variance, with the first component (PC-1) explaining 31%, the second component explaining 15%, and the third explaining 15% of the total variance. Before the event, a tendency toward separation between placebo (high values of PC-1) and *Q. amara* treated (low values of PC-1) is observed along the first PC.

Along the second PC, a separation between probiotic-treated hives (high values of PC-2) and the other two groups is highlighted, with placebo grouping together with the *Q. amara-treated* bees.

From the loadings analysis, it is possible to observe the variables mainly involved in the groupings. In particular, all the variables with a normalized loading value higher than 0.44 and lower than -0.44 were statistically significant according to Pearson's Critical Values for 25 samples (n=25). In pre-event, putrescine, propionate, succinate, trigonelline (Trig), acetate, methylguanidine (MG), 4-hydroxybenzoic acid (4-HBzA), gamma-aminobutyric acid (GABA), succinate, malonate, choline, 3-aminoisobutyric acid (3-AIBA) were higher in *Q. amara* treated group. At the same time, adenosine-X-phosphate (AXP), phosphocholine, nicotinamide adenine dinucleotide (NAD+), beta-alanine (β-Ala), valine, glutamine (Gln) and inosine monophosphate (IMP) characterized the placebo group.

The placebo-treated bees had higher levels of free carbohydrates such as fructose and glucose-1-phosphate (Glc-1-P), amino acids such as phenylalanine (Phe), leucine, tryptophan (Trp), lysine (Lys), aspartate, alanine (Ala) and uridine-xphosphate (UXP).

3.3.2. Bees' metabolic profiles related to treatments after the event

We then performed the PCA on post-event bees (Fig. S8), considering placebo, probiotic, and *Q. amara* treatment.

The first two components described 49% of the total variance, with the PC-1 explaining 31% and the PC-2 explaining 18%. Since two samples were outliers (one from the placebo and one from the *Q. amara* groups), they were not considered in this PCA.

Observing the PCA score plot (Fig. 3, left side), a tendency toward separation can also be observed in this case, however in post-event, placebo-treated bees separated along PC-1 (low values of PC-1) for *Q. amara* and probiotic-treated bees, which on the other hand, are grouped at higher levels of PC-1.

From the loading plot (Fig. S8B), it is possible to observe the variables mainly involved in the groupings. In particular, all the variables with a normalized loading value higher than 0.5 and lower than -0.5 were statistically significant (n=22), with alpha-glucose (alpha-Glu) higher in probiotic and *Q. amara* treated groups along the PC-1. At the same time, choline, Trig, and malonate were also higher in *Q. amara* and probiotic treated group but along the PC-2. On the other hand, Asp, histidine (His), Ala, putrescine, succinate, Tyr, propionate, Ile, 3-AIBA, Val, proline, GABA, Phe, MG, and UXP are higher in the placebo group. At the same time, on the second component, it is possible to observe higher levels of phosphocholine, β-Ala, Gln, trehalose, AXP, and NAD+.

Since the study aims to evaluate events mediated by the different treatments, we proceeded with pairwise PLS analysis for each treatment independently in order to evidence the effect of each treatment in relation to the placebo group.

The analysis of the loading plot suggests that some of these changes could be related to the bee microbiota since some variables, in particular putrescine and propionate, are known to be mediated by this factor (Zheng et al. 2017; Nakamura et al. 2021) and the importance of these molecules increases in the placebo model after the event. It has been shown that introducing low-carbohydrate stress significantly affected the hemolymph metabolome of *Bombus terrestris* (Wang et al. 2019). In our observations, the fire event intensely affected the flower pollen supply, leading to a significant low-carbohydrate intake for the pre-event and a completely different metabolic profile, with higher levels of amino acids in the placebo group for the probiotic and *Q. amara* fed bees. Indeed, it also can be hypothesized that under nutritional stress, placebo-fed bees responded with increased protein catabolism, as observed by Wang et al. (2019) in *Bombus terrestris* and Maity et al. (2012) in *Diporeia spp.*, and a decreased level of sugars.

3.3.3. Exposure effect on control bees

A PLS was carried out on the same data matrix (Fig. 3A, B) to understand better the evolution of bee basal metabolic profile's evolution, providing a 3-factor model with R^2=0.95 and Q^2=0.72 (Fig. 4A). From the analysis of the regression coefficients (Fig. 3B), it was possible to evidence, after the event, an increase of propionate, putrescine, succinate, Asp, β-Ala, trehalose, fumarate, as well as a reduction of dimethylamine (DMA), malonate, choline, UXP, AXP and Trig.

The propionate, putrescine, beta-alanine, trehalose, and fumarate levels were significantly higher post the event. At the same time, UXP and Trig observed were lower, as indicated by Mann-Whitney Rank Sum Test ($p<0.05$) (Fig. 3C).

Given the absence of any treatment in control bees, it is possible to evaluate the event's impact and how the hive reacted to it. Moreover, given the temporal proximity of the samplings (less than one month), it is also possible to exclude a change due to both direct (e.g., temperature) and indirect (e.g., change of flora due to seasonality) climatic aspects. In response to several types of stresses, Bees react by changing their feeding behavior to produce honey with a peculiar composition in terms of secondary metabolites (Li et al. 2018). However, little is known about how bees react to heavy metal and/or salt stress.

In this work, we observed how the placebo-treated bees showed increased levels of propionate, putrescine, Asp, β-Ala, trehalose, and fumarate and decreased choline, UXP, and Trig after the event. While none of these molecules are known for their involvement in redox defense, putrescine (Kim et al. 2018), β-Ala (Petanidou et al. 2006; Nepi et al. 2012), Trig (Ares et al. 2022; Lu et al. 2022) and UXP (Ardalani et al. 2021) are known to be related to the type of consumed pollen. Moreover, trehalose, present in the hemolymph of bees, is known to regulate the behavior of foraging bees; in particular, it lowered when bees need to reach greater distances from the hive (Akülkü et al. 2021). Therefore, the working hypothesis is that honey bees treated with a placebo changed their foraging area after the fire, changing their metabolic profile.

3.3.4. Exposure effect on probiotic fed bees

The PLS algorithm applied to placebo feed bees provided a very robust 3-factor model, with an R^2=0.99 and Q^2=0.87 (Fig. 4A).

From the analysis of the regression coefficients (Fig. 4B), it was possible to evidence, after the event, an increase of putrescine, proline, and IMP, as well as a reduction of Ile, succinate, Gln, Asp, DMA, fructose, Glc-1-P, fumarate, Tyr, Phe, Trp, AXP, and Trig.

Among the variables that were significant in the PLS model, the levels of Ile, DMA, Glc-1-P, fumarate, and Trig were also significant ($p<0.05$) at the univariate statistical analysis, all being lower after the event (Fig. 4C).

The response of prebiotic-treated bees to the event is remarkably different from the placebo-treated ones. While there was an increase in putrescine level as well as a decrease in Trig, several other significant changes were observed, such as an increase in IMP coupled with a reduction of several amino acids (Ile, Gln, Asp, Tyr, Phe, and Trp), free carbohydrates (Fructose and Glc-1-P), organic acids (succinate, fumarate) and other molecules (DMA and AXP).

As stated before, bees react to stresses through the changes in their honey composition, and, as reported in the literature, some of these changes involve the synthesis of proteins and/or the activation of genes involved in response to oxidative stress (Li et al. 2018). The difference in the free amino acid levels could be related to this phenomenon. Since these molecules are also associated with the production of some neurotransmitters like dopamine from Tyr (Sasaki and Watanabe 2022), they could also influence their behavior.

For what regards IMP, its increase could also be attributed to a change in behavior since it is the precursor of guanine nucleotides (Wang et al. 2018) and, as a consequence, it is important for signal transduction, energy transfer, glycoprotein synthesis, and other processes that are involved in cell proliferation and the overexpression of inosine-5′-monophosphate dehydrogenase has shown to be involved in the vigorous metabolism in spring bees, including the secretion of proteins.

3.3.5. Exposure effect on *Q. amara* fed bees

The PLS algorithm applied to *Q. amara*-fed bees provided a model with a 1-factor model and an R^2=0.86 and Q^2=0.50 (Fig. 5A).

From the analysis of the regression coefficients (Fig. 5B), it was possible to evidence, after the event (red), an increase of Lys, Gln, fructose, Glc-1-P, as well as a decrease of Ile, MG, His, and Trig. Of these molecules, the fructose, Glc-1-P, and IMP levels were significantly higher. In comparison, the levels of Ile, MG, and Trig were significantly lower after the event according to univariate statistical analysis ($p<0.05$) (Fig. 5C).

To evaluate the effect of *Q. amara* on bees' metabolic profiles after the event, we performed a PLS between placebo and *Q. amara*-fed bees only considering post-event. The model showed good discrimination, with validation values of R^2=0.85 and Q^2=0.61 (Fig. 6A).

Regression coefficients (Fig. 6B) showed five higher variables in the *Q. amara* group, in particular malonate, fructose, alpha-Glu, Glc-1-P, and Trig (green). In comparison, eight variables were significantly lower, namely Ile, propionate, putrescine, GABA, beta-Ala, trehalose, Tyr, and histidine (blue).

Of these molecules, the fructose, alpha-Glc, and Glc-1-P levels were significantly higher. In comparison, the levels of Ile, propionate, putrescine, GABA, Tyr, and His were significantly lower in *Q. amara-fed* bees, according to univariate statistical analysis ($p<0.05$) (Fig. S9).

Q. amara is a plant belonging to the Simaroubaceae family, and it is a renowned natural pesticide and digestive (Raji and Oloyede 2011; Flor-Peregrín et al. 2017). In particular, it is shown to act as a potential treatment against varroosis (Esquivel et al. 2014). Varroosis is a parasitic disease of the brood and adult honeybees and can weaken and even kill an entire hive (Boecking and Genersch 2008). Therefore, it was interesting to observe how bees fed with *Q. amara* aqueous extract responded to the fire. After the event, the bees showed an increase in carbohydrates (fructose and Glc-1-P), some amino acids (Lys and Gln), and IMP, as well as a decrease in Ile, His, MG, and Trig.

Comparing the two treatments and placebo, it is interesting to note that the only common trend is the decrease of Trig and, for both treatments, the increase of IMP. As stated before, the Trig changes can occur due to changes in the foraging areas, and IMP could be associated with protein production related to the defense against oxidative stress; nonetheless, for what regards *Q. amara* treated bees, it is important to highlight the increase of carbohydrates, which is perhaps related to the rise in energy expenditure linked to the need for longer flights to forage the hive (Wang et al. 2022). Since this is typical behavior of older bees, it is possible to hypothesize that *Q. amara* treated bees are more long-lived and active than placebo and probiotic-treated bees.

3.4. Explorative analysis by focused PCA

To investigate the correlation among oxidative stress responses, elemental content, and metabolomics data, an explorative method called "focused PCA" was performed (Falissard 1999). This method is advantageous when the relationship between a responsive variable (the focused one) and explanatory variables is sought (Nicoletto et al. 2018; Mander et al. 2021; Hequet et al. 2022; Legris et al. 2022). Briefly, the closer a variable is to the center of the plot, where the focused variable lies, the higher the correlation between these two variables (a different marker color highlights positive and negative correlation); variables on radii spanning similar, perpendicular, or opposite angles, are approximatively correlated, non-correlated, or anti-correlated, respectively, each other.

Focused PCA (Fig. S10) showed a significant Spearman's correlation ($p<0.05$) of PTD with valine, phospho-choline, and β-alanine and a significant anti-correlation with choline, trigonelline, and malonic acid, which look correlated each other; a significant correlation is shown with As, Co, Fe, Mn and Pb, and anti-correlation with K, P, S, and Tl. When PS is focused versus metabolites, a significant correlation with tyrosine and histidine and anti-correlation with fructose is shown; a significant anti-correlation is shown with Rb and Te when PS is focused versus the elemental content. It was possible to highlight which elements and metabolites analyzed were directly or indirectly related to the responses obtained by the two oxidative stress tests utilized.

It was observed that oxidative stress level (PTD) rises while the content of As, Pb, Fe, Mn, and Co increases, and the level of the biogenetic elements P, K, and S decreases in the bee samples. Arsenic is known to produce a toxic effect through the generation of ROS (Flora 2011), as well as the transition elements Fe, Mn, and Co that are directly connected to ROS generation in cells through Fenton or Fenton-like reactions (Leonard et al. 1998). Pb also affects non-enzymatic antioxidant capacity in bees

(Gauthier et al. 2016). Physiological stress doesn't seem connected with most elements, aside from Rb and Te, for which the connection is under investigation. A possible protective action related to trigonelline, choline, and malonic acid has been observed, focusing PCA on protein carbonyl assay. Trigonelline is recognized to have a role in the antioxidative defenses in plant cells (Minorsky 2002) and produces various benefits in human health (Liang et al. 2023), such as increasing superoxide dismutase and catalase activities and glutathione levels; though, effects on bees are not profoundly documented. Choline and malonic acid, correlated with trigonelline, could be connected through the foraged pollen. Lande et al. (2019) have reported that, even when other sources with better nutritional apportion are present, bumble bees (*Bombus* spp.) have a preference for collecting forage from flowers of plants with a high trigonelline content, which is the case of *Trigonella foenum-graecum* (fenugreek) (Wani and Kumar 2018), a clover-like leaves plant cultivated in fields within the hives foraging range. Choline content is also high in this plant (Niknam et al. 2021).

4. Conclusions

The comparison of the metabolic profiles through PCA analysis pre- and post-event highlighted how the hives treated with different feeds reacted to the environmental event. In particular, while in the pre-event PCA, the more differentiated hives were the ones treated with probiotics, in the post-event analysis, it was possible to observe a greater difference between the placebo group and the others.

Compared with control bees, lower concentrations of As, B, Ba, Cd, Co, Fe, Li, Mn, Ni, Pb, Sn, Ti, and U were found in probiotic-fed bees, and Ba, Be, Cd, Co, Fe, Li, Mn, Sn, Ti, and U in *Q. amara*-fed bees, indicating a possible protective action of probiotics and medicinal plants against the accumulation of toxic or potentially toxic elements.

The administration of probiotics and *Q. amara* to bees has also shown a protective effect against the oxidative stress caused by the fire of landfill waste. However, further studies are needed to understand better the mechanisms of action of probiotics and medicinal plants against different chemicals and the intestinal microbiome of bees.

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Fig. 1. Map of the studied area in Malagrotta (Rome province) in the Latium region, central Italy. Datum for geographical coordinates is based on the World Geodetic System 1984 (WGS84) ellipsoid. Data map: Google Earth.

Table 1. Element levels (mg kg^{-1} dry weight) in the experimental and control bee samples.

			Probiotic fed					***Quassia amara* fed**					**Placebo fed**				
			May		**June**			**May**		**June**			**May**		**June**		
Elements	**LOD**	**LOQ**	**Mean**	**SD**	**Mean**	**SD**	**p**	**Mean**	**SD**	**Mean**	**SD**	**p**	**Mean**	**SD**	**Mean**	**SD**	**p**
Al	2	6	14	1	26	1	*ns*	22	12	33	13	*ns*	23	12	28	5	*ns*
As	0.01	0.03	0.116	0.011	0.132	0.018	*ns*	0.14	0.04	0.25	0.05	*ns*	**0.11**	**0.02**	**0.25**	**0.06**	*****
B	2	5	9.0	1.6	9.4	0.7	*ns*	10.3	4.1	15.1	6.4	*ns*	15	10	14.2	2.5	*ns*
Ba	2	7	<LOD	-	<LOD	-	-	<LOD	-	<LOD	-	-	**<LOD**	**-**	**12.9**	**7**	******
Be	0.0005	0.002	0.0008	0.0001	0.0021	0.0001	*ns*	<LOD	-	<LOD	-	-	**<LOD**	**-**	**0.0020**	**0.0003**	*******
Bi	0.009	0.03	<LOD	-	<LOD	-	-	<LOD	-	<LOD	-	-	<LOD	-	<LOD	-	-
Ca	70	232	**697**	**38**	**1060**	**30**	*****	**645**	**178**	**1370**	**291**	*****	646	490	1370	310	*ns*
Cd	0.01	0.03	0.0106	0.0025	0.0159	0.0008	*ns*	0.0155	0.0078	0.032	0.020	*ns*	0.066	0.059	0.070	0.055	*ns*
Ce	0.003	0.01	0.026	0.002	0.062	0.006	*ns*	0.048	0.040	0.076	0.032	*ns*	0.045	0.042	0.061	0.006	*ns*
Co	0.001	0.003	0.073	0.007	0.052	0.016	*ns*	0.084	0.010	0.109	0.062	*ns*	0.077	0.006	0.131	0.047	*ns*
Cr	0.3	1	<LOD	-	<LOD	-	-	<LOD	-	<LOD	-	-	<LOD	-	<LOD	-	-
Cs	0.0002	0.0006	0.29	0.10	0.20	0.01	*ns*	0.28	0.12	0.27	0.10	*ns*	0.15	0.08	0.19	0.07	*ns*
Cu	0.3	1	15.4	0.1	18.4	1.9	*ns*	**16.4**	**1.5**	**19.9**	**2.1**	*****	14.8	5.9	16.4	3.1	*ns*
Fe	69	232	<LOD	-	<LOD	-	-	<LOD	-	<LOD	-	-	**<LOD**	**-**	**124**	**24**	******
Ga	0.0002	0.0006	0.0338	0.0001	0.0336	0.0001	*ns*	0.0323	0.0037	0.0343	0.0039	*ns*	0.024	0.011	0.0302	0.0057	*ns*
Hg	0.0002	0.0004	0.0201	0.0014	0.0217	0.0006	*ns*	**0.0192**	**0.0019**	**0.0219**	**0.0010**	*****	0.0194	0.0031	0.0228	0.0017	*ns*
K	5	17	12500	610	10600	180	*ns*	12100	1300	11100	1400	*ns*	7800	3200	7910	1100	*ns*
La	0.002	0.006	0.0138	0.0008	0.0329	0.0037	*ns*	0.022	0.015	0.041	0.017	*ns*	0.025	0.024	0.034	0.004	*ns*
Li	0.004	0.01	0.0109	0.0013	0.0173	0.0002	*ns*	0.0169	0.0096	0.0254	0.010	*ns*	**0.018**	**0.012**	**0.041**	**0.016**	*****
Mg	2	6	1070	61	988	82	*ns*	1010	87	1170	150	*ns*	728	370	944	160	*ns*
Mn	0.1	0.5	13	2	72	32	*ns*	**21**	**5**	**126**	**27**	******	38	14	161	100	*ns*
Mo	0.02	0.05	0.389	0.051	0.341	0.014	*ns*	0.341	0.040	0.447	0.040	*ns*	0.37	0.18	0.398	0.043	*ns*
Na	105	351	658	101	679	23	*ns*	657	46	644	65	*ns*	501	210	670	69	*ns*
Nb	0.004	0.01	<LOD	-	<LOD	-	-	<LOD	-	<LOD	-	-	<LOD	-	<LOD	-	-
Ni	0.02	0.08	0.310	0.050	0.420	0.004	*ns*	0.350	0.070	0.64	0.19	*ns*	0.33	0.19	0.71	0.30	*ns*
P	60	201	8120	392	7460	190	*ns*	7890	720	6900	560	*ns*	5520	2700	6480	1100	*ns*
Pb	0.02	0.07	0.034	0.007	0.091	0.013	*ns*	**0.040**	**0.002**	**0.128**	**0.048**	*****	**0.090**	**0.016**	**0.120**	**0.023**	*****
Rb	0.02	0.08	**183**	**10**	**138**	**1**	***ns***	192	53	168	29	*ns*	70	40	118	40	*ns*
S	71	236	6160	590	6450	180	*ns*	6570	500	6240	320	*ns*	4530	1800	5600	660	*ns*
Sb	0.03	0.09	<LOD	-	<LOD	-	-	<LOD	-	<LOD	-	-	<LOD	-	<LOD	-	-
Se	0.04	0.1	0.296	0.027	0.363	0.049	*ns*	0.239	0.026	0.283	0.019	*ns*	**0.157**	**0.035**	**0.251**	**0.021**	*****
Si	30	102	**293**	**10**	**332**	**10**	******	397	20	542	160	*ns*	334	100	330	92	*ns*
Sn	0.01	0.04	<LOD	-	<LOD	-	-	<LOD	-	<LOD	-	-	**<LOD**	**-**	**0.056**	**0.033**	******
Sr	0.2	0.5	2.2	0.1	7.4	1.1	*ns*	**2.1**	**0.5**	**8.9**	**1.4**	******	**2.9**	**1.7**	**9.5**	**2.4**	*****
Te	0.004	0.01	0.0280	0.0060	0.0310	0.0020	*ns*	0.0201	0.0049	0.0256	0.0030	*ns*	0.0085	0.0021	0.0149	0.0092	*ns*
Ti	0.1	0.4	5.0	0.1	4.2	0.1	*ns*	**3.3**	**0.2**	**5.3**	**0.9**	*****	**4.5**	**1.6**	**8.4**	**0.9**	*****
Tl	0.0002	0.0007	**0.0255**	**0.0036**	**0.0064**	**0.0016**	*****	**0.043**	**0.033**	**0.005**	**0.002**	*****	**0.028**	**0.025**	**0.0046**	**0.0032**	******
U	0.0001	0.0004	0.0074	0.0009	0.0077	0.001	*ns*	**0.0076**	**0.0028**	**0.0192**	**0.0045**	*****	0.0053	0.0048	0.027	0.012	*ns*
V	0.1	0.2	<LOD	-	<LOD	-	-	<LOD	-	<LOD	-	-	<LOD	-	<LOD	-	-
W	0.02	0.06	<LOD	-	<LOD	-	-	<LOD	-	<LOD	-	-	<LOD	-	<LOD	-	-
Zn	10	33	59	7	73	14	*ns*	67	8	87	26	*ns*	55	15	74	29	*ns*
Zr	0.004	0.01	0.0308	0.0006	0.098	0.013	*ns*	0.082	0.018	0.086	0.026	*ns*	0.040	0.027	0.094	0.026	*ns*

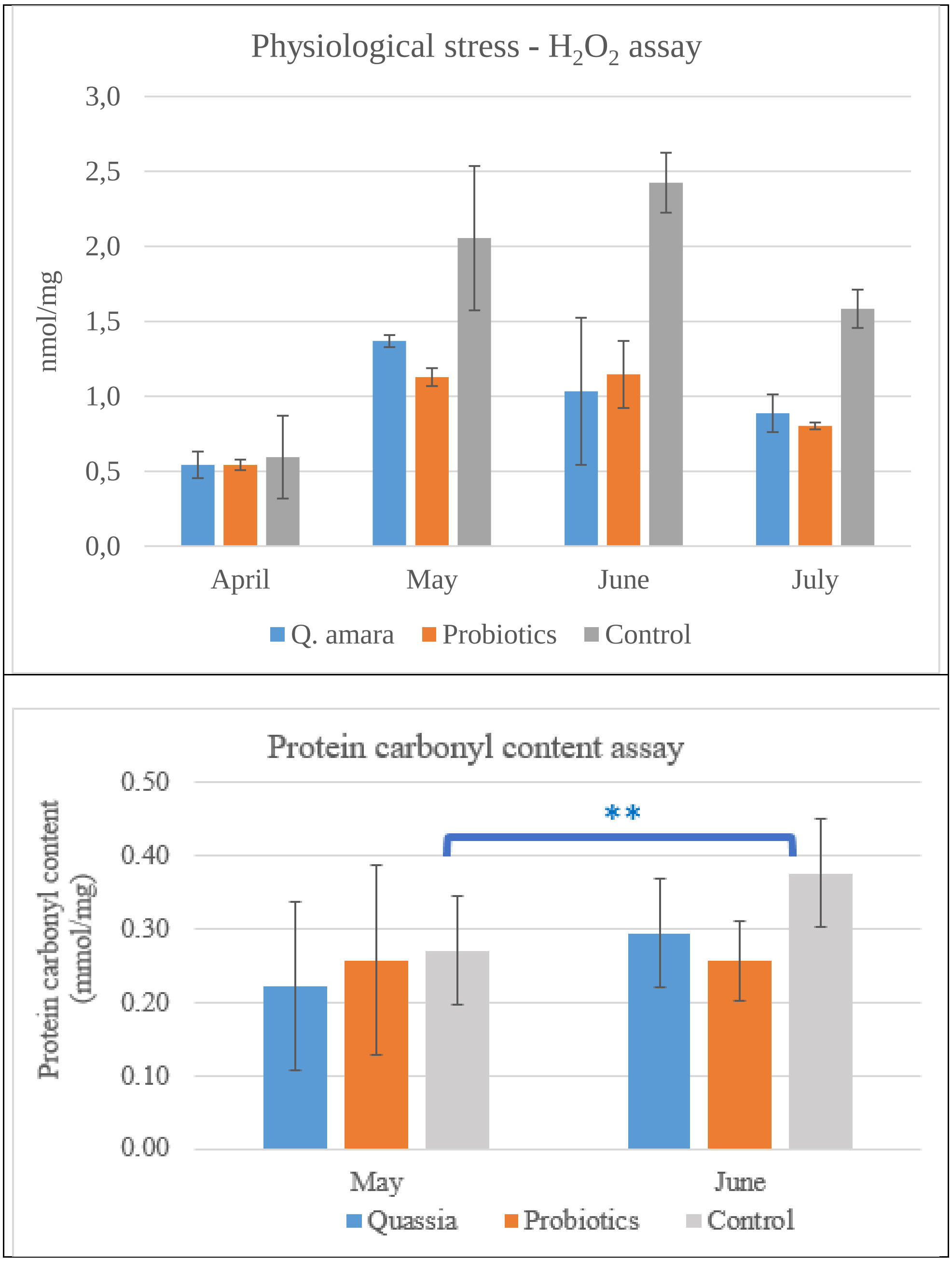
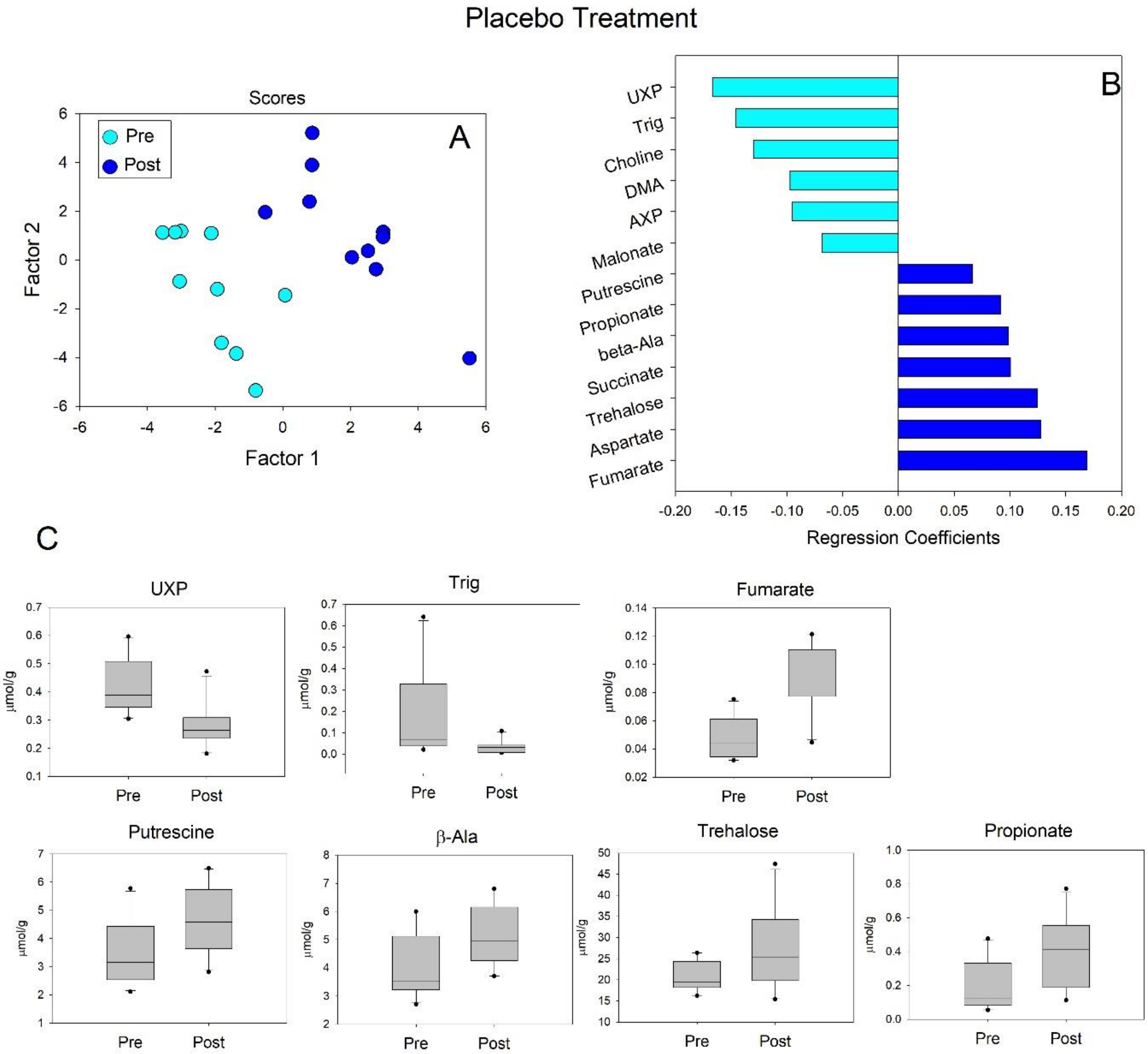


Fig. 2. Honey bee oxidative stress. Determination of both hydrogen peroxide and protein carbonyl content in bees fed with placebo (C), *Quassia amara* (Q) and probiotic (P) before (May) and after (June) the fire event. The level of significance is: ** = p <0.01.



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925 Fig. 3. Comparison performed on placebo-treated bees in pre (light blue) and post (dark blue) event. A) PLS scores plot, B) regression coefficients of significant variables that were lower (blue) and higher (red) after the event. C) Boxplot of metabolites which showed a statically significant difference between pre and post event, being equal the placebo treatment.

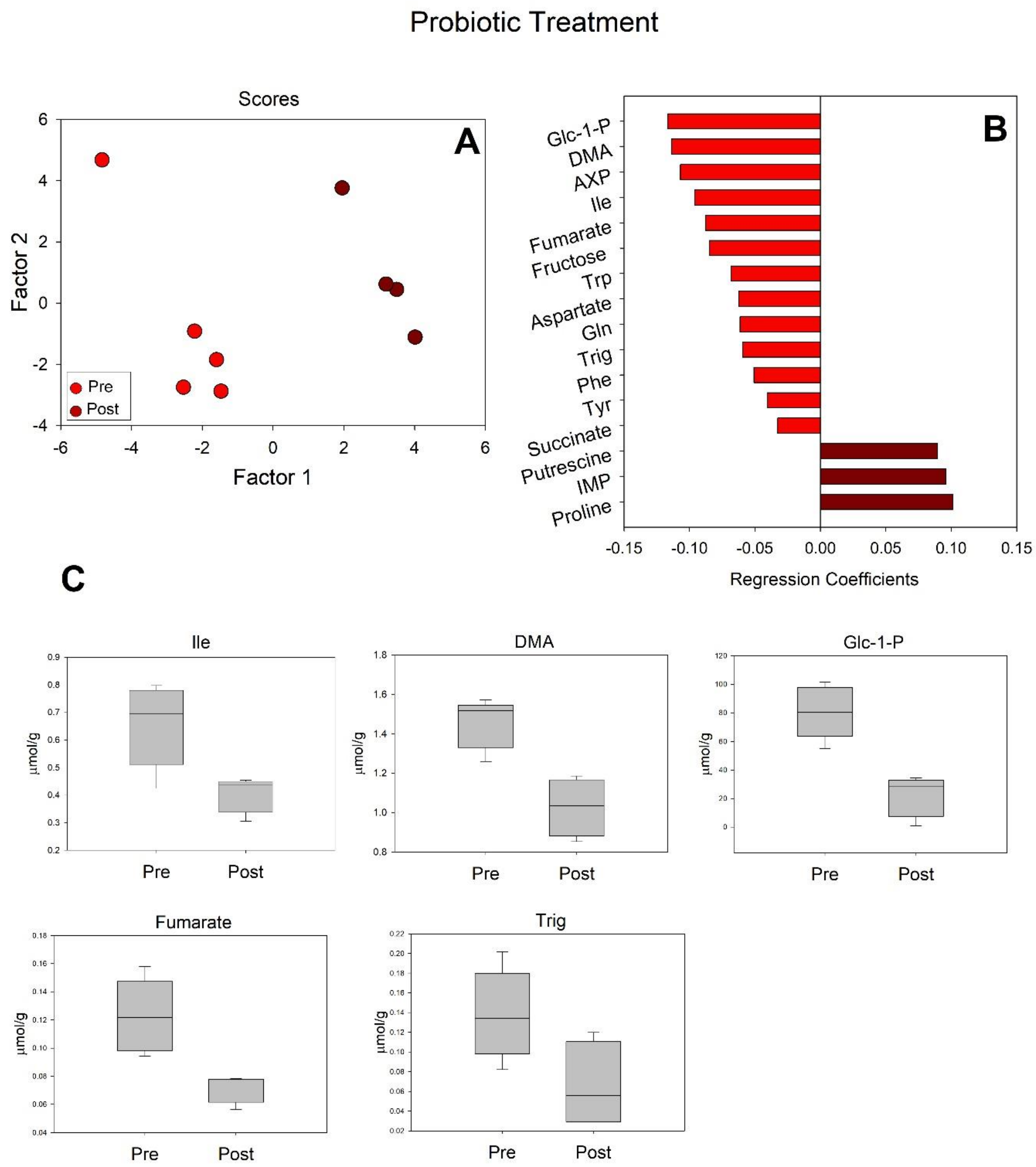

Fig. 4. Comparison performed on probiotic-treated bees in pre (red) and post (dark red) event. A) PLS scores plot, B) regression coefficients of significant variables that were lower (red) and higher (dark red) after the event. C) Boxplot of metabolites which showed a statically significant difference between pre and post event, being equal the probiotic treatment.

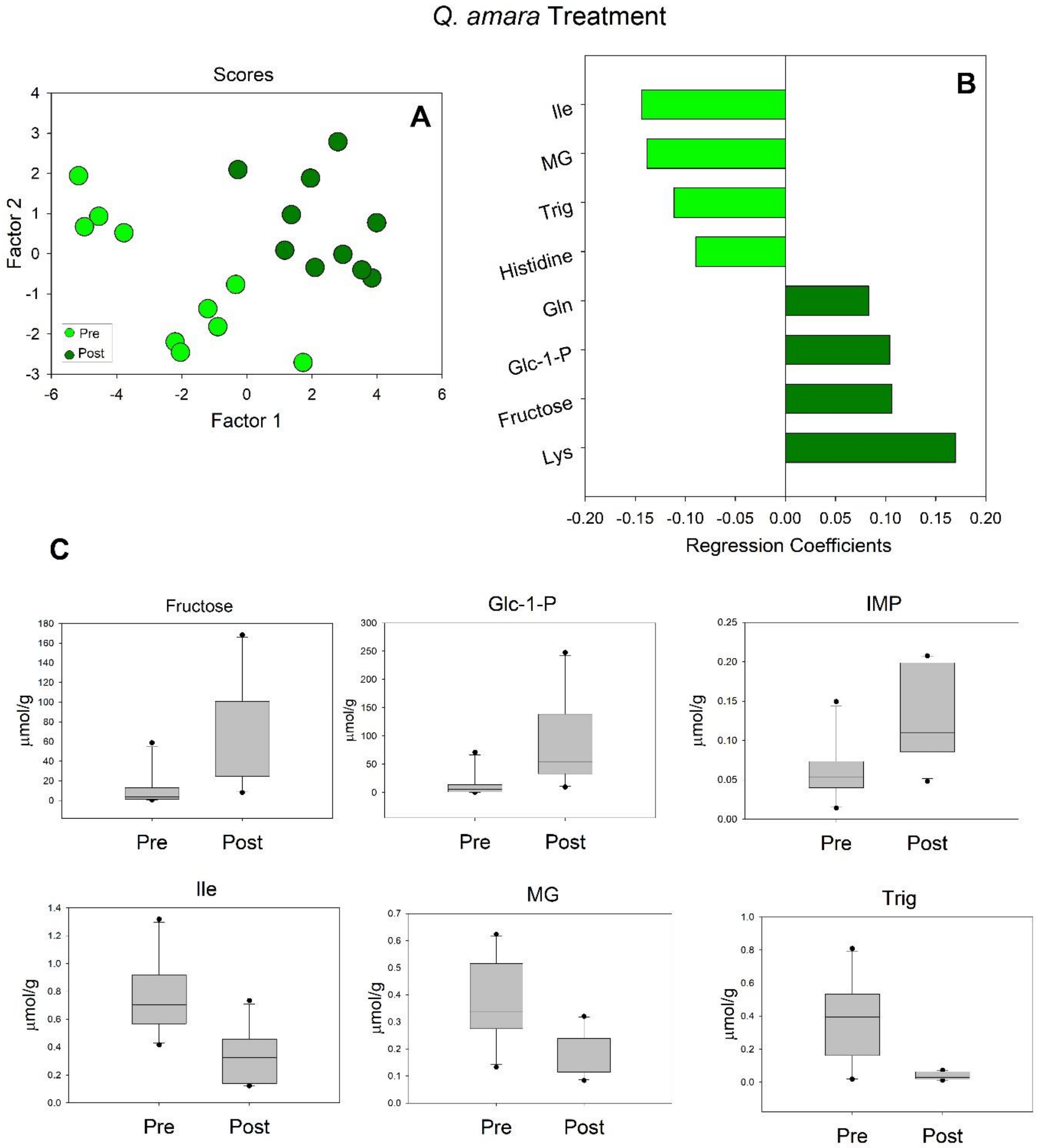

Fig. 5. Comparison performed on *Q. amara*-treated bees in pre (light green) and post (dark green) event. A) PLS score plot on *Q. amara*-fed bees considered on pre (green) and post (dark green) fire event; B) regression coefficients of significant variables that were lower (green) and higher (dark green) after the event. C) Boxplot of metabolites which showed a statically significant difference between pre and post event, being equal the *Q. amara* treatment.

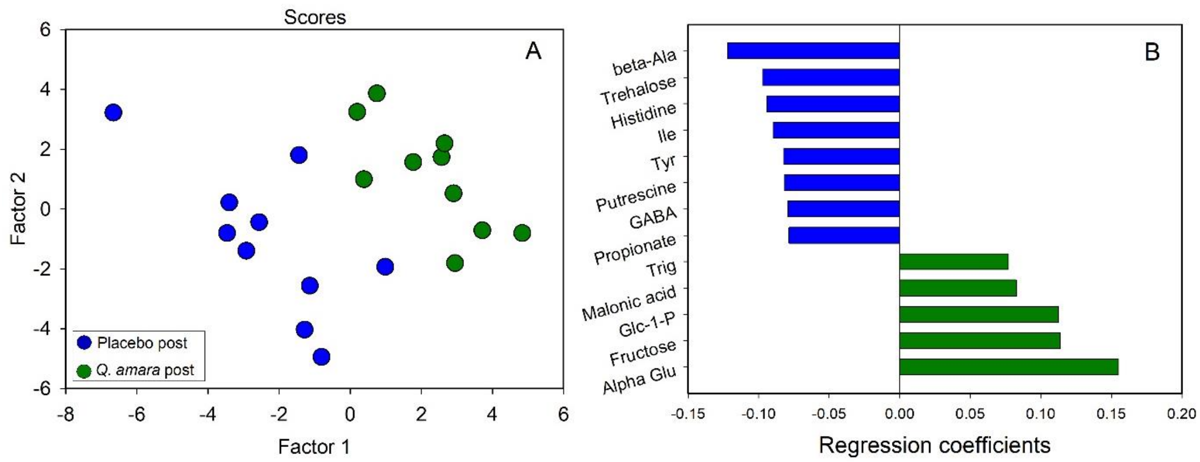

Fig. 6. PLS performed on Placebo and *Q. amara* fed bees after the event. A) Score plot on Placebo (blue) and *Q. amara*-fed bees (green) considered after the fire; B) regression coefficients of significant variables that were lower (blue) and higher (green) in *Q. amara* treated bees.