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Imidacloprid and amitraz differentially alter antioxidant enzymes in honeybee (*Apis mellifera*) hemocytes when exposed to microbial pathogen-associated molecular patterns

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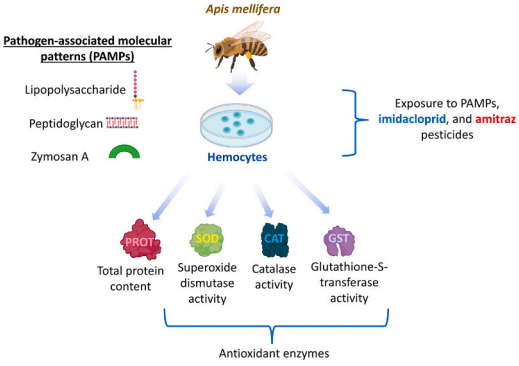
HIGHLIGHTS

- Imidacloprid and amitraz disrupt honeybee antioxidants with zymosan A.
- GST activity is significantly reduced by pesticide and fungal PAMP exposure.
- Lipopolysaccharide mitigates pesticide-induced oxidative stress in hemocytes.
- Catalase activity remains stable despite pesticide and immune stimulations.
- Honeybee oxidative defenses are weaker against fungal than bacterial PAMPs.

GRAPHICAL ABSTRACT



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ABSTRACT

Honeybees (*Apis mellifera*) are increasingly exposed to pesticides and microbial stressors, yet their combined effects on immune defenses remain unclear. Exposure to the neonicotinoid imidacloprid and the acaricide amitraz, alone and in combination, alters antioxidant enzyme activity in hemocytes when challenged with bacterial components such as lipopolysaccharide and peptidoglycan or the fungal-derived molecule zymosan A. The combination of pesticides with zymosan A synergistically suppresses superoxide dismutase and glutathione-S-transferase activity, while catalase activity remains unchanged. In contrast, lipopolysaccharide counteracts pesticide-induced oxidative stress, suggesting immune-pathway-specific modulation. The heightened vulnerability of honeybees to fungal-associated immune challenges in pesticide-contaminated environments compromises their ability to detoxify harmful substances and respond to infections. Such approaches that include comparison of different microbial interactions, pesticide cocktails, and immunity are needed. Understanding these interactions is essential for improving pesticide regulations and pollinator conservation efforts in the face of increasing environmental stressors.

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1. Introduction

The effects of pesticides on the environment have been a focus of research due to their hazardous impacts on both ecosystems and exposed organisms ranging from plants to animals including humans (Hashimi et al., 2020). Pesticides go beyond the scope of their intended use against certain pests and affect non-target organisms such as insects (Sánchez-Bayo, 2021). Rather than just hindering non-target organisms' health in the same manner as target organisms, pesticides may also alter the ecological scope by eliminating the natural enemies of pests (Sánchez-Bayo, 2021). A rapid decrease in species reaching a 70 % reduction in insects and 40 % in pollinators such as bees and butterflies is a warning sign to the environmental impacts of pollution and anthropogenic activity not to mention the extended effect of pesticides along the food chains via biomagnification (Ali et al., 2021).

The European honeybee is a highly valued pollinator worldwide, contributing significantly to 30 % global agricultural crop production and a third of human dietary supply an increasing demand (Khalifa et al., 2021). Additionally, honey production has substantial economic value in addition to its nutritive benefits ranging between 235 and 577 USD up till the 2015 estimation (Potts et al., 2016). However, honeybees are exposed to multiple risk factors simultaneously including pesticides and pathogens (Hristov et al., 2020; Doublet et al., 2015). In 2006, widespread honeybee colony collapses were reported (van Engelsdorp et al., 2009). Though no single cause was identified, the interaction of various risk factors leading to honeybee hive collapse remains unclear and complex at many levels (Cox-Foster et al., 2007; Van Engelsdorp et al., 2010).

Of the pesticides, neonicotinoids which are broad-acting insecticides and are mainly used to counter sucking insects (Bass et al., 2015). Neonicotinoid pesticides are of main concern in beekeeping and may be a cause in bee decline and colony loss (Klingelhöfer et al., 2022). Neonicotinoids are systemic neurotoxic pesticides of insects binding to the acetylcholine receptors leading to the accumulation of acetylcholine and continuous activation of the nervous system (Tomizawa and Casida, 2005; Casida and Durkin, 2013; Casida, 2018). Imidacloprid is the world's most used neonicotinoid (Klingelhöfer et al., 2022) and has been shown to negatively affect honeybee health, increasing their susceptibility to pathogens and diseases via synergism between pesticides and viruses or pathogenic fungi like *Nosema* spp. (Doublet et al., 2015; Di Prisco et al., 2013). The increased susceptibility of bees exposed to pesticides is achieved by hindering the immune system through the immunosuppressing effect of pesticides (Sánchez-Bayo et al., 2016). Neonicotinoids have been reported to decrease the immunocompetence of honeybees by affecting hemocyte count, antimicrobial response, and wound healing (Brandt et al., 2016). Furthermore, honeybees are adequate bioindicators of pesticide contamination in the environment which could extend to comprehensive risk assessments (Sukkar et al., 2025a).

Imidacloprid, the most used neonicotinoid, has been observed to impair immune responses in honeybees by altering the production of defensive molecules, such as reactive oxygen and nitrogen species (e.g., nitric oxide and hydrogen peroxide) (Sukkar et al., 2023b). It also affects immune gene expression in pathways like the Toll pathway and disrupts cellular processes such as phagocytosis (Sukkar et al., 2023a; Malladi et al., 2023; Sukkar et al., 2024). Furthermore, pesticide synergism has been documented, with imidacloprid and other chemicals potentially interacting to produce more severe effects on honeybee immunity. Amitraz is another pesticide that affects honeybee health and immunity. It is primarily used to treat mite infestations, particularly against the honeybee pest *Varroa destructor* (Rosenkranz et al., 2010). Although amitraz has been considered safe for honeybees due to its low metabolism of active compounds, some studies suggest otherwise. Synergistic effects have also been observed when imidacloprid or amitraz are present with other pesticides (Malladi et al., 2023; Shojaei et al., 2018; Dai et al., 2018).

Many microbes target honeybee hives, including several viruses, bacteria, and fungi. Viruses that infect honeybees are numerous, but the majority are positive single-stranded RNA viruses, some can be transmitted by vectors such as the *Varroa destructor* viris-1 and some are yet to be classified (Brutscher et al., 2016). As for bacteria that infect honeybees, the most notorious are American foulbrood (Matović et al., 2023) and European foulbrood (de León-Door et al., 2020), which affect honeybee larvae, leading to their rot and death. Other microbes that infect honeybees are the *Nosema* spp., which are the causal agent of nosemosis diseases. *Nosema* spp. are microsporidian unicellular obligate intracellular fungi that infect the guts of honeybees. The 3 main Nosema species are *Nosema apis*, *Nosema cerenae* and *Nosema Neumann* (Goblirsch, 2018; Chemurot et al., 2017; Fries, 1993). Notably, *Nosema ceranae* is of high concern since it doesn't show any outward clinical symptoms and is associated with collapsed hives (Marín-García et al., 2022).

In the presence of multiple stress factors, like several pesticides and pathogens, it is necessary to evaluate honeybee responses and health in complex conditions involving multiple pesticides and/or pathogens. In response to pathogens, honeybees produce reactive molecules like nitric oxide and hydrogen peroxide as a defense mechanism (Rivero, 2006; Herrera-Ortiz et al., 2011). However, these molecules can also damage the cells that produce them, requiring a delicate balance between oxidants and antioxidants to limit cellular self-damage (Felton and Summers, 1995). Superoxide dismutase (SOD) is an enzyme that catalyzes the conversion of superoxide (O_2^-) into hydrogen peroxide (H_2O_2) (Sheng et al., 2014). The concentration of H_2O_2 is regulated by catalase, an enzyme that breaks down H_2O_2 into water (Sepasi Tehrani and Moosavi-Movahedi, 2018). The balance between SOD and catalase determines the levels of reactive oxygen species, which in turn influence the extent to which H_2O_2 can destroy microbes while minimizing damage to the host organism's cells. In addition to SOD and catalase, glutathione S-transferase (GST) is another enzyme involved in antioxidant regulation, as well as in the detoxification and metabolism of pesticides, xenobiotics, drugs, and toxins (Vaish et al., 2020). GST's multifunctionality makes it a valuable target for analysis in toxicology experiments, especially in organisms exposed to both toxins and oxidative stress-inducing agents.

Honeybees, subjected to simultaneous risk factors, require toxicological tests that involves their interaction with pesticide cocktails and microbial representatives in order to understand the effect of these pesticides on the capacity of honeybee to respond to infection. In this work, we aim to evaluate the effect of pesticide combinations (amitraz and imidacloprid) on honeybee hemocytes, focusing on oxidant/antioxidant enzyme activity in the context of immune stimulation by microbial pathogen-associated molecular patterns (PAMPs). The PAMPs used represent gram-positive bacteria (peptidoglycan), gram-negative bacteria (lipopolysaccharide), and fungi (zymosan A) (Underhill, 2003; Erridge et al., 2002; Schleifer and Kandler, 1972).

2. Materials and methods

2.1. Larval hemolymph extraction

In the spring, honeybee larvae (*Apis mellifera*; Buckfast) frames were cut from hives established at the IUT Thionville-Yutz site in France. Larvae at the 5th instar stage were then extracted using a grafting tool and placed in Petri dishes. Hemolymph was extracted under sterile conditions by puncturing the posterior dorsal section of the larvae and quickly pooling the hemolymph in an Eppendorf tube using a micropipette. Each larva provided 30–35 μL of hemolymph. Hemolymph from 50 larvae was pooled for each experimental plate. The pooled hemolymph was centrifuged at 5000*g* for 5 min, washed twice with sterile phosphate-buffered saline (PBS; D8537, Sigma-Aldrich™), and then incubated with the treatments.

2.2. Exposures and treatments

All chemicals were sourced from Sigma-Aldrich™. Stock solutions were prepared accordingly. Amitraz(s.) was dissolved in hexane at a concentration of 10 mg·mL^{-1}, then diluted to 40 μg·mL^{-1} and 200 μg·mL^{-1} in PBS, with the evaporation of hexane taken into account. Imidacloprid(s.) was prepared in the same way as amitraz, but using PBS instead of hexane. The immune stimulators lipopolysaccharide, peptidoglycan, and zymosan A were dissolved in PBS to create 1 mg·mL^{-1} stock solutions.

The pesticide treatments were prepared to obtain the following final concentrations:

- Control: PBS without pesticides
- I10: 10 μg·mL^{-1} imidacloprid
- I50: 50 μg·mL^{-1} imidacloprid
- A10: 10 μg·mL^{-1} amitraz
- A50: 50 μg·mL^{-1} amitraz
- I10-A10: 10 μg·mL^{-1} imidacloprid+10 μg·mL^{-1} amitraz
- I10-A50: 10 μg·mL^{-1} imidacloprid+50 μg·mL^{-1} amitraz
- I50-A10: 50 μg·mL^{-1} imidacloprid+10 μg·mL^{-1} amitraz

All pesticide exposures were paired with 1 μg·mL^{-1} of each immune-stimulatory PAMP in PBS. A total of 36 different treatment conditions and combinations were tested. The plates were sealed with UV-sterilized tape and incubated at 20 °C for 3 h.

2.3. Total protein quantification

Total protein was measured using the Pierce™ Dilution-Free™ rapid gold BCA Protein Assay Kit (Thermo Scientific™, A55861). The principle is based on the reduction of Cu^{2+} to Cu^{1+} by proteins in an alkaline medium, which then reacts with the BCA chelator to form a complex with an observable color. The protocol was performed according to the manufacturer's manual (MAN0029413). The working solution (WR) was prepared as needed by mixing Reagent A and Reagent B from the kit with a 50:1 volumetric ratio respectively. Samples and the standard curve dilutions were added in 10 μL volumes in each well to Greiner UV-Star® 96 well plates (M3812, Sigma-Aldrich™). The standard curve is composed of bovine serum albumin (BSA) at concentrations of 0, 0.125, 0.25, 0.5, 1, 2, 5, 10 mg·mL^{-1} in each well, 200 μL of WR was added using a multi-pipette and. The reading was done within 10 min of the reaction at 480 nm wavelength using a spectrophotometer (BioTek™, Winooski, VT, USA, PowerWave XS2). The standard and the samples were normalized to protein-free blanks before calculating the concentrations ($n = 5$, $p < 0.05$). The same type of UV plates was used throughout the experiment for practicality and are not obligatory for all applied assays.

2.4. Superoxide dismutase activity assay

Superoxide dismutase (SOD) is an evolutionarily conserved enzyme present across all kingdoms of life, existing for billions of years and found in several forms in different organisms (Case, 2017). Superoxide dismutase in an enzyme that produces H_2O_2 from oxygen radicals which is a byproduct from metabolic processes and can cause oxidative damage to organisms if not catalyzed (Abreu and Cabelli, 2010; Fukai and Masuko, 2011). We measured the activity of SOD using a Superoxide Dismutase (SOD) activity assay kit (CS0009, Sigma-Aldrich™). The assay is based on the interaction between superoxide anion and the WST (Water-Soluble Tetrazolium salts) dye, resulting in the production of formazan, which is detected at λ = 450 nm. SOD catalyzes superoxide anion dismutation into oxygen and hydrogen peroxide. Hence, the intensity of coloration is inversely proportional to SOD activity. Samples were prepared for analysis by centrifuging the previously incubated plates at 2500*g* for 10 min, removing the supernatant followed by adding 30 μL of ice-cold lysis buffer to each well. The lysis buffer consisted of 0.1 M Trizma-HCl(l.) (T1819, Sigma-Aldrich™), 0.5 % Triton-X, 1 % phenylmethanesulfonyl fluoride(s.) (P7626, Sigma-Aldrich™), and 5 mM 2-mercaptoethanol(l.) (21985023, Gibco™). Lysed samples underwent centrifugation at 2500*g* for 10 min at 4 °C. Samples and standard curve dilutions were added to Greiner UV-Star® 96 well plates (M3812, Sigma-Aldrich™) containing 160 μL of WST. The reaction was initiated by adding 20 μL of diluted xanthine oxidase solution provided with the kit to each well. The samples were measured in 5 replicates with a 95 % confidence interval for statistical analysis.

Standard curve concentrations were 0.3, 0.6, 0.9, 1.5, 3 and 6 units·mL^{-1}. Blanks for samples and xanthine oxidase were also prepared and the volume was adjusted by the provided dilution buffer.

The SOD activity was calculated by the following equations:

$$\mathrm{LSR} = \frac{A_{max}}{A_{blank} - A_{sample}}$$

$$\text{SOD Activity}_{\text{units·mL}^{-1}} = \frac{\left(\mathrm{LSR} - y_{intercept}\right)(10 \cdot \mathrm{DF})}{\text{slope}}$$

where:

A_{blank} = Absorbance of the blank
A_{sample} = Absorbance of the sample
A_{max} = Maximal absorbance without SOD
LSR = Linearized SOD rate
DF = Dilution factor

The slope is generated from the standard curve. Plate-to-plate differences was accounted for pesticide groups for comparability and data was standardized accordingly.

2.5. Catalase activity assay

Similar to SOD, catalase is a crucial enzyme in regulating ROS concentration by catalyzing the breakdown of H_2O_2 when it is at high concentrations, converting it into water molecules and preventing oxidative damage. Monitoring catalase activity serves as an indicator of cellular health (Sepasi Tehrani and Moosavi-Movahedi, 2018; Nandi et al., 2019). Catalase activity was measured by a Catalase Assay Kit (MAK531, Sigma-Aldrich™). Solutions and assay reactions were prepared according to the manufacturer's instructions. Plates incubating hemocytes were centrifuged at 2500*g* for 10 min followed by the removal of supernatant leaving 10 μL in each well. Hemocyte membranes were lysed by placing plates at −20 °C for 10 min before the addition of reaction buffer. Hydrogen peroxide stock (4.8 mM) was prepared by diluting 5 μL of 3 % H_2O_2 in 914 μL ultra-pure water. Next, the stock solution was further diluted to 50 μM by adding 100 μL of 4.8 mM H_2O_2 into 9.5 mL of assay buffer for each plate. The catalase reaction was initiated by adding 90 μL of 50 μM H_2O_2 in each well and incubation for 30 min at room temperature. During incubation, standard curve H_2O_2 dilutions were prepared at concentrations of 0, 6, 12, and 20 μM. Standard curve dilutions were then added to the 96-well plate. The detection reagent was prepared by mixing 100 μL of horse radish peroxidase (HRP), 100 μL detection dye, and 10.2 mL of assay buffer. The catalase reaction was stopped by adding 104 μL of detection reagent to each well. The plate was read by spectrophotometry at λ = 570 nm. The samples were measured in 5 replicates with a 95 % confidence interval for statistical analysis. Results were calculated by the following equation:

$$\text{Catalase}_{\text{U·L}^{-1}} = \frac{\left(A_{blank} - A_{sample}\right)}{\text{slope} \cdot 30\ \text{minutes}} \cdot \mathrm{DF}$$

where:

A_{blank} = Absorbance of the blank
A_{sample} = Absorbance of the sample
DF = Dilution factor

The slope is that of the standard curve and 30 min is the reaction time.

2.6. Glutathione-S-transferase activity assay

Glutathione-S-transferases (GST) are a family of proteins involved in detoxification of toxic compounds including pesticides and in protecting organisms from their effect in addition to countering ROS (Vaish et al., 2020; Eaton, 1999). Glutathione-S-Transferase (GST) Assay Kit (CS0410, Sigma-Aldrich™) was used in this experiment. The concept of this assay is utilizing 1-chloro-2,4-dinitrobenzene (CDNB), for its versatility towards different isoenzymes. The thiol group will conjugate to CDNB, a reaction catalyzed by GST. It is this conjugated product that is detected by spectrophotometer reading at λ = 340 nm. The absorbance is directly proportional to GST activity. Analysis of the results will be based on a standard curve following the indicated procedure. Positive controls for GST were prepared at concentrations of 6.25, 3.125, and 1.56 μg·mL^{-1}, respectively, in triplicate. Samples were analyzed in quadruplicates with a 95 % confidence interval for statistical analysis.

$$\Delta A = \frac{A_f - A_i}{\text{reaction time}}$$

$$\text{GST Activity}_{\mu mol \cdot mL^{-1} \cdot min^{-1}} = \frac{\Delta A \cdot V \cdot DF}{\varepsilon_{mM} \cdot V_{enz}}$$

where:

A_f = Absorbance at the final measurement
A_i = Absorbance at the first measurement
V = Volume of the sample in mL
DF = Dilution factor
V_{enz} = Volume of the enzyme sample added in mL
ε_{mM} = 5.3 mM^{-1}·cm^{-1} (extinction coefficient for CNDB conjugate in 96-well plates)

2.7. Statistical analysis

Statistical analysis was carried out using Addinsoft® XlSTAT™ 2019.3.2. All the data was checked for normal distribution followed by Bartlett's test to verify the difference between variances. If the data was normal and variances were homogenous then significance differences are tested by ANOVA with Duncan post-hoc test. Kruskal-Wallis test was applied in place of ANOVA incase data was not normal even after transformation or if variances were not homogenous followed by a Dun post-hoc test.

Data comparison was done in 2 approaches for better visualization of differences; the first approach is to compare significant differences within groups of immune stimulators (CONTROL, LPS, ZYM, PGN), while the other approach is a comparison within different pesticide treatment groups. The statistical comparison of treatments within each group is independent from other groups unless stated otherwise. The confidence interval was set at 95 %.

GraphPad Prism® 10.41.1 was used to generate the heatmap of all experiments. All the data was normalized relative to the no-pesticide control for comparability before generating the heatmap.

3. Results and discussion

3.1. Total protein production

Total protein concentrations were standardized and compared relative to controls without pesticides (Fig. 1). The treatment of 10 μg·mL^{-1} imidacloprid (I10) resulted in a 0.19 % increase with the no-PAMP control (CONTROL) group but a 9.34 % increase in total protein with LPS which is the highest increase observed in the I10 pesticide group. In the PGN treatment, I10 resulted in a 0.61 % increase in total protein.

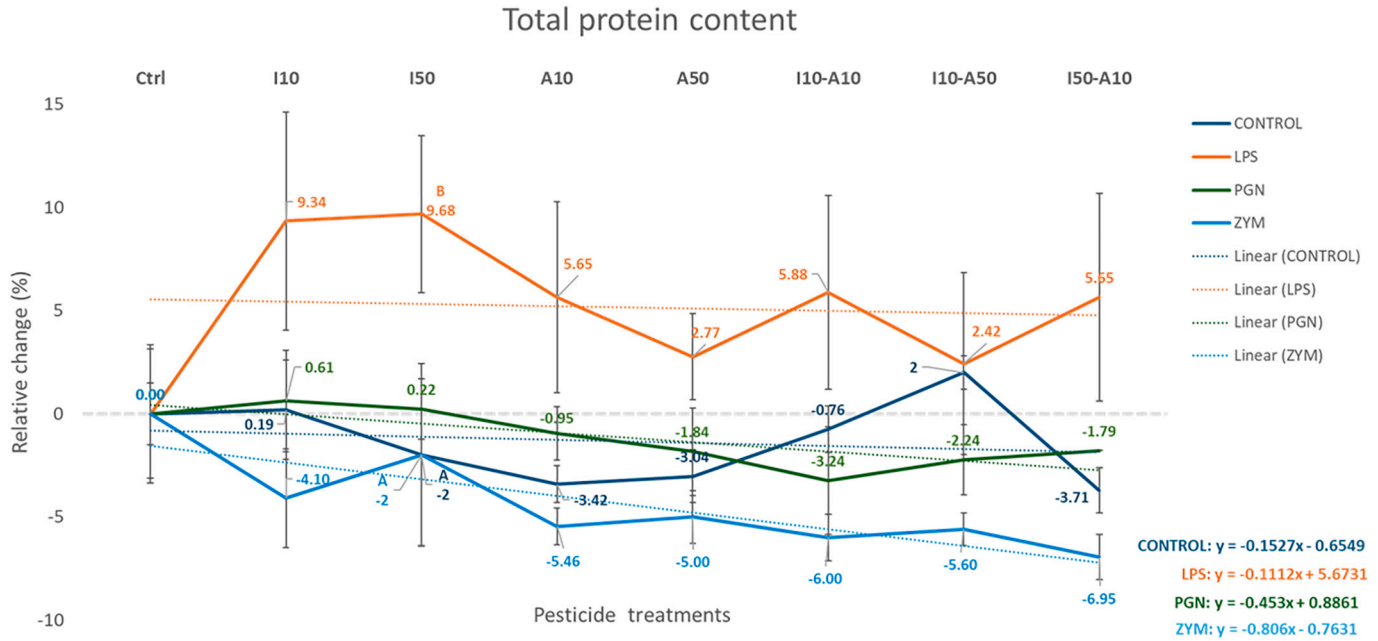

Fig. 1. Variations of total protein content in honeybee hemocytes exposed to pesticide treatments with different immune stimulators. Hemocytes were treated with 8 different pesticide conditions and 4 PAMP groups; CONTROL, 1 μg·mL^{-1} lipopolysaccharide (LPS), 1 μg·mL^{-1} zymosan A (ZYM), or 1 μg·mL^{-1} peptidoglycan (PGN). In addition to the no-pesticide control (Ctrl), hemocytes were treated with either single exposures of imidacloprid (I10 or I50), or amitraz (I10 or I50), or mixtures of different concentrations (I10-A10, I10-A50, or I50-A10). All pesticide treatment concentrations are in μg·mL^{-1}. Significant differences within the pesticide treatment but different PAMPs are indicated by different letters ($p < 0.05$, $n = 5$). Linear trends are visualized as dotted lines and their equations are indicated on the bottom right corner. Error bars represent standard errors.

However, ZYM treatment resulted in 4.1 % decrease in total protein of hemocytes.

With 50 μg·mL^{-1} imidacloprid (I50) pesticide treatment, LPS also resulted in a 9.68 % increase being significantly higher than the no-PAMP control and ZYM which both showed a 2 % decrease of total protein while the PGN was not significantly different compared to other PAMP treatments with I50.

For amitraz treatments, LPS showed the only relative increase (5.65 %) with the 10 μg·mL^{-1} amitraz treatment while all the other PAMP treatments showed a decrease in total protein with a 0.95 % decrease with PGN followed by a 3.42 % decrease in CONTROL and a 5.46 % decrease with ZYM. Similarly, the 50 μg·mL^{-1} amitraz (A50) treatment led to a 2.77 % increase with LPS, and a decrease with CONTROL (3.04 %), PGN (1.84 %), and ZYM (5 %).

Pesticide cocktail (I10-A10) led to a 5.88 % increase in total protein with LPS but to decreases with CONTROL, PGN, and ZYM respectively. Interestingly, the pesticide cocktail I10-A50 led to an increase in total protein for both LPS (2.42 %) and CONTROL (2 %) while the decrease was retained with PGN (2.24 %) and ZYM (5.6 %) making it the only treatment where the total protein in the CONTROL group increased. This observation did not extend to the I50-A10 pesticide cocktail where the total protein increased by 5.65 % with LPS and decreased by PGN, CONTROL, and ZYM respectively.

Compared to other experiments that include measurement of protein on whole bees, pesticides showed significant differences on protein levels with prolonged exposure up to 4 weeks, and seasonal differences were observed (Feazel-Orr et al., 2016). In contrast, other studies showed context-specific effects of pesticide exposures on honeybee protein content and weight (Cook, 2019). Furthermore, the effect of pesticides was observable on specific genetic and cellular parameters in hemocytes with pesticide-PAMP exposures (Walderdorff et al., 2018; Sukkar et al., 2024; Sukkar et al., 2023a).

This may indicate that the overall effect of complex pesticide-microbial interaction with the honeybee immune system is generally not observable at the total protein level and must be accompanied by in-depth analysis as well.

Although significance was only observable with the I50 pesticide treatment, when presenting the linear trends (Fig. 1), the slops demonstrated a general decrease. However, treatments that included LPS had the lowest inclination (slope = −0.1112) followed by the CONTROL (slope = −0.1527), PGN (slope = −0.453), and ZYM (slope = −0.806). This infers that LPS could alleviate the effect of pesticides and pesticide cocktails on the total protein production in *A. mellifera* hemocytes. Furthermore, peptidoglycan (PGN) and zymosan A (ZYM) appear to increase the effect of pesticides on total protein concentrations with zymosan having the most effect.

From the previously mentioned experiments, it is sound to say that measurements of total proteins from whole bees are more illustrative when it comes to the effect of pesticides than hemocyte protein production in terms of significance. This is because the behavioral (food consumption) and seasonal physiological variations of the hive are an integration of complex parameters that affect protein content. These variations are nonexistent with *in vitro* assays and cannot be integrated into the analysis directly. However, the minute differences in such parameters with *in vitro* assays could be representative when taken as a whole or when suitable predictive models are developed. Thus, it should still be considered as complementary input that could prove beneficial in continued assessments.

3.2. Superoxide dismutase (SOD) activity

Measuring SOD activity revealed a differential effect of pesticides in the presence of PAMPs (Fig. 2). The imidacloprid treatment at 10 μg·mL^{-1} (I10) led to a decrease in SOD activity by 3.69 %, 1.91 %, and 20.96 % with CONTROL, LPS, and ZYM, respectively, whereas a 13.49 % increase in SOD activity was observed with PGN. Increasing the concentration of imidacloprid to 50 μg·mL^{-1} (I50) resulted in less variation, with changes of −4.46 % (CONTROL), +1.55 % (PGN), and +0.4 %

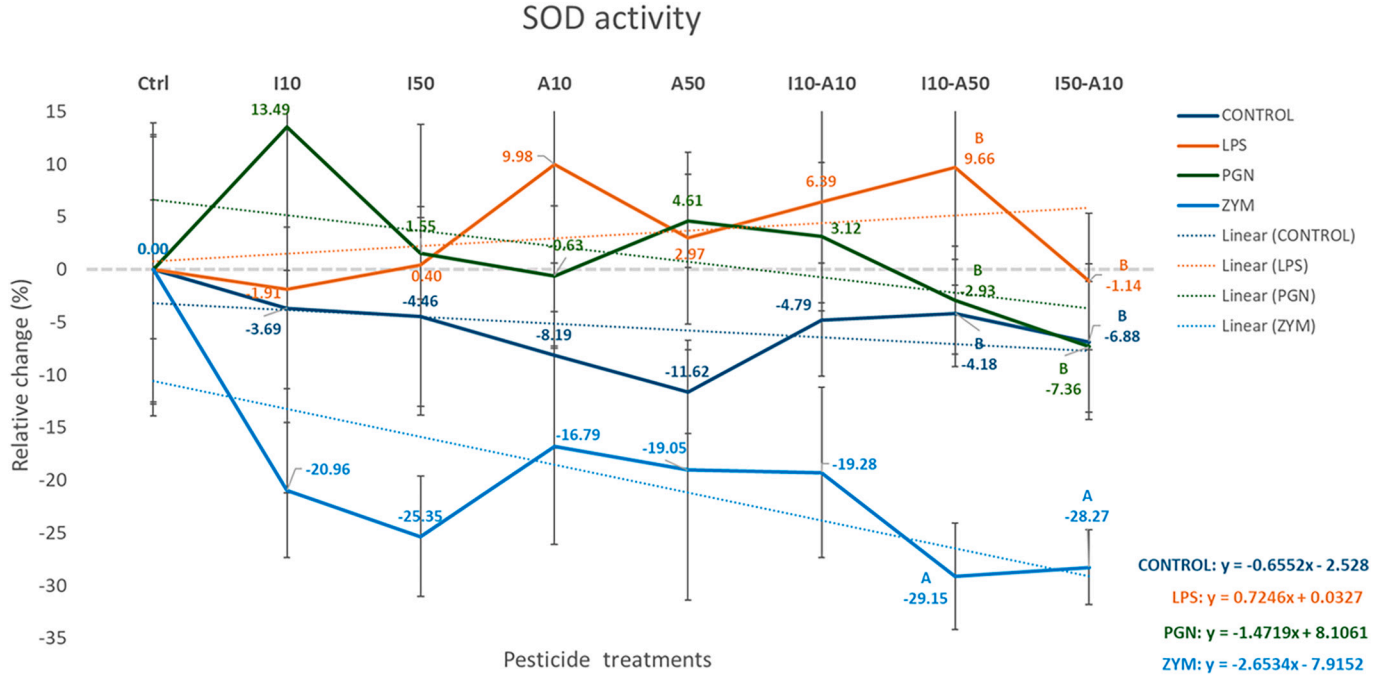

Fig. 2. Superoxide activity of honeybee hemocytes within groups of different immune stimulators and pesticide treatments. In addition to the no-pesticide control (Ctrl-no pesticide), hemocytes were treated with either single exposures of imidacloprid (I10 or I50), or amitraz (I10 or I50), or mixtures of different concentrations (I10-A10, I10-A50, or I50-A10). Hemocytes were treated with 8 different pesticide conditions and 4 immune stimulator groups; without immune stimulator (CONTROL), 1 μg·mL^{-1} lipopolysaccharide (LPS), 1 μg·mL^{-1} zymosan A (ZYM), or 1 μg·mL^{-1} peptidoglycan (PGN). All concentrations are in μg·mL^{-1}. Significant differences within the pesticide treatment but different PAMPs are indicated by different letters ($p < 0.05$, $n = 5$). Linear trends are visualized as dotted lines and their equations are indicated on the bottom right corner. Error bars represent standard errors.

(LPS), while SOD activity decreased by 25 % when exposed to I50 and zymosan (ZYM).

Amitraz exposures of A10 and A50 decreased SOD activity by 8.16 % and 11.62 % in the CONTROL group, respectively. Similarly, 16.79 % and 19.05 % decreases in SOD activity were observed with A10 and A50, respectively, in the ZYM group. However, inverse patterns were observed with LPS and PGN under amitraz exposure. The LPS group demonstrated a 9.98 % increase with A10 but only a 2.97 % increase with A50, whereas the PGN group showed a 0.63 % decrease with A10 and a 2.97 % increase with A50.

The most interesting observations occurred with pesticide cocktails. When the ratio of amitraz to imidacloprid was equal (I10-A10), no significant differences were observed; however, SOD activity decreased by 4.79 % and 19.28 % in the CONTROL and ZYM groups, respectively, while it increased by 6.39 % and 3.12 % in the LPS and PGN groups, respectively. In the case of the pesticide cocktail (I10-A50), SOD activity increased by 9.66 % with LPS but decreased by 2.93 % and 4.18 % with PGN and CONTROL, respectively. All values were significantly higher than in the ZYM group, which exhibited a 29.15 % decrease in SOD activity. Similarly, SOD activity decreased by 28.27 % when hemocytes were exposed to the I50-A10 pesticide cocktail, which was significantly lower than the SOD activity variations observed in CONTROL (−7.36 %), LPS (−1.14 %), and PGN (−6.88 %).

When establishing the linear tendencies of the different PAMP groups, we observed that LPS illustrated a positive inclination (slope = 0.7246), indicating that LPS not only induces SOD activity but also ameliorates the effect of pesticides and their combined exposure. As for the CONTROL group that was not exposed to PAMPs, we observed a negative slope of −0.6552, indicating that pesticides, to some extent, contribute to the reduction of SOD activity. Furthermore, the tendencies of pesticides with PGN and ZYM formed slopes of −1.4719 and −7.9125. Given that zymosan exposure led to a significant decrease in SOD activity with pesticide cocktails compared to other PAMPs, this infers that zymosan may act synergistically with imidacloprid and amitraz in reducing SOD activity.

These conditions of multiple pesticide exposures mimic the partial complexity of different pesticides in the environment with pathogen encounters. As observed, the SOD levels are reduced with multiple pesticides, which may lead to lower hydrogen peroxide production and lower defense capacity against fungal infections in particular (Sukkar et al., 2023b). On the other hand, there was no significant difference between LPS or PGN treatments, and the control group of any pesticide treatment groups (groups of a pesticide treatment with different immune stimulator conditions). This infers that imidacloprid and amitraz display synergistic effects on the decrease of SOD activity but with the association of zymosan A exposure. We have previously reported that zymosan A induces a potential relationship between cellular responses in the presence of pesticides (Sukkar et al., 2024). The results presented by SOD also confirm the synergism of imidacloprid and amitraz in the presence of zymosan.

When honeybees were treated with probiotics, it showed a positive effect on the SOD production (Han et al., 2023). As the microbiota in honeybee guts is important for immunity and health, it could be that honeybees have co-evolved with bacteria to not be negatively affected at the antioxidant a capacity level when they interact with bacterial PAMPs (Motta and Moran, 2024). On the other hand, pesticides were observed to increase SOD activity in honeybee head section, but the effect of the same pesticides was inversed in the midgut (Pal et al., 2022). It is necessary to evaluate the effect of pesticides on different compartments and their totality. Furthermore, when it comes to the mode of action it appears that pesticides such as imidacloprid and amitraz focus on mainly affecting mitochondrial SOD production with LPS having a similar interaction by alleviating pesticide induced dysregulation of SOD (Sukkar et al., 2025b; Gregorc et al., 2018).

3.3. Preserved catalase activity with pesticides and PAMPs

Measuring catalase activity (Fig. 3), exposure to 10 $\mu g \cdot mL^{-1}$ imidacloprid (I10) led to no significant change in CONTROL (0.23 %), LPS (−6.22 %), PGN (−0.92 %), or ZYM (−1.2 %). Exposure to 50 $\mu g \cdot mL^{-1}$ imidacloprid (I50) decreased catalase activity by 16.36 %, 8.53 %, and 10.15 % with CONTROL, LPS, and ZYM but increased by 1.27 % with PGN. The 10 $\mu g \cdot mL^{-1}$ amitraz treatment resulted in decreases by 11.92 %, 11.75 %, and 7.12 % with CONTROL, ZYM, and LPS respectively but a 1.52 % increase with PGN. With the 50 $\mu g \cdot mL^{-1}$ amitraz (A50) the catalase activity decreased by 13.62 % with CONTROL, 1.49 % with LPS, 12.62 % with ZYM but increased by 3.45 % with PGN. No significant differences were observed with single pesticide exposures despite the concentrations used.

As for pesticide cocktails, all treatments led to a decrease in catalase activity. The I10-A10 mixture led to a decrease by 11.15 %, 3.15 %, 4.95 %, and 6.27 % with CONTROL, LPS, PGN, and ZYM respectively. The I10-A50 mixture resulted in closer variation in catalase activity with decreases of 8.49 %, 7.47 %, 10.05 %, and 7.99 % with CONTROL, LPS, PGN, and ZYM respectively. The final cocktail treatment I50-A10 resulted in decreases by 6.36 %, 5.49 %, 7.37 %, and 19.06 % with CONTROL, LPS, PGN, and ZYM respectively.

Statistically, there was no observed significance in treatments whether between pesticide treatments of PAMP groups. As for the general linear trends, all PAMP exposures resulted in negative inclinations the most was ZYM followed by PGN, CONTROL, then LPS with values of −1.8635, −1.3566, −0.8834, and −0.2721 respectively indicating that ZYM and PGN tend to decrease the catalase activity with pesticide exposures while LPS tends to increase the catalase activity.

The enzymatic activities of SOD and catalase are complementary in the regulation of the antioxidant/system. A proportionality between these enzymes must be present to maintain oxidative balance. However, when we look back at the SOD activity, it is observable that the pesticide combination along with zymosan resulted in lower SOD activity but in the catalase assay there was no change in activity. Lower SOD activity means lower H_2O_2 concentration and less capability to fend off pathogens but adding that catalase activity was unchanged then the concentration of H_2O_2 will be even lower than anticipated. This was observed in previous experiments valuating imidacloprid and amitraz synergism in the presence of zymosan (Sukkar et al., 2023b). In addition, imidacloprid-dosed diets were shown to increase catalase activity in honeybees (Balieira et al., 2018) compared to control conditions confirming our results.

The tissue specificity of pesticides effects on catalase must be considered as well. The results of Pal et al. (2022) showed that there is an organ-specific effect of various pesticide categories in honeybees where the catalase activity in the head section was not affect while it was significantly lowered in the midgut.

3.4. Zymosan synergizes with pesticides in reducing GST activity

The results in Fig. 4 illustrate the effect of imidacloprid and amitraz on GST activity in honeybee hemocytes. When exposed to 10 $\mu g \cdot mL^{-1}$ imidacloprid (I10), the GST activity decreased by 26.11 % and 24.59 % in the CONTROL and ZYM groups respectively. Both CONTROL and ZYM were significantly lower than LPS which only resulted in a 1.83 % decrease in GST activity. PGN exposure was not significantly different compared to other groups exposed to I10 with a 9.8 % decrease in GST activity. However, when imidacloprid concentration was 50 $\mu g \cdot mL^{-1}$ imidacloprid (I50), the CONTROL and PGN groups decreased by 18.14 % and 6.85 % respectively and there were not significantly different from LPS or ZYM exposures. LPS (−13.73 %) and ZYM (−34.43 %) groups were significantly different regarding catalase activity. In the case of imidacloprid exposure, LPS interaction tends to act oppositely to ZYM interaction inferring that different mechanisms are in play regarding microbial interaction in honeybees.

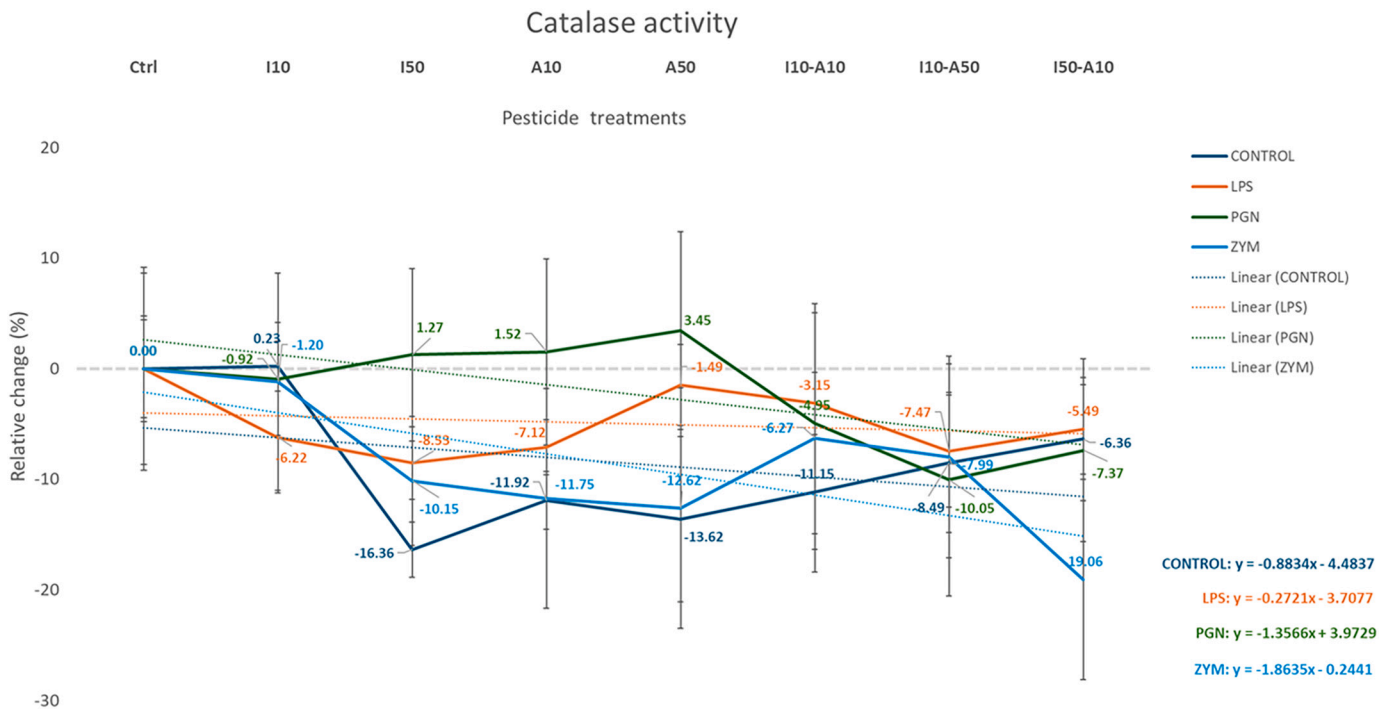

Fig. 3. Catalase activity of honeybee hemocytes within groups of different PAMPs and pesticide treatments. In addition to the no-pesticide control (Ctrl), hemocytes were treated with either single exposures of imidacloprid (I10 or I50), or amitraz (I10 or I50), or mixtures of different concentrations (I10-A10, I10-A50, or I50-A10). Hemocytes were treated with 8 different pesticide conditions and 4 PAMP groups; CONTROL, 1 μg·mL^{-1} lipopolysaccharide (LPS), 1 μg·mL^{-1} peptidoglycan (PGN), or 1 μg·mL^{-1} zymosan A (ZYM). All concentrations are in μg·mL^{-1}. Significant differences within the pesticide groups are indicated by different letters ($p < 0.05$, $n = 5$). Error bars represent standard deviations.

Amitraz treatment of 10 μg·mL^{-1} led to a 4.57 % decrease in GST activity with LPS, which is significantly different than both CONTROL (−21.24 %) and ZYM (−26.23 %) but not compared to PGN (−13.73 %). Additionally, PGN was significantly different compared to ZYM. The amitraz treatment of 50 μg·mL^{-1} (A50) led a decrease of 20.8 %, 4.57 %, 29.51 % in GST activity with CONTROL, LPS, and ZYM respectively but a 1.96 % increase with PGN. However, there was no significant difference with in the A50 treatment exposed to different PAMPs.

Pesticide cocktail I10-A50 led to a decrease in catalase activity by 9.29 %, 7.76 %, 11.76 %, and 27.87 % with CONTROL, LPS, PGN, and ZYM respectively, with zymosan being significantly different from the rest of the PAMP groups. The significance in the ZYM group was maintained compared to LPS and PGN nut not to CONTROL when hemocytes were exposed to the I50-A10 cocktail. Finally, the I10-A50 treatment resulted in significant difference between LPS (−1.37 %) and ZYM (−21.31 %) while CONTROL (−4.87 %) and PGN (27.45 %) were not significantly different compared to other PAMP exposures.

The results from pesticide cocktail treatments suggest that PAMPs act in a context specific manner rather than a dose-dependent response which is a crucial point to consider in risk assessments when evaluating multi-risk interactions.

In addition to comparing the effect of different PAMPs within the same pesticide treatments, we also analyzed the effect of the different pesticide treatments within the same PAMP group. The results demonstrated that zymosan A exposure significantly reduces GST activity in all pesticide treatments (indicated by asterisks in Fig. 4). Furthermore, the linear trends revealed that ZYM has the highest value of negative inclination of −1.776 adding confirmation to its effect. However, it should be considered that the treatments include different pesticides and not a single pesticide dose-response which could affect the robustness of using the linear trend as a reference.

In contrast to our results regarding fungal interaction, the levels of GST increased in honeybees when infected with *N. ceranae* but in the midgut and fatty body (Vidau et al., 2011). Thus, honeybees may have different detoxification potential with respect to organ and body tissue. This should be taken into account when studying multiple risk exposure. The results of the total protein where LPS had the highest concentration in all treatments (Fig. 1) could explain the higher levels on GST activity.

Generally, we can observe that zymosan A results in lower GST activity. While we aimed the verify if pesticide and PAMP interaction may cause more susceptibility to pathogens, the GST activity results suggest that interaction with fungal PAMPs could lead to more susceptibility to pesticides and toxins as well. GST is an important enzyme in detoxification and the decrease of its activity consequently results in lower detoxification potential. We note that the analysis concerns honeybee hemocytes and other compartments that may display different results not to mention at the level of the whole organism. Concerning studies on fungal interactions, *N. ceranae* affects honeybee carbohydrate metabolism, and thus fungal interaction may dysregulate cellular enzymatic processes, including detoxification enzymes and those involved in the antioxidant system (Martín-Hernández et al., 2018). Other pesticides, like flumethrin, have been shown to increase GST and catalase expression; however, the context of immune stimulation was not considered in this case (Yu et al., 2021). Indeed, the pesticide interactions in honeybees is complex and is comparable to the complexity to that in mammals (O'Neal et al., 2018).

Pesticides have shown to increase susceptibility to *Nosema* infection (Pettis et al., 2012; Wu et al., 2012). Overall, our results demonstrate that the interaction with the fungal PAMP zymosan A, in the presence of pesticides, indeed dysregulated the oxidative response and detoxification. This could lead to reduced pathogen resistance and increased susceptibility to toxins. The synergism between pesticides and fungal infections is truly concerning, as their interaction seems to amplify each other's effects.

The heatmap and images in Fig. 5 visually illustrates the effects of pesticides on the antioxidants in honeybee hemocytes. The protein content showed no change when exposed to pesticides without PAMPs and PGN, yet a visual increase was observed with LPS and a decrease

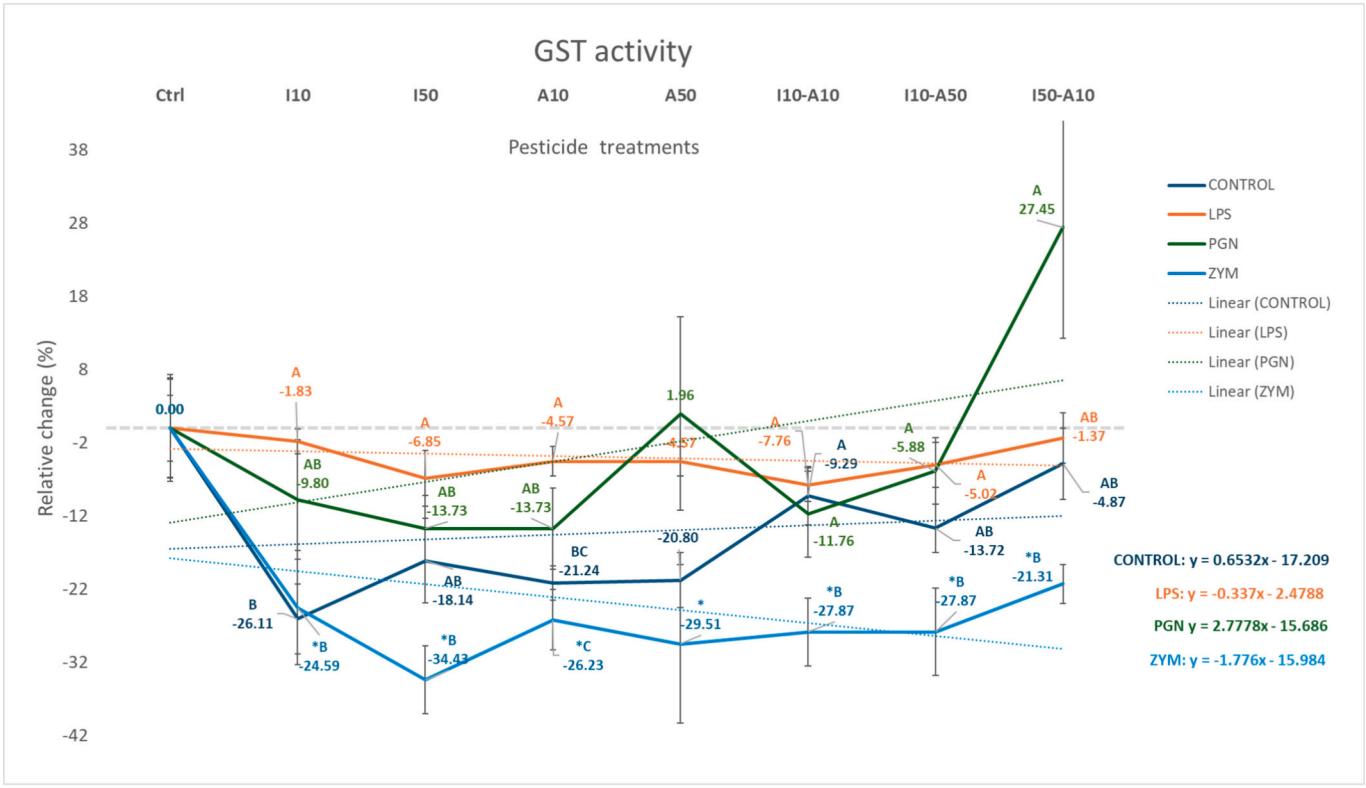

Fig. 4. Quantified GST activity in honeybee hemocytes exposed to different treatments of imidacloprid, amitraz, and PAMPs. In addition to the no-pesticide control (Ctrl), hemocytes were treated with either single exposures of imidacloprid (I10 or I50), or amitraz (I10 or I50), or mixtures of different concentrations (I10-A10, I10-A50, or I50-A10). Hemocytes were treated with 8 different pesticide conditions and 4 immune stimulator groups; without immune stimulator (CONTROL), 1 μg·mL^{-1} lipopolysaccharide (LPS), 1 μg·mL^{-1} peptidoglycan (PGN), or 1 μg·mL^{-1} zymosan A (ZYM). All concentrations are in μg·mL^{-1}. Significant differences relative to the control within groups of PAMPs are indicated by asterisk while those within the pesticide groups are indicated by different letters ($p < 0.05$, $n = 4$). Error bars represent standard errors.

with ZYM. Though statistically there were limited significant differences, the totality of these observations must be considered. The SOD appears to have slightly decreased in the control (no PAMPs) group and highly decreased when hemocytes were exposed to zymosan A while LPS and PGN view a relative increase in SOD activity as our results indicate.

It is observable that GST activity has decreased in all the used pesticide treatments and the different PAMPs indicating that GST was the most affected among the tested antioxidant enzymes. The decrease of GST was additionally more pronounced when hemocytes were exposed to imidacloprid generally and to zymosan A specifically.

Henceforth, fungal infections may pose a greater threat to honeybees than bacterial infections. We note that our experiments were conducted on honeybee 5th instar larvae, which are susceptible to American and European Foulbrood bacterial diseases, yet the bacterial PAMPs showed no significant effect compared to zymosan. Indeed, the risk of microbial infection in the presence of various environmental stressors needs further assessment. Field condition studies may be challenging due to the many variables, including honeybee breed, pest abundance, ecological niche, nutritional status, temperature and atmospheric variations, types of pesticides used in the area, and the surrounding flora. Both field and lab experiments are necessary for thorough evaluation. Lab experiments (*in vitro*) allow for specific analysis of honeybee tissues or compartments. For example, on the field level, Pettis et al. (2013) suggested the inclusion of spray programs in addition to pesticide regimes when analyzing the effect of pesticides on colonies.

A point to consider is that the effect of microbes on the immune system is often associated with effectors that alter the cellular functions of honeybees and ultimately their disease resistance, health (Li et al., 2017; Antúnez et al., 2009; Xing et al., 2021; de León-Door et al., 2020; DeGrandi-Hoffman and Chen, 2015; Chen, 2011), and potentially their ability to resist or detoxify pesticides. This underscores the need for further studies on microbial effectors and their interactions with cellular components and gene expression.

The interaction of honeybee hemocytes with immune stimulatory PAMPs and pesticides revealed that PAMPs may alter the expression of immune pathway components when pesticides are present and redirected the signaling which could explain a potential underlying mechanism (Sukkar et al., 2023a, 2023b). Also pesticides have low molecular weights that facilitates their absorption by the cells allowing them to act on different cell components such as the mitochondria and may affect ROS production via the reduction-oxidation cycling process not to mention affecting lipid peroxidation and antioxidant capacity of target cells and tissues (Sule et al., 2022) which is in consensus with the outcome present in our results.

4. Conclusion

Our results infer that interaction with fungal species dysregulates the antioxidant system in honeybees and may increase the susceptibility to infection. In addition, pesticides such as imidacloprid and amitraz synergistically act with zymosan in reducing the hemocyte internal regulation and tolerance to toxins. A two-way synergistic relationship between pesticides and zymosan is observed where zymosan renders hemocytes more susceptible to toxins by decreasing GST activity and toxins such as pesticide dysregulate the oxidative production by hemocytes limiting their ability to cause oxidative damage to pathogens.

We also observed that GST activity is mainly affected by imidacloprid single exposures and zymosan amplified the effect while pesticide mixtures did not have such an effect compared to single exposures. This could indicate a subtle antagonistic relationship as well. The presence of

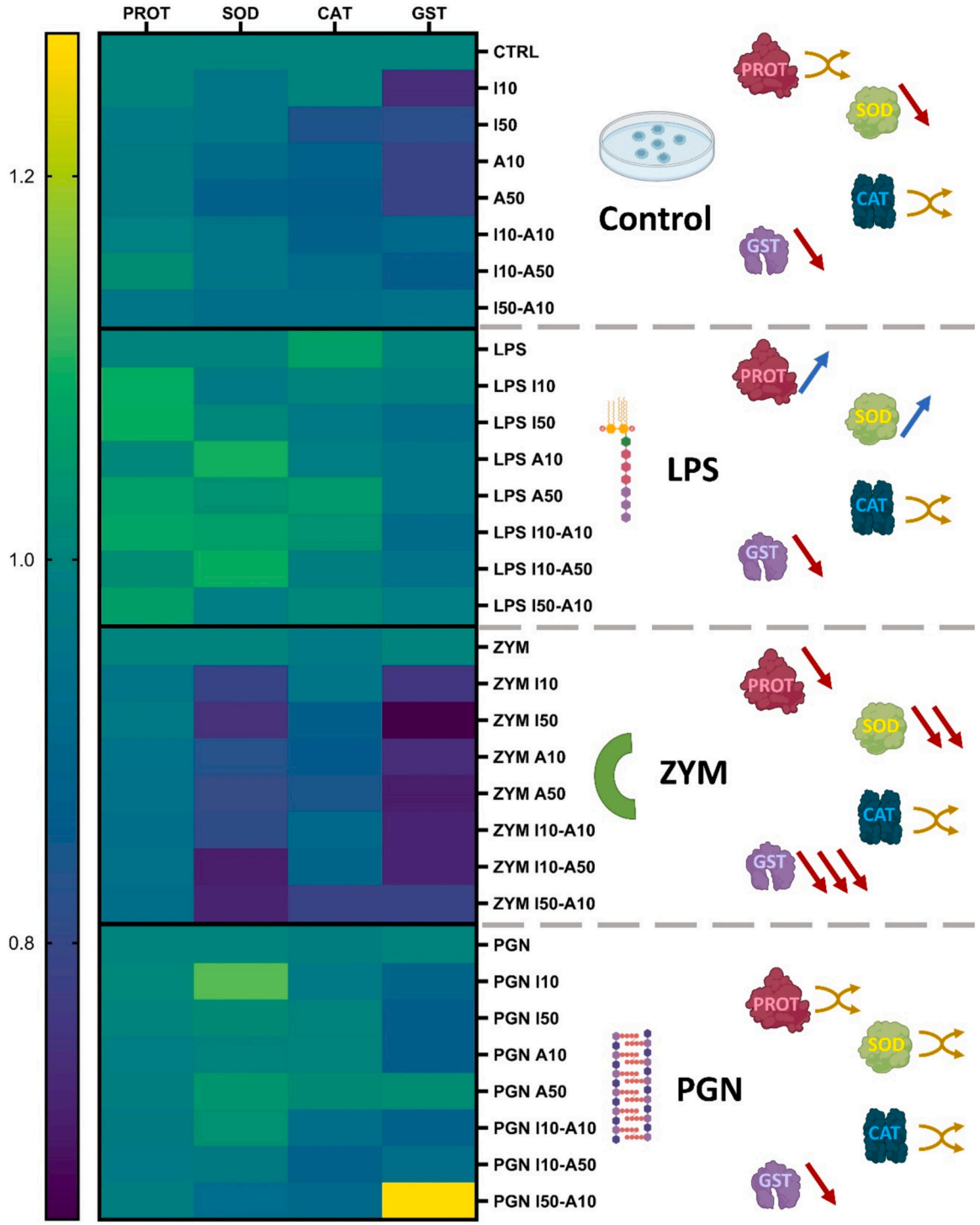

Fig. 5. Heatmap summary and visual representation of the effects of PAMP and pesticide exposure on honeybee antioxidants. Total protein content (PROT), superoxide dismutase relative activity (SOD), catalase relative activity (CAT), glutathione-S-transferase relative activity (GST) and represented in the heatmap on the left by color variations and intensity. The images on the right represent visual demonstration of pesticide effects on antioxidants via different arrows. Blur arrows indicate an increase, red arrows indicate a decrease while golden intertwined arrowed refer to variable changes with different pesticide treatments or no change. Different groups of PAMPs are indicated by control (no PAMP exposure), LPS for lipopolysaccharide, PGN for peptidoglycan and ZYM for zymosan A. Imidacloprid and amitraz pesticides are indicated by the letter (I) and (A) respectively with their concentrations in $\mu g \cdot mL^{-1}$.

multiple stressors in the same environment adds complexity to risk assessments and thus environmental impacts cannot be estimated without taking to consideration the complex interactions as presented in this study.

Further research should focus on integrating complex interaction of risk factors in the environment in addition to response of bees and other insects to these factors as they may favor pests over beneficial insects and pesticides can affect non-target organism such as bees and persist in the environment moving via the food web ultimately affecting the ecosystem and humans' well-being as well (Sukkar et al., 2025b). Additionally, only 1.4 % of insect cell lines are from Hymenopteran sources like bees (Perera et al., 2023). Thus, there is a need to develop a

sustainable resource for toxicity tests *in vitro* for honeybees via establishing cell lines of different tissues to by-pass the limitations of scarce cellular material and variability not to mention the risk of sample contamination when sterile conditions are required.

More in depth approaches are needed to understand pesticide-microbe interaction in honeybees at different developmental stages, different tissues, and different cellular compartments and their associated mechanisms. The molecular interaction of pesticide cocktails compared to single exposures need be studied to understand their behavior when they are present simultaneously. Our research sheds light on the variation of responses with different contexts and specificities demanding rigorous assessments of plant protection products like pesticides on honeybees and the environment whether already applied or for future applications.

CRediT authorship contribution statement

Dani Sukkar: Writing – original draft, Visualization, Validation, Supervision, Methodology, Formal analysis, Data curation, Conceptualization. **Lea Wagner:** Writing – original draft, Visualization, Methodology, Formal analysis, Data curation. **Antoine Bonnefoy:** Writing – review & editing, Resources, Methodology. **Jairo Falla-Angel:** Writing – review & editing, Supervision, Project administration, Investigation, Funding acquisition. **Philippe Laval-Gilly:** Writing – review & editing, Supervision, Project administration, Investigation, Funding acquisition.

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Declaration of competing interest

The authors declare that this study was conducted in the absence of any conflict of interest.

Data availability

Data will be made available on request.

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