Clothianidin induces microbial gut dysbiosis to honey bees, *Apis mellifera*

Sarah El Khoury [1], Jeff Gauthier [1], Bachar Cheaib[2], Pierre Giovenazzo [1,3], Nicolas Derome [1]

1 Université Laval, Département de Biologie, Institut de Biologie Intégrative et des Systèmes (IBIS), Québec, Canada;
2 University of Glasgow, Institute of Biodiversity, Animal Health & Comparative Medicine, Glasgow, Scotland;
3 CRSAD, Centre de recherche en sciences animales, de Deschambault, Québec, Canada

INTRODUCTION

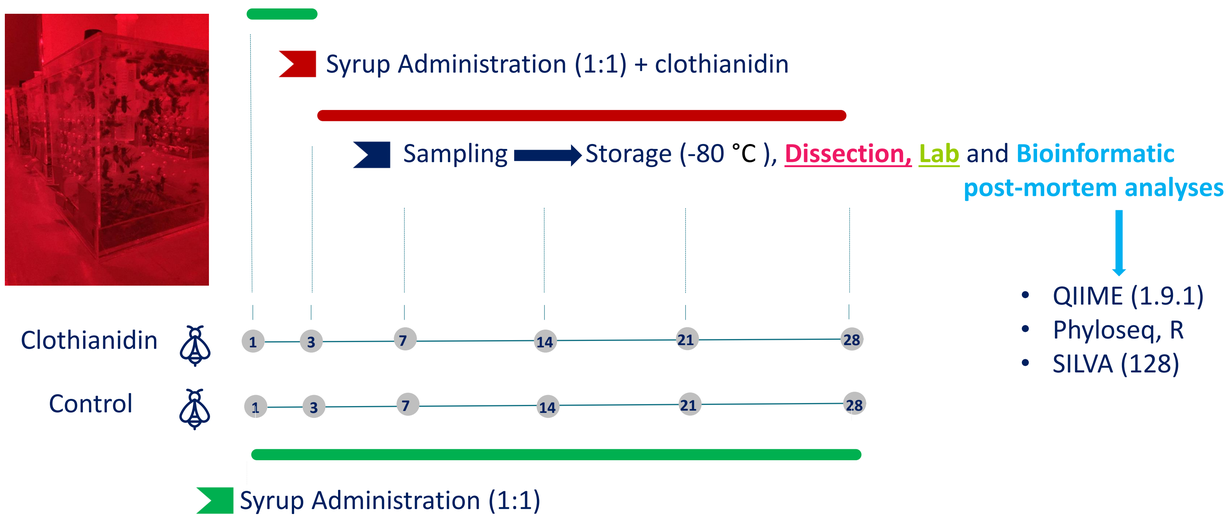
Honey bees (*Apis mellifera*) are facing a wealth of synergistically interacting stress factors affecting their lifespan, health and productivity. Neonicotinoid insecticides, as clothianidin, act on the central nervous system of insects, specifically targeting the nicotinic acetylcholine receptors inducing behavioral, memory and immunity alterations. It is now well documented that functions associated with immune response and behavior are controlled by the intestinal flora (i.e. gut microbiota) in insects [1]. Given that clothianidin is persistent in the environment, there is an urgent need to develop alternative and sustainable strategies to mitigate its toxic effects on honey bee health. Our work focused on the following questions:

- Did the host microbiota functional interactions are impacted by sublethal clothianidin exposure ?
- Is it possible to select endogenous honey bee candidate probiotics able to grow in contact with clothianidin and to degrade it ?
- Did the administration of our select candidate probiotics to bees exposed to clothianidin will help to restore the impaired functions ?

MATERIALS AND METHODS

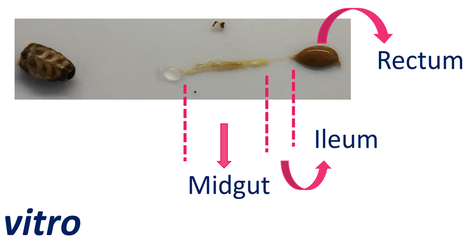
1. First *In vivo* experiment

- Young bees (3-4 days post-emergence) were used - Three concentrations (0.1; 1 and 10 ppb) have been tested - 5 cages per experimental group - 200 bees per cage. The experiment lasted for 28 days.



- QIIME (1.9.1)
- Phyloseq, R
- SILVA (128)

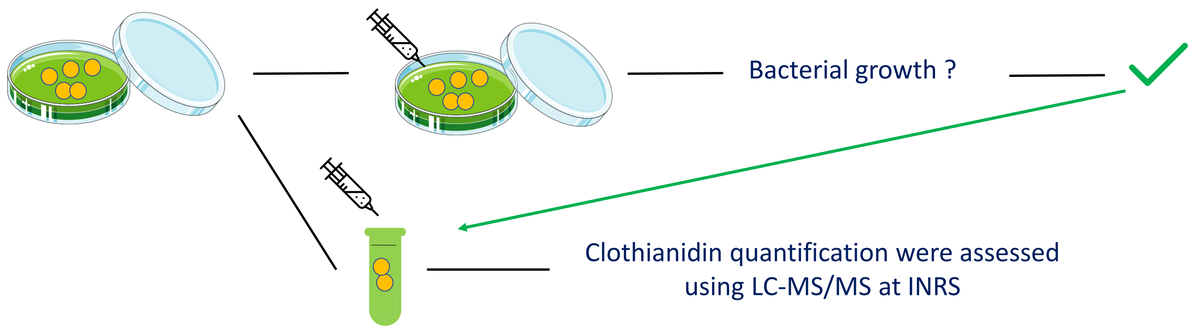
2. *In vitro*



- Lab
  - V3 region of 16S rRNA
  - 120 × 16S rRNA libraries: 2 replicates of 5 bees × 3 gut section × 5 cages per group × 4 treatments
  - Illumina MiSeq Sequencing (2 x 300 bases)

Isolation of probiotic candidates from the honey bees intestine

Incubation with 0.1; 1 and 10 ppb of clothianidin during 72 hours



3. Second *In vivo experiment*

- Young bees (3-4 days post-emergence) were used and only 0.1 ppb has been tested. 3 cages per experimental group (6 groups) - 200 bees per cage. We used the same timelines of the first *in vivo* (28 days). We sampled at Time = 7, 14, 21 and 28 days.

Experimental Group	Syrup (1:1)	Clothianidin (Clo)	Probiotic 1	Probiotic 2
1. Control	+	-	-	-
2. Pesticide	+	+	-	-
3. Probiotic 1	+	-	+	-
4. Probiotic 1 + Pesticide	+	+	+	-
5. Probiotic 2	+	-	-	+
6. Probiotic 2 + Pesticide	+	+	-	+

Table 1. Experimental Setup

RESULTS

1. First *In vivo experiment*

A. BEE SURVIVAL

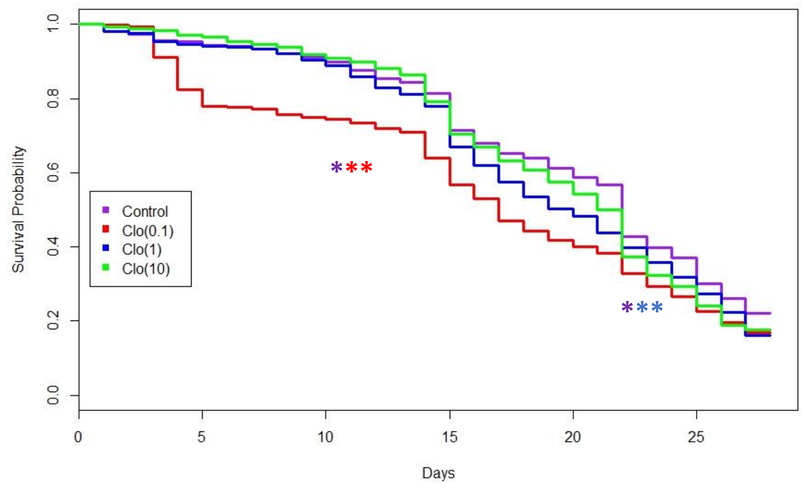


Figure 1. Kaplan-Meier Survival curves distribution of bees in each experimental group during 28 days. The red; blue and green curve represent respectively effect of 0.1; 1 and 10 ppb of clothianidin exposure on bee survival; The violet curve represents survival rate of bees supplemented only with syrup (1:1).

Cox's proportional hazards regression was performed using the *coxph* model (***= P < 0.001).

B. MICROBIAL NETWORK ANALYSES

Figure 2. Interaction networks generated based on pairwise correlations between occurrence and abundances of different bacterial genus for (**A**) control Ileum and (**B**) Ileum exposed at 10 ppb. Microbial networks were build and visualized by using the Software Cytoscape [2] based on undirected direction (undirected edges). Each color represents a distinct bacterial family (see legend). Each node represents a *denovo* OTU (operational taxonomic unit). Each edge represents significant positive correlations acquired using Spearman correlation coefficient [3] such as r > 0.6; p value < 0.05 with a fdr correction. The size of each node is proportional with the bacterial functional activity of each *denovo* OTU

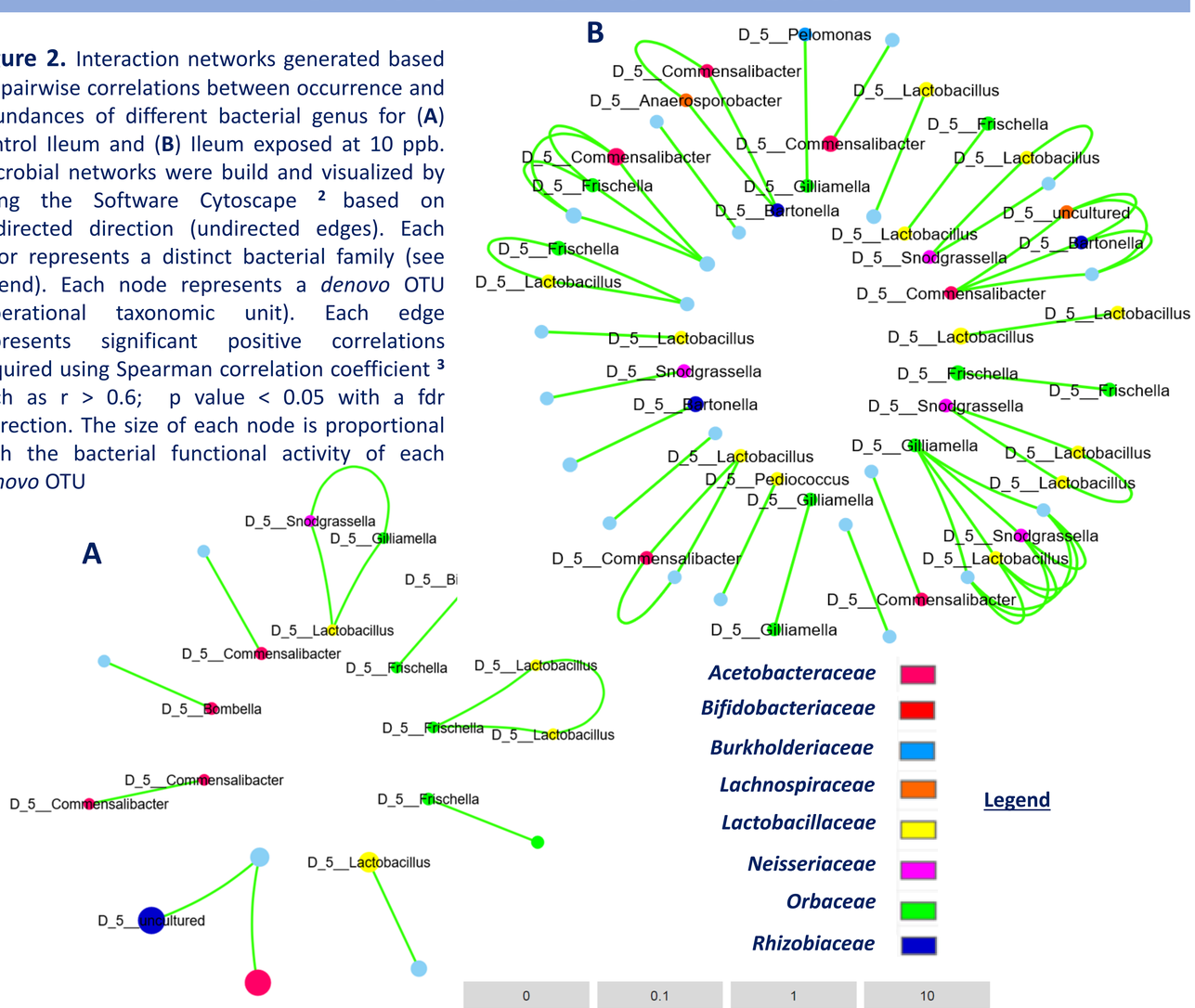
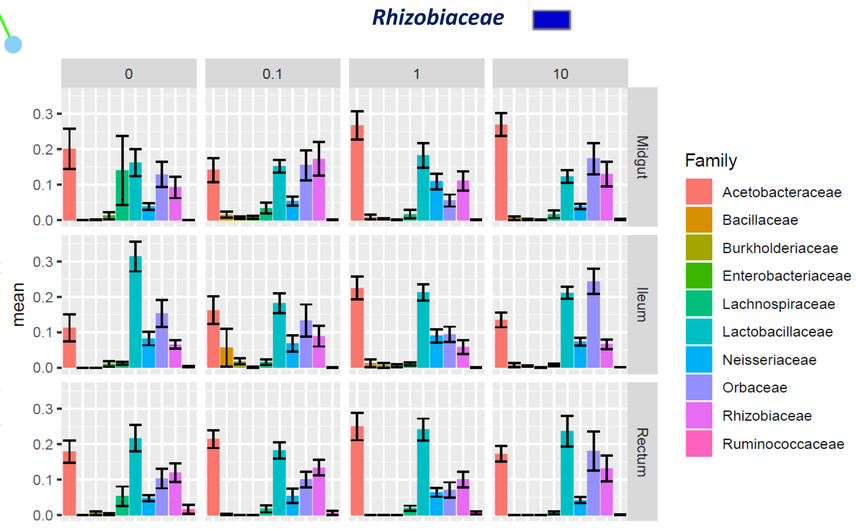


Figure 3. Histogram graph showing the mean relative abundance of the top ten most abundant families across the whole dataset at day 7 post-treatment subdivided according two conditions: Concentration (control; 0.1; 1 and 10 ppb) and gut section (midgut, ileum and rectum). Each color bar represents average occurrences of each family rank accompanied by the standard error. Each column represents n = 50 bees.

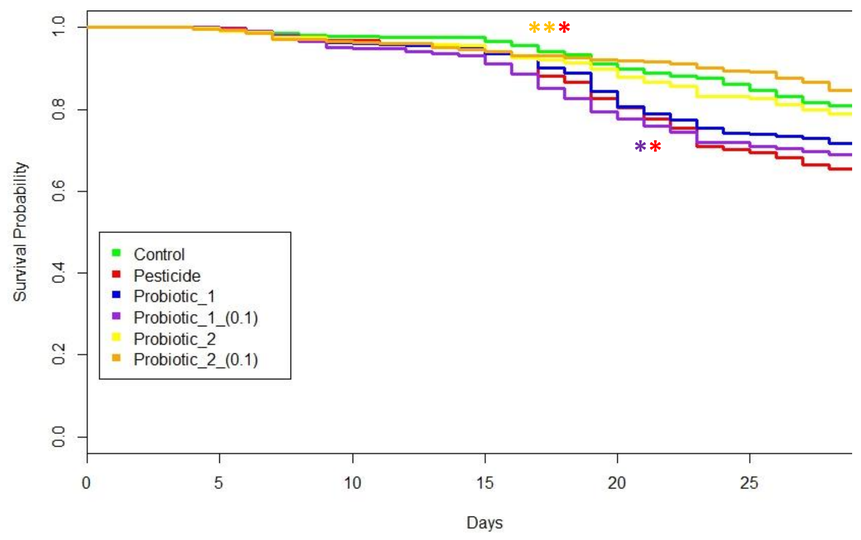


2. In *vitro*

- We isolated 69 probiotic candidates able to grow in contact with clothianidin (0.1; 1 and 10 ppb).
- Our pesticide quantification reported a complete degradation of clothianidin in contact with 6 probiotic candidates.
- Based on our results, we decided to test two probiotic candidates in the second *in vivo* experiment.

3. Second *In vivo experiment*

BEE SURVIVAL



Administration of the **candidate probiotic 2 (orange curve)** showed to improve the survival rate of bees exposed to clothianidin compared to control bees (i.e. supplemented with sugar (1:1)).

Figure 4. Kaplan-Meier Survival curves distribution of bees in each experimental group during 28 days. The green curve represents the survival rate of bees supplemented only with syrup (1:1); the red one represents the clothianidin control; the blue and the yellow curves represent respectively effect of the candidate probiotic 1 and probiotic 2 administration on bee survival; the violet and the orange curves represent respectively effect of the candidate probiotic 1 and probiotic 2 supplemented with clothianidin (0.1 ppb) on bee survival. Cox's proportional hazards regression was performed using the *coxph* model (***= P < 0.001).

CONCLUSION

Our work on clothianidin has revealed that this molecule:

- Induces **a total restructuration of the microbial community** depending: Concentration and the Gut Section of honey bees
- Induces an **Intestinal Dysbiosis** with a significant **Increase of species diversity** into the **midgut and the Ileum** at 0.1; 1 and 10 ppb clothianidin exposure
- Induces also an **Intestinal Dysbiosis** with an increase of species diversity into the **rectum** at the three concentrations of clothianidin exposure, but less significant that into the midgut and the ileum.
- **Induces modifications of correlations number** (edges) **between the *denovo* OTU involved into the microbial network**
- **Induces modifications of the bacterial functional activity**
- Induces **apparition of beneficial bacteria such as *Bacillus;* and apparition of pathogens into the microbial community.**

- **Do not inhibit the growth** of our 69 isolated **endogenous probiotic candidates**
- Is **completely degraded** by 6 endogenous probiotic candidates

More, our research showed that:

- We do not have to underestimate low concentration of chemical products introduce into our environment.
- **Use of endogenous probiotics in honey bee nutrition is therefore promising to diminish the negative impact of the neonicotinoid on honey bee colonies**

REFERENCES

[1] Hyrsl, P., Dobes, P., Vojtek, L., Hroncova, Z., Tyl, J., and Killer, J. 2017. Plant alkaloid sanguinarine and novel potential probiotic strains Lactobacillus apis, Lactobacillus melliventris and Gilliamella apicola promote resistance of honey bees to nematobacterial infection. Bull. Insectol. 70, 31–38; [2] Shannon, P., Markiel, A., Ozier, O., Baliga, N. S., Wang, J.T., Ramage, D., et al. 2003. Cytoscape: A Software Environment for Integrated Models of Biomolecular Interaction Networks. Genome Res.; 13 (11):2498±504. PMID: PMC403769. https://doi.org/10.1101/gr.1239303 PMID: 14597658; [3] Faust K and Raes J. 2012. Microbial interactions: from networks to models. Nat Rev Microbiol, 10:538–50.