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**A *Pediococcus* strain to rescue honeybees by decreasing *Nosema ceranae*-  
and pesticide-induced adverse effects.**

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**Abstract**

Honeybees ensure a key ecosystemic service by pollinating many agricultural crops and wild plants. However, since few decades, managed bee colonies have declined worldwide. This phenomenon is considered to be multifactorial, with a strong emphasis on both parasites and pesticides. Infection by the parasite *Nosema ceranae* and exposure to pesticides can contribute to adverse effects, resulting in a perturbation of the honeybee physiology. We thus hypothesized that probiotic treatment could be promising to treat or prevent these disturbances. The aim of this study was to evaluate the effects of probiotics on *N. ceranae*-infected and intoxicated honeybees (by the insecticide thiamethoxam and the fungicide boscalid). For this purpose, experiments were conducted with five probiotics. Among them, *Pediococcus acidilactici* (PA) showed the best protective effect against the parasite and pesticides. PA significantly improved the infected honeybee lifespan as prophylactic and curative treatments (respectively 2.3 fold and 1.7 fold). Furthermore, the exposure to pesticides induced an increase of honeybee mortality compared with the control group ( $p < 0.001$ ) that was restored by the PA treatment. Despite its beneficial effect on honeybee lifespan, the PA administration did not induce changes in the gut bacterial communities (neither in abundance or diversity). *N. ceranae* and the pesticides were shown to deregulate genes involved in honeybee development (vitellogenin), immunity (serine protease 40, defensin) and detoxification system (glutathione peroxidase-like 2, catalase), and these effects were corrected by the PA treatment. This study highlights the promising use of PA to protect honeybees from both pathogens and pesticides.

**Keywords:** Honeybee, *Nosema*, pesticides, *Pediococcus*, probiotics

## I. Introduction

Honeybees are valuable resources worldwide at both ecological (contribution to biodiversity by pollination) and economical (crop pollination) levels. However, since several years ago, a decline of managed European honeybee (*Apis mellifera*) populations has been reported in Europe and in the United States (Potts et al., 2010; vanEngelsdorp and Meixner, 2010). This decline involves both biotic (pathogens and parasites) and/or abiotic (pesticides and pollutants) stressors. Numerous studies have shown that a wide variety of pesticides including insecticides, fungicides and herbicides can be found in honeybees and hive matrices (pollen, honey and wax) affecting these non-target organisms even at sublethal doses (Jabot et al., 2016; Kasiotis et al., 2014; Lambert et al., 2013; Mullin et al., 2010; Simon-Delso et al., 2015). Among them, boscalid is a fungicide belonging to the carboxamide family used in agriculture to control phytopathogenic fungi. As the primary action of boscalid is the inhibition of succinate dehydrogenase of the respiratory chain (Avenot and Michailides, 2007), this fungicide can affect honeybees by decreasing ATP concentration but also pollen consumption and protein digestion (Degrandi-Hoffman et al., 2015). The use of neonicotinoids, which are neurotoxic systemic insecticides, in intensive agriculture imposes a serious threat to honeybees. Neonicotinoids are agonists of acetylcholine receptors and consequently can impact the central nervous system of insects (Casida and Durkin, 2013). The chronic consumption of neonicotinoids like thiamethoxam led to lethal and sublethal effects on honeybees by altering sensory, cognitive and/or motor functions (Henry et al., 2015). Interestingly, combined exposure to sublethal doses of neonicotinoids and the intestinal parasite *Nosema ceranae* has shown a significant decrease of honeybee survival (Aufauvre et al., 2012; Dussaubat et al., 2016; Vidau et al., 2011). *N. ceranae* is an obligate intracellular pathogen of honeybee gut, associated with a decrease of honeybee lifespan

(Alaux et al., 2010; Aufauvre et al., 2014, 2012; Goblirsch et al., 2013; Higes et al., 2008; Vidau et al., 2011). This parasite also induces sublethal effects including damages of the peritrophic membrane and impairment of gut renewal (Dussaubat et al., 2012; García-Palencia et al., 2010; Panek et al., 2018), energetic and nutritional stress (Alaux et al., 2010; Mayack and Naug, 2009), hormonal disturbances (Dussaubat et al., 2010) and immune depletion (Alaux et al., 2010; Antunez et al., 2009). The only known reliable treatment to fight *Nosema* is the terpenoid fumagillin but its use has been forbidden in Europe since 2012. The honeybee midgut is the main site of both pesticide absorption and *N. ceranae* infection. Therefore, the gut microbiota could be disturbed by these stressors alone or in combination leading to gut dysbiosis. Honeybee gut microbiota is dominated by five ubiquitous bacterial species (*Snodgrassella alvi*, *Gilliamella apicola*, *Lactobacillus* Firm-4, *Lactobacillus* Firm-5 and *Bifidobacterium asteroides*) called “core microbiota” and four species less prevalent (*Frischella perrara*, *Bartonella apis*, *Parasaccharibacter apium* and *Gluconobacter* sp.) (Moran et al., 2012). Gut microbiota benefits were highlighted at several levels: nutritional (digestion and metabolism of complex molecules as lignin, synthesis of vitamins, fatty acids and amino acids) (Engel et al., 2012), immune system (barrier effect by biofilm formation, antimicrobial peptide production) (Martinson et al., 2012; Vásquez et al., 2012) and functional levels (homeostasis with insulin and vitellogenin signalling) (Zheng et al., 2017). Gut dysbiosis could therefore affect honeybee health (Corby-Harris et al., 2014; Cox-Foster et al., 2007; Leonhardt and Kaltenpoth, 2014). Administration of selected microorganisms, in this dysbiotic context, could thus represent beneficial microbes that could be used as probiotics. If probiotics are commonly used in vertebrates (Crotti et al., 2012), few studies have focused on their impact on honeybee health (Audisio et al., 2015; Baffoni et al., 2016; El Khoury et al., 2018; Gaggia et al., 2018; Ptaszyńska et al., 2016).

In the present study, the first experiment was conducted to evaluate the potential of five yeast or bacterial candidates for their anti-*N. ceranae* activity. For this purpose, each strain was chronically administered to honeybees. The probiotics were supplied in the sucrose syrup two days before infection by *N. ceranae* to evaluate their prophylactic/preventive effects. The second experiment was then conducted with a strain of *Pediococcus acidilactici* (PA) selected from the first experiment to investigate a possible “curative” effect on *N. ceranae* infection. In this aim, we administered the PA strain two days after infection. The experiment was broadened to investigate whether this PA strain could also have a beneficial effect on bees co-exposed to low doses of two pesticides, the insecticide thiamethoxam and the fungicide boscalid. RNAs were extracted from honeybees midguts to evaluate the effects of these different treatments on the gut microbiota composition (high throughput sequencing) and on the midgut transcriptional changes (qPCR) of selected genes involved in immunity, antioxidant system and gut development.

## **II. Materials and Methods**

### **1. Honeybee rearing and experimental procedures**

A mixture of emerging honeybees from three *A. mellifera* colonies (genotype Buckfast) of the same apiary (UMR 6023, Clermont Auvergne University, Clermont-Ferrand, France) was used for both experiments. Frames of sealed brood were placed in incubators (33°C with 60% relative humidity). Emerging honeybees were collected directly on the frames and distributed in Pain-type cages into groups of 50 individuals. Five queen’s mandibular pheromones were mimicked by addition of a 5 mm piece of PseudoQueen® (Contech Enterprises Inc., Victoria, Canada) in each cage. Honeybees were maintained in incubators

for 22 days and were fed with 50% sucrose syrup (w/v) complemented with 1% (w/v) nutritional supplement (Provita' Bee, ATZ Dietetic, Mas-Cabardès, France).

The first experiment (Exp.1, **figure 1A**) was conducted with eight experimental groups: (i) uninfected and untreated honeybees (Ctrl.1), (ii) *N. ceranae*-infected honeybees (Inf.1), (iii) *N. ceranae*-infected honeybees treated with fumagillin (InfFum.1), (iv) *N. ceranae*-infected honeybees treated with *Saccharomyces cerevisiae* (InfSC.1), (v) *N. ceranae*-infected honeybees treated with *Saccharomyces boulardii* (InfSB.1), (vi) *N. ceranae*-infected honeybees treated with *Lactobacillus plantarum* (InfLP.1), (vii) *N. ceranae*-infected honeybees treated with *Bacillus pumilus* (InfBP.1) and (viii) *N. ceranae*-infected honeybees treated with *Pediococcus acidilactici* (InfPA.1). Strains were added in the sucrose syrup from the first day (D0, emergence until the end of the experiment) to evaluate their preventive effect on the infection. Honeybees were collectively infected with  $10^4$  spores/bee for two days from two days after emergence (D2). The fumagillin treatment (1 µg/mL) started two days after the infection (D4) and maintained until the end of the experiment (D22) (**figure 1A**).

The second experiment (Exp.2, **figure 1B**) was conducted with six experimental groups : (i) uninfected and untreated honeybees (Ctrl.2), (ii) uninfected honeybees treated with *Pediococcus acidilactici* (PA.2), (iii) *N. ceranae*-infected honeybees (Inf.2), (iv) *N. ceranae*-infected honeybees treated with *Pediococcus acidilactici* (InfPA.2), (v) honeybees exposed to both thiamethoxam and boscalid (ThBo.2) and (vi) honeybees exposed to both thiamethoxam and boscalid and treated with *P. acidilactici* (ThBoPA.2). In this experiment, infection was performed 2 days after emergence (D2), the treatment with pesticides started 4 days after emerging (D4 until the end of the experiment) and probiotics were given 4 days after infection (D6) (**figure 1B**). Honeybees were fed *ad libitum* with sucrose syrup



complemented or not with probiotics or pesticides according to their experimental group. The feeders were replaced every 48 h. Both the mortality and the sucrose consumption were monitored daily.

In the first experiment, infected honeybees were treated with 1 µg/mL of fumagillin as antimicrosporidial reference treatment. For intoxication experiments, honeybees were chronically exposed to low concentrations of thiamethoxam (1.5 µg/L) and boscalid (100 µg/L). Stock solutions were prepared in DMSO and diluted in sucrose syrup at a final concentration of 0.1%. Honeybees from the infected and control groups were fed with 0.1% DMSO-containing sucrose. The pesticide consumption was monitored daily by measuring sucrose consumption that was reported to the remaining bees in each cage and expressed as “ng/bee/day”.

## **2. Microbiota establishment**

In order to mimic the microbiota in honeybee digestive tract in the hive, a procedure based on Powell et al. (Powell et al., 2014) was used in the second experiment. For this purpose, 66 foragers were collected from each hive, gut and rectum were dissected and crushed in 150 µL of PBS before to be added to the syrup at day 0, 2 and 4 in each cage. In addition, to encourage a transfer of microbiota by trophallaxis to the emerging bees, three foragers from sampled colonies were collected, anaesthetized with CO<sub>2</sub>, marked with a paint dot on the thorax and placed in each cage.

## **3. *Nosema ceranae* infection procedure**

*N. ceranae* spores were obtained according to Roussel et al. (Roussel et al., 2015) and stored at RT during less than two months. The spore concentration was determined by counting on hemacytometer and *N. ceranae* species was confirmed by PCR according to the procedure described previously (Martín-Hernández et al., 2007). Honeybees were collectively infected

two days after the emergence with a dose of 10 000 *N. ceranae* spores per bee in the sucrose solution. At the end of the experiment (D22), abdomens of five honeybees per cage were dissected to evaluate the spore load according to Paris et al. (Paris et al., 2017).

#### 4. Probiotic candidate strains and culture

Strains were provided by Lallemand SAS (Blagnac, France) including (i) two yeasts, *Saccharomyces cerevisiae* (SC CNCM I-1077) and *Saccharomyces boulardii* (SB CNCM I-1079), (ii) three Gram-positive bacteria, the homofermentative *Pediococcus acidilactici* (PA CNCM MA18/5M), the heterofermentative *Lactobacillus plantarum* (LP CNCM MA18/5U) and *Bacillus pumilus* (BP AQP 4275).

Microbial strains were grown in liquid media and 100 µL of culture broth were daily sub cultured in 5 mL of their respective media and incubated at their optimum temperature under aerobic atmosphere with gentle shaking (**table S1**). The yeasts were grown in malt extract-yeast extract (YM) medium and incubated at 30°C. PA and LP were grown in MRS medium and incubated at 37°C, whereas BP was grown in *Bacillus* medium supplemented with NaCl and incubated at 30°C. The total cell count was determined by measuring the optical density at 600 nm. The sucrose syrup (1:1; w/v) was supplemented with probiotic candidates to achieve a final concentration of  $10^4$  CFU/mL. Strain survival in the sucrose syrup has been checked by inoculating  $10^4$  CFU/mL of each strain in the syrup, incubated up to 48 h at 33°C (the temperature used for honeybee rearing) and enumerated on specific agar media. Doses of probiotic candidates used in these experiments were based on previous reports (Audisio and Benítez-Ahrendts, 2011; Ptaszyńska et al., 2016). The feeders were changed every 48 h and the amount of probiotics daily consumed per honeybee was estimated from the sucrose consumption.

#### 5. Sampling, gut dissection and storage conditions

DNA and RNA extractions were performed at D0 from the intestinal tracts of six introduced foragers and six emerging bees and also from the *N. ceranae* spore solution used for the infection. At day 18, a random sampling of six bees per cage was done. Before extraction, each bee was dissected with sterilized tweezers on ice. Each sample from the intestinal tracts (from the anterior intestine to the rectum) was divided into two pools of three guts which were extracted in tubes containing 600  $\mu$ L RLT buffer (AllPrep DNA/RNA Mini kit, Qiagen, Courtaboeuf, France) and 1% of 2-mercaptoethanol and then were frozen in liquid nitrogen.

#### **6. DNA/RNA co-extraction**

Upon thawing, 60 mg of glass beads (0.1 mm, SIGMA, St. Quentin Fallavier, France) previously treated with diethylpyrocarbonate were added and mechanical grinding using three cycles of 20 s was performed (Bead-beater MM30, Retsch, Haan, Germany). After centrifuging 1 min at 600 x g at 4°C, supernatants were kept in DNase/RNase free tubes and subjected to three cycles of freeze-thaw (liquid nitrogen/65°C). Samples were then centrifuged at 8,000 x g for 10 min at 4°C and the supernatant was transferred in DNA column and centrifuged 1 min at 8,000 x g. The simultaneous purification of DNA and RNA was done according to the manufacturer (AllPrep DNA/RNA Mini kit, Qiagen). The two pools of RNA for each cage were collected in the same final tube, received two treatments with DNase (RNase-Free DNase Set, Qiagen) and were stored at -80°C. Purified DNA and RNA were quantified by NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Illkirch, France) and the RNA concentration was measured using the Agilent 2200 Tape Station and the RNA ScreenTape kit (Agilent Technologies, Les Ulis, France).

#### **7. 16S rRNA gene amplification**

The reverse transcription reaction was performed with random primers using the SuperScript III Reverse Transcriptase kit (Invitrogen™). Both DNA and cDNA coding for 16S rRNA were amplified with the universal primer 515F (5'-GTGYCAGCMGCCGCGGTA-3') and bacteria/archaea specific primer 909R (5'-CCCCGYCAATTCMTTTRAGT-3') targeting the V3/V4 region. Primers were modified by adding specific barcodes (8 nucleotides) to assign the amplicons to each condition. PCR was performed using a high-fidelity polymerase (Platinum™ Taq DNA polymerase high-fidelity, Invitrogen™), and the program was done as follows: 94°C for 3 min, and 30 cycles of 94°C for 30 s, annealing at 59°C for 30 s, elongation at 68°C for 30 s and a final elongation at 68°C for 7 min. Reactions were realized in the thermocycler Proflex (Applied Biosystems). Electrophoresis on a 2% (p/v) agarose gel was performed to check the size of PCR products. Then the amplicons were purified and concentrated using the Qiagen Gel extraction kits (Qiagen) and quantified using The Agilent 2200 Tape Station system and the D1000 ScreenTape kit (Agilent Technologies). An amplicon equimolar mixture (20 ng/μL) was constructed in a concentration for Illumina MiSeq Sequencing Technology (Run type: Paired end-Read length: 2 x 300 bp) by GATC Biotech.

## 8. Taxonomic affiliations

Sequences were analysed using vsearch tool (<https://github.com/torognes/vsearch>). The MiSEQ data were assembled, sequences having ambiguous bases "N" were removed as well as if they had a mismatch in the forward and reverse primers or a length shorter than 200 bp. The putative chimaeras were removed using vsearch (denovo command). The bacterial reads were clustered into operational taxonomic units (OTUs) with 97% similarity using vsearch (Kim et al., 2011). The cleaned reads were clustered to identify a representative sequence for each OTU and were inserted into phylogenetic trees for taxonomic annotation. They were then affiliated by similarity and phylogeny with reference sequences extracted

from the SSURef SILVA database (Pruesse et al., 2007). These sequences were extracted according to the criteria: (i) length > 1,200 bp, (ii) quality score >75% and (iii) pintail value > 50. Closest OTUs with reference sequences according to similarity approach (vsearch tool), were used to build trees with FastTree (Price et al., 2010). Taxonomic assignment was performed according to the Last Common Ancestor (LCA) affiliation using the pipeline PANAM (Phylogenetic Analysis of Next-generation AMplicons <https://github.com/panammeb/>) and is described in details in Taib et al. (Taib et al., 2013). To limit bias linked to differences in the number of sequences between samples, relative abundance was calculated for a semi-quantitative approach and sequences <1% were removed from the analysis.

## 9. qPCR

Quantitative PCR experiments were carried out in a thermocycler Realplex2 (Eppendorf) to analyze the fold-change of five genes (**table S2**). QPCR reactions were performed in 20  $\mu$ L using 10  $\mu$ L Absolute Blue qPCR SYBER GreenMix (Thermo Scientific), 10 pmol of each primer and 10 ng cDNA on 96-well plates (Eurogentec RT-PL96-MQ). Amplification was conducted under the following protocol: 94°C for 10 min, 10 cycles of 94°C for 40 s, 52°C for 40 s, 72°C for 30 s, followed by one cycle of 95°C for 5 s and 65°C for 1 min, then 40°C for 10 s. QPCR data were expressed as the threshold cycle (Ct) values normalized to RpS5a and calculated using the  $2^{-\Delta\Delta Ct}$  method following standard protocols (Schmittgen and Livak, 2008).

## 10. Statistics

Statistical analyses were performed using the statistical software R 3.2.5 (<https://cran.r-project.org/>). Survival analysis was performed using the Cox regression (proportional hazard model). For all statistical comparisons across different treatments, normality (Shapiro-Wilk) and the homogeneity of variances (Bartlett test) were verified. To determine whether the

composition and structure of midgut bacterial communities differed significantly among treatments, statistical comparisons were made across the different conditions with Kruskal-Wallis test followed by Dunn's test and the Benjamini-Hochberg correction. Correlation analyses between bacterial or yeast genera and the mortality were performed using nonparametric Spearman's rank tests. Different estimators were used to infer the taxa richness of the bacterial communities: the number of observed species (sequencing depth) and Shannon diversity indexes and ANOVA 1 was performed followed by two-tailed Student's t-test.

### III. Results

#### 1. Experiment 1: Selection of probiotic candidates.

The first experiment was conducted with eight experimental groups corresponding to uninfected or infected and probiotic-treated honeybees. Strain survival in the sucrose syrup was considered as satisfactory for *S. cerevisiae* (SC), *S. boulardii* (SB), *P. acidilactici* (PA) and *B. pumilus* (BP) for which either growth (for SC and SB) or a maximum reduction of 0.5 log<sub>10</sub> CFU (for PA and BP) was observed after 48 h at 33°C. A lower survival was measured for the *L. plantarum* (LP) strain with a reduction by 1.0 log<sub>10</sub> CFU (data not shown).

The cumulative microbial strain consumption was the same in all treatments with an average of 2.10<sup>3</sup> CFU/bee. No significant difference in sucrose consumption was observed between treatments (data not shown). *N. ceranae* infection led to a significant decrease in honeybee survival as it decreased until 20.9% for the infected group (Inf.1) whereas the control group (Ctrl.1) one reached to 84.8% ( $p < 0.001$ ) (**figure S1**). Infected honeybees treated with LP, SC or BP had a significant higher survival rate (44.6%, 48.6% and 66.6% of surviving bees at day 20, respectively) than the infected group, but their survival rates remained lower than the control group (84.8% of surviving bees). More interestingly, both

PA and SB induced a high increase of the honeybee survival compared with the infected group (2.3-fold,  $p<0.001$  and 2.2-fold,  $p<0.001$  respectively for PA and SB) and showed no significant difference with the control group. Their effects on survival were similar to that measured in the fumagillin-treated group which was used as a positive control against *N. ceranae* infection. According to these results, PA was selected as the probiotic strain of interest for the second experiment (Exp.2).

## **2. Effects of the probiotic PA on bee mortality, sucrose consumption and *N. ceranae* spore load.**

In the second experiment (Exp.2), the probiotic PA was tested on honeybees infected by *N. ceranae* or co-exposed to two pesticides: the insecticide thiamethoxam and the fungicide boscalid. The sucrose consumption was significantly higher in the infected group compared with the control group (2-fold,  $p=0.021$ ) (**figure 2**). Infected honeybees treated with PA (InfPA.2) also had a higher consumption than the control group (Ctrl.2, 1.3-fold) but this increase was significantly less important compared with the infected group (Inf.2, 1.4-fold). No difference was observed on sucrose consumption in the groups of bees exposed to pesticides (ThBo.2, ThBoPA.2) and the amount of consumed pesticides was on average of 2.94 ng/bee/day for thiamethoxam and 196.5 ng/bee/day for boscalid. The consumption of PA was on average of  $2.5 \times 10^2$  CFU/24 h per bee.

The survival of honeybees only treated with PA (PA.2) showed no significant difference with the control group (Ctrl.2,  $p=0.870$ ) suggesting that PA did not exhibit any toxic effect (**figure 3**). The co-exposure of honeybees to the insecticide thiamethoxam and to the fungicide boscalid (ThBo.2) led to a significant decrease in survival compared with the control group (Ctrl.2) with respectively 59.0% and 88.9% of survival rate ( $p<0.001$ ). The treatment with PA fully restored the survival of honeybees exposed to pesticides (87.3% of survival bees,

$p < 0.001$  for comparison between ThBo.2 and ThBoPA.2); no significant difference was observed between ThBoPA.2 and Ctrl.2 group. Infection by *N. ceranae* induced a decrease of 3.0-fold the rate of honeybee survival in the Inf.2 group compared with the control group ( $p < 0.001$ ). The survival rate of the infected group treated with PA (InfPA.2) significantly increased compared with the Inf.2 group (1.7-fold,  $p < 0.001$ ) although the survival remained lower than the Ctrl.2 group (1.3-fold,  $p < 0.001$ ). Furthermore, treatment with PA induced a significant decrease of the spore load compared with the Inf.2 group (5.4-fold,  $p < 0.001$ ) (figure 4).

### 3. Response of bacterial microbiota to different stressors and probiotic treatment

In order to detect effects of the different treatments on the midgut bacterial community, a sampling was performed at day 16 (corresponding to significant effects of infection and intoxication) and RNA was extracted from the midguts to perform a metagenomic analysis. After filtering, the average number of sequences was 2,513,863 from 27 samples (9 conditions x 3 replicates), each sample comprising a pool of RNA corresponding to six honeybees. In all treatments, dominant bacterial taxa were composed of four classes (Bacilli, Gammaproteobacteria, Alphaproteobacteria and Betaproteobacteria), five orders (Lactobacillales, Rhizobiales, Rhodospirillales, Neisseriales and Orbales) and four genera (*Lactobacillus*, *Bartonella*, *Orbus* and *Gilliamella*, data not shown) (figure 5). Honeybee gut microbiota were similar in control and forager groups suggesting that the implantation of the gut microbiota in experimental conditions was quite successful (figure S2). The infection by *N. ceranae* and the co-exposure to pesticides revealed no significant difference on the gut microbiota composition compared with the control group. The study of the composition of bacterial community structure was also performed by analyzing the alpha diversity (through the Shannon index) and the beta diversity (Factorial Analysis Correspondence) but no



significant difference was observed between the different treatments and the control (**figure 6**). It is important to note that 20-40% of the sequences could not be affiliated to any genus, as we used the LCA (lowest common ancestor) assignment method that demonstrated to be more accurate (Taib et al., 2013) but enabling a lower assignation in our case.

#### **4. Effects of treatments on targeted host gene expression**

The mRNA expression of five genes involved in different functions was followed by quantitative PCR (**figure 7**). Downregulations were observed in the expression of two genes involved in the detoxification and antioxidant systems : the gene coding for the catalase (Gene ID 443552) and for the glutathione peroxidase-like 2 (Gene ID726269). The expression level of these two genes was significantly downregulated in infected (Inf.2) and co-intoxicated (ThBo.2 and ThBoPA.2) honeybees, except for infected honeybees treated with the probiotic PA (InfPA.2) which had a similar expression level with the control group (Ctrl.2). The transcript levels of two genes involved in immunity were also reduced in the gut in response to the infection by *N. ceranae*. The gene coding for the defensin (Gene ID406143) was significantly downregulated in Inf.2, ThBo.2 and ThBoPA.2 groups whereas its expression was significantly upregulated in infected honeybees treated with PA (InfPA.2). The expression level of the gene coding for the serine protease 40 (Gene ID409626) was significantly lower in infected honeybees (Inf.2) than in the control group (Ctrl.2) while no difference was observed between InfPA.2 and Ctrl.2 groups. On the contrary, honeybees co-exposed to thiamethoxam and boscalid (ThBo.2) had a significant higher expression level of the serine protease 40 encoding gene than Ctrl.2 but no significant difference was observed between ThBoPA.2 and Ctrl.2. Finally, the vitellogenin encoding gene was significantly downregulated in Inf.2 and upregulated in ThBo.2 whereas no significant difference was observed in both PA-treated groups (InfPA.2 and ThBoPA.2).

#### IV. Discussion

Since the antibiotic fumagillin has been withdrawn from the European market, there is no other available treatment to fight the parasite *N. ceranae*. Given the importance of the gut microbiota, increasingly number of studies investigated on the efficiency of probiotic treatments (Audisio et al., 2015; Baffoni et al., 2016; Corby-Harris et al., 2014; Gaggia et al., 2018; Ptaszyńska et al., 2016). In the present work, a PA strain appeared to be the most efficient probiotic against *N. ceranae* (Exp.1). The treatment with PA has not disturbed the midgut microbiota community (neither abundance or diversity). Furthermore, PA was not identified in the metagenomic analysis suggesting that it was not established in the gut microbiota or only as a minor component. This probiotic treatment has completely restored the survival rates of infected honeybees in Exp.1 suggesting that PA treatment efficiency is enhanced when it is administered before the infection. The survival rate improvement by PA has already been shown in both curative and prophylactic administrations with a survival probability enhance of 20-30% in honeybees treated with a commercial product containing the same strain (El Khoury et al., 2018). Similar effects were highlighted with the prophylactic administration of Biogen-N, a probiotic formulation containing PA among other strains (Kaznowski et al., 2005) and in other species like piglets (Di Giancamillo et al., 2008; Dowarah et al., 2018), red tilapia (Ferguson et al., 2010), rainbow trout (Merrifield et al., 2010) or chickens (Jazi et al., 2018).

As previously reported, *N. ceranae* induced an increase of sucrose consumption arguing for an energetic stress (Mayack and Naug, 2009; Vidau et al., 2011). This increase was counterbalanced when honeybees were treated with PA and we can hypothesize that this could be due to a greater digestibility as it was demonstrated in chickens infected by the pathogenic bacteria *Salmonella Typhimurium* (Jazi et al., 2018) or in piglets (Dowarah et al.,

2018). PA has also shown an efficiency to reduce the oxidative stress in different species including *Litopenaeus stylirostris* infected by *Vibrio nigripulchritudo* (Castex et al., 2010) and *Oncorhynchus mykiss* infected by *Streptococcus* (Hoseinifar et al., 2017). In our study, the expression of genes encoding catalase and glutathione peroxidase involved in antioxidant reaction and xenobiotic detoxification was decreased in infected bees suggesting a disruption of the oxidative balance as previously reported by Aufauvre *et al.* (Aufauvre et al., 2014) and Paris *et al.* (Paris et al., 2017). The oxidative balance is essential for the honeybee health since reactive oxygen species (ROS) could be both beneficial (immune defence, signal transduction, cell cycle regulation) and dangerous (DNA, lipid or protein damages) for the honeybees (Chiu and Dawes, 2012; Finkel, 2011). The treatment with PA restored their expression levels, suggesting that this probiotic strain may be involved in a mechanism, that needs to be deciphered, which would contribute to reduce the oxidative stress deleterious to honeybees. Other honeybee functions were altered by *N. ceranae* infection, including the downregulation of the genes coding for serine protease 40 and defensin, which is in line with previously reported data (Antunez et al., 2009; Aufauvre et al., 2014; Chaimanee et al., 2013) but in discordance with another study (Li et al., 2017). This discrepancy may be due to differences in honeybee susceptibility to *N. ceranae* as previously described (Kurze et al., 2015). Indeed, the honeybees analysed in the work of Li et al. (Li et al., 2017), contrary to our study, could therefore correspond to *Nosema*-tolerant honeybees. The analysed genes are linked to the immune response since the serine protease 40 is involved in the regulatory cascade reaction which activates the prophenoloxidase and Toll pathways leading, for the latter, to the production of antimicrobial peptides like defensin. The expression level of the serine protease 40 was restored and gene coding for the defensin was overexpressed when honeybees were fed with PA. This result suggests that PA treatment may have a protective

action against *N. ceranae* infection. In the same vein, the treatment with PA induced a 5-fold reduction of the *N. ceranae* spore load. Taken together, these results suggest that the probiotic PA might be used to prevent infection by the parasite *N. ceranae*.

Honeybees are also chronically exposed to multiple abiotic stressors like pesticides. Indeed, a multitude of pesticides were detected in honeybees and hive matrices including pollen, honey and wax (Jabot et al., 2016; Kasiotis et al., 2014; Lambert et al., 2013; Mullin et al., 2010; Simon-Delso et al., 2015) and could affect them at both lethal and sub-lethal levels. In this study, we have observed the effects of the chronical co-exposure to an insecticide (thiamethoxam) and a fungicide (boscalid) at low doses. The association of these two pesticides appeared to be deleterious for honeybees with a significant increase of mortality. Interestingly, the treatment with the PA strain completely restored honeybee survival rate. The mechanism through which this treatment acts on pesticide intoxication is unknown, but we showed that the PA treatment restored the expression of two genes which were altered by the pesticide co-exposure, those coding for serine protease 40 and vitellogenin. Previous studies have shown that induction of vitellogenin transcript could be used as a biomarker for neonicotinoid exposure (Christen et al., 2017). Changes in expression levels of these two genes showed the beneficial action of PA treatment. Furthermore, Lactobacilli have shown potentials to sequester and degrade environmental toxins. They could sequester, but not metabolize, organophosphate pesticides (parathion and chlorpyrifos) and this sequestration was associated with decreased intestinal absorption and insect toxicity in appropriate models (Trinder et al., 2016). This could also be the case for PA in our experiments and this potential ability deserves to be studied more in detail.

In our opinion, the use of the PA strain may represent a prophylactic and natural tool to protect honeybees from both *N. ceranae* infection and pesticide exposure. However, studies

in natural conditions need to be undertaken to assess the efficiency of PA at the colony level in different landscapes against nosemosis and intoxications.

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**Figure 1. Experimental design.** Emerging honeybees were collected from three different colonies and placed in cages in groups of 50 individuals. **A.** A preliminary experiment (**Exp.1**) was done to evaluate the effects of five different probiotics (PA, BP, SC, SB and LP) on bee mortality and sucrose consumption. Probiotics were administered two days before the infection by *N. ceranae* to investigate preventive effects. Fumagillin treatment was also given to infected honeybees as a positive control against *N. ceranae*. **B.** During the second experiment (**Exp.2**), the probiotic *Pediococcus acidilactici* (PA) was given to honeybees two days after infection by *N. ceranae* or exposure to pesticides (thiamethoxam + boscalid). Effects of probiotics on bee mortality, sucrose consumption and spore load were monitored, and sampling of individuals at day 16 was designed to metagenomics analysis of the gut microbiota. SC: *Saccharomyces cerevisiae*, SB: *Saccharomyces boulardii*, PA: *Pediococcus acidilactici*, BP: *Bacillus pumilus*, LP: *Lactobacillus plantarum*.

**Figure 2: Sucrose consumption by infected or pesticide-exposed honeybees treated with the probiotic strain *Pediococcus acidilactici* (PA).** The sucrose consumption was daily monitored during the experiment (g/bee/day  $\pm$  standard deviation sd): uninfected and untreated (Ctrl.2), infected (Inf.2), infected and treated with PA (InfPA.2), intoxicated with both thiamethoxam and boscalid (ThBo.2) or intoxicated with pesticides and treated with PA (ThBoPA.2).

**Figure 3. *Pediococcus acidilactici* (PA) effect on the survival of honeybees infected by *N. ceranae* or co-exposed to pesticides.** This graph represents the cumulative proportion of surviving honeybees: untreated and uninfected (Ctrl.2), treated with PA (PA.2), infected (Inf.2), infected and treated with PA (InfPA.2), co-exposed to pesticides (ThBo.2) and co-

exposed to pesticides and treated with PA (ThBoPA.2). Data were analysed from 150 honeybees per condition among Kaplan-Meier method.

**Figure 4. Spore loads in honeybees infected by *N. ceranae* and treated or not with *Pediococcus acidilactici* (PA).** At the end of the experiment (d22), the spore production was evaluated from ten abdomens of honeybees per cage. The data show the mean number of spores per honeybee abdomen  $\pm$  standard deviation (sd) for each condition: infected and treated or not with PA (InfPA.2). Asterix indicate significant differences ( $\alpha=5\%$ ).

**Figure 5. Cumulative relative abundances of bacterial classes (A) or orders (B) from honeybee gut microbiota.** The relative abundances of bacterial cDNA sequences from the 6 experimental groups are shown at two taxonomic levels: class and order.

**Figure 6. Diversity analysis of the midgut microbiota of infected- or pesticide exposed-honeybees treated or not with *Pediococcus acidilactici* (PA).** Diversity of honeybees untreated and uninfected (Ctrl.2), co-intoxicated with pesticides (ThBo.2), infected (Inf.2), treated with PA (PA.2), infected and treated with PA (InfPA.2) or co-intoxicated with pesticides and treated with PA (ThBoPA.2). **A.** The Factorial Correspondence Analysis (FCA) is an indicator of  $\beta$ -diversity and no significant difference was observed between the different treatments. **B.** On the same way, no significant difference was observed between the  $\alpha$ -diversity of the different treatments.

**Figure 7. Expression levels of honeybee genes in response to different treatments at day 16.** This graph represents the mean of the log fold change of genes involved in the detoxification system (Catalase and Glutathione peroxidase-like 2) and genes involved in immunity (vitellogenin, serine protease 40 and defensin): intoxicated by pesticides (ThBo.2),



infected by *N. ceranae* (Inf.2), intoxicated and treated with PA (ThBoPA.2), infected and treated with PA (InfPA.2). Asterix indicate significant differences ( $\alpha=5\%$ ).

### Highlights

- The honeybee physiology is disturbed by *Nosema ceranae* and pesticides.
- A *Pediococcus* strain can rescue honeybees from *N. ceranae*- and pesticide adverse effects.
- The *Pediococcus* strain can act by stimulating the honeybee immune and detoxication systems.

Graphical abstract

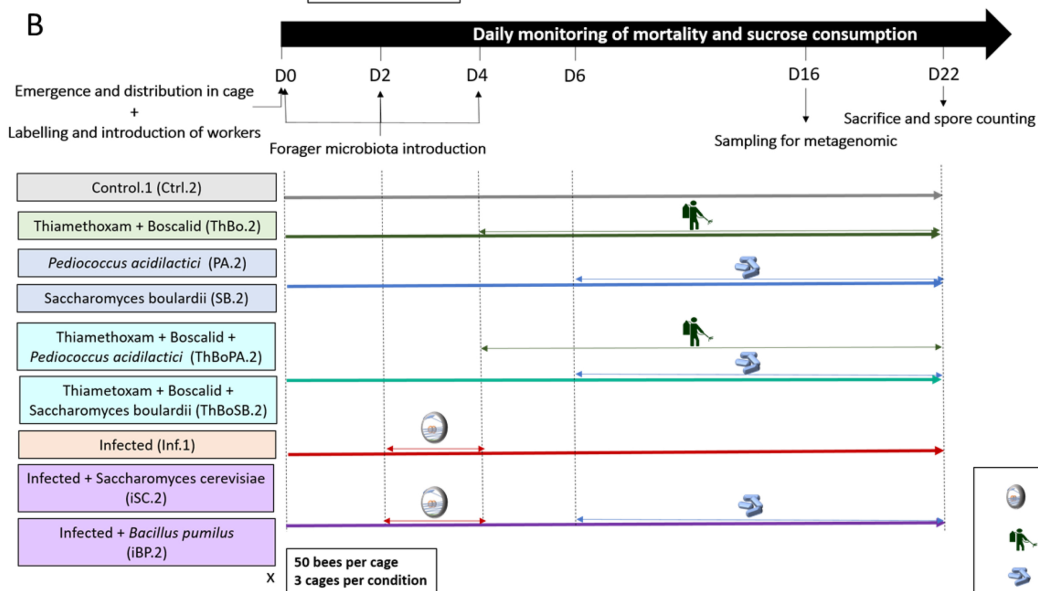
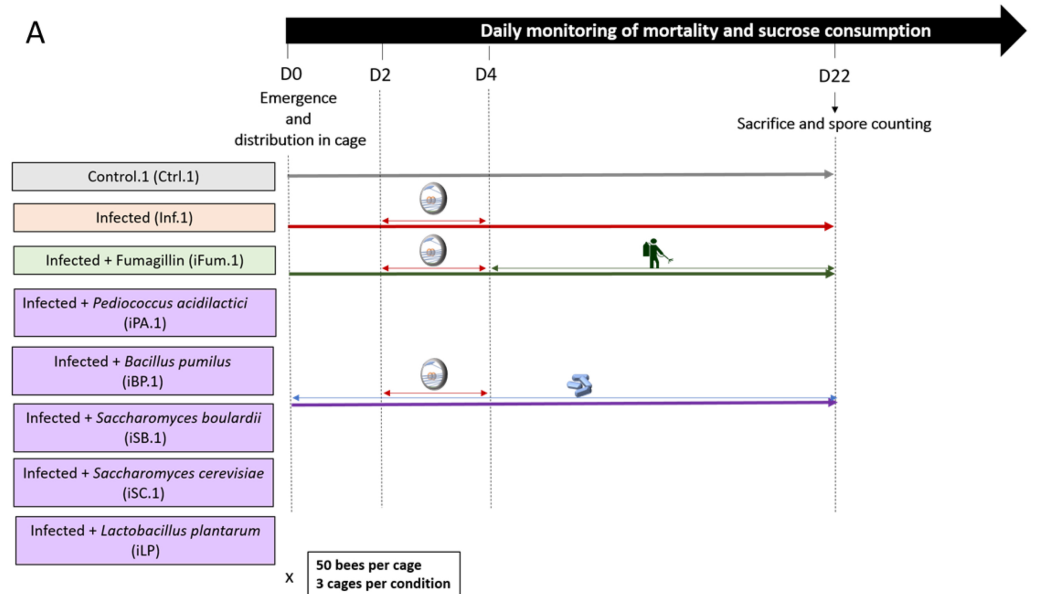


Figure 1

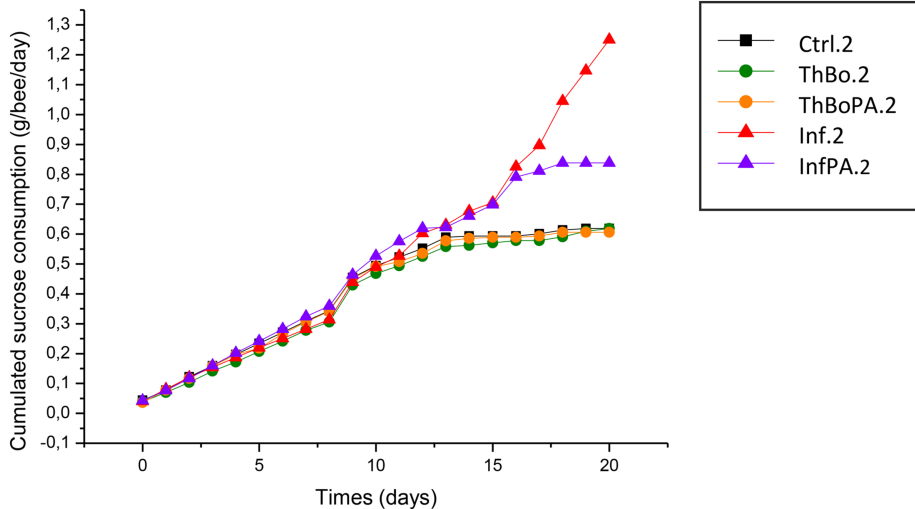


Figure 2

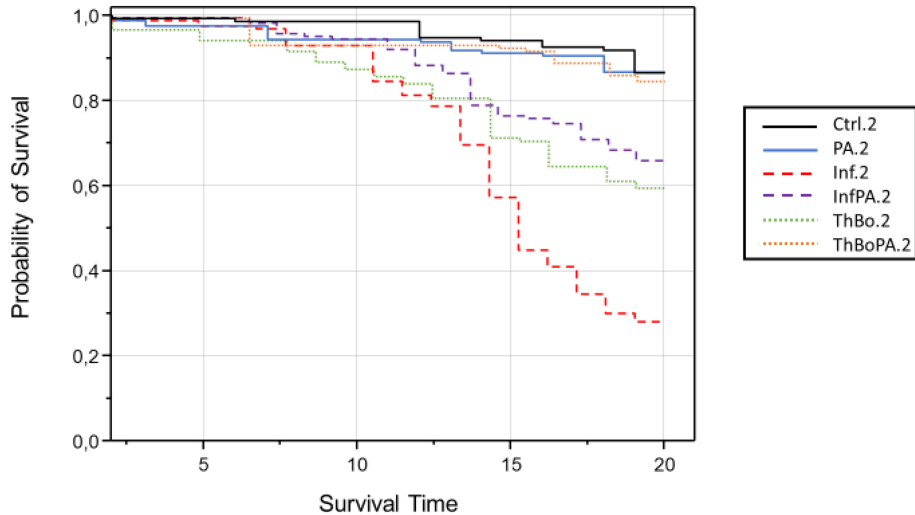


Figure 3

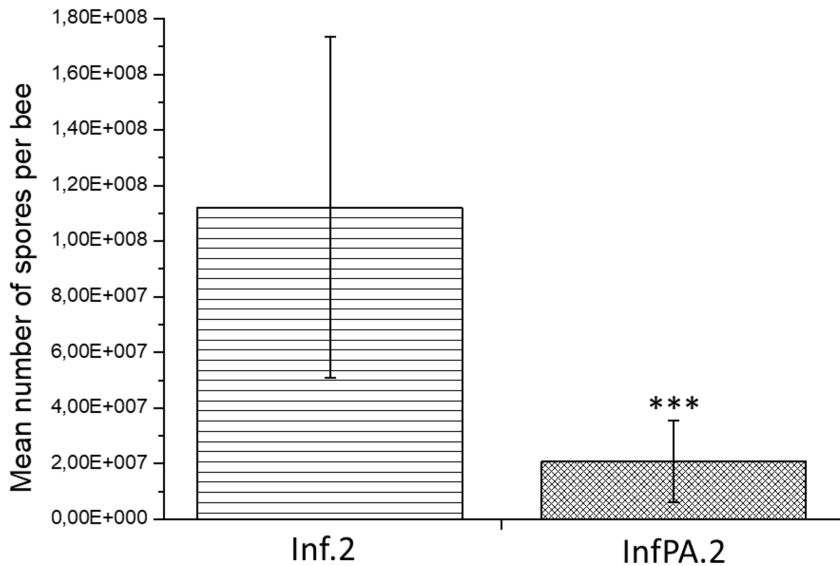
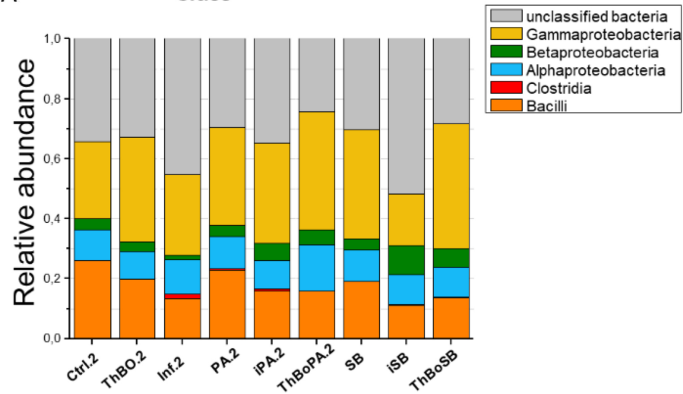


Figure 4

A

Class



B

Order

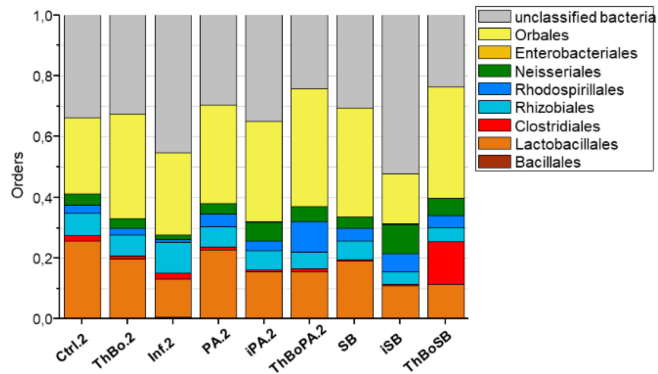


Figure 5

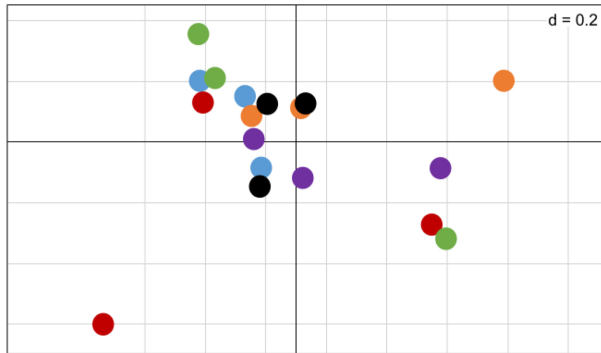
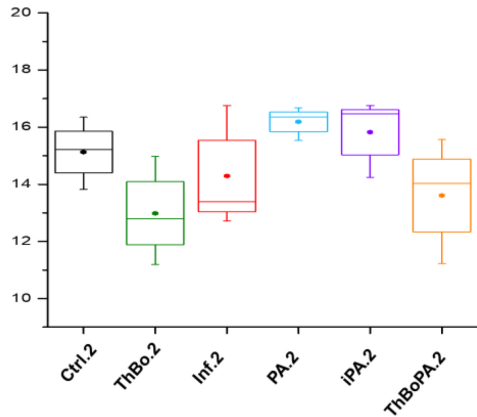
**A****B**

Figure 6

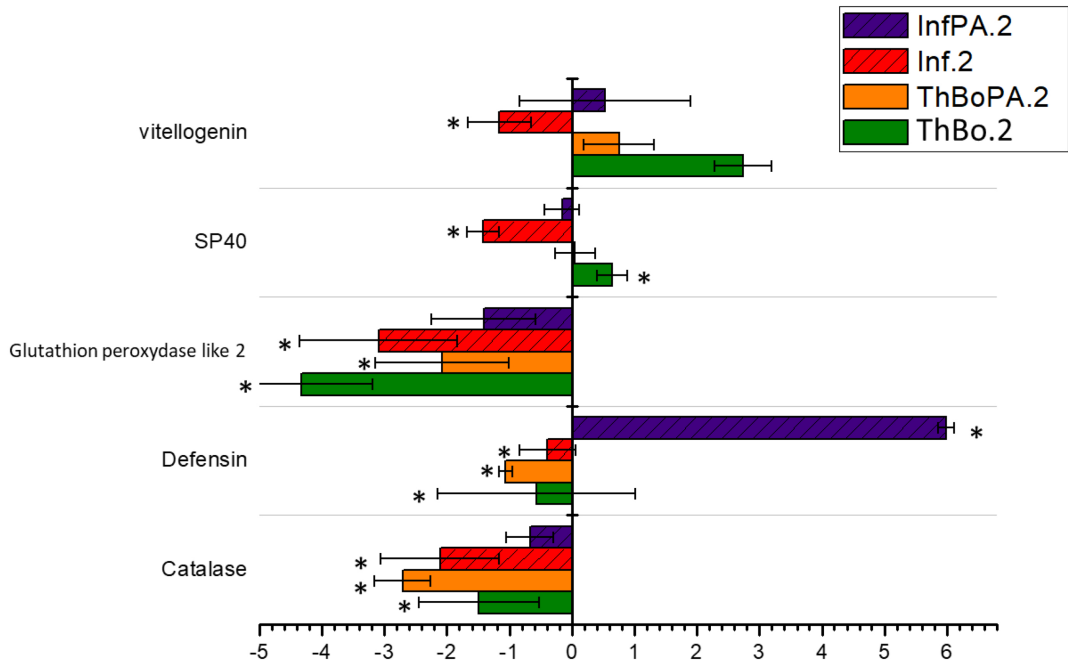


Figure 7