Dietary Contamination With Neonicotinoid (Clothianidin) Gradient Triggers Specific Dysbiosis Signatures of Microbiota Activity Along the Honeybee (Apis Mellifera) Digestive Tract

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Abstract

Background

Honeybees (Apis mellifera) are facing a number of interacting stress factors affecting their general health as well as their nervous and immune systems. Recent evidence proposed the clothianidin neonicotinoid as a potential disrupting factor for the microbiota-immunity axis. In this research, we conducted in vivo experiments to measure the impact of three sublethal clothianidin concentrations (0.1, 1 and 10 ppb) on honeybee survival, syrup consumption and the honeybee gut microbiota dysbiosis.

Results

Clothianidin exposure significantly increased mortality in the three concentrations compared to controls. Interestingly, the lowest clothianidin concentration was associated to the highest food intake and the highest mortality. Clothianidin exposure significantly induced variation in the taxonomic composition of the gut microbiota. The co-abundance network analysis reveals local dysbiosis signatures specific to each gut cohorts (midgut, ileum and rectum) driven by specific taxa.

Conclusions

Our findings confirm that clothianidin exposure triggers a disruption of the honeybee's symbiotic defense systems such as resistance to colonization, translating into a surge of potentially pathogenic taxa. Furthermore, the importance of low activity taxa with weak transcriptional activity in maintaining a stable honeybee gut microbiota. Finally, the early detection of gut dysbiosis in honeybee is an early promising alarming signal towards a microbiome approach in hive management the impact for assessing the impact of sublethal xenobiotics exposure.

Background

Honeybees are important pollinators that benefit nature and human agriculture [1]. However, they are permanently in contact with chemical agents intensively used for crop protection against vector enemies [2], e.g. neonicotinoids [3]. Being soluble in water and persisting into the environment [4, 5], neonicotinoids are mainly bioaccumulated in honeybees via pollen and water intake [6]. Neonicotinoids were found on honeybee body [7] and inside the honeybee, more specifically within gut cells [8]. These bioaccumulated xenobiotics were documented to alter honeybee physiology [9, 10], memory [11], neuronal communication [12, 13], immunity [14]. Given that gut microbes are of primary importance in regulating the above mentioned beneficial functions in honeybees [15–18], recent studies started to focus in understanding relationship between symbiotic microbes and host health [15]. Recent works observed that exposure to neonicotinoids (thiacloprid [19], nitenpyram [20] and imidacloprid [21]) exerted adverse effects on gut microbiota. In addition, the clothianidin (CAS 210880-92-5) was suspected to potentially disrupt the microbiota-immunity axis [21–23]. Thus, there is an urgent need to understand to which extent those toxic compounds impact the homeostasis of the honeybee gut microbiota.
The honeybee gut anatomy is partitioned in four distinct sections: crop, midgut, ileum and rectum. Characterized by a low taxonomic diversity [24], healthy honeybee gut microbiota harbours between five to nine phylotypes, representing more than 98% of the bacterial 16S rRNA gene sequences [25] and subdivided into core members (i.e. present in almost all individuals), non-core members [26] and low abundance taxa, suspected to play an important role in a microbial community [27]. Recent studies on gut microbiota evidenced its involvement in crucial health related functions such as detoxification [28], host immunity [15] and pathogens prevention [29]. The control of innate immunity by the gut microbiota was evidenced in *Drosophila*, through the NF-κB pathway [30]. In honeybees, the NF-κB pathway expression was observed to decrease following sublethal clothianidin exposure (0.1 to 10 ppb (µg/L)) [22]. Given that the gut microbiota is a major component of the honeybee immunity [18, 31], we hypothesized that the microbiota-immunity axis is potentially disrupted by sublethal clothianidin concentrations.

In addition, the disturbance of the microbiota-immunity axis would depend on the ability of the gut microbiota to metabolize pesticides. For instance, *Drosophila melanogaster* gut microbiota degrade chlorpyrifos [32], while *Apis mellifera* gut microbiota can not metabolize imidacloprid [33]. Degradation might differ depending on the chemical agent, the microbial strains involved [34, 35], and the generated metabolites, which may be more toxic than the parent molecule [36]. Thus, we hypothesized that clothianidin induces gut dysbiosis in honeybees, and we aimed to investigate to which extent the different sections of the gut microbiota respond to the sublethal clothianidin concentrations, ranging from 0.1 to 10 ppb. Based on the importance of maintaining stable positive correlations between gut microbiota members [37] in order to limit colonization by pathogens [38], we monitored the dynamics of negative/positive correlations between core, non-core and low activity taxa, as landmarks of microbiota dysbiosis [39–42].

In this work, we implemented a reverse-transcribed 16S rRNA based metataxonomic approach to characterize the gut microbiota's functional dynamics. The 16S rRNA metataxonomic approach (i.e. quantifying taxa's overall gene expression with their relative 16S rRNA transcript copy number) allows evaluating the functionally active taxonomic diversity and the relative contribution of each bacterial strain to the overall microbiota activity, therefore providing relevant insights in deciphering their functional relationship [43]. The 16S rRNA transcript expression levels (hereby called activity), were used to construct co-expression networks in order to detect and quantify the community activity changes during gut microbiota dysbiosis [44]. Furthermore, we evaluated to which extent the monitoring ratio of positive/negative correlations in microbial taxonomic networks is a valuable approach to detect and/or quantify the dysbiosis process when the host organism is facing a stress factor. Most of the studies targeted one microbial niche (i.e. one type of host tissue) and one level of the stress factor [39, 45] or a combination of individuals with various levels of a given factor [40, 46] most of these using 16S rDNA metataxonomics. To get a more comprehensive assessment of dysbiosis monitoring, we targeted three gut sections and the microbiota response to a logarithmic gradient of three xenobiotic doses. To our knowledge, this is the first work to study the impact of clothianidin on the honeybee gut microbiota. This
study aims to provide further insights with respect to the urgent need to understand correlations between
the host, its microbiota and exposure to xenobiotics.

**Material And Methods**

**Neonicotinoid compound and quantification method used**

Clothianidin (CAS Number 210880-92-5) was supplied by Sigma-Aldrich Inc. (Ontario, Canada). Clothianidin quantifications were obtained by liquid chromatography-tandem mass spectrometry (LC-MS/MS) at the INRS (Institut National de la Recherche Scientifique, Quebec, Canada). Clothianidin occurrence in bees was measured as described in Paradis et al. [47]. For each experimental condition, 10 individual bees were homogenized and pooled for LC-MS/MS analysis (in triplicates).

**Experimental setup**

*In vivo* experiments on honeybees were conducted between July and August 2017 at the Centre de Recherche en Sciences Animales de Deschambault (CRSAD, Quebec, Canada). All bees used for this study originated from two European honeybee colonies (*Apis mellifera* L.) headed by sister queens. Newly emerging bees were obtained as described in Williams et al. [48]. Two hundred bees were randomly distributed in each cage (5 cages per group) for a total of 4,000 bees. Cages were kept in a controlled dark room (30 °C ± 1°C and 50% ± 5% relative humidity) for 28 days. Each cage was supplied with an inverted sterile syringe (20 mL, BD, Franklin Lakes, New Jersey, U.S.A.) containing 50% w/v sucrose syrup. Cages were randomly distributed between groups and clothianidin administration began on day 3. Each exposed group was supplied with 50% w/v sucrose solution supplemented with the tested clothianidin concentration. Experimental groups were defined as follows: three clothianidin concentrations (0.1, 1 and 10 ppb) and a control group (50% w/v sucrose solution without clothianidin). Mortalities were recorded daily in each cage. Once a week, 20 bees were sampled from each cage and stored at -80 °C.

**Feeding rate**

Every syringe was weighted daily before and after feeding bees to measure average syrup consumption per bee and group. Syrup consumption per bee was calculated as the total measured syrup consumption per cage divided by the number of living bees per cage. Prior to ANOVA, the assumption of normality was measured using a Shapiro-Wilk test [49]. Because data did not respect this condition, treatment effect on syrup consumption was analysed using a Kolmogorov-Smirnov test [50] confirmed with a Wilcoxon test [51].

**Survival analysis**

To estimate the honeybee’s survival rate, we used the Kaplan-Meier formula [52] using the *survival* R package [53]. Statistically significant risk differences between treatments were detected with a Cox’s proportional hazards regression using the *coxph* model implemented in the *survival* R package, as previously described [54].
**RNA extraction**

Tissue sampling targeted three honeybee gut sections (midgut, ileum and rectum) at T = 7. For RNA extraction, samples from the same cage were pooled (5 gut sections from 5 bees per cage) and RNA was extracted using the TriReagent method (Ambion, Thermo Fisher Scientific). Intestinal tissues were placed into a 2 mL microtube containing 1 mL of TriReagent. Each sample was crushed with a sterilized grinder, and then incubated at room temperature (RT) for 5 minutes. Then, 200 µL of fresh chloroform per 1 mL of TriReagent was added to each sample and vortexed for 15 seconds. Samples were incubated for a second time at RT for 12 minutes and vortexed at half-time, then centrifuged for 15 minutes at 12,000 g at 4°C. Next, 400 µL of the upper aqueous phase was transferred in a new 1.5 mL microtube. Then, 250 µL of isopropanol and 250 µL of hypersaline solution (1.2 M Trisodium citrate; 0.8 M NaCl) were added per mL of TriReagent. A few inversions followed to mix solutions together, which were incubated at RT for 10 minutes. Then, samples were centrifuged for 15 minutes at 12,000 g at 25°C and we removed the supernatant. Then 1 mL of 75% ethanol was added per mL of TriReagent, followed by a centrifugation for 15 minutes at 12,000 g at 25°C. The supernatant was discarded. The RNA pellet was then air-dried and dissolved in 30 µL of nuclease free water.

**16S rRNA gene sequencing**

**cDNA synthesis**

RNA samples were reverse transcribed into complementary DNA (cDNA) with the qScript™ cDNA SuperMix method (QuantaBio, VWR, Ontario, Canada) by following the manufacturer protocol [55]. Then, partial 16S rDNA amplicons of the hypervariable V3-V4 regions were obtained in a two-step dual indexing procedure.

**Two-step 16S rDNA amplicon library preparation**

First, the V3-V4 hypervariable region was amplified by PCR using universal primers

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[347-F (5'-ACACTCTTTCCCTACAGGAGCTCTTCCTCCGATCT-GGAGGCAGCAGTRRGGAAT-3') and 803-R (5'-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-CTACCRGGGTATCTAATCC-3')] (Sigma-Aldrich Life Science) which were tailed on the 5' end with part of the Illumina TruSeq adaptors. The first PCR was conducted in a total volume of 50 µL: Reaction Buffer (Q5) (5X) 10 µL; dNTPs (10 mM) 1 µL; 347-F 2.5 µL; 803-R 2.5 µL; High GC enhancer (Q5) (5X) (NEB) 10 µL; Q5 High-Fidelity DNA Polymerase (NEB) 1 µL; H2O 20 µL and DNA template 3 µL. After initial denaturation at 98 °C for 2 minutes, amplification was performed using 35 cycles of 10 seconds at 98 °C, 30 seconds at 60 °C and 30 seconds at 72 °C followed by a final extension at 72 °C for 2 minutes. PCR reactions were purified using the PCR cleanup kit (Axygen, Inc., Fischer Scientific, Ottawa, Canada). Quality of purified PCR products was checked on a DNA 7500 BioAnalyzer chip (Agilent) and quantified using the Nanodrop 1000 Spectrophotometer. Then, a second PCR was performed to attach the remaining adapter sequences (regions that anneal to the flow cell and library specific barcodes). The second PCR was conducted in a total volume of 50 µL: Reaction
Buffer (Q5) (5X) 10 µL; dNTPs (10 mM) 1 µL; generic forward primer (5’-AATGATACGGCGACCACCGATCTACAC-[index1]-ACACTCTTTCCCTACACGAC-3’) (Sigma-Aldrich) 1 µL; reverse primer (5’-CAAGCAGAAGACGGCATACGAGAT-[index2]-GTGACTGGAGTTCAGACGTGT-3’) (Sigma-Aldrich) 1 µL; High GC enhancer (Q5) (5X) (NEB) 10 µL; Q5 High Fidelity DNA Polymerase (NEB) 1 µL; H2O 24 µL and DNA template (10–15 ng/µL) 3 µL. After initial denaturation at 98 °C for 2 minutes, amplification was performed using 12 cycles of 10 seconds at 98 °C, 30 seconds at 60 °C and 30 seconds at 72 °C followed by a final extension at 72 °C for 2 minutes.

**Paired-end Illumina sequencing**

Barcoded amplicons were pooled in equimolar concentrations and sequenced at the “Plate-forme d’Analyses Génomiques” of Laval University (Quebec, Canada) using the Illumina MiSeq Paired-End technology (2 × 300 bases). Please note that primers used in this work contain Illumina specific sequences protected by intellectual property (Oligonucleotide sequences © 2007–2013 Illumina, Inc. All rights reserved. Derivative works created by Illumina customers are authorized for use with Illumina instruments and products only. All other uses are strictly prohibited).

**Bioinformatics analysis**

**Sequence clustering and taxonomic assignment**

In total, 120 samples (2 replicates of 5 bees × 3 gut section × 5 cages per group × 4 treatments) were sequenced individually. Reads were processed through the dada2 [56] pipeline: quality control of reads was processed through the filterAndTrim() function by using the following parameters: 270 for the read truncation length, 2 as the Phred score threshold for total read removal, and a maximum expected error of 2 for forward reads and 4 for reverse reads. Filtered reads were then fed to the error rate learning, dereplication and ASV inference steps using the functions learnErrors(), derepFastq() and dada(). Chimeric sequences were removed using the removeBimeraDenovo() function with the “consensus” method parameter. Taxonomic classification was done through the using blast matches from the NCBI 16S Microbial database using a lowest common ancestor (LCA) algorithm, similar to the method used in MEGAN [57] to determine the taxonomy when matches were under a 98% identity threshold. Data was subsetted by condition (gut section + concentration). Correlation matrices were constructed using the Hmisc R [58] package with P-values corrected with the false discovery rate (FDR) method [59].

**Network analysis**

Co-abundance networks were built using R to identify significant taxon-taxon correlations in each gut section per experimental condition. Twelve microbial networks were generated from pairwise correlations between sum of abundances for each taxa genus and significant correlations, and visualised using the software Cytoscape (version 3.7.2) [60]. Each node represents a bacterial genus. The size of each node is proportional with the genus bacterial activity. The darker a node color is, the more it is interconnected in the network. Each edge represents significant positive or negative correlations acquired using Spearman’s correlation coefficient such as -1 < r < -0.4 (negative, red edge) and 1 < r < 0.4 (positive, green edge); p
value < 0.05 with FDR correction. For network interpretation, we only accounted bee gut taxa that occur in most replicated samples (n > 7 on 10 samples per condition). We defined low activity taxa: genera with a very low activity (< 0.01%) of the total sample activity that occurs in very few numbers of samples (n < 3 on 10 per condition).

To further assess significant gut bacterial perturbations induced by clothianidin, we measured the distribution of the following network topologic parameters: Degree (DG), Neighborhood Connectivity (NC) and Closeness Centrality (CC) obtained with the NetworkAnalyser function built in Cytoscape. DG is a local quantification representing the communication activity inside a network. The more the DG of a node increases, the more a node is connected locally inside the network [61, 62]. NC is a quantitative score giving the average connectivity of a specific node on the overall network. The more the NC increases, the more this node will impact the global network dynamics [63, 64]. CC is a qualitative measure representing the mean of the shortest path length. A high CC means that the node is central inside the network and can interact with the other nodes [65].

Results

**Honeybee survival is unexpectedly lower when exposed to lesser concentrations of clothianidin**

Honeybee survival was inversely proportional to the clothianidin concentration: bees exposed to the 0.1 ppb had the highest mortality relatively to 1 and 10 ppb groups. Similarly, bees exposed to 1 ppb had more mortality compared to bees exposed to 10 ppb (Fig. 1). For bees exposed to 0.1 ppb, survival probability was 16.3% less than the unexposed group on days 7, 14 and 21 (Supplementary Table S1). Similarly, honeybees exposed to 1 ppb showed significant differences of survival probabilities from the second week of the experiment. Contrarily, at 10 ppb, significant differences of survival probabilities began only at the end of the experiment with a survival probability of 4.6% less than the control group on day 27 of the experiment (Coxph model).

**Within-group survival is highly variable during early exposure to clothianidin**

We observed significant differences between survival probabilities for each experimental group at T = 7. For the control group, significant differences of survival probabilities were more important at T = 7 (Supplementary Table S2). For the 0.1 ppb group, significant differences of survival probabilities were more constant during the experiment (Supplementary Table S3). For the 1 ppb group, significant differences of survival probabilities increased from T = 14 (Supplementary Table S4). For the 10 ppb group, significant differences of survival probabilities were higher at T = 7, then decreased from T = 14 until T = 28 (Supplementary Table S5).

**Clothianidin influences the feeding rate of honeybees**
Syrup consumption differed over time in all groups (Fig. 2) with a significant difference between control and exposed groups (Kolmogorov-Smirnov, P < 0.05), suggesting a significant treatment effect on feeding rate. Significant differences were further validated with a Wilcoxon test (P < 0.05) (Supplementary Table S6). Bees from the 0.1 ppb group consumed more syrup than the others experimental groups.

**Clothianidin quantification in experimental honeybees**

Clothianidin concentration (per bee) accumulated differently across all experimental groups (Supplementary Table S7). At T = 7, bees from the 0.1 ppb group accumulated more clothianidin than 1 and 10 ppb groups. At T = 14, clothianidin concentration increased on the 10 ppb group in comparison with 0.1 and 1 ppb). Clothianidin concentration from the 0.1 and 1 ppb groups decreased over time. We observed significant differences across experimental groups for clothianidin quantification per bee (Kruskal-Wallis, P < 0.05) (Supplementary Table S8).

**Clothianidin disturbs taxon-taxon interactions in the honeybee gut microbiota**

Midgut. At the genus taxonomic rank, the number of ASVs varied from 45 (control midgut) to 35, 36 and 33 respectively after 0.1, 1 and 10 ppb of clothianidin exposure (Fig. 3; Supplementary Fig.S1). Our results indicated how exposed midgut ASVs were significantly less connected (DG) relatively to the control midgut network (0.1 ppb: P = 0.0045; 1 ppb: P ≤ 0.001; 10 ppb: P = 0.002) (Supplementary Fig.S7). We observed a decrease of significant positive correlations from 249 (control midgut) to 119; 112 and 101 respectively at 0.1, 1 and 10 ppb of clothianidin exposure, and an increase of significant negative correlations from 25 (control midgut) to 53; 39 and 39 respectively at 0.1, 1 and 10 ppb of clothianidin exposure. Concerning honeybee gut core members: *Lactobacillus*, *Snodgrassella* and *Gilliamella* activity was variable across concentrations. For all clothianidin treatment concentrations: *Bifidobacterium* (core members) activity increased; *Frischella* (core member) activity decreased and *Flavobacterium* (low activity taxa) decreased. At 0.1 ppb, we observed a drastic decrease of *Parassacharibacter* (core member) activity. We observed a gain in significant correlations with the following low activity taxa: *Moraxella, Oceanobacillus, Blautia, Lysinibacillus* (0.1 ppb); *Lysinibacillus, Lawsonella* (1 ppb); *Thiohalobacter, Lawsonella* (10 ppb) and *Bacillus, Ralstonia* (three concentrations). We observed a loss of correlations with *Devosia* (0.1 and 1 ppb) and *Leifsonia* (three concentrations).

We observed significant differences (higher) for the CC (P = 0.03 at 0.1 ppb) and (lower) for NC (P ≤ 0.001 at 0.1, 1 and 10 ppb) relatively to the control midgut network (Supplementary Fig.S4 and S10). Our results support significant difference for the CC between the microbial networks exposed to clothianidin (P = 0.012 at 1 and 10 ppb, relatively to 0.1 ppb) (Supplementary Fig.S4). Also, we observed a significant (higher) NC difference (P ≤ 0.001; respectively at 0.1) and a significant lower (NC) (P = 0.025; respectively at 10 ppb) relatively to 1 ppb (Supplementary Fig.S10).

At 0.1 ppb, *Lactobacillus* was positively correlated with *Bifidobacterium, Pediococcus* and with low activity taxa: *Ralstonia, Thiohalobacter, Lysinibacillus*, and negatively correlated with *Bombella,*
Parassacharibacter and with low activity taxa: *Moraxella, Prevotella, Oceanobacillus* (Fig. 3). Still at 0.1 ppb, *Moraxella* and *Lysinibacillus* were characterized by a high CC and NC (Supplementary Table S10). *Prevotella, Ralstonia, Thiohalobacter, Oceanobacillus* were also characterized by a high NC (Supplementary Table S10).

Ileum. At the genus taxonomic rank, the number of ASVs varied from 35 (control ileum) to 42, 40 and 30 respectively after 0.1, 1 and 10 ppb of clothianidin exposure (Fig. 4; Supplementary Fig. S2). Our results showed how exposed ileum networks were significantly more connected (DG) compared to the control ileum network (*P* ≤ 0.018; *P* = 0.002 respectively at 1 and 10 ppb) (Supplementary Fig. S8). We observed an increase of significant positive correlations from 96 (control ileum) to 156; 194 and a decrease of significant positive correlations to 70 respectively at 0.1, 1 and 10 ppb of clothianidin exposure, and a decrease of significant negative correlations from 62 (control ileum) to 55; 26 respectively at 0.1, 1 and 10 ppb of clothianidin exposure (Fig. 4; Supplementary Fig. 2A-B-C). Concerning honeybee gut core members: *Lactobacillus* and *Gilliamella* (core member) activity was variable across all treatment concentrations. For all concentrations: *Bifidobacterium* (core members) activity increased; *Snodgrassella* and *Frischella* (core members) activity decreased. We observed a gain of significant correlations with low activity taxa: *Leifsonia* (0.1 ppb); *Moraxella, Prevotella* (1 ppb); *Lawsonella, Lysinibacillus* (0.1 and 1 ppb) and *Bacillus, Staphylococcus, Ralstonia* (three concentrations). At 0.1 ppb, we observed a slow increase of *Pseudomonas* and *Flavobacterium* taxa activity.

We observed significant higher difference for CC (*P* = 0.023) and NC (*P* ≤ 0.001) respectively at 1 ppb, relatively to the control ileum network (Supplementary Table S13, S15); and significant lower difference for CC (*P* = 0.001) and NC (*P* ≤ 0.001), respectively at 10 ppb, relatively to the control ileum network (Supplementary Table S13, S16). Our results also support significant difference between the microbial networks exposed to clothianidin. In fact, our results support significant higher difference for CC (*P* = 0.001) respectively at 1 ppb relatively to 0.1 ppb (Supplementary Fig. S5; Table S14-S15); significant lower difference for CC (*P* ≤ 0.001) respectively at 10 ppb relatively to 1 ppb (Supplementary Fig. S5; Table S15-S16); significant higher difference for NC (*P* ≤ 0.001) respectively at 1 ppb relatively to 0.1 ppb (Supplementary Fig. S11; Table S14-S15); significant lower difference for NC (*P* ≤ 0.001) at 10 ppb relatively to 0.1 ppb (Supplementary Fig. S11; Table S14-S16); and significant lower difference (*P* ≤ 0.001) respectively at 10 ppb relatively to 1 ppb (Supplementary Fig. S11; Table S15-S16).

At 1 ppb, *Commensalibacter, Bombella* and *Ralstonia* were characterized by a high CC; and *Bartonella* by a high NC (Supplementary Table S15). *Ralstonia* experienced an increase of DG, CC and NC (Supplementary Table S15). In addition, *Bifidobacterium* was positively correlated with low activity taxa: *Prevotella* and *Moraxella*; and negatively correlated with probionts like *Gilliamella, Frischella*; and with a low activity taxa: *Lysinibacillus* (Fig. 4).

Rectum. At the genus taxonomic rank, the number of ASVs varied from 34 (control rectum) to 25; 23 and 22 respectively after 0.1, 1 and 10 ppb of clothianidin exposure (Fig. 5; Supplementary Fig. S3). Our results indicated how exposed rectums were significantly less connected (DG) compared to the control
rectum network (P ≤ 0.001; P = 0.004 and P = 0.001 respectively at 0.1; 1 and 10 ppb) (Supplementary Fig.S9). We observed a strong decrease of significant positive correlations from 148 (control rectum) to 38; 57 and 49 respectively at 0.1, 1 and 10 ppb of clothianidin exposure, and a drastic decrease of significant negative correlations from 108 (control rectum) to 34; 17 and 27 respectively at 0.1, 1 and 10 ppb of clothianidin exposure. Concerning honeybee gut members: Lactobacillus, Snodgrassella and Gilliamella (core members) activity was variable across treatment concentrations. For all concentrations: Bifidobacterium (core member) activity increased, while Frischella (core member) activity decreased. We observed a gain of significant correlations with low activity taxa: Bacillus, Lysinibacillus, Leifsonia (0.1 ppb); Lysinibacillus, Oceanobacillus (1 ppb); Bacillus, Psychrobacter (10 ppb) and Pseudaminobacter (three concentrations).

We observed a significant lower difference for CC (P ≤ 0.001, P = 0.028 and P = 0.008; respectively at 0.1, 1 and 10 ppb) and for NC (P ≤ 0.001; respectively at 0.1, 1 and 10 ppb) relatively to the control rectum network (Supplementary Fig.S6; Table S17-S20). Our results support a significant higher CC difference between the microbial networks exposed to clothianidin (P = 0.003 and P = 0.008; respectively at 1 and 10 ppb relatively to 0.1 ppb) (Supplementary Fig.S6; Table S18-S20).

At 10 ppb, Frischella was negatively correlated with Snodgrassella, Bifidobacterium, Commensalibacter, and with low activity: Streptococcus, Pseudaminobacter, and Staphylococcus (Fig. 5). Contrastingly, Frischella gained positive correlations with Lactobacillus (Fig. 5).

**Discussion**

In this study, exposure to various concentrations of clothianidin left different signatures of microbiota dysbiosis in the three gut sections of honeybees. Changes of correlations reveal pathogen activity spikes correlated to different patterns of mutualist imbalance according to gut section. Such dysbiosis patterns were expected as gut sections are colonized by specific microbial communities (Fig. 1–3; Supplementary Fig.S1-S3), each microbial community differentially shaping the environmental conditions of each gut section [66]. In the midgut and the rectum, a decrease of ASVs activity correlation was detected in all exposed groups to clothianidin. In the ileum, two types of variations occurred: significant correlations among ASVs increased under 0.1 and 1 ppb groups; and decreased for the 10 ppb group.

We expected that a clothianidin exposure gradient would significantly increase positive and negative correlations between pathogens [41, 67]; and a change of correlation type between core members [42]. Despite disrupted microbial activity correlations, dominant core and non-core members were still active in all test groups, as reported in a previous study [68].

Our work highlights the importance of low activity taxa in the gut microbiota stability as illustrated by the loss of Devosia and Leifsonia; and exposed in previous studies [62, 69–72]. Despite their low bacterial activity, these taxa are characterized by high degree in the correlational networks: DG, CC and NC within the control midgut network (Supplementary Table S9).
To understand why the lowest clothianidin concentration induced the lowest honeybee survival, we investigated whether a specific signature of microbiota dysbiosis could be associated to the low clothianidin (0.1 ppb) treatment group. The loss of positive correlations with the increasing neonicotinoid concentrations along the gut section, is a landmark of gut dysbiosis [73]. As stated above, the most extensive adverse impact in terms of correlational network structure, was recorded at 0.1 ppb (Fig. 3; Supplementary Fig.S1A,S2A). Noticeably, the gain of DG and the high CC and NC for *Lawsonella*, a human pathogen [74] supports its important central role inside the ileum (Supplementary Table S13-14), in addition to the important decrease of DG and NC in the rectum (Supplementary Table S17-18) for *Bifidobacterium*, *Pediococcus* and *Commensalibacter*: known as probiotics [15, 75, 76]. Then, numerous correlations occurred with low activity taxa in the midgut (Fig. 3) with the high NC (Supplementary Table S10) for *Moraxella* [77] suggesting a potential pervasive adverse effect on the overall network. Also, the majority of correlations (negative and/or positive depending the gut section) with two beneficial strains, *Pediococcus* and *Lactobacillus* involved potential pathogens such as *Thiohalobacter* [78] and *Prevotella* [79].

The significant impact of different pesticide concentrations on the overall network may depend on the microbial strain that first metabolizes the molecule, which influences the syntrophic exchange network. For instance, honeybee gut microbiota exposed to two fipronil concentrations did not respond similarly: the lowest concentration (0.25 µg/kg) affected *Bifidobacterium* sp. abundance, with no significant bees mortality increase, whereas the highest concentration (1 µg/kg) did not affect *Bifidobacterium* sp. abundance, but induced a significant bee mortality increase [8]. Daisley et al. [32] showed in gnotobiotic *Drosophila* that the pesticide chlorpyrifos was more toxic than its metabolite (chlorpyrifos oxon). In our case, the final metabolites’ toxicity, according to the initial clothianidin concentration, could differentially impact the honeybee physiology, and in turn, survival. Interestingly, clothianidin quantification with LC-MS/MS suggests a slower degradation at 0.1 ppb (Supplementary Table S7-S8) which could result from a different clothianidin metabolism pathway, potentially translating into lower survival. Previous works reported clothianidin degradation by *Flavobacterium* and *Pseudomonas* sp. [80] and imidacloprid degradation by *Leifsonia* sp. [34]. These taxa were mostly impacted at 0.1 ppb for *Flavobacterium* and *Pseudomonas* across all gut sections (Fig. 3; Supplementary Fig.S4B; Fig. 5A; Table S10;14;18) and for *Leifsonia* in the ileum and rectum (Fig.S4B; Fig. 5A; Supplementary Table S14, S18). These taxa are widely connected with the overall network (high NC). In addition, *Flavobacterium* and *Pseudomonas* played a central position inside the network (high CC).

Within all these three gut sections, we observed a gain of edges/significant correlations (positive and/or negative) for the genera *Bifidobacterium*, *Frischella*, *Gilliamella*, *Lactobacillus*, *Parasaccharibacter* and *Snodgrassella*. All these bees symbionts are known to be involved in either host immunity or maintaining a homeostatic microbiota [38, 81–84]. For example, *Gilliamella apicola* [85] and *Lactobacillus* [86] are responsible for short-chain fatty acid production, and their diminishing activity may likely alter the host’s immunity [87]. Moreover, the functional complementarity between *Snodgrassella alvi* and *G. apicola* ensures homeostatic microbiota in the intestine ecosystem [84]. *Frischella perrara* [83] and *Parasaccharibacter* spp. [81] were documented as important key factors in the immune system.
Investigating the local effect of clothianidin gradient on the gut microbiota structure, we found a gain of correlations (positive and/or negative) among low activity taxa. These taxa who showed to be involved in: 1) disturbed environment and/or in pathogens activities includes genera like *Moraxella* [77], *Lawsonella* [88], *Thiohalobacter* [78], *Ralstonia* [89], *Leifsonia* [90], *Prevotella* [79], *Psychrobacter* [39, 91] and *Pseudaminobacter* [92]; and 2) in healthy environment as probiotic and/or in antimicrobial activity. These low activity taxa are belonging to the genera *Oceanobacillus* [93], *Blautia* [94, 95], *Bacillus* [54, 96] and *Lysinibacillus* [93, 97].

Within the midgut, exposure to 0.1 ppb clothianidin (Fig. 3) was more harmful relatively to the other concentrations as supported by an increase of DG and CC; mainly impacting *Lactobacillus* genus activity (Supplementary Table S9-S10). Increase of these topological parameters (DG and CC) support how the *Lactobacillus* genus became centrally connected inside the network at 0.1 ppb. *Lactobacillus* genus is known in improving the immune system, resistance against pathogens [21, 40, 98, 99] and reduced pesticide toxicity [100] supporting how *Lactobacillus* spp. activity is favorable for the host.

Complementarily, the loss of connectivity (diminishing DG) for *Bifidobacterium* and *Pediococcus* (diminishing CC) (Supplementary Table S9-S10). supports clothianidin adverse impact on these genera known for their probiotic properties [15, 75, 76, 101, 102]. However, the increase of NC for *Bifidobacterium* suggests a likely favorable cooperation inside the network. Network node metrics suggest a pathogenic shift compensated by mutualistic correlation following exposure to clothianidin. Noticeably, clothianidin induced a shift from a positive to a negative correlation activity between *Frischella* (decreasing activity) and *Pediococcus* (increasing activity). This competition shift suggests a dysbiosis pattern [42].

Within the ileum, 1 ppb treatment (Fig. 4B) was more adverse relatively to the other concentrations, mainly targeting *Bifidobacterium* genus activity, supported by an increase of DG, CC and NC (Supplementary Table S13, S15).

Within the rectum, 10 ppb treatment (Fig. 5B) was more adverse relatively to the other concentrations, mainly impacting *Frischella* genus activity, with a gain of nine significant local correlations supported by an increase of DG and CC, and a loss of connectivity with the overall network, as supported by a decrease of NC (Supplementary Table S17, S20).

Taken together, pathogens, and correlations rise are not surprising as clothianidin induced negative correlations between core and non-core members within each gut section. Given that all these symbionts are either involved in host immunity and/or microbiota equilibrium [38, 81–84] and that clothianidin neonicotinoid could potentially disrupt the microbiota-immunity axis [21–23], our results suggest that clothianidin exposure induced dysbiosis is at least targeting, the microbiota-immunity axis.

More specifically, the impact due to pathogen invasion was variable along the gut section and gradient of exposure. *Ralstonia* genus impacted much more the midgut network (0.1 ppb) (Fig. 3) and the ileum (1 ppb) (Fig. 4B). The high CC and NC for *Ralstonia* endorse its DG and important NC (Supplementary Table S10, S15) within the overall network suggesting this strain exerts an important negative impact on
microbiota structure following clothianidin exposure. Our results provide additional evidence from the lens of bacterial activity that honeybees exposed to neonicotinoids are more sensitive to microbial gut pathogens [33, 103, 104]. In addition, the dysbiosis patterns may suggest a shift from opportunistic to infectious symbionts by stressors [105], which could potentially result from an impairment from resistance to colonization (i.e. the microbiota resistance to pathogen invasion [106]).

Previous studies already highlighted fluctuations of honeybee microbiota. Application of coumaphos, tau-fluvalinate [107] and tetracycline [108] showed to increase *Giamella apicola* prevalence. Different experimental approaches may induce microbial composition variability [38] as observed in our work with *G. apicola* and *Snodgrassella alvi*. The authors [107, 108] used entire bee gut, sampled from hive, whereas we used gut sections, sampled from cages. *Snodgrassella alvi* abundance decreasing in the ileum is consistent with [109, 110] who reported the same observations in the bee gut after glyphosate exposure. Moreover, *S. alvi* is responsible in the upregulation of the gene's expression related to antimicrobial peptide [111] and as honeybee gut biofilm pioneer, *S. alvi* destabilization may create an overall disbalance of gut microbiota. In our experiment, it is likely the decreasing *Snodgrassella activity* helped in adversely impacting the immune system of *Apis mellifera*.

We observed a decreasing in *Frischella perrara* activity, while exposure to other pesticides was variable as it either induced (nitenpyram) [20] or not (imidacloprid) an increase of *F. perrara* abundance [33]. *F. perrara* is known to have a key role in honeybee immunity in limiting microbial resistance [83], therefore *F. perrara* disbalance might affect the honeybee gut immunity, leading to microbiota dysbiosis. Finally, the increasing activity of *Bifidobacterium* is in agreement with previous studies [19–21] that tested nitenpyram and thiacloprid exposure on honeybees.

Altogether, the initial pesticide concentration has differentially impacted the microbiota correlation network. For any given concentration, different members could first handle the molecule and thus determine the kind of metabolites excreted, and in turn determine its toxicity, which may not be necessarily proportional to the concentration. Further analysis on metabolites generated by *Flavobacterium* spp., *Leifsonia* spp. and *Pseudomonas* spp. grown in media with a clothianidin concentration gradient might give more explanations on their ability to modulate clothianidin toxicity on honeybee.

Finally, it is documented that honeybees are more attracted to contaminated than non-contaminated syrup [112]. A dose-dependent attraction was observed for Nitenpyram where food consumption was negatively correlated with pesticide concentration: normal with 3–30 µL/L, low with 300 µL/L [20]. In our case, honeybees exposed to 0.1 ppb consumed significantly more syrup, translating into higher clothianidin bioaccumulation in the 0.1 ppb group, relatively to the other groups (Fig. 2, Supplementary Table S6). Previous studies highlighted how nutritional stress shapes the gut microbiota composition [113, 114] inducing long term negative impacts on honeybee health [114]. Therefore, it is very likely that higher bioaccumulated clothianidin in younger bees (with an immature microbiota) would have long-term health effect.
Conclusion

This work highlights a new facet in understanding the interplay between gut microbiota activity, food intake and pesticide exposure. Our work provides unprecedented insights regarding the impact of clothianidin gradient on the correlations between bee gut members, and its potential link with the microbiota-immunity axis: The clothianidin-induced destabilisation of the immune balance between probionts, commensals and pathogenic strains, translated into the rise of pathogenic strains. Overall, our results suggest that extent of gut microbiota dysbiosis depend on both xenobiotic exposure level, and gut section. Finally, taxon-taxon co-occurrence networks appeared as a valuable tool to measure the impact of pesticide exposure on microbiomes community structure.

Abbreviations

ASV: Amplicon Sequence Variant
CAS: Chemical Abstracts Service
CC: Closeness Centrality
cDNA: complementary DNA
DG: Degree

e.g.: exempli gratia
FDR: False Discovery Rate
Fig. Figure
i.e.: id est (means : in other words)
LCA: Lowest Common Ancestor
LC-MS/MS: Liquid Chromatography-tandem Mass Spectrometry
NCBI: National Center for Biotechnology Information
NC: Neighborhood Connectivity
NF-κB: Nuclear Factor-kappa B
PCR: Polymerase Chain Reaction
P: Probability
Ppb: Parts-per-billion (10^{-9})
RNA: RiboNucleic Acid

RT: Room Temperature

T: Time

16S rDNA: 16S ribosomal DNA

16S rRNA: 16S ribosomal RNA

Declarations

AVAILABILITY OF DATA

The raw sequence reads analysed during the current study are available in the NCBI BioProject ID repository under the number PRJNA678327.

COMPETING INTERESTS

The authors have no conflict of interest to declare.

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AUTHOR CONTRIBUTIONS

Conceptualization, N.D., P.G. and S.E.; Funding acquisition, N.D. and P.G.; Experimental work, S.E.; Statistical data analysis, S.E. and B.C.; Bioinformatics, S.E., J.G., S.B. and B.C., Original draft, S.E.; Writing - review & editing, all authors.

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Figures

![Graph showing survival probability over days for different treatments](image-url)
Kaplan-Meier Survival curves of bees in each experimental group during the 28 days cage bee experiment. The y-axis represents the Kaplan-Meier estimates of the survival probabilities. The x-axis represents the experimental days. The red, blue and violet curves represent survival probabilities of honeybees exposed to 0.1, 1 and 10 ppb clothianidin respectively. The green curve represents the survival rate of honeybees supplemented with 50%w/v sucrose solution only.
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Figure 2

Syrup consumption in each experimental group during the 28 days cage bee experiment. Clothianidin effect on syrup consumption was analysed using a non-parametric (Kolmogorov-Smirnov) test confirmed with a Wilcoxon test with R. The x-axis represents the experimental days. The red, blue and violet curves represent the syrup consumption of honeybees exposed respectively to 0.1, 1 and 10 ppb clothianidin. The green curve represents the syrup consumption of honeybees supplemented with 50%w/v sucrose solution only.
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Figure 3

Interaction networks were generated based on pairwise correlations between the relative abundances of different bacterial genera for the midgut exposed at 0.1 ppb. Each node represents a bacterial genus. The size of each node is proportional with the bacterial functional activity of each genus. The more a node is dark, the more it is interconnected. Each edge represents significant positive or negative Spearman correlation coefficients (-1 < r < -0.4) (negative, red) and (1 < r < 0.4) (positive, green); (FDR-adjusted p-value < 0.05).
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Interaction networks were generated based on pairwise correlations between the relative abundances of different bacterial genera for the A) ileum exposed at 0.1 ppb; B) ileum exposed at 1 ppb; and C) the ileum exposed at 10 ppb. Each node represents a bacterial genus. The size of each node is proportional with the bacterial functional activity of each genus. The more a node is dark, the more it is interconnected. Each edge represents significant positive or negative Spearman correlation coefficients (-1 < r < -0.4) (negative, red) and (1 < r < 0.4) (positive, green); (FDR-adjusted p-value < 0.05).
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Figure 5

Interaction networks were generated based on pairwise correlations between the relative abundances of different bacterial genera for A) the rectum exposed at 0.1 ppb; and B) for the rectum exposed at 10 ppb. Each node represents a bacterial genus. The size of each node is proportional with the bacterial functional activity of each genus. The more a node is dark, the more it is interconnected. Each edge
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**Supplementary Files**

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