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Understanding Neonicotinoid Related Immunosuppression: A Potential for Beneficial Bacterial Intervention

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Abstract

The use of neonicotinoid pesticides has been implicated in the recent decrease of honey bee (*Apis mellifera*) populations. In this thesis, a *Drosophila melanogaster* model was used to characterize immune impairment associated with imidacloprid (neonicotinoid) exposure and test the ability of beneficial bacteria (lactobacilli) to alleviate these harmful effects. The experiments outlined in chapters 2 and 3 demonstrate that imidacloprid alters the gut microbiota by exploiting the cooperation between gut immune pathways. The pesticide reduces expression of *Duox* via dysregulation of the immune deficiency pathway resulting in decreased hydrogen peroxide production. This contributes to the microbiota changes but also depletes antimicrobial peptide expression through reduced nitric oxide signalling. By supplementing *Drosophila* with certain strains of lactobacilli, this immune impairment was mitigated. In summary, these studies show how a widely used pesticide contributes to honey bee losses by dysregulating their immune system; however, these effects can be countered by lactobacilli intervention.

Keywords

Pesticide, *Drosophila*, microbiota, dual oxidase, immune deficiency pathway, reactive oxygen species, antimicrobial peptides, *Lactobacillus*, probiotics, honey bee.

Summary for Lay Audience

Honey bees are vital pollinators that help to maintain the global food supply. Despite their benefits to the global community, these insects are experiencing considerable population decline. While numerous causal factors have been identified, pesticides have been recognized for their unintentional toxicity to non-target insects. In particular, neonicotinoid pesticides are widely used despite warnings of collateral damage. The goal of this thesis is to understand the mechanisms whereby low doses of neonicotinoids harm honey bees. As it is difficult to do experiments on bees themselves, *Drosophila melanogaster* possess similar properties that make it a sufficient model organism. Capitalizing on the genetic tractability of these flies, we showed that neonicotinoids suppress the gut immune system, which then makes the honey bee susceptible to being killed by harmful bacteria. The dual oxidase (Duox) pathway is the first line of defence, which produces hydrogen peroxide to kill invading microorganisms. It was found that a commonly used neonicotinoid—imidacloprid—impaired this pathway by reducing the production of hydrogen peroxide. Imidacloprid induced this by interacting with the immune deficiency pathway, the second line of gut defence. This resulted in insufficient hydrogen peroxide produced to kill harmful bacteria. Additionally, the reduction in hydrogen peroxide causes a decrease in the generation of nitric oxide and subsequent nitric oxide signalling to distal organs, which results in diminished antimicrobial peptide production. It was found that by feeding the flies with specially chosen lactobacilli (beneficial bacteria), the damage caused by the pesticide to the immune system was less severe. This work forms the basis of testing supplementation with beneficial bacteria as a means to reduce the demise of honey bee populations. Development has led to the creation of a BioPatty that contains the lactobacilli plus essential nutrients for the bees. Therefore, by using basic science principles and an appropriate fruit fly model, we can generate a mechanistic rationale to test an intervention in a real-world setting. While cessation of pesticide use should be the ultimate goal, until then, the application of probiotic lactobacilli may contribute to saving the honey bees and our food supply.

Co-Authorship Statement

The experiments and data analyses within this thesis were primarily conceived, performed, and analyzed by John Chmiel with supervision and guidance from Gregor Reid. The manuscripts presented were written by John Chmiel with input from the co-authors.

Chapter 1: General introduction.

John Chmiel, Brendan Daisley, Graham Thompson, and Gregor Reid conceived the concepts of the manuscript. John Chmiel drafted the manuscript and collected the toxicology data. Brendan Daisley contributed to sections of the manuscript and provided scientific input. Andrew Pitek prepared the figures for the manuscript and provided practical honey beekeeping input. Graham Thompson provided scientific input. All authors read, revised, and approved the final manuscript.

Chapter 2: Deleterious effects of neonicotinoid pesticides on *Drosophila melanogaster* immune pathways.

John Chmiel, Brendan Daisley, and Gregor Reid conceived the experiments. John Chmiel performed experiments, collected data, and analyzed data for all experiments with minor exceptions. Brendan Daisley assisted with some experiments and manuscript preparation. Jeremy Burton provided scientific input and access to the high-performance liquid chromatography (HPLC) instrument. All authors read, revised, and approved the final manuscript.

Chapter 3: **Imidacloprid impairs nitric oxide-mediated antimicrobial peptide production in *Drosophila melanogaster*.**

John Chmiel and Gregor Reid conceived the experiments. John Chmiel performed experiments, collected data, and analyzed data for all experiments with minor exceptions. Brendan Daisley provided scientific input and assisted with some experiments. Paul Akouris and Anna-Lena Spierling assisted with *Drosophila* maintenance. Mark Stasiewicz, Bethany Rogers, and Brendan Daisley assisted with *Drosophila* microorganism collection and identification.

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List of Abbreviations

AMP	Antimicrobial peptide
ATP	Adenosine triphosphate
BHI	Brain heart infusion (media)
bp	Base pair
Cad99C	Cadherin 99C (gene)
CFU	Colony-forming units
CO_2	Carbon dioxide
CYP	Cytochrome P450
ddH_2O	Double-distilled water
Def	Defensin (gene)
DMSO	Dimethyl sulfoxide
Dpt	Diptericin (gene)
Drs	Drosomycin (gene)
Duox	Dual oxidase (gene)
DUOX	Dual oxidase (protein)
Duox	Dual oxidase pathway
Ecc15	*Erwinia carotovora* subspecies *carotovora* 15
EFSA	European Food Safety Authority
EPA	Environmental Protection Agency
GAL4	Galactose-responsive transcription factor
GF	Germ-free
GOX	Glucose oxidase
GPCR	G protein-coupled receptor
H_2O_2	Hydrogen peroxide
HOCl	Hypochlorous acid
HPLC	High-performance liquid chromatography
IMD	Immune deficiency pathway
IMI	Imidacloprid
IP_3	Inositol triphosphate
IRC	Immune-regulated catalase (gene)
ITS	Internal transcribed spacer
JAK/STAT	Janus kinase/signal transducers and activators of transcription

JNK	c-Jun N-terminal kinase
LAB	Lactic acid bacteria
LB	Lysogeny broth
LD_{50}	Lethal dose 50
LGR-1	*Lactobacillus rhamnosus* GR-1
LX3	Probiotic combination containing (*L. plantarum* Lp39, *L. rhamnosus* GR-1, and *L. kunkeei* BR-1)
mAChR	Muscarinic acetylcholine receptor
MAN	Mannitol (media)
MAPK	Mitogen-activated protein kinase
MKP	Mitogen-activated protein kinase phosphatase
MRS	De Man, Rogosa, and Sharpe (media)
nAChR	Nicotinic acetylcholine receptor
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated b cells
NO	Nitric oxide
NO_2^-	Nitrite
NOS	Nitric oxide synthase (gene)
NOX	NADPH oxidase (protein)
OPH	Organophosphate-hydrolyzing protein
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
Pe	*Pseudomonas entomophila* DSM 28517
PER	Proboscis extension reflex
PGRP	Peptidoglycan recognition protein
PIP_2	Phosphatidylinositol biphosphate
PLC-β	Phospholipase C-β
PO	Phenoloxidase
PPO	Prophenoloxidase
qPCR	Quantitative polymerase chain reaction
ROS	Reactive oxygen species
UAS	Upstream activator sequence
WT	Wild-type
YPD	Yeast peptone dextrose (media)

Chapter 1

1 General Introduction

The material in this chapter has been reproduced/adapted from a review article published in Frontiers in Ecology and Evaluation and has a content license that can be found in Appendix A.

Chmiel JA, Daisley BA, Pitek AP, Thompson GJ, Reid G. Understanding the effects of sublethal pesticide exposure on honey bees: a role for probiotics as mediators of environmental stress. *Front Ecol Evol*. 2020;8(22):1-19. doi:10.3389/fevo.2020.00022

1.1 Neonicotinoids in modern agriculture

1.1.1 What are neonicotinoids

Neonicotinoids are a pesticide class that is used in modern agricultural practices to reduce herbivorous insect burden and improve crop yield. Compared to traditional pesticides (for example, organophosphates, carbamates, and pyrethroids), neonicotinoids demonstrate superior qualities, including improved water solubility (1), lower toxicity to mammals (2), ample specificity to insects (3), and reduce pesticide quantity needed to obtain an effective response (4). Neonicotinoids are systemic pesticides, which means that they enter plant circulation and are transported throughout rather than remaining on the surface.

In 2014, neonicotinoids were valued at $3 billion (USD) and composed more than 25% of the global pesticide market (5). The most commonly used neonicotinoids are imidacloprid, clothianidin, and thiamethoxam (6), with imidacloprid highest of all in fruits and vegetables, from both domestic and imported sources, in the United States (5). In the Canadian Prairies, clothianidin and thiamethoxam are the most commonly found neonicotinoids in crop pollen (7).

Neonicotinoid pesticides can be conveniently applied to crops through foliar spray, soil drenching, granules, or seed dressing. However, because they are systemic pesticides,

seed dressing is often preferred because it reduces drift and off-target spreading of the chemical. Seed applications are commonly used for maize (corn), soybeans, and oilseed rape (canola) (8).

1.1.2 Neonicotinoids mode of action

Neonicotinoids are a class of insecticide that interacts with postsynaptic nicotinic acetylcholine receptors (nAChRs) (9). A majority of these chemicals resemble nicotine, with a few resembling acetylcholine. They bind to insect nAChRs as agonists (10), which then induces a depolarization response and subsequent neural activation. This causes convulsions and loss of coordination, ultimately leading to the death of the insect. Despite the presence of nAChRs in vertebrates, neonicotinoids are less toxic to these organisms because the composition of their nAChR subunits differs from that of invertebrate nAChRs (2).

1.1.3 Generations of neonicotinoid pesticides

There are currently 13 commonly used neonicotinoids, which span across four generations of development (**Figure 1-1**). This was started in the 1970s by the Shell Chemical Company, with the discovery of nithiazine, a heterocyclic nitromethylene-based chemical that showed high toxicity toward insects (11). This was ground-breaking because nithiazine did not function like traditional pesticides, which typically act as acetylcholinesterase inhibitors. Instead, it functioned through a novel mechanism that acted as a postsynaptic acetylcholine receptor agonist (12). However, the low photostability of nithiazine limited its in-field effectiveness and prompted the development of neonicotinoid pesticides (13).

In the 1980s, Nihon Bayer Agrochem synthesized imidacloprid. This featured improvements on the photosensitivity and insect toxicity of nithiazine (14) and led to the chemical being the archetype of first-generation neonicotinoids, characterized by pyridine-like rinks. Advancements using the structure of imidalcoprid led to the discovery of several other first-generation neonicotinoids (thiacloprid, nitenpyram, and acetamiprid) whose chemical structures are shown in **Figure 1-1**.

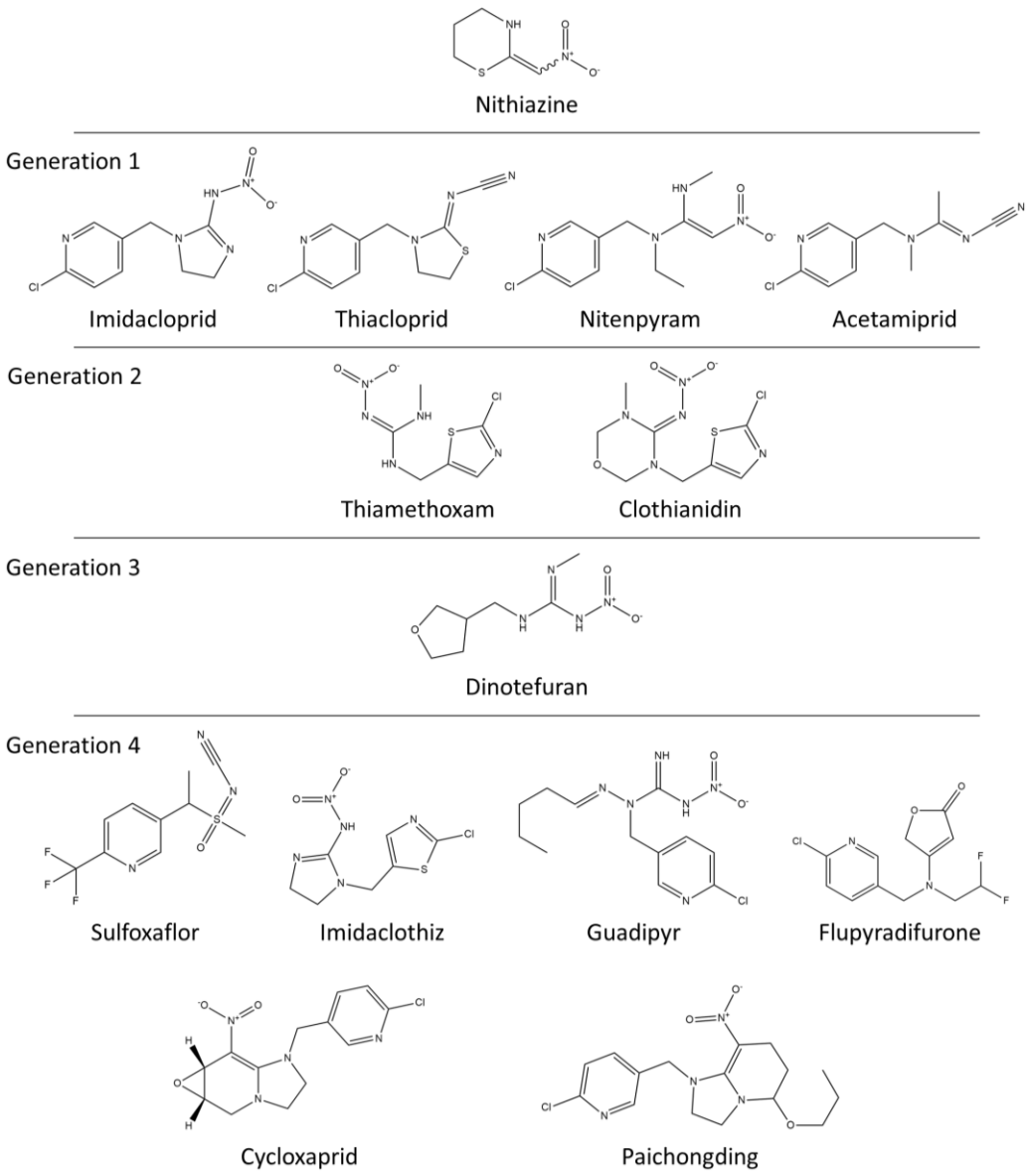

Figure 1-1. Chemical structures of neonicotinoids.

Production of first-generation neonicotinoids swiftly led to the discovery and development of second-generation neonicotinoids, thiamethoxam (15) and clothianidin (16). These chemicals have a distinctive thianicotinyl group. Thiamethoxam was selected for further development because of its ease of synthesis and improved insecticidal activity compared to first-generation neonicotinoids, acetamiprid and nitenpyram (17). Through further research, it was found that thiamethoxam was metabolized to clothianidin in both insects and plants (18), which may explain why these chemicals are usually found together in environmental samples (19) and how the presence of both increases lethality (20).

Dinotefuran is the sole member of the third-generation of neonicotinoids on the market, and it is characterized by furanicotinyl (based off of the (±)-tetrahydro-3-furylmethyl moiety) (21). The structure of dinotefuran differs from other neonicotinoids and more closely resembles acetylcholine rather than nicotine (21, 22). Although dinotefuran interacts with nAChRs, it appears that the mode of action differs slightly from other neonicotinoids (22). Similar to second-generation neonicotinoids, dinotefuran shows improved water solubility over imidacloprid (23).

Continued research has led to the fourth generation of neonicotinoids, which are less defined by chemical structure and more so by the chronological development of these chemicals (24). However, this begs the question of why is a classification system needed if the new neonicotinoids improve on established chemical moieties (24)? This new category is extremely broad, with approximately 600 synthesized compounds (25). Now, the most commonly used fourth-generation neonicotinoids are guadipyr, sulfoxaflor, flupyradifurone, imidaclothiz, cycloxaprid, and paichongding. The fourth-generation compounds have been further divided into a subclass of *cis*-neonicotinoids, which include cycloxaprid and paichongding (25). The *cis*-configuration of neonicotinoids shows improved insecticidal activity against neonicotinoid-resistant insect pests (26).

1.1.4 Regulation of neonicotinoids throughout the world

Throughout the world, neonicotinoids are highly controversial pesticides because of their association with honey bee population decline. As such, there have been multiple

movements to restrict their use. A net result is that some countries have introduced strong stipulations to control their application. In particular, the European Union has been a strong proponent of restricting neonicotinoid use, starting in January 2013 when the European Food Safety Authority (EFSA) published multiple articles outlining the unacceptably high risks that thiamethoxam (27), clothianidin (28), and imidacloprid (29) pose to bees. From these studies, the European Commission in 2018 restricted the use of thiamethoxam (30), clothianidin (31), and imidacloprid (32) to seed treatment of plants that must remain in greenhouses and banned all use of the pesticides on field crops. Since their restrictions, the approvals of thiamethoxam (33) and clothianidin (34) have not been renewed past their expiration date of 2019; thus, they are effectively banned in Europe. Approval of imidacloprid has been renewed until July 2022 (35); however, its use is still restricted to greenhouses.

In Canada, restrictions in neonicotinoid use have begun due to increased pollinator death (36). In response to this report, Health Canada began collaborations in 2012 with the United States Environmental Protection Agency (EPA) and California Department of Pesticide Regulation to re-evaluate the status of thiamethoxam, clothianidin, and imidacloprid based on the safety of pollinators (37). In 2013, Health Canada issued a notice of intent, which suggested that neonicotinoids were affecting pollinator and bee health (38). This notice outlined some additional protective measured for neonicotinoid use and opened the discussion on other pesticide management options. In 2019, Health Canada issued a news release concluding that imidacloprid, clothianidin, and thiamethoxam were posing an unacceptable risk to bees and other pollinators (39). As a result, guidelines were updated to outline modifications to seed treatment protocols and reduced spraying in crops that bees were attracted to before and during the bloom of specific crops by April 2021 (37). Currently, the use of thiamethoxam, clothianidin, and imidacloprid is still permitted in Canada but heavily regulated.

While Pesticide use in Canada is typically governed at the federal level through the Pest Control Products Act (40), provinces are able to further regulate these substances within their own borders. Ontario was one of the first to do so. On July 1, 2015, Ontario defined a new class of pesticides (Class 12), which included pesticides that are used to treat corn

seed or soybean seeds and contain imidacloprid, clothianidin, or thiamethoxam (41). They also stated plans to reduce the use of Class 12 pesticides in the Ontario agriculture industry (42). In 2015, Quebec began outlining restrictions limiting the use of neonicotinoids province-wide (43). The City of Vancouver acknowledged the need to limit neonicotinoid use and passed a by-law in 2015 that restricted the use of neonicotinoids within city boundaries (44).

Regulations on neonicotinoids were more controversial in the United States. In March 2012, a group of beekeepers and environmental- and consumer-based organizations, represented under the Center for Food Safety, sent an Emergency Petition to the EPA declaring that clothianidin was an imminent hazard to insect pollinators and bee health (45). The EPA responded in July 2012, refuting the claim that clothianidin was an 'imminent hazard' to bees; however, they acknowledged their own efforts in evaluating the risk of neonicotinoids to pollinators (46). The Center for Food Safety challenged the EPA in court on March 21, 2013, stating that the EPA violated the United States Federal Insecticide, Fungicide and Rodenticide Act when it approved the use of pesticides containing clothianidin or thiamethoxam (47). In May of 2017, the court found that the EPA had indeed violated the United States Endangered Species Act when it approved various pesticides that contained clothianidin and thiamethoxam, which have been known to cause harm to bees (43).

The disagreement between the EPA and the Center for Food Safety has led to new regulations for neonicotinoid use in the United States. Currently, the EPA proposes to reduce the application rate and restrict the use of thiamethoxam (48), clothianidin (48), imidacloprid (49), and dinotefuran (50) to specific crop stages. However, these proposed regulations do not apply to acetamiprid (51). Ultimately, the EPA has acknowledged the threat that neonicotinoid exposure presents to pollinators and bees, and aims to limit their exposure to these chemicals. Despite the documented harmful effects of neonicotinoids on honey bees and beneficial insects, the current Federal administration-headed EPA has approved new uses for the neonicotinoid, sulfoxaflor (52).

1.2 Impact of neonicotinoids on honey bees

1.2.1 Honey bee population decline

Popular interest in the biology of the common European honey bee (*Apis mellifera*) has surged in recent years due to the stark population decline of this important pollinator (53). Managed colonies of *Apis mellifera*, strictly speaking, are an invasive insect species to the Americas (54), but contribute hugely to its food supply and overall to the production of roughly a third (~35%) of the global food supply (55). In Canada, this single insect species is tied to a ~$2.5 billion (CAD) industry of pollination services, whereby colonies are strategically situated in orchards and fields to promote farmer yields via the cross-fertilization of flowering crops (56). In the United States, the value of bee-mediated pollination is even larger (57). Despite the value of honey bees to the agri-food industry, we have yet to fully understand how their populations cope with natural- and agriculture-induced stress, or to what extent this stress explains recent increases to reported mortalities (53).

Although no single factor can provide a universal explanation for the apparent decline of honey bee populations, one overriding theme to emerge from the global research effort is that more than one factor combines to overwhelm bee health. Among them, pesticide exposure (58, 59), pathogens (60), and habitat loss (61, 62) are prime factors that disproportionately contribute to the decline. Sublethal pesticide exposure has been a popular focus of political discussion, which has highlighted the potential conflict between parties that rely on the production and use of commercial pesticides and those who advocate for their regulation and alternative means of crop pest control. Moreover, the risk of pesticides to honey bees is especially alarming due to their long chemical half-lives (19) and presence in food (63) and honey (64).

1.2.2 Mode of pesticide exposure for honey bees

Herbivorous pest insects are the intended target of systemic application of agriculture insecticides. Nonetheless, honey bees are insects just the same and thus cannot help but to be vulnerable through incidental exposure. The application of pesticides to crops occurs in two main ways: spraying and seed coating, both of which have effects on honey bee

exposure. Spraying is typically accomplished through aerial application, but vehicle-based sprayers or manual spray units are also used. These are effective for pest control but can inadvertently affect honey bees through direct topical contact or secondary exposure via bee consumption of contaminated pollen, nectar, or water (65–68). Furthermore, spray-based application allows pesticides to disseminate into the broader environment and contaminate surrounding habitats, including orchards and fields that are not sprayed (69, 70). The concept of seed coatings was used to avoid affecting off-site targets by more carefully controlling pesticide delivery to the full crop as it emerges from germination. However, pesticides are active in plant tissue, including nectar and pollen (71–74), therefore exposing honey bees.

Honey bees can deliberately be exposed to miticides and fungicides by beekeepers through basic hive management practices that aim to combat pests and pathogens within the hive. Although beekeepers have the best intentions, this practice can harm the bees.

In total, managed honey bee colonies can be exposed to a diverse set of pesticides, which can only be determined by detailed toxicological sampling (75). These chemicals affect bees through any combination of ingestion, contact exposure, or ambient intake through respiratory openings (spiracles). Contact exposure and ingestion as routes of contamination are well studied and reveal pesticide-specific effects on honey bee health (59, 69, 76, 77). Honey bee respiration, which occurs in respiratory spiracles that are found along the thorax and abdomen of adults, is thought only to be a minor route of pesticide uptake (78). Ultimately, these modes of exposure are responsible for the accumulation within individual bees, which can lead to bioaccumulation of pesticides throughout the hive (**Figure 1-2**).

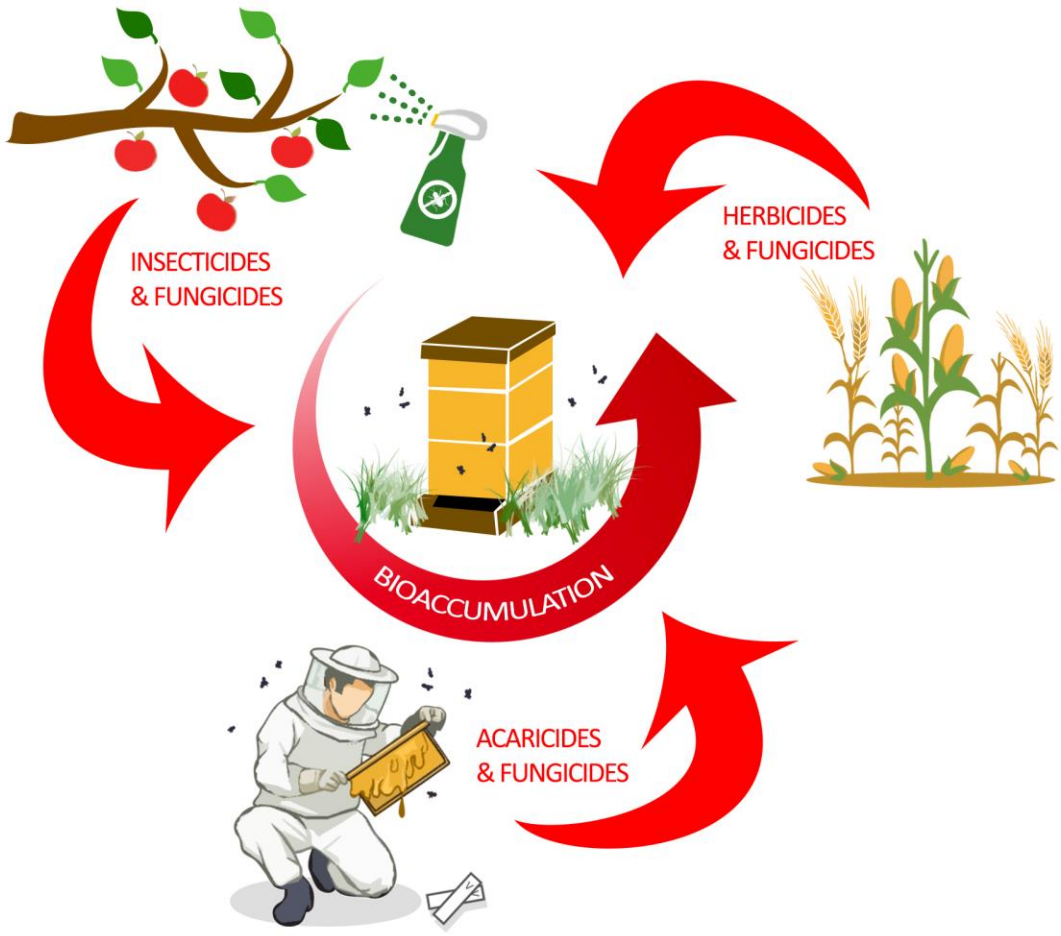

Figure 1-2. Bioaccumulation of pesticides in a honey bee colony.

In summary, a wide variety of pesticides affect honey bees through agricultural practices and modern beekeeping. Typically, farming and other agricultural practices are responsible for exposing honey bees to insecticides, herbicides, and fungicides. As honey bees forage for nectar and pollen, they are incidentally exposed to pesticides and facilitate pesticide accumulation in the hive by physically transferring these contaminated food sources to unexposed bees. However, honey bees can also be intentionally exposed to acaricides and fungicides by beekeepers in efforts to control mite burden and fungal diseases in the hive. Ultimately, pesticide bioaccumulation in the hive has the potential to negatively impact all honey bee ranks.

1.2.3 Dose-dependent toxicity of neonicotinoids to honey bees

The risk to honey bees as a result of pesticide exposure is evaluated by considering both the incidence of exposure and toxicity of pesticides used. Incidence is quantified by examining the usage rates of pesticides, mode of application, and environmentally relevant concentrations of pesticides in a crop-space. A widely used metric for quantifying pesticide-specific toxicity of adult honey bees is the lethal dose (LD) at which half the population dies, or the LD_{50}. This latter metric uses acute exposure (24 – 96 hours) of adult honey bees to predict a toxic dose. Estimates of LD_{50} can vary by length of exposure and mode of delivery, so knowing the oral- and dermal-specific LD_{50} of individual pesticides can make a useful predictor of pesticide-associated risk. Further, by comparing LD_{50} obtained for pest and beneficial insect species, we can better assess the trade-off between intended target species and any collateral damage to pollinators. When combined with pesticide application rates, toxicity values are useful for calculating the risk of pesticide use against the damage caused to pollinators. The Hazard Quotient (HQ = application rate/LD_{50}) is a viable metric to calculate field use risk of pesticide application but can be erroneous alongside variable LD_{50} values (76).

Despite the potential of comparative analysis, the variation that is associated with published estimates of LD_{50} for neonicotinoids is substantial for both contact (**Table 1-1**) and oral (**Table 1-2**) versions of this metric. This variation can reduce their value in risk assessment. The seemingly high variation in LD_{50} estimates, which can range up to 100-fold, may stem in part from differences in sample size, precision of measurement, and experimental protocol. Even for toxicological studies with a high degree of statistical power, the variance associated with LD_{50} can be large (79). This suggests that the genuine effect of pesticides on insect survivorship may vary intensely between populations, regardless of how it is measured. Biological sources of variation can stem from differences in age (young, nurse-age workers versus older, foraging-age workers), genotype (natural variation as well as apicultural strains), caste (workers, queens, drones), or life stage (larvae versus adults) (80, 81).

Table 1-1. Range and median of contact LD_{50} values of pesticides for adult honey bees.

Pesticide	Contact LD50 (µg/bee)	Range	Number of reports
Acetamiprid	17.045	1.69 – 276.85	6
Clothianidin	0.03	0.021418 – 0.04426	5
Cycloxaprid	ND		
Dinotefuran	0.0378	0.0006 – 0.075	2
Flupyradifurone	69.25	15.7 – 122.8	2
Guadipyr	51.82	N/A	1
Imidacloprid	0.04645	0.0128 – 0.19	18
Imidaclothiz	ND		
Paichongding	ND		
Nitenpyram	0.138	N/A	1
Thiacloprid	38.82	14.6 – 122.4	3
Thiamethoxam	0.04	0.024 – 0.124	5
Sulfoxaflor	0.255	0.130 – 0.379	2

N/A, not applicable; ND, no data

Table 1-2. Range and median of oral LD_{50} values of pesticides for adult honey bees.

Pesticide	Oral LD50 (µg/bee)	Range	Number of reports
Acetamiprid	11.815	0.0215 – 72.9	4
Clothianidin	0.00344	0.002608 – 0.0269	14
Cycloxaprid	ND		
Dinotefuran	ND		
Flupyradifurone	2.951	1.2 – 6.823	6
Guadipyr	ND		
Imidacloprid	0.049	0.0048 – 0.536	20
Imidaclothiz	ND		
Paichongding	ND		
Nitenpyram	ND		
Thiacloprid	19.955	17.32 – 22.59	2
Thiamethoxam	0.004358	0.00416 – 0.0112	10
Sulfoxaflor	0.146	N/A	2

N/A, not applicable; ND, no data

Additional sources of variation can occur due to the composition of the pesticide formulations that are used. While different amounts of solvents used for toxicology analysis can affect pesticide toxicology (82), pesticide adjuvants (other ingredients found in pesticide formulations that are thought to be inert) can also influence pesticide toxicity (83). An emerging interest is the potential for synergistic toxicity between multiple pesticides that are applied in combination. These can increase overall honey bee mortality in unpredictable ways (58, 84, 85), yet they are often overlooked in LD_{50} studies, which typically determine the toxicity of individual pesticides in standard laboratory solvents.

1.2.4 Neonicotinoids affect metabolism in honey bees

Like most insects, honey bees use an array of enzymes to detoxify pollutants and other harmful chemicals that they encounter, including pesticides (86). Unfortunately, honey bees are genetically depauperate in a number of key detoxification genes, with the remainder of relevant genes expressed at low levels (87). Some key detoxifying genes that appear underrepresented in the honey bee genome compared to the well-studied insect model, *Drosophila melanogaster* include many of the cytochrome P450 monooxygenases (Phase I detoxification—oxidation, reduction, and hydrolysis of xenobiotics), glutathione-S-transferases (Phase II detoxification—increase water solubility of xenobiotics for excretion), and carboxyl/cholinesterases (insecticide resistance) (87). Although honey bees possess similar amounts of detoxification genes compared to other members of the Apidae family, they have far fewer than pest insects, thus making them more susceptible to pesticides (88). The diminished repertoire of detoxifying genes in the honey bee might stem from compensatory mechanisms associated with their highly social behaviour, including herd immunity (89, 90) and a ‘social detoxification system,’ which focuses on how hive behavioural dynamics can reduce the burden of toxin substances on the detoxification system of individual members (91). It is uncertain if the relatively small innate capacity of the honey bee is fully compensated by social effects or if the bees remain genetically more sensitive to the toxic effects of pesticides.

Honey bees can clear imidacloprid, with studies reporting results from partial to complete clearance (92–94). Using ^{14}C-imidacloprid, honey bees were found to rely on Phase I

detoxification genes to metabolize the pesticide (94). The resultant major metabolites are olefin, 5-hydroxy-imidacloprid, 4,5-dihydroxy-imidacloprid, 6-chloronicotinic acid, and a urea derivative (94, 95). These metabolites have similar to less toxicity compared to imidacloprid (96, 97).

Honey bees exposed to neonicotinoids display altered metabolic profiles. While exposure to imidacloprid broadly up-regulates cytochrome P450 gene expression (98–100), presumably in response to the xenobiotic, it also disrupts ATP production (101). Nicodemo et al. (101) demonstrated that imidacloprid reduces oxygen consumption and impairs mitochondrial function. This reduction in aerobic respiration is accompanied by an increase in glycolysis and citric acid cycle-related gene expression in exposed honey bees (102, 103). Thus, pesticide exposure may be favouring low efficiency means of ATP production (glycolysis and citric acid cycle) over higher efficiency oxidative phosphorylation. Interestingly, the use of near-infrared light (670 nm) to restore mitochondria function can mitigate ATP reduction, diminish physiological impairments, and improve survival in bumblebees (*Bombus terrestris*) (104).

1.2.5 Neonicotinoids negatively affect motor function, behaviour, and cognition

Honey bees are highly social insects. They rely on individual cognition to navigate their environment and respond to changing conditions and colony needs. Forager bee cognition is demonstrated by their ability to encode memories of resources, which are typically found within a 2 – 6 km radius of the hive (105, 106). These memories are then transmitted through waggle dances to other foragers to encourage the process of collecting hive resources, which promotes the success of a colony (107). Exposure to pesticides appears to impair the foraging response in a dose-dependent relationship.

Acute neonicotinoid exposure induces a series of symptoms that are consistent with hyper-responsive neural impairments (96). These are observed as excitation symptoms, which include increased time in the air, increased flight distances, and an inability to right themselves when placed on their backs (108–110). By contrast, chronic exposure induces hypo-responsive neurological impairments (96), including decreased flight speed and

duration, and impaired navigation (110–112). Thus, initial exposure to neonicotinoids can overstimulate honey bees and induce a hyper-responsiveness, leading to exhaustion or hypo-responsiveness. One implication of this would be that neonicotinoid exposure drives foragers to go far distances, where they eventually become exhausted and lose their spatial awareness and cannot return to the hive. This reduces hive resources. As a result, nurse bees may begin foraging at a younger age, thus creating a group of precocious foragers, which then reduces the number of nurse bees available for rearing brood (113).

Honey bees likely cannot tell if food is contaminated with pesticides (109, 114); thus, they are not averse to it. Fortunately, pesticide exposure reduces the trophallactic transfer of food from donor to recipient (115, 116). Although this may reduce the spread of pesticide-contaminated food within a colony, the change in social behaviour may also compromise other forms of communication, including the waggle dance (which allows successful foragers to inform others in the colony on the direction and distance to food and water or new nesting sites) (117), or reduce larval feeding altogether (118).

The most pronounced pesticide-induced cognitive impairments are on olfactory learning, visual learning, and memory. Olfactory learning occurs when honey bees learn to associate an odour with an award, which is often tested using the proboscis extension reflex (PER). Honey bees exposed to imidacloprid show reduced PER activity compared to unexposed bees (119–121). Pesticides affect visual and associative learning in honey bees (122). For example, Han et al. (120) found that using their T-tube maze, less than half of bees treated with imidacloprid were able to successfully make the correct decision in a visual learning task. As visual learning is used to remember food locations and predators, this may explain why Eastern honey bees (*Apis cerana*) exposed to sublethal imidacloprid do not show aversion to the predator hornet, *Vespa velutina* (123). Imidacloprid may reduce the visual association and cognitive fear response when coming upon a predator. It seems likely that pesticides can have direct effects on the brain.

On a cellular level, neonicotinoids interfere with neuronal polarization in mushroom bodies, a segment of the honey bee brain that is associated with learning, memory, and

sensory integration (124). Mushroom bodies are composed of Kenyon cells (neural cells). When these cells are exposed *in vitro* to imidacloprid, they show a modified synaptic profile, which is characterized by a slow depolarization, followed by increased excitability, then inhibition of the action potential (125). Imidacloprid is a partial agonist of nicotinic acetylcholine receptors; thus, it could be acting on these receptors and blocking a natural acetylcholine response, thereby altering the neural cell action potential. This may explain some of the impairment to the aforementioned cognitive processes. In addition, there appear to be differences in the brain proteome and microRNA (miRNA) expression of bees exposed to pesticides (126, 127), which could lead to changes in brain development and structure that result in differential signalling.

An alternate process to explain neural impairment following pesticide exposure is that pesticides may interfere with the perception of a stimulus rather than the cognition of one. Imidacloprid exposure has been shown to reduce calcium signalling in the antennal lobe in response to an odours stimulus (128). This results in problems perceiving the stimulus as opposed to difficulty coding and recalling the stimulus (cognition). Ultimately, pesticide-induced cognitive-related deficits may be a result of a combination of impairments to the honey bee brain.

1.2.6 Neonicotinoids obstruct reproduction and development of honey bees

Exposure to pesticides can slow the reproductive cycle of queens (**Figure 1-3**). This is illustrated by exposure to sublethal doses of thiamethoxam during development, resulting in reduced body weight and a lower probability of queen success (129). Likewise, laboratory experiments show that queens exposed to field-realistic concentrations of neonicotinoids carry fewer viable spermatozoa and lay fewer fertilized eggs that would normally develop into diploid (female) workers (130–132). Queens that underperform are eventually targeted by workers for replacement (133), but in the short-term reproductive succession is costly to the colony. Furthermore, queens exposed to sublethal doses of neonicotinoids have reduced mating compared with unexposed queens (134).

Drones are male bees whose sole purpose is to mate with virgin queen bees. They are also affected by pesticides. Sublethal concentrations of neonicotinoids and

phenylpyrazoles can reduce sperm viability (135–138), which can hamper the fertilization of queens and the production of diploid workers. Together, reduced sperm transfer and fertilization may limit the production of a genetically diverse workforce, which may compromise the division of labour (139) and response to disease (140).

While pesticides are known to interfere with reproduction, they have also been implicated in changes to larval development. Honey bee larvae reared *in vitro* with thiamethoxam (1/10 of LC_{50}) show atypical progression through developmental stages, including skipping some stages and reduced larval weight (141). This is corroborated by field data showing similar atypical developmental progression upon pesticide exposure (142). At the molecular level, honey bees exposed to imidacloprid show changes in miRNA transcription, which are responsible for development (98). In particular, a reduction in the miRNA, *mir-14*, has been observed (98); although its exact function in honey bees is unknown, in *D. melanogaster* it has been shown to modulate metabolism, nutritional status, and larval survival (143, 144). Thus, pesticide exposure impairs individual development, contributing to reduced colony strength.

Honey bee larval development is guided by hormone signalling and jelly supplementation. Exposure to neonicotinoids reduces the expression of vitellogenin, an essential protein that is required for honey bee development (146, 147). As brood develop, they primarily consume jelly, which is a nutritionally rich food source produced and delivered by nurse bees. Sublethal neonicotinoids reduce the size of the hypopharyngeal and mandibular glands where it is synthesized (148, 149), which in turn decreases jelly secretions and may lead to reduced longevity and smaller honey bee populations (150). The jelly produced may further be deficient in major royal jelly proteins (126) that are vital for honey bee development and physiology (151). These changes in hormone signalling and reduced nutritional value of jelly can contribute to the atypical development of honey bee larvae exposed to pesticides. By limiting the amount of viable brood and the rate at which these few larvae develop, pesticide exposure effectively reduces the overall workforce and success of the colony.

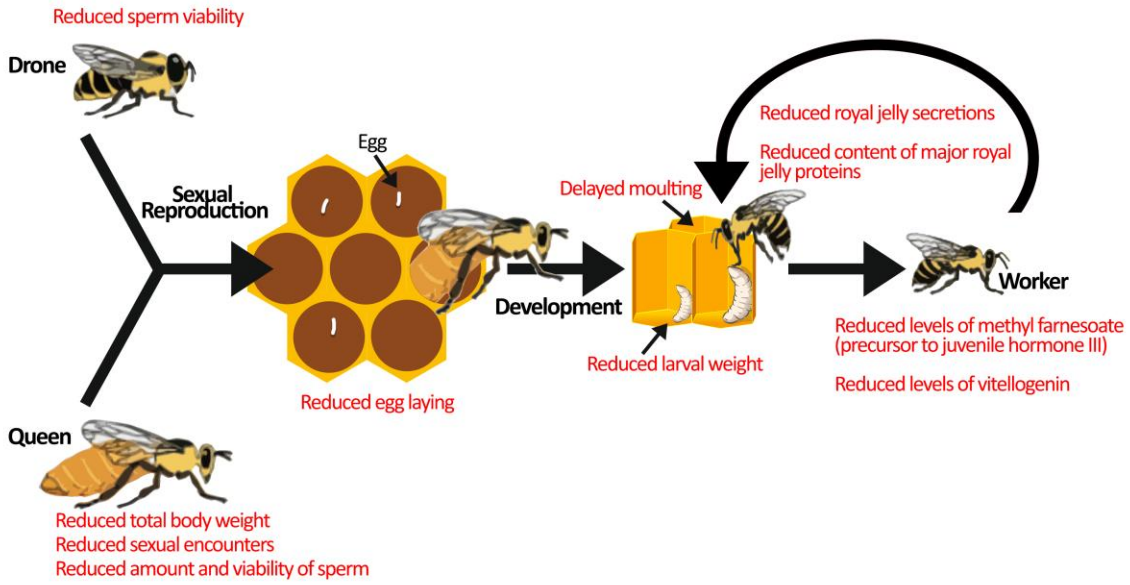

Figure 1-3. Pesticides interfere with colony reproduction.

Drones and queen sexual reproduction is the source of genetic diversity in the hive. This is important for pathogen resistance and colony survival. Sublethal pesticide exposure reduces sexual reproduction by affecting the drones and the queen. Drones exposed to pesticides have lower sperm viability, while queens display reduced sexual encounters, sperm amount, and sperm viability. Moreover, pesticide exposed queens have smaller body weights, which may explain the reduction in sperm amount and egg-laying. Developing larvae exposed to pesticides demonstrate atypical progression through developmental phases, reduced larval weight, and delayed moulting. These may be a result of direct pesticide exposure, but pesticides could also be indirectly affecting larvae. Nurse bees exposed to pesticides produce a reduced amount of royal jelly secretions, with lower nutritional value, potentially explaining the indirect effects of pesticides on honey bee larvae. Image of larvae in the hive is adapted from Maori et al. (145) under Creative Commons Attribution 4.0 International (https://www-sciencedirect-com/science/article/pii/S10972765 19301844).

1.2.7 Neonicotinoids disrupt honey bee immunity

Honey bees exposed to pesticides have increased loads of bacterial, fungal, and viral pathogens (73, 130, 152–157). This has raised concern over the potential of synergistic interactions between pesticides and pathogens that exacerbate mortality in honey bees (158–162). Vidau et al. (163) demonstrated that honey bees previously infected with *Nosema ceranae* were more sensitive to subsequent pesticide exposure. Fungal parasites like *Nosema* might therefore increase pesticide-related mortality by altering the expression of detoxification enzymes. As the adult honey bee gut microbiota develops 4 – 6 days after eclosion and is composed of bacteria from older bees and the hive environment (164), colonization by disease-causing microorganisms could alter resistance to pesticides (165, 166). Conversely, pesticides may cause immunosuppression in honey bees, rendering them more susceptible to pathogens. To better understand the possible synergism between pesticides and pathogens, it is essential to consider individual immunity and social immunity.

Individual honey bee immunity is divided into humoral and cellular immune responses, both of which are impaired by sublethal neonicotinoid exposure (**Figure 1-4**). The humoral response is initiated by recognition of pathogen-associated molecular patterns (PAMPs), which triggers signalling through one of the four insect immune pathways: 1) the Toll pathway, 2) the Immune Deficiency (IMD) pathway, 3) the c-Jun N-terminal kinase (JNK) pathway, and 4) the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway (167). Activation of these pathways leads to the production of antimicrobial peptides (AMPs), namely proteases, complement-like proteins, or broad-range microbiocidal proteins. In insects, these signalling pathways and proteins are conserved. However, honey bees harbour fewer paralogues, gene copies, and splice variants of immune genes compared to *Drosophila* and *Anopheles* (89).

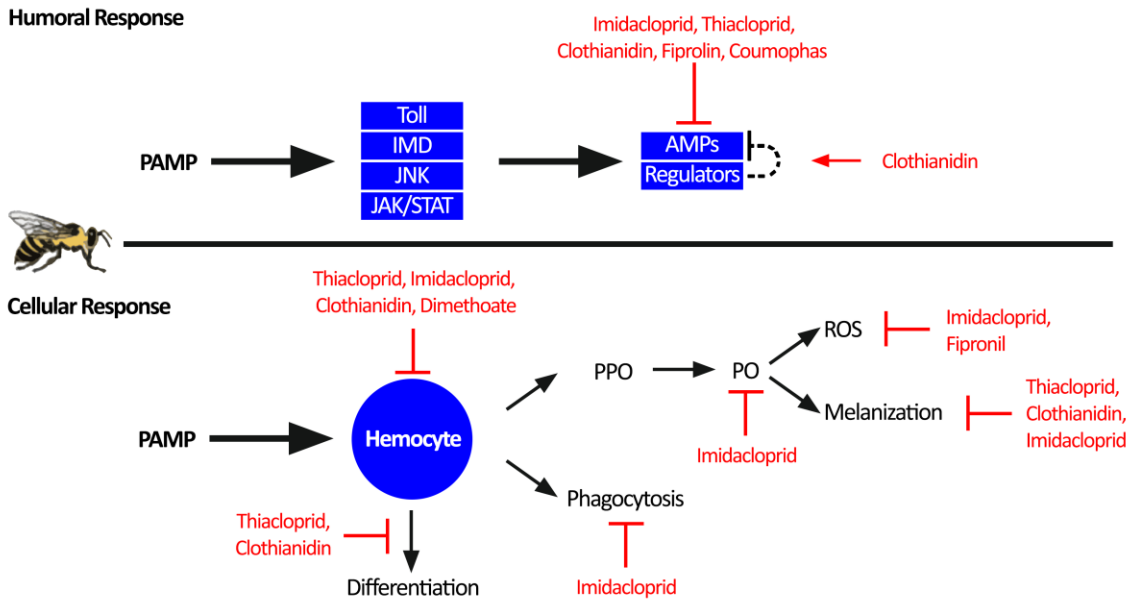

Figure 1-4. Individual honey bee immunity impairment by pesticides.

Honey bee immune response toward pathogen-associated molecular patterns (PAMPs) can be divided into humoral response and cellular response. The former generates antimicrobial peptides (AMPs) through activation of the four immune pathways: Toll, immune deficiency pathway (IMD), c-Jun N-terminal kinase (JNK), and Janus kinase/signal transducers and activators of transcription (JAK/STAT). Sublethal pesticide exposure impairs the humoral immune response by reducing the production of AMPs. The cellular immune response is orchestrated through hemocyte function. Hemocytes can facilitate melanization of pathogens and wounds through activation of prophenoloxidase (PPO) to phenoloxidase (PO) and reactive oxygen species (ROS) as a by-product. In addition, hemocytes can phagocytosis and clear invading pathogens, as well as differentiation into other immune cells. Multiple aspects of the cellular immune response are impaired by sublethal pesticide exposure.

Exposure to pesticides reduces global AMP generation, thus further compromising an already depauperate immune system (126, 168–170). Although the specific mechanisms by which AMP production is reduced are largely unknown, Di Prisco et al. (155) demonstrated that honey bees exposed to clothianidin had increased expression of a leucine-rich repeat protein (*Amel/LRR*), which is similar to the *D. melanogaster* gene *CG1399*, a negative regulator of NF-κB signalling (Toll and IMD). Therefore, by increasing the expression of negative immune regulators, this pesticide acted to reduce AMP production, leading to higher infection titres of deformed wing virus (155). Although that study only represents one specific mechanism for one class of pesticide, it is possible that combined exposure to multiple classes of pesticide may further dysregulate the immune response leading to drastic outcomes on pathogen load and mortality.

Activation of the cellular immune response triggers the migration of hemocytes, leading to the engulfment of the pathogen and activation of prophenoloxidase (PPO) to phenoloxidase (PO). Active PO catalyzes the production of a melanin polymer capsule around the pathogen (melanization response). Reactive oxygen species and nitric oxide intermediates are also created, with both being important in pathogen defence (171, 172). Neonicotinoid exposure impairs this melanization response (173, 174), potentially due to the reduction of PO activity (99) or through the decrease of reactive oxygen species and nitric oxide (171, 172). Consequences of this would be reduced pathogen isolation and clearance, and slower wound healing, both of which could increase viral loads and systemic infections (174).

Neonicotinoid exposure, which reduces intestinal stem cell proliferation (175), increases midgut apoptosis (176) and potentially weakens the gut barrier, exacerbates systemic infections. Hemocytes also function as phagocytic cells in the honey bee hemolymph; however exposure to neonicotinoids reduces hemocytes phagocytic activity (171) and hemolymph antimicrobial activity (173). These pesticide-exposed hemocytes also display altered differentiation profiles and reduced total cell counts (173, 174, 177), factors that can lower the magnitude of the melanization response. The mechanisms of pesticide effects on hemocytes and cellular immunity remain elusive. Studies on *D. melanogaster*

and *Chilo suppressalis* demonstrate that the nervous system can regulate hemocyte proliferation (178), and neurotransmitters have a role in modulating hemocyte phagocytosis (179, 180), perhaps suggesting that pesticides act through the nervous system to dysregulate hemocytes. Future studies are required to explore the mechanisms of pesticide-induced impairment of hemocytes, with a focus on pesticide dysregulation of neuro-immune cell signalling.

Social immunity, where individuals contribute to group health, can arise through individual secretion of peptides that effectively sterilize the hive environment. Glucose oxidase (GOX) is secreted from the hypopharyngeal glands and catalyzes the production of hydrogen peroxide (H_2O_2) to sterilize the hive. Alaux et al. (158) demonstrated that there is a synergistic interaction between imidacloprid exposure and *Nosema* infection, whereby GOX activity is reduced. Defensin 1 (*Def 1*) is a social immunity peptide that is secreted into the hive environment and is particularly effective against Gram-positive bacteria as well as fungi. Studies show that *Def 1* expression may increase (thiamethoxam) (168), decrease (fipronil) (169), or remain unchanged (acaricides) (170) in response to the exposure of different types of pesticides.

Honey bees also practice various hygienic behaviours that reduce pathogen load within colonies, most notably self- or mutual-grooming and removal of dead bees. Wu-Smart and Spivak (132) found that worker bees treated chronically with imidacloprid displayed significantly reduced hygienic removal of freeze-killed brood. Likewise, de Mattos et al. (181) showed that synthetic acaricides (coumaphos, amitraz, and tau-fluvalinate), caused workers to groom less, which led to higher *Varroa destructor* loads.

1.3 *Drosophila* as a model organism for honey bees

1.3.1 Overview

Drosophila, also known as the fruit fly, is a classic model organism that has been used in research since the beginning of the 20th century (182). Originally, the flies were used for analyses of inheritance because phenotypical differences could be easily identified, and the generation time is short. As scientific techniques advanced, *Drosophila* became

fundamental for discoveries in molecular genetics and biochemical pathway investigation.

While only having four pairs of chromosomes, exploitations of the *Drosophila* genome are practical and drive improvements to the mechanistic understanding of signalling pathways. In particular, the galactose-responsive transcription factor/upstream activator sequence (GAL4/UAS) allows for the binary manifestation of recombinant expression vectors that can control endogenous host gene articulation.

Drosophila can also be used to study host-microbe interactions. The presence of a well-developed and easily manipulated innate immune system allows for direct insights into microbial sensing and immune response in other organisms. Take, for example, the inquiry into the immune functions of Toll receptors in *Drosophila* (183, 184). This directly led to the discovery of Toll-like receptors in humans (185, 186). More recently, investigations have focused on the importance of the microbiota on host health (187). Considering the extent to which *Drosophila* models are used in scientific study, fruit flies are an excellent model for research into xenobiotics and host-microbe interactions in honey bees (188, 189).

1.3.2 Gut immunity in *Drosophila*

Gut immunity pathways are essential in defence against invading pathogens. Of these, the first line of protection against enteric pathogens is the Dual oxidase (Duox) pathway (190, 191). This pathway is a redox-based immune response that generates hydrogen peroxide (H_2O_2), a potent antimicrobial reactive oxygen species (ROS), via the Dual Oxidase (DUOX) protein (191, 192). While H_2O_2 is antimicrobial on its own, in the presence of chloride it forms hypochlorous acid (HOCl) (191).

To better understand the Duox pathway, it is essential to consider distinctions between the expression and activation components. While activation leads to expression, this on its own does not lead to activation. Peptidoglycan-dependent expression of *Duox*, which is independent of phospholipase C-β (PLC-β), thus would not activate the Duox pathway and cause generation of reactive oxygen species (ROS) (190, 193). The peptidoglycan-

dependent expression of *Duox* is induced by the cross-talk between the immune deficiency (IMD) pathway, whereby peptidoglycan recognition proteins (PGRPs) bind peptidoglycan and signal through IMD, MEKK1, MKK2, p38, and ATF2, to induce expression of *Duox* (193, 194). This signalling cascade is independent of the IMD pathway terminal nuclear factor kappa-light-chain-enhancer of activated b cells (NF-κB), Relish (193).

Activation of the Duox pathway is triggered through the recognition of uracil (predicted to activate a G protein-coupled receptor (GPCR)) or yeast (through an unknown receptor) (190, 195). For the former, endosome formation succeeds ligand recognition (196). Nevertheless, Duox pathway activation proceeds in a PLC-β-dependant manner whereby PLC-β converts phosphatidylinositol biphosphate (PIP_2) to inositol triphosphate (IP_3), which releases calcium stores from the endoplasmic reticulum (190). This calcium mobilizes to the EF-hand domain of the DUOX protein, where it causes DUOX-specific production of ROS (190). Meanwhile, the MEKK1, MKK2, p38, and ATF2 pathway is activated to increase the expression of *Duox* (193).

PLC-β-mediated activation of the Duox pathway is finetuned through the production of negative regulators. Activation of PLC-β leads to the expression of *CanB* (a calcineurin family calcium-dependent phosphatase) and *Mkp3* (mitogen-activated kinase phosphatase-3), which dephosphorylate p38, subsequently reducing *Duox* expression (193). The production of immune-regulated catalase (IRC) scavenges and detoxifies ROS (192).

The IMD pathway also controls pathogen entry into the gut. It controls pathogens that can overcome ROS, by producing antimicrobial peptides (AMPs). The IMD pathway is activated by recognition of peptidoglycan by the pattern recognition receptors, PGRP-LC and PGRP-LE. The former is a transmembrane extracellular receptor, while PGRP-LE is a small intracellular receptor (197). Upon activation of these receptors, a signal cascade begins under the control of the IMD protein, which leads to the activation of the NF-κB, Relish. This then induces expression of AMPs, including *Diptericin*, *Ceropin*, and *Attacin*, along with the expression of negative regulators, including *Caudal*, *Pirk*, and

PGRP-LF (198). Together through the finetuning of the Duox and IMD pathways, *Drosophila* is able to maintain microbial homeostasis and counter invading enteric pathogens.

1.3.3 *Drosophila* gut microbes

The gut of *D. melanogaster* harbours a simple microbiota that consists of only a few bacterial taxa. The majority of bacteria found in the gut belong to the Proteobacteria or Firmicutes phylum (188). The dominant genera are *Gluconobacter* and *Acetobacter*—acetic acid-producing Proteobacteria; and *Lactobacillus*—lactic acid-producing Firmicutes. Interestingly, there are stark differences between the composition of bacteria in wild-caught and laboratory-reared flies. While wild-caught *D. melanogaster* are primarily dominated by *Gluconobacter* and *Acetobacter*, and show minimal *Lactobacillus*; laboratory-reared flies display limited *Gluconobacter*, but competing proportions of *Acetobacter* and *Lactobacillus* (199). The majority of these findings can be explained by differences in food sources; however, the microbiota of *Drosophila* can vary amongst laboratories despite using the same food source (200). Notably, *Drosophila* can transfer microbes from their gut to their food (201), which, in a laboratory setting, could affect the number of bacteria present in the gut based on food changing cycles (202).

Discussion of the bacteria found in *Drosophila* is not complete without considering the presence of the endosymbiotic bacteria, *Wolbachia*, which infects many laboratory stocks (203). This intracellular bacterium lives in both somatic and germline cells and is vertically transmitted to the offspring through the maternal lineage (204). The debate about the implications of *Wolbachia* on host health is ongoing, with studies finding both mutualistic and parasitic consequences associated with its presence (205).

A lesser studied component of the *Drosophila* microbiota are fungi, specifically yeast. While most research focuses on the importance of dietary yeast (both living and dead) (206), limited studies describe the presence of yeast in the microbiota. *Hanseniaspora* spp. appears to be the most dominant yeast based on samples from naturally occurring substrates throughout the world, followed by *Saccharomyces* and *Candida* (207). The

yeast taxa also vary based on food source (207). Other yeast taxa found in *Drosophila* include *Cryptococcus*, *Saccharaomycopsis*, *Kloeckera*, and *Pichia* (201). The association of yeast in the gut is complicated because the digestive tract of the fly is a hazardous place for yeast; they likely overcome this by going dormant (208).

1.3.4 Advantages of a *Drosophila model*

There are many complexities that need to be considered when studying honey bees, which can be mitigated by using a *D. melanogaster* model. While honey bees are eusocial insects that rely heavily on the division of labour, *D. melanogaster* are non-social insects, which reduces the need for groups because social structure is unnecessary. Unlike honey bees, *Drosophila* does not need stimulants, like pheromones, to maintain homeostasis (209). *Drosophila* can easily be studied in the laboratory where extraneous variables (for example, weather, infection, or environmental toxins) can be minimized; however, bees kept in laboratory cages can exhibit different responses compared with field colonies (210).

In addition to practicality, the *D. melanogaster* model offers strong genetic tractability and well-established cell biology. Although the honey bee genome (211) and *D. melanogaster* (212) genome were sequenced a few years apart, *Drosophila* genetic studies began as far back as 1910 (213). With the advent of molecular cloning, *Drosophila* genetics has grown to include an extensive repository of knockouts, knockdown, and overexpression mutants that allow for a mechanistic understanding of molecular pathways. One of the most commonly used expression systems in *Drosophila* (GAL4/UAS) was adapted from yeast in the 1980s and is still used today (214).

Another advantage of the *D. melanogaster* model is the low-diversity and predictability of the gut microbiota. This allows for practical microbiota composition monitoring, whether through qPCR or culture-based enumeration. The microbiota can also be easily abolished to generate germ-free flies, effectively eliminating microbes as a variable and allowing the exclusive study of the host responses. Combining the practicality of use with the repertoire of established gene knockouts and the modularity of the gut microbiota, *D.*

melanogaster is a powerful model to investigate how xenobiotics alter host-microbe interactions.

1.4 Probiotic potential of lactobacilli

1.4.1 Benefits of lactobacilli in honey bees

One novel solution to combatting honey bee decline may be through supplementation beneficial microbes, such as lactic acid bacteria (LAB; such as *Lactobacillus* and *Bifidobacterium* spp.) believed capable of mitigating the harmful effects of pesticides and pathogens. The basis for this is several-fold, but the most discernible benefit is by reducing pesticide absorption via degradation (215–218) and sequestering ingested pesticides, thereby allowing them to pass through the digestive tract rather than be absorbed (219). In other model organisms, LAB have been shown to reduce toxicity and have a protective effect on the host (220, 221), thus establishing a basis to investigate this potential in honey bees.

Supplementing honey bees with beneficial bacteria can reduce *Nosema* spore counts (222–225) and *P. larvae* bacterial load (225–227). *In vivo* evidence from a *D. melanogaster* model of pesticide exposure has shown that supplementation with LAB improves the immunity of pesticide-exposed flies via immune stimulation (228, 229). Likewise, certain LAB are able to stimulate AMP production in honey bees and improve survival during *Paenibacillus larvae* infection (227, 230). Together these studies demonstrate that beneficial bacteria can *indirectly* contribute to pathogen resistance by stimulating the immune system and assisting the host in overcoming the infection. These form part of the basis for the present thesis.

Some lactobacilli strains can *directly* inhibit pathogen growth, thus enhancing overall honey bee resistance to infection. For example, isolates of *L. kunkeei* have been shown to inhibit *N. ceranae*, *P. larvae,* and *Serratia marcesscens* (225, 226, 231, 232). *Lactobacillus kunkeei* is known to produce biofilms in honey bees, thereby facilitating its vertical transmission from one generation to the next (233). Another LAB, *Lactobacillus apis* R4B^{T}, can inhibit *P. larvae* and *M. pluntonius*, *in vitro* (234). Some *Bifidobacterium* species inhibit *P. larvae* and *S. marcesscens*, and when found adequately in the

microbiota they are associated with reduced pathogen load (226, 232, 235). Honey bee-derived *Lactobacillus johnsonii* CRL1647 is a well-documented LAB that has been shown to reduce the abundance of *Nosema* and *Varroa* in the hive (236). Although the mechanism for *direct* pathogen inhibition is not completely clear, it is likely the production of organic acids (223), bacteriocins (237), or other antimicrobial proteins (238). This forms a strong basis to mitigate the immune impairment caused by sublethal pesticide exposure. Supplementation with lactobacilli could prove to be an alternative to antibiotic prophylaxis to reduce pathogen burden.

In addition, beneficial bacteria can bolster colony developments that are notably decreased by pesticide exposure. Honey bees supplemented with LAB typically produce more honey, have more pollen stores, and have increased brood counts (236, 239–241). For example, *L. johnsonii* CRL1647 stimulates egg-laying, which can increase the hive population (239). These positive effects have been partially attributed to organic acid production (223), but could also be due to microbiota restoration as 'non-thriving' hives typically have lower levels of *Lactobacillus* and *Bifidobacterium* (242).

1.4.2 Methods of probiotic supplementation in honey bees

The long-standing challenge to supplementing honey bees with beneficial bacteria is in the delivery method (**Figure 1-5**). A number of commercial bee supplements containing dried LAB claim to work by 'dusting' frames with the bacteria, which may also promote grooming. However, the efficacy has not been confirmed nor has survival of the organisms during shelf-life. Moreover, dusting is prone to uneven distribution and is negatively impacted by moisture and humidity.

More commonly, beneficial bacteria are added to sucrose-based syrup solutions. Numerous studies have utilized this method with results showing a reduction in *Nosema ceranae* loads (243), lowered overwintering death rates (244), and increased brood populations and harvestable honey by ~46% and ~60%, respectively (240). However, the lacklustre viability and activity of bacteria in sucrose-based solutions (>90% drop in original CFU after 96 hours at 30°C) due to osmotic stress (245) questions the practicality

of this approach. In addition, this method of supplementation may not transfer bacteria to younger bees and larvae (246).

Another option is to infuse beneficial bacteria into pollen-substitute patties. This has the advantage of improving honey bee nutrition. Pollen-substitute patties per se have been shown to benefit honey bee health through reducing titers of deformed wing virus (247) and increasing hemolymph protein content (248). Evaluating pollen substitutes as a delivery method, Kaznowski et al. (249) demonstrated that hives supplemented with probiotic-infused pollen substitutes had better overall survival, higher dry mass, and increased crude fat levels of bees when compared to groups receiving only the pollen-substitute. Another study showed that honey bees receiving probiotic bacteria delivered via pollen-substitutes have better developed peritrophic membranes (responsible for nutrient utilization and pathogen protection) compared to vehicle controls (250). Some points to consider are that pollen-substitute patties may attract unwanted opportunistic insects (for example the small hive beetle, *Aethina tumida*) and it may not be consumed if other pollen sources exist. Nonetheless, pollen substitutes are already used by beekeepers with the hope of providing nutritional adequacy.

Along with the introduction of any live microorganism to the hive comes the risk of inducing hive microbial dysbiosis (251). A few documented cases exist in which negative effects were observed from supplying honey bees with ostensibly beneficial bacteria. Ptaszyńska et al. (245) reported that supplementation with *L. rhamnosus* (no strain type provided) increased honey bee susceptibility towards Nosemosis C. In the same year, the same group demonstrated that co-administration with three LAB (*Lactobacillus acidophilus*, *Lactobacillus delbrueckii*, and *Bifidobacterium bifidum*—no strain designations provided) led to a decrease in total yeast concentrations in adult honey bee guts, but an increase in *N. ceranae* spores (252). It is difficult to ascertain the biological relevance of these findings as crucial details are missing from analyses, including 1) strain-type information of lactobacilli used, 2) confirmation that live bacteria actually reached their target destination in the adult honey bee gut, and 3) whether or not the apparent increase in *Nosema* spp. led to any measurable changes in individual or hive-level health outcomes. Johnson et al. (253) found no net positive or negative effect on

hive health or performance following supplementation with lactobacilli in a high-fructose corn syrup vehicle. These collective findings illustrate the need to carefully select biological agents for in-hive supplementation.

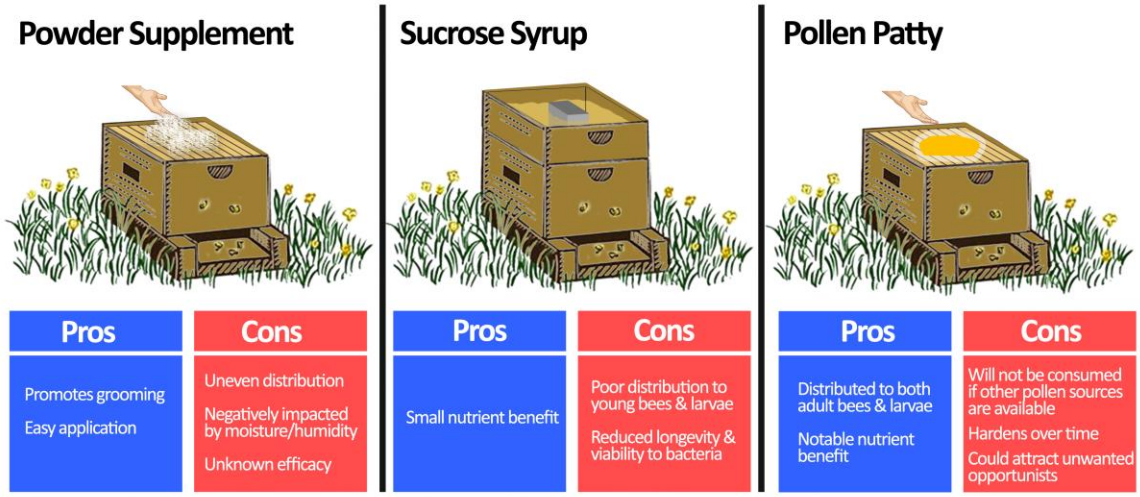

Figure 1-5. Comparison of methods for beneficial bacteria supplementation.

Beneficial bacteria are usually combined with a vehicle to supplement honey bees in one of three ways: powder supplementation, sucrose syrup, or pollen patty. Powder supplementation can be easily performed by spreading a probiotic infused dust on the beehive, which also promotes bees to groom. However, it is prone to uneven distribution, negative impacts of moisture, and unknown efficacy as an application method. Sucrose syrup supplementation can be achieved by adding probiotics directly to conventional sucrose feeders for the hive. Although this method benefits from a small nutrient enhancement, the sucrose solution is not usually distributed well to all members of the hive, and it is an unfavourable environment for bacteria. Pollen patty supplementation involves adding beneficial bacteria directly to a traditional pollen supplement. In addition to the added nutrient benefit, pollen patty supplementation will be distributed throughout the hive to both adult bees and larvae. However, if sufficient nutrient sources already exist, then the pollen patty may be disregarded by the hive. Moreover, it is prone to hardening over time and could attract unwanted pests. Langstroth beehive image modified from Net Art under the Creative Commons Attribution 2.0 Generic License (https://netart.us/box-shaped-beehive-coloring-page/).

1.5 Rationale and hypothesis

Sublethal neonicotinoid exposure is known to impair the immune system of honey bees, which can alter their microbiota and increase their susceptibility to infection (254). A majority of studies note that neonicotinoids reduce AMP expression, but there is limited research identifying a mechanism of immunosuppression (254). Despite the research showing that neonicotinoids alter ROS generation, it has not been identified if neonicotinoids interfere with the Duox pathway, a key regulator of enteric pathogens and the microbiota (171, 190). Identifying and characterizing the interactions between neonicotinoids and the immune system will allow for the development of an intervention that can mitigate the elicited immunosuppression. Given some preliminary results showing benefits of probiotic lactobacilli, and the successful development and verification of *Lactobacillus plantarum* Lp39, *Lactobacillus rhamnosus* GR-1, and *Lactobacillus kunkeei* BR-1 in improving immunity in honey bees (227), it was hypothesized that the neonicotinoid imidacloprid will alter the signalling of the principle gut immune pathways (Duox and IMD) and this interaction can be mitigated through probiotic supplementation. This thesis will describe the use of a *D. melanogaster* model to examine the mechanism of pesticide exposure on the host, and the potential for remediation by probiotic intervention.

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Chapter 2

2 Deleterious effects of neonicotinoid pesticides on *Drosophila melanogaster* immune pathways

The material in this chapter has been published in mBio as a full-length primary article and has a content license that can be found in Appendix B.

Chmiel JA, Daisley BA, Burton JP, Reid G. Deleterious effects of neonicotinoid pesticides on *Drosophila melanogaster* immune pathways. *mBio*. 2019;10(5):e01395-19. doi:10.1128/mBio.01395-19

2.1 Abstract

Neonicotinoid insecticides are common agrochemicals that are used to kill pest insects and improve crop yield. However, sublethal exposure can exert unintentional toxicity to honey bees and other beneficial pollinators by dysregulating innate immunity. Generation of hydrogen peroxide (H_2O_2) by the dual oxidase (Duox) pathway is a critical component of the innate immune response, which functions to impede infection and maintain homeostatic regulation of the gut microbiota. Despite the importance of this pathway in gut immunity, the consequences of neonicotinoid exposure on Duox signalling has yet to be studied. Here, a *Drosophila melanogaster* model was used to investigate the hypothesis that imidacloprid (common neonicotinoid) can affect the Duox pathway. The results demonstrated that exposure to sublethal imidacloprid reduced H_2O_2 production by inhibiting transcription of the *Duox* gene. Furthermore, the reduction in *Duox* expression was found to be a result of imidacloprid interacting with the midgut portion of the immune deficiency pathway. This impairment led to a loss of microbial regulation, as exemplified by a compositional shift and increased total abundance of *Lactobacillus* and *Acetobacter* spp. (dominant microbiota members) found in the gut. In addition, certain probiotic lactobacilli were able to ameliorate Duox pathway impairment caused by imidacloprid, but that this effect was not directly dependent on the Duox pathway itself. This study is the first to demonstrate the deleterious effects that neonicotinoids can have on Duox-mediated generation of H_2O_2 and highlights a novel coordination between two important innate immune pathways present in insects.

2.2 Introduction

Neonicotinoid insecticides are a class of neuro-active agrochemicals used to control pest organisms. They are currently the most widely used (~20% of the global market) insecticides in the world, owing largely to affordability, flexible application, and long-lasting systemic activity in plant tissue (1). Imidacloprid (IMI), with a half-life exceeding 1,000 days in some cases (2), is the most commonly used neonicotinoid and has been detected in 52% and 66% of all fruits and vegetables in the United States and China, respectively (3). Further supporting its ubiquity in the environment, imidacloprid was recently found present in 51% of honey samples globally-sourced through a citizen science project (4).

Despite their success as a pesticide, neonicotinoids pose a threat to honey bees and other beneficial pollinators, and may contribute to declining pollinator populations (5, 6). Honey bees exposed to neonicotinoids have growth defects (7), motor deficiencies (8), and behavioural abnormalities (9, 10). Moreover, neonicotinoids at sublethal concentrations have been shown to cause immunosuppression and increased susceptibility to fungal and viral pathogens in honey bees (11–13). Therefore, by reducing immune function and increasing susceptibility to infection, exposure to low-dose pesticides are believed to pose a threat to beneficial pollinators.

The insect gut microbiota is simultaneously controlled by the immune deficiency pathway (IMD) and the dual oxidase pathway (Duox) (14–17). The IMD pathway is used to control Gram-negative bacteria through peptidoglycan recognition and subsequent Relish-mediated induction of antimicrobial peptides (AMPs) expression (18, 19). The Duox pathway is divided into an expression and an activation pathway. The expression pathway is mediated by p38 activation through the mitogen-activated protein (MAP) kinase pathway (20). Activated p38 causes phosphorylation of activating transcription factor-2 (ATF2), which is a transcription factor for the *Duox* gene. Duox pathway activation is induced by recognition of pathogen secreted uracil and yeast (21, 22). This drives PLC-β-mediated calcium efflux, which triggers the subsequent conformational changes required in DUOX for H_2O_2 generation. In the presence of chloride, DUOX can convert hydrogen peroxide (H_2O_2) to HOCl, a potent antimicrobial compound (23).

Together, the IMD and Duox pathways control the insect gut microbiota in both honey bees (24, 25) and *Drosophila melanogaster* (15).

Honey bees are intrinsically difficult to work with under controlled laboratory settings because of their stringent requirement for queen pheromone replacement and social hierarchy. *Drosophila melanogaster* is a suitable organism to model the effects of pesticides on the innate immune system of bees as both insects possess homologous nicotinamide acetylcholine receptors (the primary target of neonicotinoids) and share highly conserved innate immune systems (12, 26). A major advantage of this model is that the genome of *D. melanogaster* is well characterized and easily manipulated. This allows for generation of pathway mutants, which aids in the understanding of how factors, like pesticides, influence immune functionality of insects. Moreover, *D. melanogaster* possesses a simple microbiota that is dominated by culturable bacteria, low in diversity, and can be easily monitored via either culture-based CFU enumeration or molecular methods like qPCR-based quantification and 16S rRNA gene sequencing to determine composition (27).

It has been shown that loss of function mutations in the Duox or IMD pathways causes increased microbial load and reduced longevity (15). Interestingly, oral supplementation with certain probiotic *Lactobacillus* spp., can modulate these pathways to increase activation even in times of immunosuppression (28, 29). We have previously demonstrated that supplementation with *Lactobacillus plantarum* Lp39 could mitigate imidacloprid-induced susceptibility to septic infection with *Serratia marcescens*, a Gram-negative bacterial pathogen (29). Nevertheless, the relationship between the Duox pathway and the insect microbiota is still poorly understood, and the effect of neonicotinoids on the Duox pathway and the microbiota is inadequately characterized. Here, a *D. melanogaster* model (with a simplified microbiota largely dominated by Gram-positive *Lactobacillus* spp. and Gram-negative *Acetobacter* spp.) was used as a tractable and high-throughput model to investigate the relationship between the Duox pathway, regulation of the insect microbiota, and the effect of sublethal imidacloprid exposure. It was hypothesized that sublethal imidacloprid exposure will alter Duox pathway signalling and thereby affect microbicidal H_2O_2 production in *D. melanogaster*.

2.3 Materials and Methods

2.3.1 Chemicals

Imidacloprid (catalogue number: 37894) was obtained from Sigma-Aldrich. Stock solutions were prepared at 100 mg/mL in dimethyl sulfoxide (DMSO; Sigma, catalogue number: D8418) and stored at 4°C until usage.

2.3.2 *Drosophila melanogaster* husbandry

Wild-type (WT) Canton-S (stock number: 1; RRID:BDSC_1), w^{1118} (stock number: 3605; RRID:BDSC_3605), *daughterless* GAL4 (*da*-GAL4; stock number: 55850; RRID:BDSC_55850), $PGRP\text{-}LE^{112}$ (PGRP-LE$^{-/-}$; stock number: 33055; RRID:BDSC_33055), $PRGP\text{-}LC^{\Delta E}$ (PGRP-LC$^{-/-}$; stock number: 55713; RRID:BDSC_55713), and $norpA^{7}$ (PLC-β$^{-/-}$; stock number: 5685, RRID:BDSC_5685) were obtained from Bloomington Drosophila Stock Center (NIH P40ODO18537) at Indiana University. The previously described UAS-*dDuox*-RNAi (*Duox*-RNAi) fly line (approximately 50% reduction of *Duox*) (23) and R156 imd^{1} (IMD$^{-/-}$) fly line (30) were also used in this study. *D. melanogaster* were maintained using media with 1.5% (wt/vol) agar, 1.73% (wt/vol) yeast (Sigma-Aldrich, catalogue number: 51475), 7.3% (wt/vol) cornmeal, 7.6% (vol/vol) corn syrup, and 0.58% (vol/vol) propionic acid at 25°C with 12-hour light/dark cycles. For experimental procedures, IMI media were supplemented with pesticide, and vehicle media were supplemented with dimethyl sulfoxide (DMSO) prior to agar solidification. All experiments were performed in wide polypropylene *D. melanogaster* vials (catalogue number: GEN32-121 and GEN49-101, Diamed Lab Supplies Inc., Mississauga, ON, Canada). Adult flies used for experiments were 3 to 5 days old unless otherwise stated. UAS/GAL4 crosses were performed by mating male *da*-GAL4 with virgin female UAS-*dDuox*-RNAi knockdown flies or virgin female w^{1118} flies as control. The GAL4 driver, *da*-GAL4, is an all tissue driver, which has ubiquitous GAL4 expression. WT Canton-S flies were supplemented with 10 μM imidacloprid, as previously determined to be sublethal (29). The sublethal dose of imidacloprid for *Duox*-RNAi and GAL4/w^{1118} flies was determined to be 1 μM (**Supplementary, Figure 2-6**).

2.3.3 Generation and rearing of germ-free *D. melanogaster*

Germ-free flies were prepared and reared on sterile media (31). Eggs were collected, rinsed with water to remove excess debris, and dechlorinated with 2.7% (vol/vol) sodium hypochlorite for 2 – 3 minutes, followed by two rinses of 70% ethanol. Finally, eggs were rinsed with sterile water for 10 minutes and placed on sterile media to grow. Germ-free conditions were verified by homogenizing and plating *D. melanogaster* larvae on brain heart infusion (BHI), MRS, and mannitol (MAN) agar (3 g Bacto Peptone Number:3, 5 g yeast extract, 25 g mannitol, 15 g agar, 1 L H_2O) incubating them at 30°C for 2 days.

2.3.4 DNA extraction for qPCR-based quantification of *D. melanogaster* gut bacteria

Three- to five-day-old Canton-S flies were placed on media containing 10 μM of imidacloprid or vehicle for 5 days. Five female flies were surface sterilized with 70% ethanol for 1 – 2 minutes and washed with sterile water. Flies were kept at –20°C until DNA extraction was performed. DNA was extracted using the method from Staubach et al. (32) with the Qiagen QIAmp DNA Mini Kit (Qiagen, catalogue number: 51304). Briefly, flies were homogenized in 180 μL of ATL buffer containing 20 μL of proteinase K at 56°C for 30 minutes to soften the exoskeleton. Following this incubation, flies were homogenized by bead beating at 4,800 rpm with 0.1 mm (zirconia/silica; BioSpec, catalogue number: 11079101z), 0.5 mm (zirconia/silica; BioSpec, catalogue number: 11079105z), and 1 mm (glass) beads using a BioSpec 3110BX Mini Beadbeater 1 (Fisher Scientific, catalogue number: NC0251414) for 3 – 5 minutes, and another incubation for 30 minutes at 56°C. Next, 200 μL of lysis buffer AL was added, and samples were incubated at 70°C for 30 minutes and then 95°C for 10 minutes. The rest of the extraction followed the manufacturer's protocol. The quality of DNA was evaluated using DeNovix DS-11 Spectrophotometer and determined to have A260/280 and A260/230 absorbance ratios between 1.7 – 1.9 and 1.7 – 2.2, respectively.

2.3.5 Culture-based enumeration of *D. melanogaster* gut bacteria

Three female flies were surface sterilized with 70% ethanol then homogenized with three 2 mm glass beads in 300 μL of PBS using a BioSpec 3110BX Mini Beadbeater 1 (Fisher

Scientific, catalogue number: NC0251414). Homogenates were then serially diluted in PBS and plated on MRS and MAN agar. MRS plates were grown anaerobically at 30°C for 48 hours, and MAN plates were grown aerobically at 30°C for 48 hours. Subsequent colony-forming units on MRS and MAN plates were counted and confirmed to be *Lactobacillus* spp. or *Acetobacter* spp., respectively, based on morphological characteristics and Gram stain analysis.

2.3.6 *D. melanogaster* gut abundance of yeast

Three to five-day-old Canton-S flies were exposed to vehicle (DMSO), 10 μM imidacloprid, 2% (wt/vol) *Saccharomyces cerevisiae* (Fleischmann's® Traditional Active Dry Yeast) with vehicle, or 2% *S. cerevisiae* with 10 μM imidacloprid on previously described media without the addition of propionic acid to allow the yeast to survive. Tubes consisted of 25 – 30 flies that were then kept under standard conditions for 5 days. Five female flies were surface sterilized and collected in 500 μL of PBS, then homogenized for 30 seconds at 4,800 rpm with three 2 mm glass beads. Homogenates were serially diluted and plated on YPD agar (10 g yeast extract, 20 g peptone, 20 g dextrose, 15 g agar, 1 L ddH_2O) with 100 μg/mL rifampicin as previously described (22), then incubated at 30°C for 24 – 48 hours.

2.3.7 Determination of H_2O_2-specific ROS in *D. melanogaster*

Hydrogen peroxide was quantified using Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen, catalogue number: A22188) as previously demonstrated but with minor modifications (30). Three female adult *D. melanogaster* were collected and homogenized in 300 μL of PBS with three 2 mm glass beads beating for 10 seconds at 4,200 rpm. For Canton-S flies, heads were removed because of the intense red eye pigment. Samples were centrifuged at 12,000 × *g* for 3 minutes (room temperature) and 50 μL of supernatant was used for the assay following the manufacturer's protocol with spectrophotometry quantification at 560 nm or excitation/emission 535/595 nm using a BioTek Eon microplate reader or Eppendorf PlateReader AF2200, respectively. Hydrogen peroxide concentrations were normalized to total protein and plotted as relative H_2O_2 to the vehicle. Total protein was quantified using a bicinchoninic acid (BCA)

Protein Assay Kit (Invitrogen, catalogue number: 23227) following the manufacturer's microplate protocol. Protein was measured from samples that were obtained from the H_2O_2 determination protocol and used to normalize H_2O_2 quantification. Samples were centrifuged at 12,000 × *g* for 3 minutes (room temperature), and 25 µL was used for quantification as per the manufacturer's microplate protocol using a BioTek Eon microplate reader (BioTek, Eon) at 562 nm.

2.3.8 Adult *D. melanogaster* survival assays

Five to ten-day-old flies were used for all adult survival experiments as described previously (29) with modifications. Prior to the experimental start point, flies were gently anesthetized with CO_2 and transferred from standard rearing media to an empty vial containing a 100 µL ddH_2O-soaked Whatman filter disc (25 mm; Sigma-Aldrich) and starved for 120 minutes to normalize feeding frequency. For lethal exposure experiments, flies were briefly anesthetized with CO_2 and transferred to vials with 5% sucrose agar (5% sucrose [wt/vol] and 1.5% agar [wt/vol]) containing 10 µM imidacloprid or vehicle (DMSO). Any early deaths (< 1 hour) were assumed to be from the transfer process and removed from subsequent analyses. Survival was monitored daily at 24-hour intervals from the experimental start point.

2.3.9 RNA extraction and reverse transcription

Five female adult *D. melanogaster* were homogenized in 550 µL of TRIzol reagent (Ambion, catalogue number: 15596018) using three 2 mm glass beads beating twice for 30 seconds at 4,800 rpm with a BioSpec 3110BX Mini Beadbeater 1 (Fisher Scientific, catalogue number: NC0251414). Tubes were centrifuged at 13,000 rpm for 10 minutes at 4°C to pellet debris. Supernatant was collected, and 0.2 volumes of chloroform were added, followed by centrifugation at 13,000 rpm for 15 minutes at 4°C. The upper aqueous layer was collected, and 0.7 volumes of isopropanol was added to precipitate the RNA, followed by centrifugation at 13,000 rpm for 15 minutes at 4°C. The RNA pellet was washed with 1 mL of 70% ethanol in diethyl pyrocarbonate-treated ddH_2O and centrifuged at 13,000 rpm for 15 minutes at 4°C. Following removal of supernatant, the RNA was air-dried and then re-suspended in 30 µL of nuclease-free water. The quality of

RNA was evaluated using a DeNovix DS-11 Spectrophotometer and determined to have A260/280 and A260/230 ratios between 1.7 – 2.2 and 1.8 – 2.4. cDNA was synthesized from 1,500 ng of total RNA using a High-Capacity cDNA Reverse Transcription Kit following manufacturer's instructions (Applied Biosystems, catalogue number: 4368813).

2.3.10 qPCR analysis

Reverse transcribed cDNA was diluted 6× and isolated *D. melanogaster* DNA was diluted 10× in nuclease-free water and used for qPCR reactions with the *Power* SYBR Green Kit (Applied Biosystems, catalogue number: 4368702). The following primers were used in this study (**Supplementary, Table 2-1**). For analysis of gene expression, *RpLP0* used as the endogenous reference gene because it was identified as the most stably expressed reference gene (29). The *Duox* primers were designed in this study and are exon-spanning for *Duox* mRNA (NM_001273039.1). For qPCR analysis of total bacteria and the ratio of *Acetobacter* to *Lactobacillus*, *Dros_rt_1* (*Drosophila* actin gene) was used as the endogenous control. The vehicle (DMSO) group was used as the calibrator in all qPCR analysis experiments, except for the LGR-1 supplementation experiments, where the vehicle groups were used as the calibrators for the respective imidacloprid exposure groups. Reagent volumes for 10 µL reactions (performed in triplicate technical replicates) consisted of 2.5 µL of diluted DNA or cDNA, 5 µL of *Power* SYBR (2×), and 2.5 µL of forward and reverse primer mix (3.2 µM each stock). Reaction conditions were 50°C for 2 minutes, then 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. qPCR was performed on a QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific) and analyzed using the associated QuantStudio Design and Analysis Software v1.4.3 (Thermo Fisher Scientific). Gene expression ($2^{-\Delta\Delta Ct}$) was calculated using fold change, and statistics were performed on the $-\Delta\Delta Ct$ values (33). PCR efficiencies were calculated using LinRegPCR version 2016.1 and determined to be above 1.80. Primer specificity was tested using gel electrophoresis (**Supplementary, Figure 2-7A–C**) and monitored by analyzing the melt curves.

2.3.11 LGR-1 imidacloprid tolerance assay

LGR-1 was grown overnight in MRS and subcultured (1:100) into 96-well plates (Falcon, catalogue number: 35177) containing MRS with or without vehicle (DMSO) or 100 ppm imidacloprid. Plates were incubated at 37℃ for 24 hours and measured every 30 minutes at 600 nm using a microplate reader (BioTek, Eon).

2.3.12 Pesticide metabolism/binding assay

High-performance liquid chromatography (HPLC) analysis of culture supernatant was employed to test if LGR-1 was able to reduce the amount of imidacloprid in culture supernatant. LGR-1 grown in minimal media (2.5 g/L yeast extract, 1.5 g/L K_2HPO_4, 0.5 g/L KH_2PO_4, 0.5 g/L $(NH_4)_2SO_4$, 0.5 g/L NaCl, 0.4 g/L $MgSO_4 \cdot 7H_2O$, 0.05 $CaCl_2$, 0.03 g/L $FeSO_4 \cdot 7H_2O$) and minimal media alone were spiked with 100 ppm of imidacloprid and incubated anaerobically for 24 hours at 37℃, with shaking (175 rpm), and protected from light. The solutions were then centrifuged at 5,000 rpm (4,500 × *g*) for 10 minutes at room temperature. Supernatants were removed and filter sterilized using 0.45 μm filters prior to HPLC analysis.

All samples and standards were analyzed using an Agilent 1100 HPLC equipped with a degasser (G1379A), quaternary pump (G1311A), autosampler (G1313A), and diode array detector (G1315B). All analyses were performed on an Agilent Poroshell 120 EC-C18 (4.6 × 150 mm I.D., 4 μm particle size) column kept at ambient temperature. Acetonitrile (Fisher, catalogue number: A996-4) and water (Fisher, catalogue number: W5-4) used were HPLC grade. Mobile phase consisted of an isocratic mixture of acetonitrile/water (40:60 vol/vol) at a flow rate of 1.0 mL/min. Sample injection volume was 5 μL and detection was performed at 270 nm. Run times were 5 minutes with imidacloprid eluting at ~2.3 minutes. Data were analyzed using ChemStation A. 10.02. The peak area of samples was compared with the peak area of the external calibration curve (1 – 200 ppm) to determine imidacloprid quantification.

2.3.13 Statistical analysis

All statistical comparisons were performed using GraphPad Prism 7.0 software. Nonparametric data were statistically compared with an unpaired, two-tailed Mann-Whitney test. Data were tested for normality using the Shapiro-Wilk test for data with unique values or D'Agostino and Pearson test for data with tied values. Normally distributed data were compared with an unpaired, two-tailed t test. Experiments with two factors were statistically compared with a two-way analysis of variance (ANOVA), complemented with Sidak's multiple comparisons test.

2.4 Results

2.4.1 Imidacloprid exposure causes loss of microbial regulation in *Drosophila melanogaster*

Quantitative PCR was used to determine the change in bacterial load in response to imidacloprid exposure. Wild-type (WT) Canton-S exposed to imidacloprid showed significantly higher −ΔCt values compared to control flies, which corresponds with a higher bacterial load (Mann-Whitney test, U = 1.000, $P < 0.05$; **Figure 2-1A**). The imidacloprid-exposed flies also demonstrated a significant increase in the ratio of *Acetobacter* spp. to *Lactobacillus* spp. compared to control flies (Mann-Whitney test, U = 1.000, $P < 0.05$; **Figure 2-1B**). Time-course CFU enumeration showed that the CFU of *Acetobacter* spp. and *Lactobacillus* spp. began to increase as early as 3 days after imidacloprid exposure (**Figure 2-1C** and **Figure 2-1D**). A significant increase in both *Acetobacter* spp. (two-way ANOVA, $P < 0.001$; **Figure 2-1C**) and *Lactobacillus* spp. (two-way ANOVA, $P < 0.0001$; **Figure 2-1D**) were observed at day 6 and 9 of imidacloprid exposure.

Drosophila melanogaster exposed to imidacloprid was shown to have significantly higher abundance of total endogenous yeast per fly compared with control exposed flies (unpaired, two-tailed t test, t = 5.836, df = 22, $P < 0.0001$; **Figure 2-1E**). When *D. melanogaster* was administered 2% (wt/v) *Saccharomyces cerevisiae* along with vehicle or imidacloprid treatment, flies exposed to both imidacloprid and the 2% yeast supplement had significantly higher CFU of yeast per fly compared to *D. melanogaster*

given only the 2% yeast supplement (unpaired, two-tailed t test, t = 3.661, df = 22, $P < 0.01$; **Figure 2-1F**).

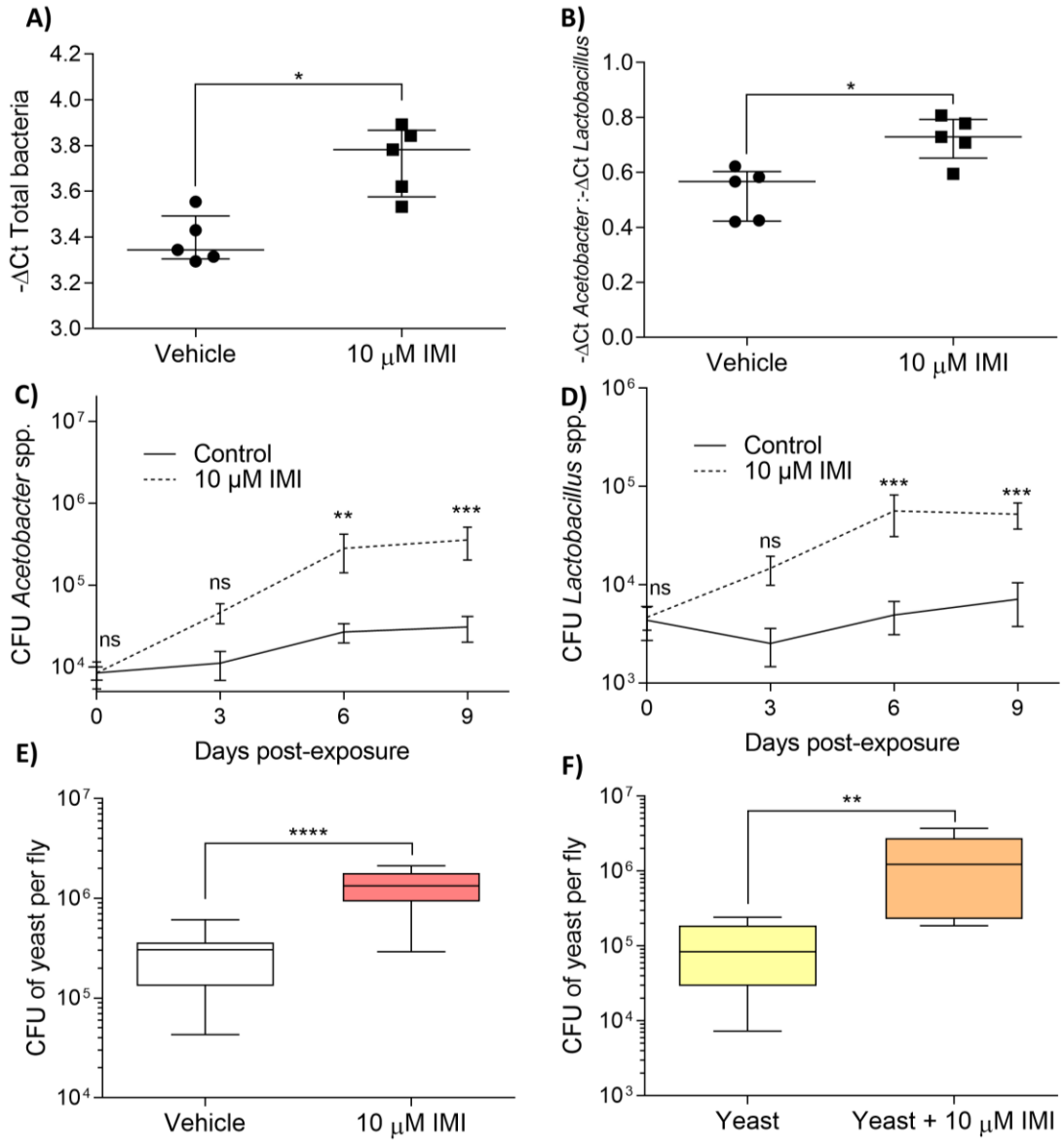

Figure 2-1. Imidacloprid exposure causes loss of microbial regulation in *Drosophila melanogaster*.

Three to five-day old WT Canton-S flies were transferred to food vials containing vehicle (DMSO) or imidacloprid (IMI; 10μM) for five days. Flies were then surface sterilized, DNA was extracted, and bacteria were quantified using qPCR microbial quantification relative to *Dros_rt_1* (*Drosophila* actin gene) . Data are displayed as mean −ΔCt of total bacteria **(A)** or mean −ΔCt *Acetobacter* spp./−ΔCt *Lactobacillus* spp. **(B)**. From 5 biological replicates (each consisting of 5 flies). Error bars represent median with interquartile range (Mann-Whitney test). **(C – D)** WT Canton-S time course CFU enumeration over 9 days of dominant gut bacteria per fly. Flies were surface sterilized

and plated on MAN agar for *Acetobacter* spp. **(C)** and MRS agar for *Lactobacillus* spp. **(D)**. Data displayed as mean CFU per fly ± SD (two-way ANOVA) at each time point of 3 biological replicates (n = 18 per time point for each group). **(E – F)** Three to five-day old WT Canton-S flies were transferred to food vials containing either vehicle (DMSO) or imidacloprid (10μM) **(E)** or 2% (w/v) dried yeast (*Saccharomyces cerevisiae)* or 2% (w/v) dried yeast with 10 μM imidacloprid **(F)** for five days. Flies were then surface sterilized and plated on YPD with 100 μg/mL of rifampicin. Data displayed as mean yeast CFU per fly ± SD (unpaired, two-tailed t test) of 12 biological replicates (each consisting of 5 flies). In box plot diagrams, boxes represent first and third quartile values while black lines denote medians. Whiskers encompass maximum and minimum values. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. ns = not significant.

2.4.2 Imidacloprid exposure affects Duox-mediated H_2O_2 production in *Drosophila melanogaster*

Since H_2O_2 is the primary metabolite produced downstream of the Duox pathway, its concentration was used to monitor pathway activity. Wild-type (WT) Canton-S flies exposed to sublethal (10 μM) imidacloprid had significantly reduced whole-body H_2O_2 compared to vehicle-exposed flies (unpaired, two-tailed t test, t = 7.092, df = 32, $P < 0.0001$; **Figure 2-2A**). This was also observed in germ-free (GF) flies, where imidacloprid-exposed GF flies had significantly reduced whole-body H_2O_2 compared to vehicle-exposed GF flies (unpaired, two-tailed t test, t = 4.633, df = 22, $P < 0.001$; **Figure 2-2B**).

To test if the Duox pathway is necessary to resist imidacloprid-induced toxicity, *Duox* RNA interference knockdown (*Duox*-RNAi) flies were exposed to imidacloprid and assessed for survival. *Duox*-RNAi flies exposed to imidacloprid demonstrated a significant reduction (log-rank [Mantel-Cox], chi-square = 40.04, degrees of freedom [df] = 1, $P < 0.0001$) in survival compared to control-crossed (GAL4/w^{1118}) flies (**Figure 2-2C**). There were no observable differences (Mann-Whitney test, U = 6, $P = 0.6857$) in whole-body H_2O_2 of *Duox*-RNAi flies exposed to either imidacloprid or vehicle (**Figure 2-2D**). Similar to our findings in WT flies, there was a significant decrease (Mann-Whitney test, U = 0, $P < 0.05$) in whole-body H_2O_2 of control cross (GAL4/w^{1118}) flies exposed to imidacloprid compared with vehicle-exposed control cross flies. In addition, there was no significant change in the ratio of *Acetobacter* spp. to *Lactobacillus* spp. of *Duox*-RNAi flies exposed to 1 μM imidacloprid or vehicle (unpaired, two-tailed t test, t = 0.05109, df = 8, $P = 0.9605$; **Figure 2-2E**). Meanwhile, there was a significant increase in the ratio of *Acetobacter* spp. to *Lactobacillus* spp. for control crossed (GAL4/w^{1118}) flies exposed to 1 μM imidacloprid compared with vehicle exposure (unpaired, two-tailed t test, t = 2.557, df = 8, $P < 0.05$).

As it appeared that the Duox pathway is involved in imidacloprid toxicity, we looked at expression of Duox pathway-related genes in wild-type flies exposed to imidacloprid (**Figure 2-2F**). Canton-S flies exposed to sublethal imidacloprid displayed a significant reduction in expression of *Duox* (Mann-Whitney test, U = 2, $P < 0.001$), *p38c* (Mann-

Whitney test, U = 7, $P < 0.01$), and *MAP kinase phosphatase 3* (*Mkp3*; Mann-Whitney test, U = 12, $P < 0.05$). These flies also displayed no change in *Cadherin 99C* (*Cad99C*; Mann-Whitney test, U = 39.5, $P = 0.9528$) expression.

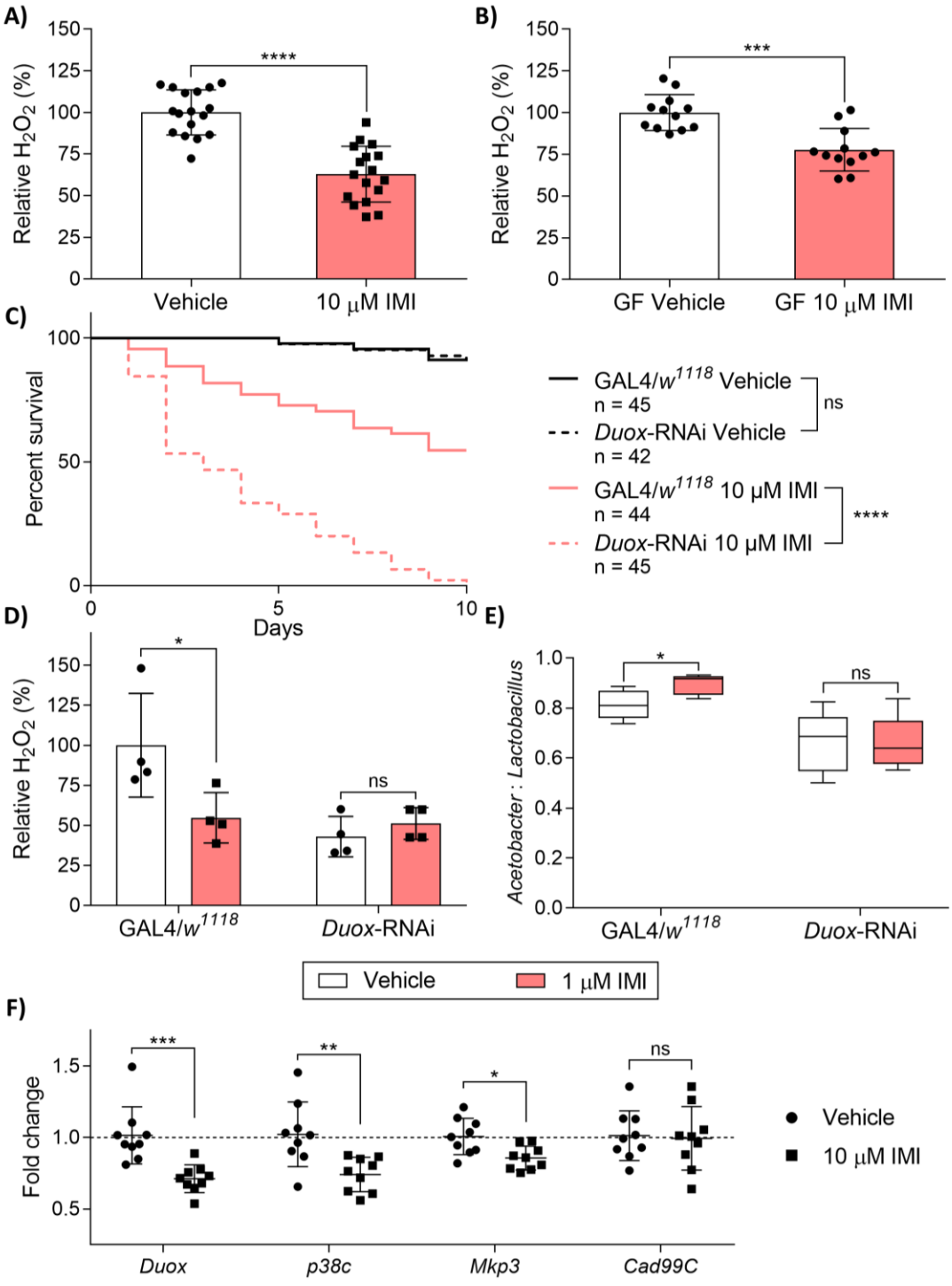

Figure 2-2. Imidacloprid exposure affects Duox-mediated H_2O_2 production in *Drosophila melanogaster*.

Whole body H_2O_2 concentrations of three female flies was measured using Amplex Red and normalized to total protein. **(A – B)** Three to five-day old conventional WT Canton-S

flies **(A)** and germ-free (GF) WT Canton-S **(B)** were placed on vehicle (DMSO) or imidacloprid (IMI; 10μM) for five days. Data displayed as mean relative H_2O_2 (%) ± SD (unpaired, two-tailed t test) of 17 biological replicates and 12 biological replicates (each consisting of 3 flies), respectively. **(C)** Survival curves for GAL4/w^{1118} and *Duox*-RNAi on imidacloprid (10μM) or vehicle (DMSO) for 5 days. Data are displayed from at least 3 independent experiments (n = 15 – 25 for each group). Statistical analyses are shown from log-rank (Mantel-Cox) tests. **(D – E)** Three to five-day old GAL4/w^{1118} and *Duox*-RNAi were exposed to 1 μM imidacloprid. **(D)** Whole body H_2O_2 concentrations of three female flies was measured from flies exposed for 5 – 7 days. Data points represent mean relative H_2O_2 (%) ± SD (Mann-Whitney tests) compared to GAL4/w^{1118} of 4 biological replicates (each consisting of 3 flies). **(E)** CFU enumeration of *Acetobacter* spp. : *Lactobacillus* spp. from flies exposed for 24 hours. Flies were surface sterilized and plated on MAN agar for *Acetobacter* spp. and MRS agar for *Lactobacillus* spp.. Data are displayed as mean *Acetobacter* spp. CFU divided by total bacteria (*Acetobacter* spp. CFU + *Lactobacillus* spp.) CFU ± SD (unpaired, two-tailed t tests) of 5 biological replicates, each consisting of 3 flies. **(F)** Gene expression of *Duox*, *p38c*, *Mkp3*, and *Cad99C* in WT Canton-S flies exposed to imidacloprid (10μM) or vehicle (DMSO) for 5 days. Data points are displayed as mean fold change (relative to *RpLP0*) of 5 pooled female flies in each group (n = 9). Error bars represent mean ± SD (Mann-Whitney test). In box plot diagrams, boxes represent first and third quartile values while black lines denote medians. Whiskers encompass maximum and minimum values. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. ns = not significant.

2.4.3 Imidacloprid disrupts *Duox* expression via dysregulation of the IMD pathway

To understand how imidacloprid affects the expression of *Duox* and H_2O_2 generation, *norpA*^{7} (PLC-β$^{-/-}$) flies were exposed to 10 μM imidacloprid with no resultant change (Mann-Whitney test, U = 27, P = 0.6454) in *Duox* expression (**Figure 2-3A**). These flies also demonstrated no significant difference (unpaired, two-tailed t test, t = 0.4027, df = 12, P = 0.6943) in whole-body H_2O_2 (**Figure 2-3B**).

Cross talk between the IMD and Duox pathways allows for co-regulation of these two pathways. In particular, these two pathways converge on p38c, which is activated by the IMD pathway and regulates *Duox* transcription (34). Therefore, the potential of imidacloprid to interfere with the cross-talk between these pathways was assessed. The R156 *imd*^{1} (IMD$^{-/-}$) flies were first exposed to imidacloprid and no significant difference was found (Mann-Whitney test, U = 21, P = 0.7104) in *Duox* expression (**Figure 2-3C**) or total-body H_2O_2 concentrations (unpaired, two-tailed t test, t = 1.388, df = 18, P = 0.1821; **Figure 2-3D**). Investigating upstream in the IMD pathway signalling cascade, *PGRP-LE*^{112} (PGRP-LE$^{-/-}$) flies were exposed to 10 μM imidacloprid or vehicle, again with no resultant significant difference (Mann-Whitney test, U = 23, P = 0.3823) in *Duox* expression (**Figure 2-3E**) and no significant difference in total body H_2O_2 (unpaired, two-tailed t test, t = 1.015, df = 22, P = 0.3212; **Figure 2-3F**). The *PGRP-LC*$^{\Delta E}$ (PGRP-LC$^{-/-}$) flies were exposed to 10 μM imidacloprid or vehicle, and this did show a significant decrease (Mann-Whitney, U = 0, P < 0.001) in *Duox* expression in imidacloprid-exposed flies (**Figure 2-3G**) and a significant reduction (unpaired, two-tailed t test, t = 2.199, df = 18, P < 0.05) in total-body H_2O_2 (**Figure 2-3H**).

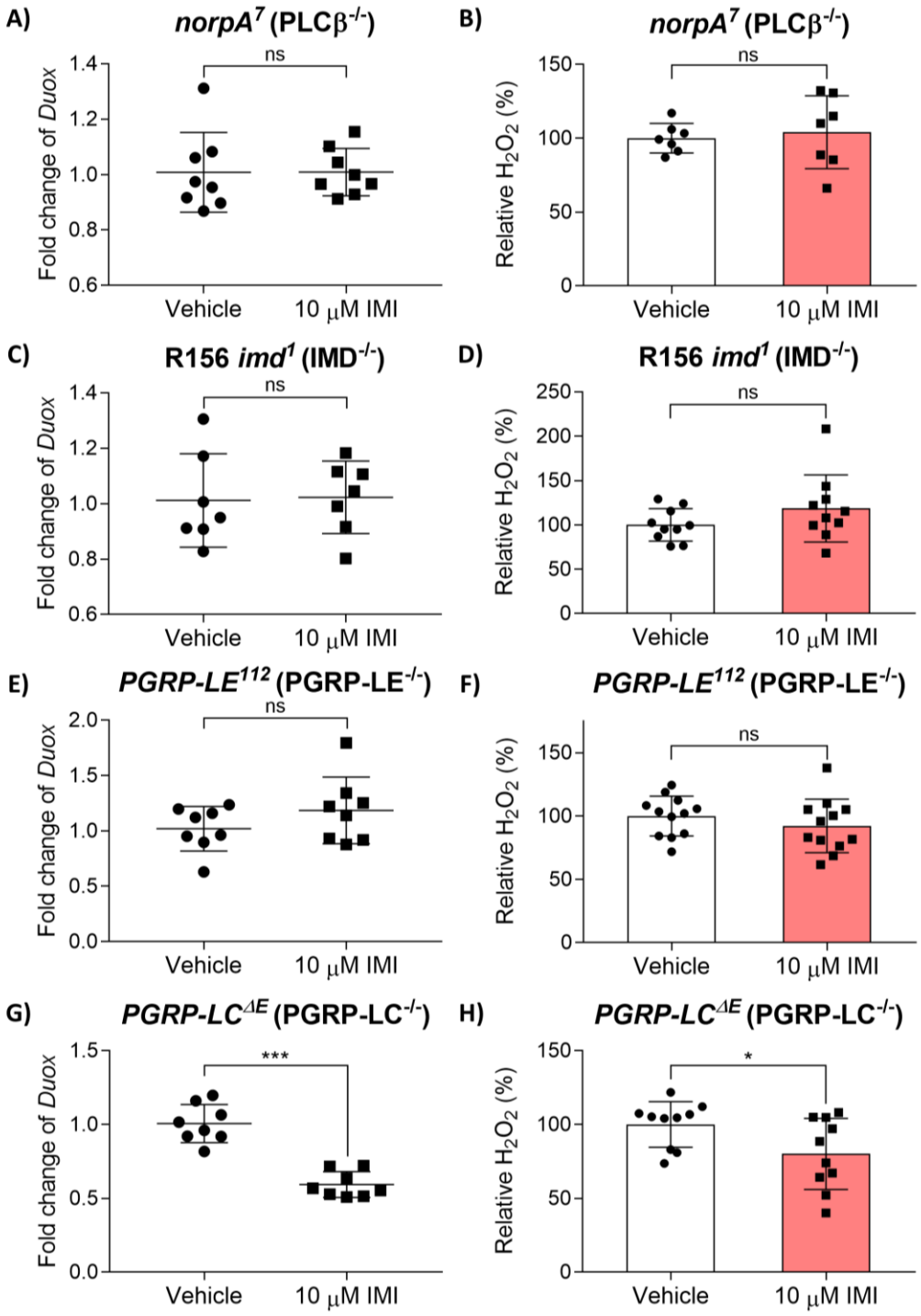

Figure 2-3. Imidacloprid impairs Duox pathway expression via the IMD pathway.

(A-B) *norpA*^{7} (PLCβ$^{-/-}$) flies exposed to 10μM imidacloprid (IMI) or vehicle (DMSO) for 5 days. **(A)** *Duox* gene expression data points are displayed as mean fold change (relative to *RpLP0*) of 8 biological replicates with 5 pooled female flies in each group. Error bars

represent mean ± SD (Mann-Whitney test). **(B)** Whole body H_2O_2 displayed as mean relative H_2O_2 (%) ± SD (unpaired, two-tailed t test) of 7 biological replicates (each consisting of 3 flies). **(C-D)** R156 *imd*^{1} (IMD$^{-/-}$) flies exposed to 10μM imidacloprid or vehicle (DMSO) for 5 days. **(C)** *Duox* gene expression data points are displayed as mean fold change (relative to *RpLP0*) of 7 biological replicates with 5 pooled female flies in each group. Error bars represent mean ± SD (Mann-Whitney test). **(D)** Whole body H_2O_2 displayed as mean relative H_2O_2 (%) ± SD (unpaired, two-tailed t test) of 10 biological replicates (each consisting of 3 flies). **(E-F)** *PGRP-LE*^{112} (PGRP-LE$^{-/-}$) flies exposed to 10μM imidacloprid or vehicle (DMSO) for 5 days. **(E)** *Duox* gene expression data points are displayed as mean fold change (relative to *RpLP0*) of 8 biological replicates with 5 pooled female flies in each group. Error bars represent mean ± SD (Mann-Whitney test). **(F)** Whole body H_2O_2 displayed as mean relative H_2O_2 (%) ± SD (unpaired, two-tailed t test) of 12 biological replicates (each consisting of 3 flies). **(G-H)** *PGRP-LC*$^{\Delta E}$ (PGRP-LC$^{-/-}$) flies exposed to 10μM imidacloprid or vehicle (DMSO) for 5 days. **(G)** *Duox* gene expression data points are displayed as mean fold change (relative to *RpLP0*) of 8 biological replicates with 5 pooled female flies in each group. Error bars represent mean ± SD (Mann-Whitney test). **(H)** Whole body H_2O_2 displayed as mean relative H_2O_2 (%) ± SD (unpaired, two-tailed t test) of 10 biological replicates (each consisting of 3 flies). In box plot diagrams, boxes represent first and third quartile values while black lines denote medians. Whiskers encompass maximum and minimum values.* $p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$. ns = not significant.

2.4.4 *Lactobacillus rhamnosus* GR-1 supplementation mitigates imidacloprid induced impairment of the Duox pathway in *Drosophila melanogaster*

To assess if human probiotic strain *Lactobacillus rhamnosus* GR-1 (LGR-1) would be a suitable supplement, its ability to survive in culture with the addition of imidacloprid was tested. There were no apparent differences in the growth profile of LGR-1 grown in MRS supplemented with 100 μM imidacloprid compared to growth in MRS alone (**Figure 2-4A**). The LGR-1 was not able to significantly reduce the concentration of imidacloprid when grown *in vitro* (Mann-Whitney test, U = 6, $P = 0.6857$; **Figure 2-4B**).

Wild-type (WT) Canton-S were pre-supplemented with LGR-1 or phosphate buffered saline (PBS) for 48 hours, then placed on vehicle (dimethyl sulfoxide [DMSO]) or 10 μM imidacloprid to assess the ability of the bacterium to mitigate the sublethal effects of imidacloprid. When LGR-1 supplemented WT Canton-S flies were exposed to a sublethal concentration (10 μM) of imidacloprid, they showed no change in the gut *Acetobacter* spp. to *Lactobacillus* spp. ratio (unpaired, two-tailed t test, t = 0.7744, df = 17, $P = 0.4493$; **Figure 2-5A**). The PBS-supplemented flies showed a significant increase in *Acetobacter* spp. (unpaired, two-tailed t test, t = 4.215, df = 16, $P < 0.001$; **Figure 2-5A**). Looking at the Duox pathway, LGR-1 supplemented flies fed sublethal imidacloprid demonstrated no significant difference in *Duox* expression (Mann-Whitney test, U = 20, $P = 0.5962$; **Figure 2-5B**) and H_2O_2 (Mann-Whitney test, U = 68, $P = 0.2800$; **Figure 2-5C**) compared with LGR-1 supplemented vehicle exposed flies. As seen with previous experiments, PBS-supplemented flies exposed to imidacloprid showed reduced *Duox* expression (Mann-Whitney test, U = 2, $P < 0.05$; **Figure 2-5B**) and reduced H_2O_2 (Mann-Whitney test, U = 8, $P < 0.0001$; **Figure 2-5C**) compared to PBS-supplemented vehicle flies.

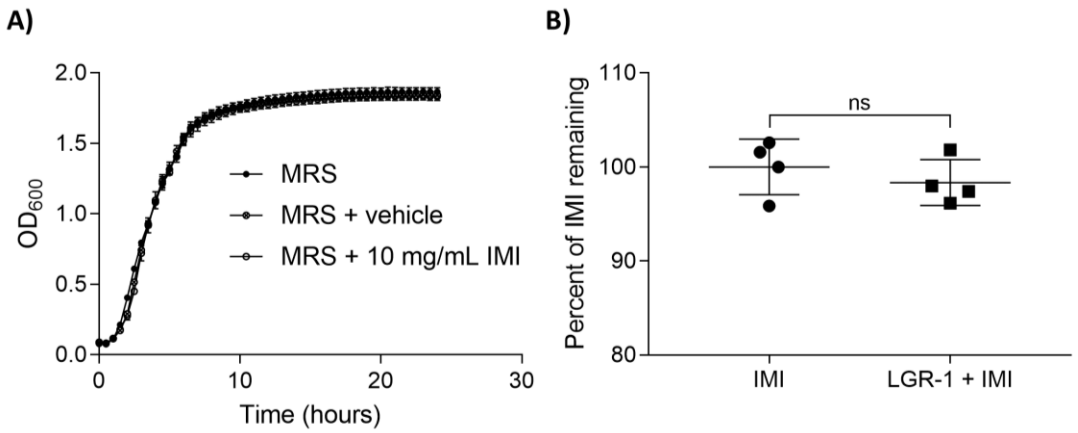

Figure 2-4. LGR-1 can survive with imidacloprid but not remove it from solution.

Growth curve of LGR-1 in MRS and MRS supplemented with Vehicle (DMSO) or 10 mg/mL imidacloprid (IMI). Data points are depicted as means ± SD of 3 biological replicates. **(B)** Percent imidacloprid remaining in culture of LGR-1 grown in minimal media with yeast extract for 24 hours. Data are displayed as mean percent imidacloprid remaining ± SD of 4 biological replicates (Mann-Whitney test). * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. ns = not significant.

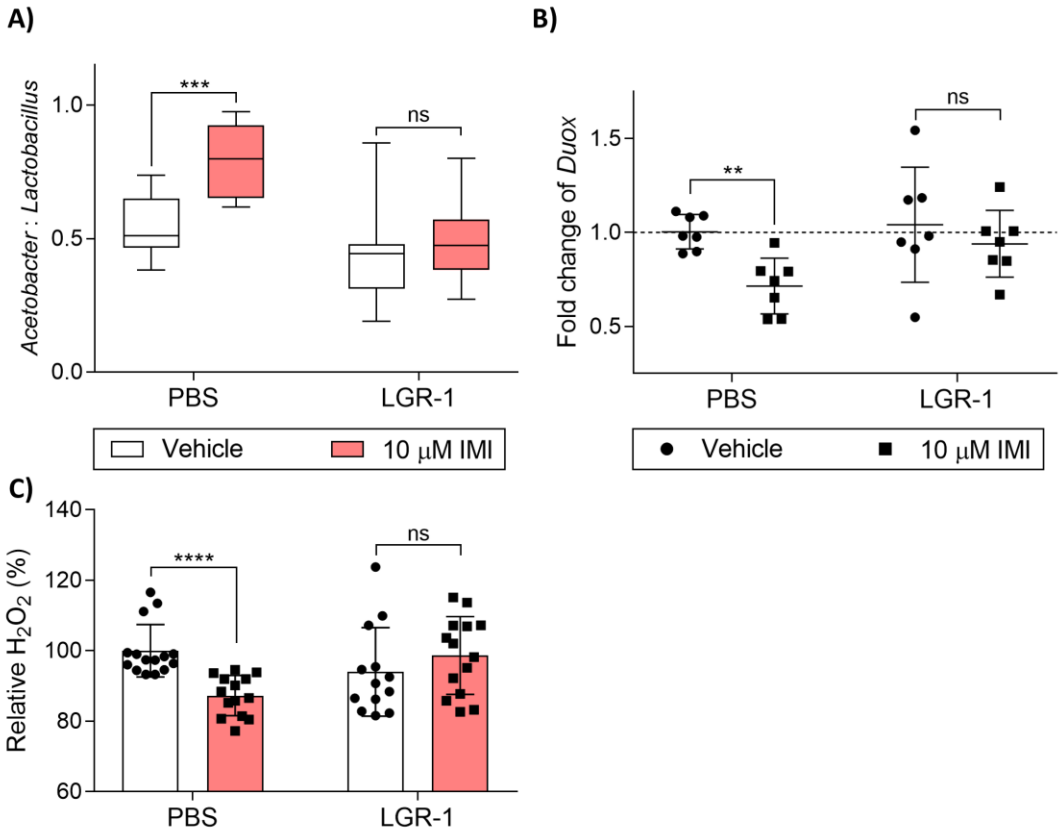

Figure 2-5. Probiotic supplementation improves immunosuppression of *Drosophila melanogaster* exposed to imidacloprid.

(A) CFU enumeration of *Acetobacter* spp. : *Lactobacillus* spp.. Flies were surface sterilized and plated on MAN agar for *Acetobacter* spp. and MRS agar for *Lactobacillus* spp.. Data are displayed as mean *Acetobacter* spp. CFU divided by total bacteria (*Acetobacter* spp. CFU + *Lactobacillus* spp.) CFU ± SD (unpaired, two-tailed t tests) of 10 biological replicates (PBS Vehicle), 8 biological replicates (PBS 10 μM imidacloprid; IMI), 9 biological replicates (LGR-1 Vehicle), and 10 biological replicates (LGR-1 10 μM imidacloprid), each consisting of 3 flies. **(B)** *Duox* gene expression displayed as mean fold change (relative to *RpLP0*) of 7 biological replicates with 5 pooled female flies in each group. Error bars represent mean ± SD (Mann-Whitney tests). **(C)** Whole body H_2O_2 displayed as mean relative H_2O_2 (%) ± SD (Mann-Whitney tests) compared to PBS vehicle of 15 biological replicates (PBS Vehicle), 14 biological replicates (PBS 10 μM imidacloprid), 13 biological replicates (LGR-1 Vehicle), and 14 biological replicates (LGR-1 10 μM imidacloprid), each consisting of 3 flies. In box plot diagrams, boxes represent first and third quartile values while black lines denote medians. Whiskers encompass maximum and minimum values. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. ns = not significant.

2.5 Discussion

This study demonstrated that sublethal imidacloprid exposure interferes with the Duox pathway in *D. melanogaster*. Imidacloprid-induced immunosuppression was observed by an increase in total bacteria and yeast, which has been associated with impaired Duox (22) and IMD (29) pathway function. There was a shift in the gut microbiota from a homeostatic balance of *Lactobacillus* spp. and *Acetobacter* spp. towards an *Acetobacter*-dominated gut microbiota upon exposure to imidacloprid. However, this was not the case for *Duox*-RNAi flies exposed to imidacloprid, indicating that the Duox pathway may be critical for mediating the gut- perturbing effects of imidacloprid. *Acetobacter* colonization has been attributed to triacylglyceride reduction (35) and shortening of lifespan in *D. melanogaster* (36). Furthermore, *Acetobacter* spp. are known to accelerate larval development via increased insulin signalling (37), which has coined the idea that colonization with *Acetobacter* confers a "live fast, die young" lifestyle (36).

Hydrogen peroxide and other reactive oxygen species (ROS) are essential molecules generated by the immune system to control gut homeostasis (38). The H_2O_2 was reduced in both GF and conventional WT Canton-S flies exposed to imidacloprid, which suggests that imidacloprid is directly interacting with the host to elicit Duox impairment and that this effect is not a result of an altered microbiota. Corroborating this, honey bee hemocytes exposed to imidacloprid show reduced H_2O_2 levels *in vitro* (39). Despite the potential regulatory interactions that occur between different microbial species, reduced H_2O_2 levels in the lumen of the intestinal tract are suspected to be the most likely candidate responsible for the observed shift in the gut microbiota. Interestingly, Duox pathway knockout flies have increased amounts of *Acetobacter* (16), further supporting the role of Duox in controlling Gram-negative spp. in the gut. Given that many lactobacilli are inherently resistant to ROS (40), we propose that reduced H_2O_2 levels during imidacloprid exposure would permit the growth of ROS-susceptible organisms (like *Acetobacter* spp.), and thereby reduce the relative abundance of *Lactobacillus* spp. via competitive exclusion.

Reactive oxygen species are a product of many metabolic processes in *D. melanogaster*; therefore, it is important to confirm that imidacloprid is impairing Duox pathway

production of ROS and not one of the other generators of ROS. There was no significant difference between H_2O_2 concentration of *Duox*-RNAi flies exposed to imidacloprid and vehicle, which suggests the Duox pathway is affected by imidacloprid exposure. Corroborating these findings that show reduced *Duox* expression by imidacloprid, it appears that the decrease in H_2O_2 observed in imidacloprid-exposed WT Canton-S flies is a result of decreased *Duox* expression and is not mediated through direct impairment of the DUOX protein. Furthermore, activation-related components of the Duox pathway appear to be unaffected by imidacloprid. In particular, *Cadherin 99C* (*Cad99C*) expression, which has been shown to be induced by uracil (activator of Duox pathway) (21), remained unchanged between vehicle- and imidacloprid-exposed WT flies. In essence, it appears that Duox pathway functionality is intact, but expression is reduced, thus leading to reduced H_2O_2.

The Duox pathway is regulated by its own activation (22) and at the expression level by the IMD pathway (20). Since *Duox* expression was reduced, experiments were performed to determine how imidacloprid affects Duox pathway signalling. Expression of *Mkp3* (negative regulator of *Duox* expression) (20) and *p38c* (activator of ATF2 transcription factor leading to *Duox* transcription) (34) was reduced in imidacloprid-exposed flies. Moreover, there was no change in *Cad99C* (regulated by hedgehog signalling and associated with Duox pathway activation) (41). These results suggest that expression of *Duox* is not being inhibited by a negative regulator, nor by inadequate activation, but is impaired at the level of transcriptional activation of *Duox*. PLC-β knockout ($norpA^7$) flies exposed to imidacloprid showed no change in *Duox* expression or H_2O_2 concentration, likely because it functions downstream of Duox. Therefore, imidacloprid is not directly acting on the Duox pathway to cause reduced *Duox* gene expression.

The IMD pathway was investigated because it can modulate *Duox* expression through peptidoglycan-dependent activation of p38 (20, 42). The R156 imd^1 ($IMD^{-/-}$) flies exposed to imidacloprid showed no change in *Duox* expression or H_2O_2 concentrations compared with vehicle-exposed flies. These flies lack a functional IMD protein; therefore, the absence of a change in *Duox* expression and H_2O_2 in imidacloprid-exposed *Drosophila* suggests that the IMD pathway is involved in mediating imidacloprid-

induced suppression of *Duox*. The IMD pathway activation is achieved by peptidoglycan recognition receptors PGRP-LC and PGRP-LE. PGRP-LC mainly functions in the foregut, hindgut, and fat body as a surface receptor found on the impenetrable cuticle (43). The PGRP-LE functions primarily in the midgut as an intracellular receptor that binds molecules that cross the permeable peritrophic matrix (43, 44). The PGRP-LC$^{-/-}$ flies exposed to imidacloprid showed a reduction in *Duox* expression and H_2O_2 levels, indicating that imidacloprid is not acting through this receptor to impair the Duox pathway. Rather, PGRP-LE$^{-/-}$ flies exposed to imidacloprid showed no change in *Duox* expression and no change in H_2O_2 concentration, indicating that imidacloprid may be acting through PGRP-LE to hinder the Duox pathway. Given the interconnectedness of the two pathways, this makes sense as both the Duox pathway and PGRP-LE function to control gut immunity (28, 44).

In brief, the data indicate that imidacloprid is interacting with the IMD pathway in the gut, thereby influencing the Duox pathway by reducing *Duox* expression and H_2O_2 generation. These results are corroborated by studies showing that neonicotinoids interfere with NF-κB signalling and increase susceptibility to pathogen challenge in *D. melanogaster* and honey bees (12, 29, 45).

Supplementation with LGR-1 restored the balance in the gut microbiota and mitigated imidacloprid-induced changes in the Duox pathway. Despite the ability of LGR-1 to inherently produce ROS (46), its effectiveness is likely attributed to its role in stimulating the host immune system. Gram-positive bacteria can be detected by PGRP-SD (47), which in turn can activate PGRP-LE and the subsequent IMD pathway (48). This activation of the IMD pathway can lead to p38-dependent Duox pathway expression (34), thereby alleviating the immune impairment induced by imidacloprid. Notably, LGR-1 is not able to metabolize or sequester imidacloprid thus promoting the notion of immune stimulation. Though it is cautionary to directly extrapolate the *Drosophila* findings to honey bees, similarities in immune response to neonicotinoids (49) and bacterial probiotics (50) suggests that lactobacilli supplementation could bolster honey bee resistance to neonicotinoids.

In summary, this study shows that (i) exposure to imidacloprid causes loss of microbial regulation by increasing Gram-negative bacteria and yeast, both regulated primarily by the Duox pathway; (ii) imidacloprid exposure impairs *Duox* expression leading to reduced antimicrobial H_2O_2; (iii) imidacloprid-induced Duox pathway impairment might be acting through the IMD pathway in the midgut; and (iv) LGR-1 supplementation mitigates imidacloprid-mediated Duox pathway impairments. Further work is merited on understanding the mechanism in which imidacloprid interferes with the IMD pathway, investigating how lactobacilli mitigate imidacloprid-induced suppression of Duox, and extending our findings to off-target species like honey bees.

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2.8 Supplementary

Table 2-1. qPCR primers used in this study.

Primer	Sequence	Amplicon size (bp)	Efficiency
Dros_rt_1 (*Drosophila* actin gene) (51)	F: 5’ GGAAACCACGCAAATTCTCAGT 3’ R: 5’ CGACAACCAGAGCAGCAACTT 3’	140	1.96
Universal bacterial primer (52)	F: 5’ ACTCCTACGGGAGGCAGCAGT 3’ R: 5’ ATTACCGCGGCTGCTGGC 3’	172	1.85
Acetobacter spp. (51)	F: 5’ TAGTGGCGGACGGGTGAGTA 3’ R: 5’ AATCAAACGCAGGCTCCTCC 3’	134	1.96
Lactobacillus spp. (51)	F: 5’ AGGTAACGGCTCACCATGGC 3’ R: 5’ ATTCCCTACTGCTGCCTCCC 3’	108	1.98
RpLP0 (29)	F: 5’ CCGAAAAGTCTGTGCTTTGTTCT 3’ R: 5’ CGCTGCCTTGTTCTCCCTAA 3’	83	1.85
Duox (this study)	F: 5’ CATGCGCTCCTTCCACAATG 3’ R: 5’ CACCAAGAAGAAACAGCCGC 3’	146	1.82
p38c (34)	F: 5’ TACCTATCGCGAGATCCGTCT 3’ R: 5’ ATGTACTTCAGTCCCCGCAGT 3’	225	1.84
Mkp3 (20)	F: 5’ GTGACGCTCGCCTACTTGAT 3’ R: 5’ GAAGTGGAAGTTGGGCGATA 3’	102	1.82
Cad99C (21)	F: 5’ TCTTCGTGAAGCCAGTGGAC 3’ R 5’ ACGATAGCGGGTTACCGTGC 3’	123	1.84

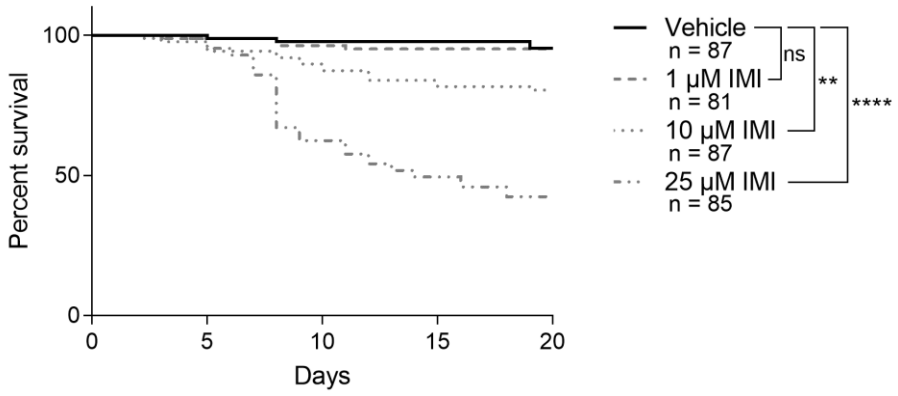

Figure 2-6. Determination of sublethal imidacloprid dose for *w*^{1118} flies.

Three to five-day old *w*^{1118} flies were exposed to vehicle (DMSO) or various concentrations of imidacloprid (IMI) to assess the sublethal dose. Data are displayed from at least 3 independent experiments (n = 25 – 30 for each group). Statistical analyses are shown from log-rank (Mantel-Cox) tests. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. ns = not significant.

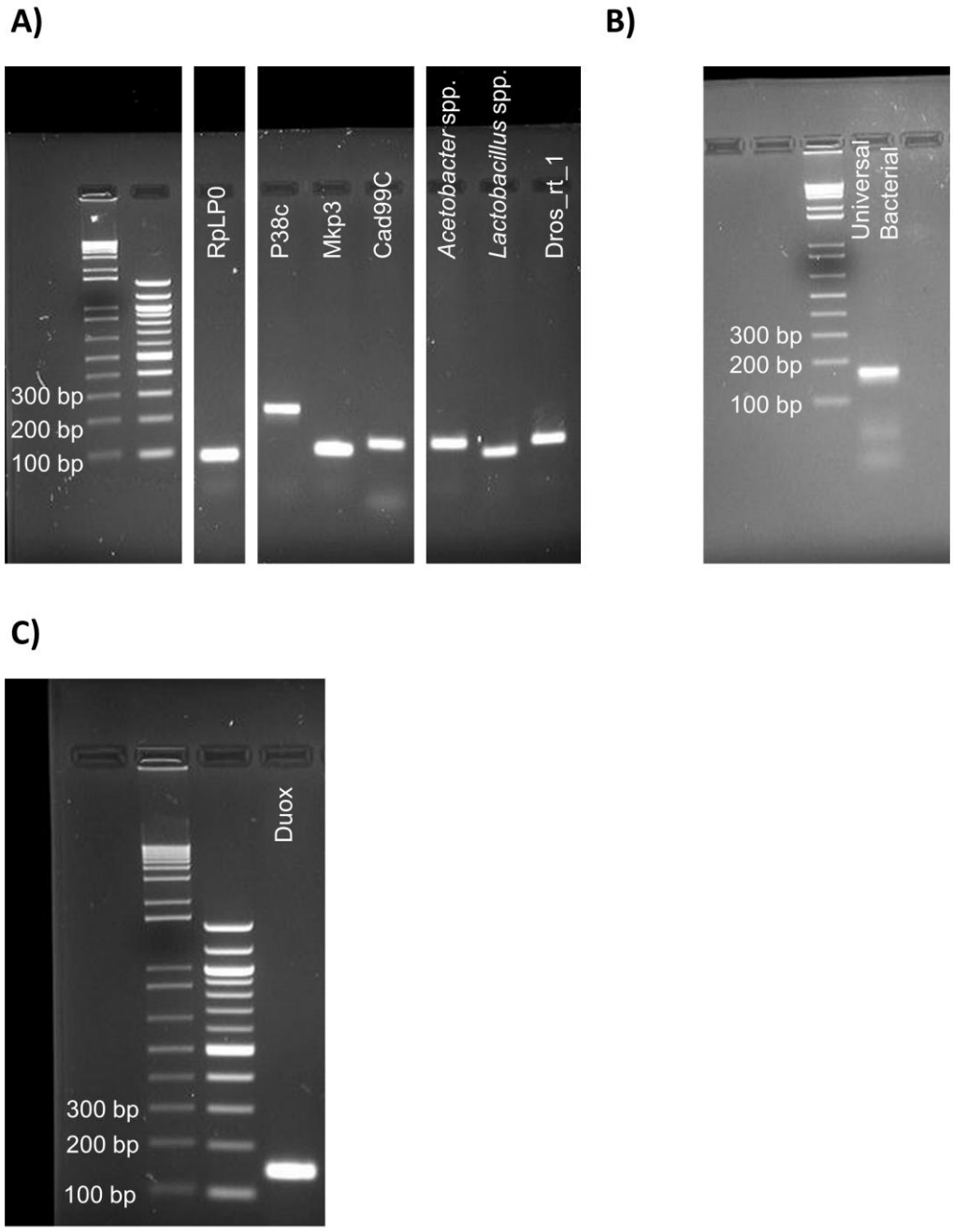

Figure 2-7. Gel electrophoresis verification of qPCR primer specificity.

(A) Primers shown: RpLP0, p38c, Mkp3, Cad99C, *Acetobacter* spp., *Lactobacillus* spp., and Dros_rt_1 (*Drosophila* actin). **(B)** Primer shown: Universal Bacterial primer. **(C)** Primer shown: Duox.

Chapter 3

3 Imidacloprid impairs nitric oxide-mediated antimicrobial peptide production in *Drosophila melanogaster*

3.1 Abstract

The extensive use of neonicotinoid insecticides in modern agriculture is the primary strategy used to control pests and improve crop yield. However, incidental exposure of beneficial pollinators (e.g. honey bees) with these agricultural insecticides has been speculated to be a leading causal factor in the pollinator population decline by dysregulating their immune system and altering the microbiota. Using a *Drosophila melanogaster* model, experiments were undertaken to understand how commonly used neonicotinoid insecticide, imidacloprid, interferes with the insect innate immune system and if probiotic bacteria can improve tolerance to the pesticide. The hypothesis was that imidacloprid exposure alters nitric oxide (NO) signalling and that a three-strain probiotic combination (LX3), which has been shown to improve honey bee immunity, is able to alter the toxicity of imidacloprid in a *D. melanogaster* model. The results demonstrated that imidacloprid does not directly affect the microbes that are found in the gut of *D. melanogaster*, which further exemplifies that neonicotinoids induce immune impairment. Imidacloprid exposure reduced NO generation in flies, which leads to a reduction in antimicrobial peptide (AMP) generation. In an oral infection model, *D. melanogaster* exposed to both imidacloprid and oral pathogen insult displayed reduced survival compared to either treatment on their own, suggesting that the change in AMP production affects pathogen clearance in the flies. Together, these results indicate that imidacloprid is reducing the amount of NO in adult *D. melanogaster*, which reduces AMP generation, ultimately leading to immune impairment. We also found that the three-strain probiotic combination did not mitigate lethal imidacloprid toxicity. By better understanding the pernicious effects of pesticides on the immune system of pollinators and evaluating potential solutions to combat honey bee population decline, strategies can be developed to improve the long-term survival of these critical insect species.

3.2 Introduction

Honey bees (*Apis mellifera*) are critical to maintaining an adequate food supply for the growing global population. Through pollination services alone, these insects contribute approximately $225 billion (USD) annually to the global economy and aid in the production of almost a third of the global food supply (1, 2). Despite their importance, honey bee populations are continuing to decline, with pesticide exposure being a prominent contributor to these losses (3). While it is evident that pesticides can impair immune pathways (and ultimately increase pathogen burden), there is limited support to understand the role of pesticides on the microbiota of honey bees (4). In honey bees, the microbiota is important for immunity (5), behaviour (6), metabolic function (7), and overall health (8). Furthermore, 'thriving' hives appear to have higher levels of *Lactobacillus* and *Bifidobacterium* compared to 'non-thriving' hives (9). Evident by these observations, the microbiota is an essential aspect of honey bee health. Though research is limited, the majority of studies have demonstrated that exposure to neonicotinoid pesticides alters the microbiota of honey bees (10–12).

Neonicotinoids are a controversial class of neuro-active insecticides that are routinely used in modern agricultural practices. These chemicals have been implicated in the decline of honey bees and other pollinators, and are highly regulated throughout the world (13, 14). Notably, imidacloprid is one of the most studied neonicotinoids; despite the documented adverse effects of this chemical, it is still used today.

The inherent difficulties of working with honey bees can be circumvented through the use of a *Drosophila melanogaster* model of insect toxicity. The combination of the genetic tractability of this established model and the ability to do high-throughput experimentation allows for mechanistic analyses of insect physiology and host-microbe interactions (15). Exemplifying the similarities between *D. melanogaster* and honey bees, the gut microbiota of *D. melanogaster* is also altered upon neonicotinoid exposure (16, 17). These two species maintain some similarities in the composition of their gut microbiota, albeit with *D. melanogaster* fostering a simpler microbiota. Honey bees harbour an established set of core microbes, which include *Lactobacillus* Firm-5, *Lactobacillus* Firm-4, *Bifidobacterium* spp., *Gilliamella apicola*, and *Snodgrassella alvi*

(18). They also possess varying amounts of *Frischella perrara*, *Bartonella apis* (alphaproteobacteria), and some members of the Acetobacteraceae family (18, 19). On the other hand, *D. melanogaster* are primarily colonized by bacteria from the Lactobacillaceae (*Lactobacillus* sp.) and Acetobacteraceae (*Acetobacter*, *Gluconobacter*, and *Commensalibacter* spp.) families (20).

Xenobiotic-induced disturbances to the microbiota in *D. melanogaster* could have a multitude of explanations; the most probable being innate immune pathway impairments. Previous work has shown that imidacloprid impairs the Dual oxidase (Duox) pathway by acting through the immune deficiency (IMD) pathway (17). The Duox pathway is responsible for first-line gut defence, and it produces antimicrobial hydrogen peroxide (H_2O_2) to control invading microbes. However exposure to imidacloprid reduces the production of H_2O_2 (17, 21). Hydrogen peroxide also acts as a signalling molecule, particularly for nitric oxide (NO) signalling and subsequent IMD pathway activation in distant organs (22). Upon reactive oxygen species (ROS) generation from pathogen assault, *NOS* (*nitric oxide synthase*) is upregulated by epithelial cells in the gut and produces NO in a Ca^{2+}-dependant reaction that utilizes L-arginine (23, 24). The NO then triggers the production of Relish-dependent (NF-κB) antimicrobial peptides (AMPs) in the fat body by relaying the signal through the hemocytes (22, 23). The fat body of *D. melanogaster* is analogous to the mammalian liver, and functions as a detoxifying and immune response organ (25, 26). It is unknown if the imidacloprid-induced impairment of the Duox pathway, which reduces H_2O_2, might also contribute to reduced NO signalling and subsequent AMP expression.

While disruptions to insect immunity are a compelling source of these microbiota changes, it is important to consider the xenobiotic-microbe interactions that may also occur. Many bacteria and yeast are able to metabolize neonicotinoids (27–30) or are harmed by its presence (31), which could explain the change in microbial composition. Of interest to both honey bees and *D. melanogaster*, growth of an *Acetobacter* sp. has been shown to increase in the presence of glyphosate, suggesting that this bacterium may use it as a carbon source (32). While some *Lactobacillus* spp. are able to degrade certain pesticides, (33–35), others (isolated from various origins) are not able to degrade

imidacloprid (16). It is not known if commensal *Lactobacillus* or *Acetobacter* spp. can utilize imidacloprid as a growth substance and drive changes to the microbiota.

In pursuit of a solution for honey population decline, probiotic supplementation has emerged as a practical and viable option. In particular, supplementation with the LX3 combination (*Lactobacillus plantarum* Lp39, *Lactobacillus kunkeei* BR-1, *Lactobacillus rhamnosus* GR-1) has been shown to enhance honey bee immunity and improve survival against a bacterial pathogen. This makes the LX3 combination a strong contender to mitigate the immunosuppression that is observed when honey bees are exposed to pesticides (36). In addition, bacteria are able to reduce the toxicity of xenobiotics by modulating host detoxification gene expression or through direct detoxification and sequestration of xenobiotics (35, 37, 38). Despite this, studies have not assessed if probiotics can improve neonicotinoid tolerance through either of these mechanisms.

In this study, the overall goal was to better understand the host-microbe-xenobiotic interactions in a simplified *in vivo* model. Specifically, the aim was to understand how imidacloprid interacts with the immune system and commensal microbes of *D. melanogaster*. The second aim was to test if probiotics improve tolerance to imidacloprid.

3.3 Materials and Methods

3.3.1 Chemicals

Imidacloprid (catalogue number: 37894) was obtained from Sigma. Stock solutions were prepared at 100 mg/mL in dimethyl sulfoxide (DMSO; Sigma, catalogue number: D8418) and stored protected from light at 4°C until usage. Acetonitrile (Fisher, catalogue number: A996-4) and water (Fisher, catalogue number: W5-4) used for HPLC analysis were HPLC grade.

3.3.2 *Drosophila melanogaster* husbandry

Wild-type (WT) Canton-S (stock number: 1; RRID:BDSC_1), w^{1118} (stock number: 3605; RRID:BDSC_3605), $y^{1}w^{67c23}$ (stock number: 6599; RRID:BDSC_6599), UAS-*pirk* (*pirk* overexpression; stock number: 15039; RRID:BDSC_15039), *daughterless* GAL4 (*da*-GAL4; stock number: 55850; RRID:BDSC_55850), $norpA^{7}$ (PLC-$\beta^{-/-}$; stock number:

5685, RRID:BDSC_5685), *Tak1*^{2}(TAK1$^{-/-}$; stock number: 26272; RRID:BDSC_26272) were obtained from Bloomington Drosophila Stock Center (NIH P40ODO18537) at Indiana University. *D. melanogaster* were maintained using media with 1.5% (wt/vol) agar, 1.73% (wt/vol) yeast (Sigma-Aldrich, catalogue number: 51475), 7.3% (wt/vol) cornmeal, 7.6% (vol/vol) corn syrup, and 0.58% (vol/vol) propionic acid at 25°C with 12-hour light/dark cycles. For experimental procedures, IMI media were supplemented with pesticide, and vehicle media were supplemented with dimethyl sulfoxide (DMSO) prior to agar solidification. All experiments were performed in wide polypropylene *D. melanogaster* vials (catalogue number: GEN32-121 and GEN49-101, Diamed Lab Supplies Inc., Mississauga, ON, Canada). Adult flies used for experiments were 3 to 5 days old unless otherwise stated.

UAS/GAL4 crosses were performed by mating male *da*-GAL4 with virgin female UAS-*pirk* knockdown flies or virgin female $y^{1}w^{67c23}$ flies as control. The GAL4 driver, *da*-GAL4, is an all tissue driver, which has ubiquitous GAL4 expression. Overexpression of *pirk* was found to be upregulated over 100-fold (**Supplementary, Figure 3-5**).

3.3.3 Commensal microorganism identification

Commensal microorganisms were isolated from Caton-S and w^{1118} flies. Briefly, 5 female flies were surface sterilized with 70% ethanol, added to 500 µL of PBS with four 2 mm glass beads, homogenized by bead beating in a BioSpec 3110BX Mini Beadbeater 1 (Fisher Scientific, catalogue number: NC0251414), then plated on MRS (BD Difco, catalogue number: B11059) or MAN (mannitol agar; 3 g Bacto Peptone Number:3, 5 g yeast extract, 25 g mannitol, 15 g agar, 1 L H_2O), and incubated at 30°C for up to 72 hours anaerobically and aerobically, respectively. Microorganisms were maintained on MRS, MAN, or SDA at appropriate culture conditions.

Once isolated, microorganisms were Gram-stained for initial screening and colony morphology. Bacteria were identified by sequencing the 16S rRNA gene, using the established pA/pH primers (39). Unknown isolate DNA was extracted by microwaving a small colony for 3 minutes and then adding the complete PCR master mix. Complete pA/pH master mix totaled 50 µL and was composed of 1× PCR buffer, 3 mM $MgCl_2$, 400

nM dNTP, 0.8 mg/mL bovine serum albumin, 400 nM pA, 400 nM pH, and 5 U Taq polymerase at final concentrations. Reaction conditions were as follows: initial denaturation at 95℃ for 5 minutes; followed by 35 cycles of 95℃ for 30 seconds, 55℃ for 30 seconds, and 68℃ for 1 minute; final extension at 72℃ for 10 minutes. Eukaryotic microorganisms were identified by sequencing the internal transcribed spacer (ITS) region using ITS1/ITS4 primers (40) with slight modifications. Complete ITS1/ITS4 master mix totaled 50 μL and contained 1× PCR buffer, 1.5 mM $MgCl_2$, 200 nM dNTP, 500 nM ITS1, 500 nM ITS4, and 2.5 U Taq polymerase at final concentrations. Reaction conditions were as follows: initial denaturation at 95℃ for 5 minutes; followed by 35 cycles of 95℃ for 30 seconds, 50℃ for 30 seconds, and 72℃ for 1 minute; final extension at 72℃ for 10 minutes. After bacteria and eukaryotes were sequenced, bands were gel purified and sequenced at the London Regional Genomics Centre (Robarts Research Institute, London, Canada). Identified isolates, morphology, and optimal culture conditions can be found in **Table 3-1**.

3.3.4 Commensal microorganism imidacloprid tolerance assay

Commensal microbes were grown in their particular growth conditions (**Table 3-1**). After incubation, microorganisms were subcultured (1:100) into 96-well plates (Falcon, catalogue number: 35177) containing respective growth media with vehicle (DMSO) or 100 ppm IMI. Biological replicates were plated in triplicate technical replicates. Plates were incubated at 30℃ for up to 72 hours and measured every 30 minutes at 600 nm using a microplate reader (BioTek, Eon).

3.3.5 Commensal microorganism metabolism/binding assay

High-performance liquid chromatography (HPLC) analysis of culture supernatant was employed to test if a commensal microorganism or *L. kunkeei* BR-1 was able to reduce the amount of imidacloprid in the culture supernatant. Microorganisms were grown in their respective media (**Table 3-1**) spiked with 100 ppm of IMI and incubated aerobically at their 30℃ for 24 hours, with shaking (175 rpm) and protected from light. After incubation, bacterial suspensions were centrifuged at 5,000 rpm (4,500 × *g*) for 10

minutes at room temperature. Supernatants were removed and filter sterilized using 0.45 μm filters prior to HPLC analysis.

All samples and standards were analyzed using an Agilent 1100 HPLC equipped with a degasser (G1379A), quaternary pump (G1311A), autosampler (G1313A), and diode array detector (G1315B). All analyses were performed on an Agilent Poroshell 120 EC-C18 (4.6 × 150 mm I.D., 4 μm particle size) column with a Poroshell 120 EC-C18 (4.6 mm, 4 μm particle size) guard column kept at ambient temperature. Acetonitrile (Fisher, catalogue number: A996-4) and water (Fisher, catalogue number: W5-4) used were HPLC grade. The mobile phase consisted of an isocratic mixture of acetonitrile/water (40:60 vol/vol) at a flow rate of 1.0 mL/min. Sample injection volume was 5 μL, and detection was performed at 270 nm. Run times were 5 minutes with imidacloprid eluting at ~2.4 minutes. Data were analyzed using ChemStation A. 10.02. The peak area of samples was compared with the peak area of the external calibration curve (1 – 200 ppm) to quantify imidacloprid.

3.3.6 RNA extraction and reverse transcription

Five female adult *D. melanogaster* were homogenized in 550 μL of TRIzol reagent (Ambion, catalogue number: 15596018) using eight 2 mm glass beads beating twice for 30 seconds at 4,800 rpm with a BioSpec 3110BX Mini Beadbeater 1 (Fisher Scientific, catalogue number: NC0251414). Tubes were centrifuged at 13,000 rpm for 10 minutes at 4°C to pellet debris. Supernatant was collected, and 0.2 volumes of chloroform were added, followed by centrifugation at 13,000 rpm for 15 minutes at 4°C. The upper aqueous layer was collected, and 0.7 volumes of isopropanol were added to precipitate the RNA, followed by centrifugation at 13,000 rpm for 15 minutes at 4°C. The RNA pellet was washed with 1 mL of 70% ethanol in diethyl pyrocarbonate-treated ddH_2O and centrifuged at 13,000 rpm for 15 minutes at 4°C. Following removal of the supernatant, the RNA was air-dried and then re-suspended in 30 μL of nuclease-free water. The quality of RNA was evaluated using a DeNovix DS-11 Spectrophotometer and determined to have A260/280 and A260/230 ratios between 1.7–2.2 and 1.8–2.4. cDNA was synthesized from 1,500 ng of total RNA using a High-Capacity cDNA Reverse

Transcription Kit following manufacturer's instructions (Applied Biosystems, catalogue number: 4368813).

3.3.7 qPCR analysis

Reverse transcribed cDNA was diluted 10× in nuclease-free water and used for qPCR reactions with the *Power* SYBR Green Kit (Applied Biosystems, catalogue number: 4368702). The following primers were used in this study (**Supplementary, Table 3-2**). *Diptericin A* (*DptA*) and *Defensin 1* (*Def*1) were designed in this study using Genbank sequences NM_057460.4 and NM_078948.3, respectively. For analysis of gene expression, *RpLP0* used as the endogenous reference gene because it was identified as the most stably expressed reference gene (16). The vehicle (DMSO) group was used as the calibrator in all qPCR analysis experiments. Reagent volumes for 10 µL reactions consisted of 4.5 µL of diluted cDNA, 5 µL of *Power* SYBR (2×), and 0.5 µL of forward and reverse primer mix (each at 500 nM final concertation). Reaction conditions were 50℃ for 2 minutes, then 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. qPCR was performed on a QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific) and analyzed using the associated QuantStudio Design and Analysis Software v1.4.3 (Thermo Fisher Scientific). Gene expression ($2^{-\Delta\Delta Ct}$) was calculated using fold change, and statistics were performed on the $-\Delta\Delta Ct$ values (41). PCR efficiencies were calculated using LinRegPCR version 2016.1.

3.3.8 Nitrite quantification

Nitric oxide was evaluated by measuring nitrite using the Griess reagent (Fluka, catalogue number: 03553) (42). To quantify nitrite in *D. melanogaster*, four w^{1118} female flies were homogenized in 400 µL of phosphate buffer (0.1 M phosphate [pH 7.4], 0.015 M KCl) with four 2 mm glass beads by bead beating at 7 m/s for 30 seconds. Fly homogenates were centrifuged at 10,000 × *g* for 10 minutes at 4℃. After centrifugation, 50 µL of supernatant was combined with 50 µL of Griess' reagent (Sigma, catalogue number: 03553) into a 96-well plate (Falcon, catalogue number: 35177) and incubated at room temperature in the dark for 10 minutes, then absorbance was read at 520 nm using a

microplate reader (BioTek, Eon), and compared to a standard curve of sodium nitrite (0 – 100 μM).

Nitrite quantification was normalized to total protein, which was quantified using a bicinchoninic acid (BCA) Protein Assay Kit (Invitrogen, catalogue number: 23227) Briefly, after centrifugation for the nitrite assay, 25 μL of supernatant was used for the BCA assay following the manufacturer's microplate protocol and measuring absorbance as 562 nm using a microplate reader (BioTek, Eon).

3.3.9 Buoyancy assay

A buoyancy assay was used to estimate the fat levels of larvae (43). First instar Canton-S eggs were collected and placed on vehicle (DMSO) or 10 μM imidacloprid food and allowed to develop. Once the larvae became third instar wandering larvae, they were collected and placed in 10 mL of 9% sucrose prepared in PBS. Larvae were gently mixed and allowed to equilibrate for 5 minutes. After equilibration, the number of floating larvae was enumerated.

3.3.10 Oral infection

Pathogenic bacteria, *Erwinia carotovora* subspecies *carotovora* 15 (*Ecc15*) and *Pseudomonas entomophila* DSM 28517 (*Pe*) were grown overnight in LB at 37°C shaking (150 rpm). After incubation, bacteria were washed twice with PBS, and infection inoculum was prepared by concentrating bacteria 100× in 5% sucrose with vehicle (DMSO) or 10 μM imidacloprid.

Prior to infection, adult w^{1118} flies were starved 1 hour on 1% agar then moved to a vial containing a 1% agar base with a filter disk on top immersed with 100 μL of the infection inoculum, which was replaced daily, and mortality was assessed twice per day.

3.3.11 Adult *D. melanogaster* IMI survival assays

Three- to five-day-old *D. melanogaster* were used for all survival experiments. Prior to the experimental start point, flies were gently anesthetized with CO_2 and transferred from standard rearing media to a vial containing 1% agar and starved for 60 minutes to

normalize feeding frequency. Flies were then transferred to standard media containing vehicle (DMSO) or the appropriate amount of IMI. Any early deaths (< 1 hour) were assumed to be from the transfer process and removed from subsequent analyses. Survival was monitored daily at 24-hour intervals from the experimental start point.

3.3.12 Probiotic supplementation

Three- to five-day-old Canton-S were supplemented for two days with 100 μL of PBS or LX3 (containing *L. plantarum* Lp39, *L. kunkeei* BR-1, *L. rhamnosus* GR-1), which was allowed to dry on top of fly media. LX3 was prepared as previously described (36). After supplementation, flies were directly transferred to fly food containing vehicle (DMSO) or 100 μM imidacloprid. Any early deaths (< 1 hour) were assumed to be from the transfer process and removed from subsequent analyses. Survival was monitored daily at 24-hour intervals from the experimental start point.

3.3.13 Statistical analysis

All statistical comparisons were performed using GraphPad Prism 7.0 software. Nonparametric data were statistically compared with an unpaired, two-tailed Mann-Whitney test. Data were tested for normality using the Shapiro-Wilk test for data with unique values or D'Agostino and Pearson test for data with tied values. Data with two populations that were non-parametric were analyzed using a Mann-Whitney test, while parametric data were analyzed using a t test. Data that were non-parametric and greater than two groups were analyzed with a Kruskal-Wallis test (with Dunn's multiple comparisons). Mantel-Cox tests were used to analyze overall survival data. Grehan-Breslow-Wilcoxon tests were used to assess early timepoint deaths.

3.4 Results

3.4.1 The growth of *D. melanogaster* commensal microorganisms is not affected by imidacloprid

Drosophila melanogaster microorganisms were isolated from adult Canton-S and w^{1118} *D. melanogaster* (**Table 3-1**). The main genera (*Lactobacillus* and *Acetobacter*) were the only bacteria isolated. The *Lactobacillus* genus was well represented by the two common

species: *Lactobacillus plantarum* and *Lactobacillus brevis*. However, the *Acetobacter* genus was mostly limited to *Acetobacter persici*. Unanticipated, three eukaryotic organisms were isolated from the flies, two of which were identified as the algae *Prototheca* spp. and the other isolate was identified as the yeast *Pichia manshurica*.

Bacterial isolates show no substantial growth changes when grown in the presence of 100 ppm imidacloprid (**Figure 3-1A**). *Lactobacillus* (DM-8, DM-13, and DM-18) demonstrate almost identical growth with and without imidacloprid. Other microorganisms from the Acetobacteraceae family demonstrate similar trends compared to the *Lactobacillus*. Although with the exception of DM-34 and DM-35, which show increased variability in growth.

Microorganisms from *D. melanogaster* do not appear to be able to metabolize imidacloprid (**Figure 3-1B**). *Lactobacillus* isolates (DM-8, DM-13, and DM-18) appear to have the same or more imidacloprid remaining after incubation compared to the media only control. Acetobacteraceae (DM6, DM-10, DM-23, DM-34, DM-35, and DM-36) and eukaryotic (DM-1, DM-2, and DM-3) isolates appear to have the same amount of imidacloprid remaining after incubation as their respective controls.

Table 3-1. *Drosophila melanogaster* microbial isolates.

Isolate name	Microorganism	Culture conditions		
		Temperature (°C)	Media	Oxygenation
DM-1	*Pichia manshurica*	30	MAN/SDA	Aerobic
DM-2	*Prototheca* spp.	30	MAN/SDA	Aerobic
DM-3	*Prototheca* spp.	30	MAN/SDA	Aerobic
DM-4	*Lactobacillus plantarum*	30	MRS	Anaerobic
DM-5	*Lactobacillus plantarum*	30	MRS	Anaerobic
DM-6	*Acetobacter persici*	30	MAN	Aerobic
DM-7	*Acetobacter persici*	30	MAN	Aerobic
DM-8	*Lactobacillus plantarum*	30	MRS	Anaerobic
DM-9	*Acetobacter persici*	30	MAN	Aerobic
DM-10	*Aceotbacter indonesiensis*	30	MAN	Aerobic
DM-11	*Acetobacter persici*	30	MAN	Aerobic
DM-12	N/A	30	MAN	Aerobic
DM-13	*Lactobacillus plantarum*	30	MRS	Anaerobic
DM-14	*Acetobacter cerevisiae* or *Acetobacter persici*	30	MAN/SDA	Aerobic
DM-15	*Lactobacillus plantarum*	30	MRS	Anaerobic
DM-16	*Acetobacter persici*	30	MAN	Aerobic
DM-17	*Lactobacillus plantarum*	30	MRS	Anaerobic
DM-18	*Lactobacillus brevis*	30	MRS	Anaerobic
DM-19	*Lactobacillus plantarum*	30	MRS	Anaerobic
DM-20	*Acetobacter persici*	30	MAN	Aerobic
DM-21	*Lactobacillus plantarum*	30	MRS	Anaerobic
DM-22	*Lactobacillus plantarum*	30	MRS	Anaerobic
DM-23	N/A	30	MAN	Aerobic
DM-24	*Lactobacillus plantarum*	30	MRS	Anaerobic
DM-25	*Lactobacillus brevis*	30	MRS	Anaerobic
DM-26	*Lactobacillus plantarum*	30	MRS	Anaerobic
DM-27	*Lactobacillus plantarum*	30	MRS	Anaerobic
DM-28	*Lactobacillus brevis*	30	MRS	Anaerobic
DM-30	*Lactobacillus plantarum*	30	MRS	Anaerobic
DM-31	*Lactobacillus plantarum*	30	MRS	Anaerobic
DM-32	*Lactobacillus brevis*	30	MRS	Anaerobic
DM-33	*Lactobacillus plantarum*	30	MRS	Anaerobic
DM-34	*Asaia astilbis*	30	MAN	Aerobic
DM-35	*Acetobacter indonesiensis*	30	MAN	Aerobic
DM-36	*Commensalibacter intestini*	30	MAN/SDA	Aerobic

N/A, not available; MRS, De Man, Rogosa, and Sharpe; MAN, mannitol; SDA, Sabourand dextrose agar

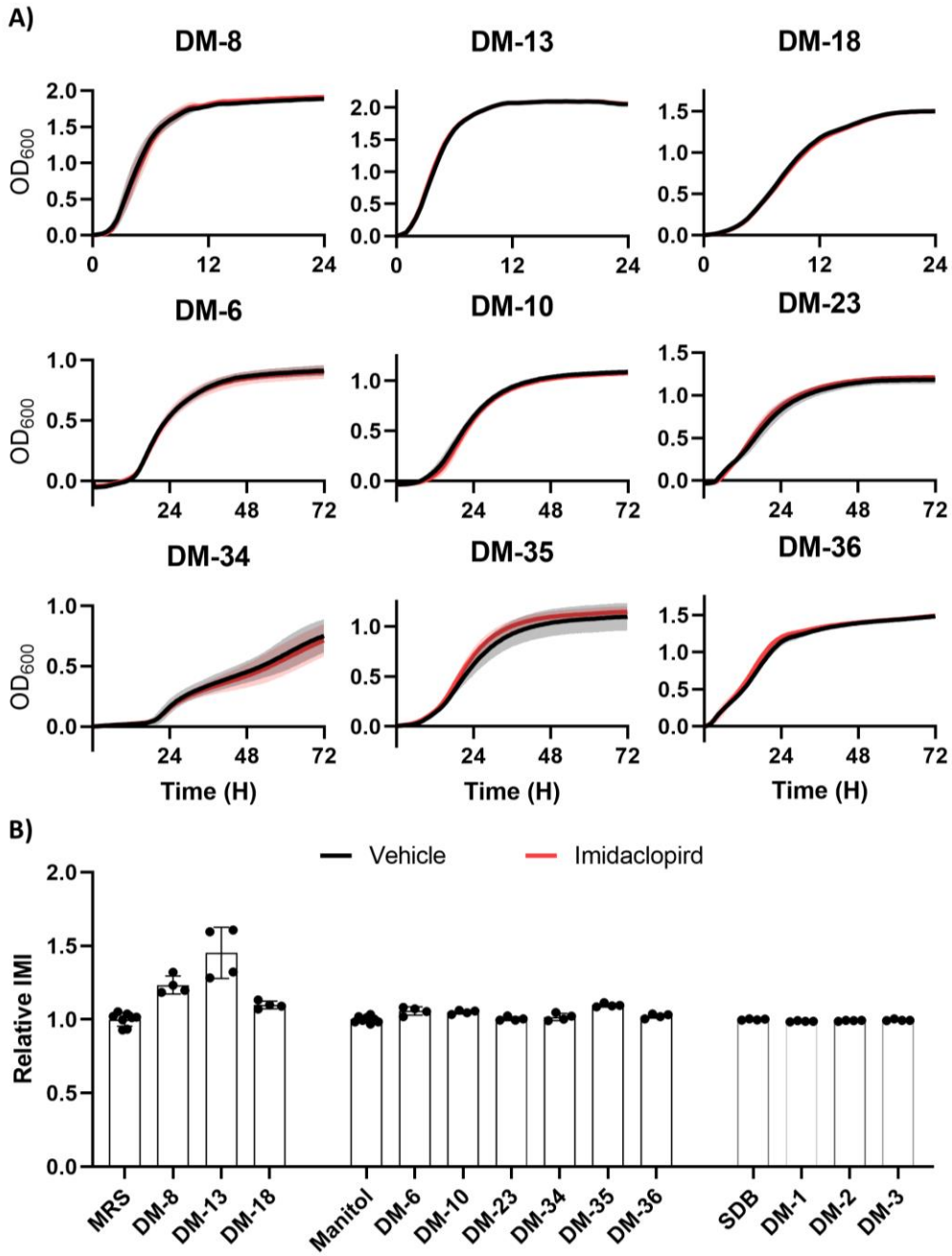

Figure 3-1. Imidacloprid does not affect the growth of commensal microorganisms.

(A) Representative growth curves of *Drosophila melanogaster* isolates in the presence of vehicle (DMSO; black solid line) or 100 ppm imidacloprid (red solid line). Data are displayed as mean OD600 (solid line) ± SD (shaded region) of four biological replicates. **(B)** Relative amount of imidacloprid (IMI) in bacterial culture supernatant following 24-hour incubation. Data are displayed as mean (amount IMI [ppm]/average amount IMI [ppm] in media) ± SD.

3.4.2 Imidacloprid exposure impairs immune response in *Drosophila melanogaster*

Gene expression of immune-related genes in Canton-S flies exposed to vehicle (DMSO) was compared to Canton-S exposed to sublethal (10 μM) imidacloprid (**Figure 3-2A**). Imidacloprid exposed flies displayed a significant decrease in *DptA* (*Diptericin A*; Mann-Whitney test, U = 12, $P < 0.05$), *Def1* (*Defensin 1*; Mann-Whitney, U = 12, $P < 0.05$), and *NOS* (*Nitric oxide synthase*; Mann-Whitney, U = 1, $P < 0.001$). They also showed a decrease in *DptB* (*Diptericin B*), although it was not significant (Mann-Whitney, U = 17, $P = 0.0745$). There was no significant change in *IRC* (*Immune regulated catalase*; Mann-Whitney, U = 36, $P = 0.7304$) or *Drs* (Mann-Whitney, U = 33, $P = 0.5457$).

To test if the decreased expression of *NOS* affects the nitric oxide response, Griess reagent was used to quantify nitrite, which is a proxy for nitric oxide (**Figure 3-2B**). *Drosophila melanogaster* exposed to imidacloprid demonstrate a significant reduction in relative nitrite (NO_2^-) (Kruskal-Wallis, $P < 0.0001$).

Fat composition can be estimated by the proportion of larvae floating in a buoyancy assay (**Figure 3-2C**). Larvae grown on 10 μM imidacloprid were significantly decreased in the percent of floating larvae compared to vehicle grown larvae (Mann-Whitney, U = 0, $P < 0.05$).

Immune parameters appeared to be compromised with exposure to imidacloprid. To investigate if these impairments increased susceptibility to oral assault, flies were given food containing 10 μM imidacloprid, with or without oral insult (**Figure 3-2D**). *Drosophila melanogaster* fed imidacloprid and challenged orally with *Ecc15*, displayed reduced overall survival (log-rank [Mantel-Cox], chi-square = 81.23, df = 1, $P < 0.0001$) and increased early timepoint deaths (Gehan-Breslow-Wilcoxon test, chi-square = 64.64, df = 1, $P < 0.0001$) compared to flies fed only imidacloprid. Similarly, *D. melanogaster* fed imidacloprid and challenged orally with *Pe* also displayed reduced overall survival (log-rank [Mantel-Cox], chi-square = 81.68, df = 1, $P < 0.0001$) and increased early timepoint deaths (Gehan-Breslow-Wilcoxon test, chi-square = 64.32, df = 1, $P < 0.0001$) compared to flies fed only imidacloprid. It should be noted that flies fed imidacloprid

demonstrated reduced overall survival (log-rank [Mantel-Cox], chi-square = 9.800, df = 1, $P < 0.01$) and increased early timepoint deaths (Gehan-Breslow-Wilcoxon test, chi-square = 9.780, df = 1, $P < 0.01$) compared to flies fed vehicle.

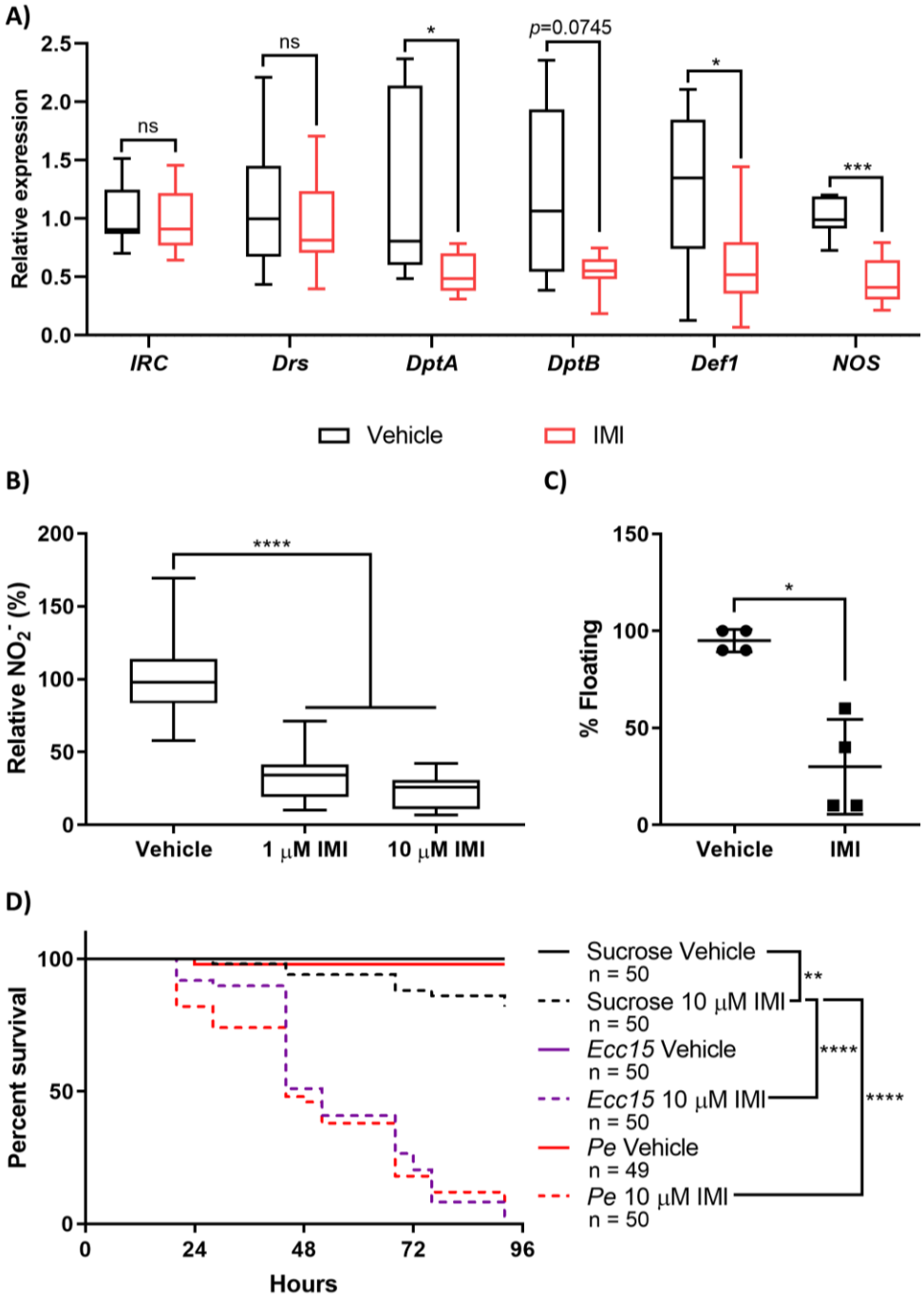

Figure 3-2. Exposure to imidacloprid impairs the immune response of *Drosophila melanogaster*.

(A) Relative expression of *IRC*, *Drs*, *DptA*, *DptB*, *Def1*, and *NOS* in Canton-S flies exposed to vehicle (black) or 10 μM imidacloprid (IMI; red) for 5 days. Data represent median fold change (relative to *RplP0*) of 8 – 9 biological replicates. All comparative statistics were performed on the ΔΔCt values (Mann-Whitney tests). Outliers were tested

by performing a Grubbs' test ($\alpha = 0.05$) on linearized ΔCt values of each individual gene. One outlier was removed from the vehicle *DptA* group and one outlier was removed from the vehicle *DptB* group. **(B)** Relative nitrite (NO^-_2) measured using the Griess test. Data are displayed as median relative NO^-_2 (%) (Kruskal-Wallis test). Vehicle has 32 biological replicates and both 1 μM IMI and 10 μM IMI have 16 biological replicates. In box plot diagrams, boxes represent first and third quartile values while black lines denote medians. Whiskers encompass maximum and minimum values. **(C)** Buoyancy assay of 3rd instar wandering Canton-S larvae grown on either vehicle (DMSO) or 10 μM IMI food. Data are displayed as mean % floating ± SD from four biological replicates, each containing 10 larvae (Mann-Whitney test). **(D)** Survival curves of w^{1118} flies exposed to vehicle (DMSO) or 10 μM IMI, and either given sucrose (vehicle), or oral infection with *Erwinia carotovora* subspecies *carotovora* 15 (*Ecc15*) or *Pseudomonas entomophila* DSM 28517 (*Pe*). Data are displayed from at least 3 independent experiments (n = 15 – 25 for each group). Statistical analyses are shown from log-rank (Mantel-Cox) tests. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. ns = not significant.

3.4.3 Functional immune pathways are required for survival on imidacloprid food

To assess if immune pathways are necessary to resist imidacloprid toxicity, the survival of immune knockout or knockdown flies was tested in the presence of lethal concentrations of imidacloprid (**Figure 3-3**). The *norpA*^{7} (PLC-β$^{-/-}$) flies exposed to 100 μM imidacloprid had lower overall survival (log-rank [Mantel=Cox], chi-square = 127.8, df= 1, $P < 0.0001$) and increased early timepoint deaths (Gehan-Breslow-Wilcoxon test, chi-square = 110.4, df = 1, $P < 0.0001$) compared to background Canton-S flies (**Figure 3-3A**). The *Tak1*^{2} (TAK1$^{-/-}$) flies exposed to 100 μM imidacloprid had reduced overall survival (log-rank [Mantel=Cox], chi-square = 54.29, df= 1, $P < 0.0001$) and increased early timepoint deaths (Gehan-Breslow-Wilcoxon test, chi-square = 42.28, df = 1, $P < 0.0001$) compared to background Canton-S flies (**Figure 3-3B**). The UAS-*pirk* (overexpression of *pirk*) flies exposed to 50 μM imidacloprid had not change in overall survival (log-rank [Mantel=Cox], chi-square = 1.717, df= 1, $P = 0.1901$) or early timepoint deaths (Gehan-Breslow-Wilcoxon test, chi-square = 0.04321, df = 1, $P = 0.8353$) compared to control cross ($y^{1}w^{67c23}$) flies (**Figure 3-3C**).

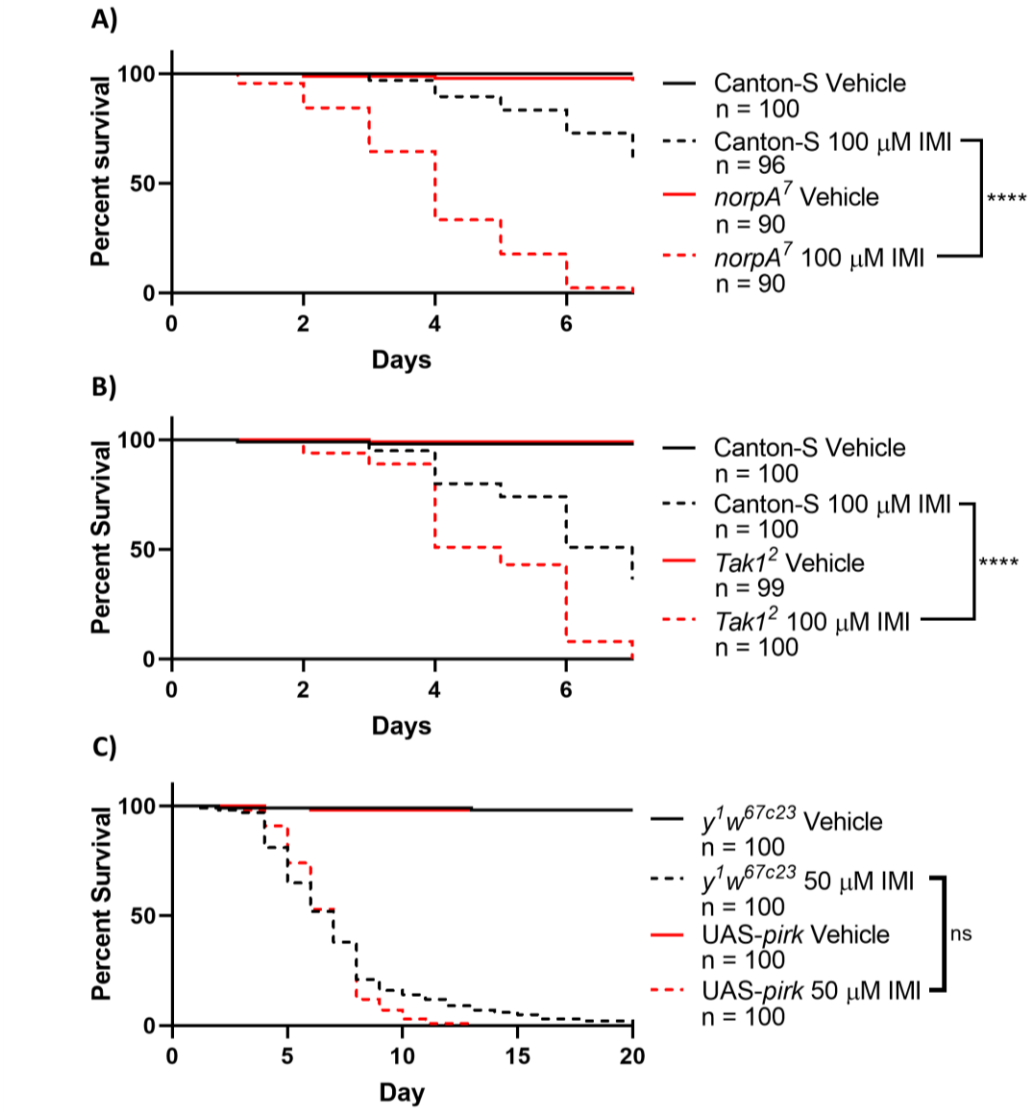

Figure 3-3. Survival curves of mutant *Drosophila melanogaster* exposed to lethal concentrations of imidacloprid.

(A) Canton-S (background) and *norpA*^{7} flies were exposed to 100 μM imidacloprid (IMI) or vehicle (DMSO). **(B)** Canton-S (background) and *Tak1*^{2} flies were exposed to 100 μM imidacloprid or vehicle (DMSO). **(C)** *y*^{1}*w*^{67c23} (control cross) and UAS-*pirk* flies were crossed with *daughterless*-GAL4 for whole body expression of UAS/GAL4 system. F1 generation flies were exposed to 50 μM imidacloprid or vehicle (DMSO). All data are displayed from at least 3 independent experiments (n = 20 – 30 for each group). Statistical analyses are shown from log-rank (Mantel-Cox) tests. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. ns = not significant.

3.4.4 Probiotic supplementation is unable to mitigate mortality from lethal imidacloprid exposure

Because neonicotinoid toxicity is the root cause of honey bee health impairments, the ability of LX3 to directly and indirectly reduce the toxicity of imidacloprid (**Figure 3-4**). Although it had previously been shown that *L. plantarum* Lp39 and *L. rhamnosus* GR-1 are unable to remove imidacloprid from culture supernatant (16, 17), is not known if *L. kunkeei* BR-1 has this potential. *L. kunkeei* BR-1 did not appear to remove imidacloprid from the supernatant (**Figure 3-4A**). Supplementation with LX3 did not improve overall survival (log-rank [Mantel=Cox], chi-square = 1.375, df= 1, $P = 0.2410$) or early timepoint deaths (Gehan-Breslow-Wilcoxon test, chi-square = 0.02276, df = 1, $P = 0.8801$) of *D. melanogaster* exposed to lethal imidacloprid compared to the lethal imidacloprid alone (**Figure 3-4B**).

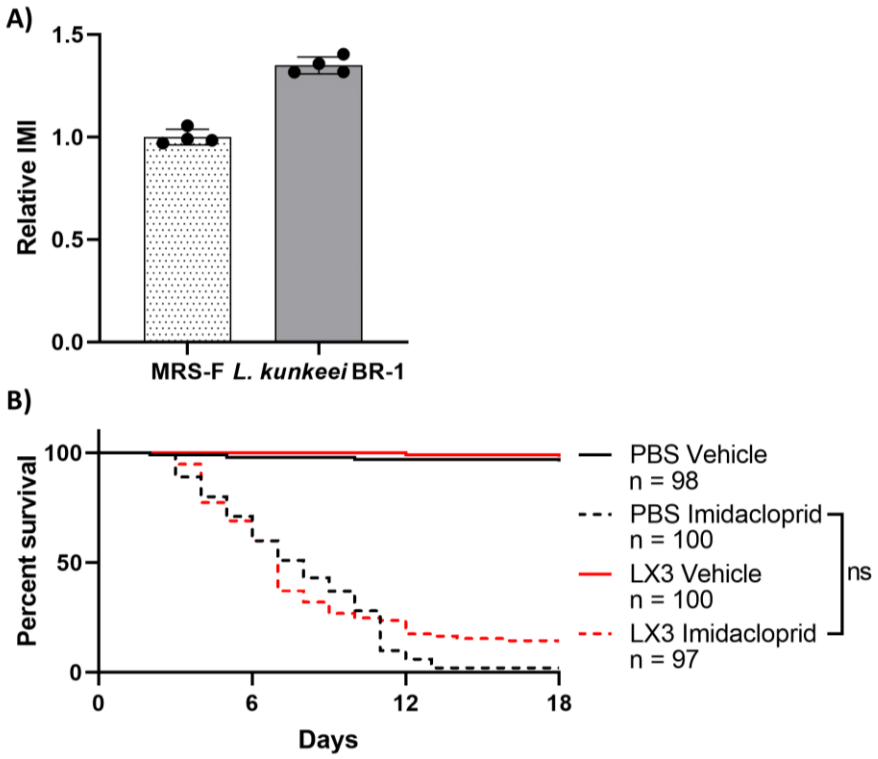

Figure 3-4. Probiotic supplementation is unable to mitigate mortality from lethal imidacloprid exposure.

(A) Relative amount of imidacloprid (IMI) in bacterial culture supernatant following 24-hour incubation. Data are displayed as mean (amount IMI [ppm]/average amount IMI [ppm] in media) ± SD. **(B)** Survival curve of probiotic flies exposed to lethal concentrations of imidacloprid. Canton-S flies were supplemented with PBS or LX3 for 2 days and then transferred to 100 µM imidacloprid or vehicle (DMSO). Data are displayed from at least 3 independent experiments (n = 20 – 30 for each group). Statistical analyses are shown from log-rank (Mantel-Cox) tests. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. ns = not significant.

3.5 Discussion

In this study, the neonicotinoid insecticide, imidacloprid, did not appear to be degraded by or harmful to the commensal gut microbes of *D. melanogaster,* but did affect the immune response through the nitric oxide (NO) signalling pathway. This suggests that the previously documented changes in the composition of the gut microbiota of *D. melanogaster* (17) are not xenobiotic-microbe mediated. Rather, imidacloprid elicits a host response.

A decrease in relative nitrite (NO_2^-) levels were found upon exposure to the pesticide. Although the change in nitrite levels is not a definitive measure of NO, nitrite is a final product of NO oxidation and provides a suitable representation of NO levels in *D. melanogaster* (42, 44). Combining the change in nitrite concentrations with the decrease in expression of *nitric oxide synthase* (*NOS*), it is surmised that imidacloprid lowered reduced NO in *D. melanogaster* and likely impaired NO pathway signalling.

Nitric oxide is a key signalling molecule that has several roles in immune pathway regulation. Specifically, NO contributes to the Relish-dependent regulation of *Diptericin* (*Dpt*), an IMD pathway effector in the fat body (22, 23). Exposure to imidacloprid reduces the expression of *Dpt* and *Defensin* (*Def*), but not *Drosomycin* (*Drs*), suggesting that imidacloprid is affecting the IMD pathway and not the Toll pathway (45). A reduction in *DptA*, a known immune effector was found. As *DptB* has roles in the immune system and behaviour, this potentially explains the non-significant reduction (46). These findings complement previous research showing AMP expression is reduced in *D. melanogaster* and honey bees in the presence of neonicotinoids (47, 48).

The unchanged expression of *IRC* indicates that any reduction in ROS is not due to catalase-mediated elimination. Given the interconnectedness of these immune pathways, it appears that reduced NO signalling is contributing to the decrease in AMP expression and ultimately inducing immune impairment that leaves *D. melanogaster* susceptible to oral pathogen insult.

The immune system was shown to mitigate imidacloprid toxicity. The *norpA*^{7} (PLC-β$^{-/-}$) flies had reduced survival on toxic concentrations of imidacloprid. These insects do not have a functional phospholipase C-β (PLC-β), which is needed for Duox-dependent H_2O_2 production (49). The reduced survival of *norpA*^{7} flies substantiates the same findings in *Duox*-RNAi (*Duox* knockdown) flies, which also have reduced ROS (17, 21). The TAK1 protein is required for IMD pathway activation and may have implications in the Duox pathway as a crosstalk kinase (25, 50). As *Tak1*^{2} (TAK1$^{-/-}$) flies have reduced survival when fed imidacloprid, this indicates the importance of the protein in imidacloprid tolerance. Interestingly, overexpression of *pirk* (UAS-*pirk*) has no effect on imidacloprid toxicity. *Pirk* is a negative regulator of the IMD pathway that stops the signalling cascade at the PGRP-LC/-LE and IMD complex (51); thus we would suspect that overexpression of *pirk* would reduce IMD pathway signalling and increase susceptibility to the pesticide (16). However, while *pirk* impairs the PGRP/IMD complex, it does not necessarily affect the expression of downstream IMD effectors, which implies that the pathway might still be functional to some extent, and would explain the observations (51).

The buoyancy assay results demonstrate that larvae grown in the presence of imidacloprid have lower fat content compared to larvae grown in vehicle. While these require further experimentation, they allow for speculations into the consequence of altered larval density. The most probable explanation is that these larvae have a smaller fat body, which would increase their density and reduce the number of floating larvae (25, 26). As the fat body contributes to adult size, it is likely that when these flies eclose, they will be smaller than their vehicle control counterparts (52). Indeed, honey bee larvae allowed to develop in the presence of sublethal thiamethoxam (neonicotinoid) levels display reduced larval weight (53). Another consideration is that the fat body is an essential site for AMP generation (54). Thus, if larvae develop into adults with a smaller fat body, their immune response and AMP generation might be hampered.

Supplementation with LX3 was not able to reduce the lethal toxicity of imidacloprid. The results indicate that the LX3 strains did not sufficiently stimulate host detoxification of the pesticide. In humans, the gut microbiota is able to modulate the expression of host detoxification enzymes (55). However, in *D. melanogaster*, germ-free larvae had reduced

amounts of imidacloprid metabolites and slightly more unaltered imidacloprid than controls (56). Despite not removing imidacloprid, the LX3 combination may be able to modulate the expression of metabolic genes.

To explore this further, the LX3 strains were found not to degrade imidacloprid when grown in isolation (16, 17). Further studies are required to test the whole strain combination to see if the bacteria compensate for the metabolic needs of each other (57). As of now, several insect cytochrome P450 genes (CYP) have been identified to degrade imidacloprid (58), and while bacteria can degrade the compound (27), no genes have been identified to correlate with these findings. In contrast, organophosphate insecticides are degraded by the organophosphate-hydrolyzing protein (OPH), which is encoded by the *opd* gene and also found in some lactobacilli (33, 59). Considering this, the LX3 combination may still convey detoxification of other pesticides.

In conclusion, these investigations have demonstrated that (i) the gut microbes of *D. melanogaster* are not affected by imidacloprid exposure; (ii) exposure to this pesticide reduces immune signalling and the generation of AMPs, which leads to depleted survival when challenged when a pathogen insult; (iii) immune pathways are required for imidacloprid survival; and (iv) LX3 is not able to improve survival with toxic levels of imidacloprid.

Further experiments should utilize the advantages of the germ-free *D. melanogaster* model to focus on understanding the direct interactions between imidacloprid and the host that induce immunosuppression. Although LX3 was not able to metabolize imidacloprid, additional work is warranted in testing if LX3 can degrade other pesticides, which would eliminate these harmful chemicals from the honey bee hive environment.

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3.8 Supplementary

Table 3-2. qPCR primers used in this study.

Primer	Sequence	Amplicon size (bp)	Efficiency
RpLP0 (16)	F: 5’ CCGAAAAGTCTGTGCTTTGTTCT 3’ R: 5’ CGCTGCCTTGTTCTCCCTAA 3’	83	1.86
IRC (60)	F: 5’ AAAGCGACTGGAGGACAATC 3’ R: 5’ GAAGTTGAGCGTGTGAAAGG 3’	74	1.88
Drs (16)	F: 5’ TACTTGTTCGCCCTCTTCGC 3’ R: 5’ CACCAGCACTTCAGACTGGG 3’	185	1.81
DptA	F: 5’ GCCACGAGATTGGACTGAAT 3’ R: 5’ TAGGTGCTTCCCACTTTCCA 3’	91	1.81
DptB (16)	F: 5’ CCACTGGCATATGCTCCCAAT 3’ R: 5’ CAAGGTGCTGGGCATACGAT 3’	190	1.81
Def1	F: 5’ AGTTCTTCGTTCTCGTGGCT 3’ R: 5’ GATCCACATCGGAAACTGGC 3’	78	1.79
NOS (22)	F: 5’ CCGCACGACAAAATACC 3’ R: 5’ GCGTTAGTTGGGCAAG 3’	265	1.81

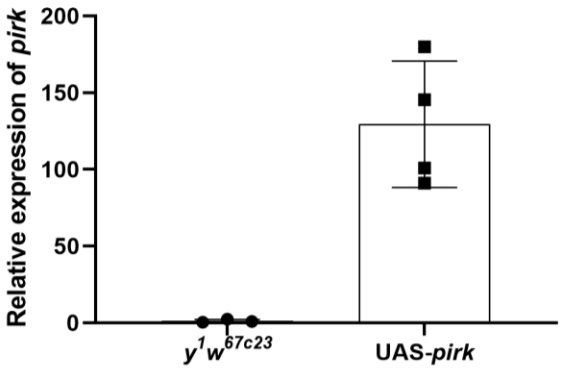

Figure 3-5. Overexpression of *pirk*.

Relative expression of *pirk* in F1 generation of y^1w^{67c23} (control cross) and UAS-*pirk* flies crossed with *daughterless*-GAL4. Data represent median fold change (relative to *RplP0*) of 3 or 4 biological replicates.

Chapter 4

4 General discussion

4.1 Proposed mechanism of imidacloprid-induced immunosuppression

While pesticide-induced immune impairments are well documented (1), limited studies have deciphered the process by which these chemicals exert their harmful effects on the immune system. Thus, it makes it difficult to conceive a solution when the problem at hand is not completely understood.

One study in particular has deeply examined neonicotinoid-induced immunosuppression. Di Prisco et al. (2) demonstrated that neonicotinoids increase expression of *CG1399* (*Dmel\LRR*), a leucine-rich repeat protein that is a negative regulator of NF-κB and subsequently reduces expression of antimicrobial peptides (AMPs), thereby amplifying susceptibility to viral infection. This thesis builds on these findings and proposes that the neonicotinoid pesticide, imidacloprid, reduces Duox-specific hydrogen peroxide (H_2O_2) in an IMD pathway-dependent mechanism. The reduction in H_2O_2 results in reduced generation of nitric oxide (NO), which limited organ-to-organ signalling and antimicrobial peptide (AMP) production (**Figure 4-1**).

In Chapter 2, imidacloprid exposure was shown to alter the composition of the gut microbiota in *D. melanogaster*, with an increase in total bacteria, a higher proportion of *Acetobacter*: *Lactobacillus*, and increased yeast in imidacloprid exposed flies. These commensal microbes were not able to consume imidacloprid as a nutrient source, nor did the chemical hamper their growth. Therefore the conclusion was that imidacloprid was impairing the immune system, and the microbiota could be used as a diagnostic marker of immunosuppression, as well as immune deficiency (3–5).

Considering the observed changes to the microbiota and the published literature outlining that neonicotinoids impair the IMD pathway, it was reasonable that total bacteria increased, specifically *Acetobacter* spp. because they are Gram-negative and the host's response to them is mainly controlled by the IMD pathway (2, 6). However, the increase

in yeast was unusual as it did not coincide with a dramatic increase in Gram-positive bacteria, which would indicate that the Toll pathway, the main immune response against Gram-positive bacteria and fungi, is not affected by imidacloprid (7, 8). As the Duox pathway controls yeast and bacteria that secrete uracil, it is a likely candidate for an impaired immune response (3, 9).

Both conventional and germfree wild-type flies had a reduction in H_2O_2 in the presence of imidacloprid, but *Duox*-RNAi knockdown flies did not. Wild-type flies exposed to either vehicle or imidacloprid demonstrated no change in expression of *immune regulated catalase* (*IRC*), which functions to eliminate H_2O_2 (10). The *Duox*-RNAi flies also displayed no change in the ratio of *Acetobacter*: *Lactobacillus*. Taken together, these results suggest that imidacloprid decreases the ability of the Duox pathway to produce H_2O_2, thereby altering the composition of the gut microbiota in *D. melanogaster*.

Wild-type flies exposed to imidacloprid demonstrated reduced expression of *Duox*, *p38c*, and *Mkp3* (a negative regulator of *Duox*), suggesting that the loss of H_2O_2 is a result of reduced *Duox* expression, which is not caused by overactivation of the Duox pathway negative regulator, MKP3 (11). In addition, there was no change in expression of *Cad99C*, a cadherin gene shown to be upregulated when the Duox pathway is activated (12). Flies with a knockout of phospholipase C-β, a protein required for Ca^{2+} mobilization and subsequent DUOX production of H_2O_2, demonstrated no change in *Duox* expression or production of H_2O_2 (3). Altogether, these findings indicate that imidacloprid is altering the expression, not the activation, of the Duox pathway to reduce H_2O_2.

Expression of the Duox pathway is controlled either by Duox pathway activation itself or through the IMD pathway which does not stimulate H_2O_2 production (3, 11). Using IMD pathway knockout flies, it was determined that imidacloprid was interacting with the receptor, PGRP-LE of the IMD pathway, to reduce expression of *Duox*. The PGRP-LE is found in the midgut of *D. melanogaster*, which is where the Duox pathway functions and controls the composition of the gut microbiota (12, 13).

Hydrogen peroxide is not only a critical microbiocidal substance but also a potent signalling molecule, specifically in NO signalling (14). Imidacloprid exposed flies had reduced expression of *nitric oxide synthase* (*NOS*) and decreased levels of nitrite, a proxy for NO (15). These flies also had a diminished expression of *Diptericin*, a Relish-dependent (NF-κB) AMP (16). When taken together, these results show that imidacloprid exposure reduces NO signalling and successive AMP production.

In summary, **Figure 4-1** demonstrates that imidacloprid is interacting with PGRP-LE of the IMD pathway, which decreases the expression of *Duox* thus reducing basal levels of H_2O_2. Reduced basal H_2O_2 does not allow for adequate expression of *NOS*, which decreases NO production and subsequent Relish-dependent expression of *Diptericin*. Ultimately, this immunosuppressive state, which is the result of imidacloprid exposure, causes an alteration in the microbiota that can be used to characterize imidacloprid exposure.

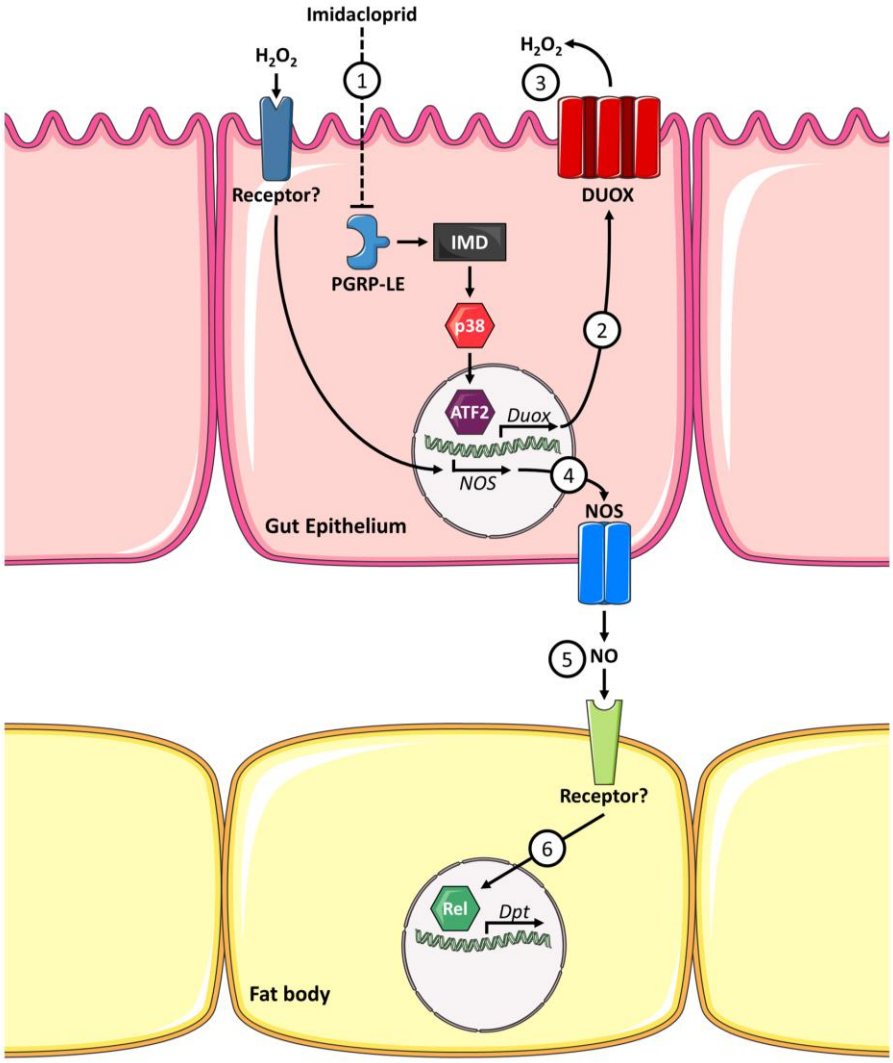

Figure 4-1. Proposed mechanism of imidacloprid immunosuppression in *Drosophila melanogaster*.

(1) Imidacloprid interacts with PGRP-LE in the gut epithelium to reduce IMD pathway signalling. **(2)** Impaired IMD pathway signalling reduces *dual oxidase* (*Duox*) expression via the p38-ATF2 signalling cascade. **(3)** Reduced *Duox* expression causes a reduction in basal hydrogen peroxide (H_2O_2) levels. **(4)** A decrease in H_2O_2 causes a decrease in *nitric oxide synthase* (*NOS*) expression, which is mediated through an unclear mechanism. **(5)** Reduced expression of *NOS* by the gut epithelium causes a reduction in nitric oxide (NO), a key signalling molecule (NO). **(6)** Reduced NO production causes a reduction in the equivocal NO-mediated signalling to the fat body, which decreases the Relish- (Rel; NF-κB) dependent expression of the antimicrobial peptide, *Diptericin* (*Dpt*). Images were modified from Servier Medical Art by Servier under the Creative Commons Attribution 3.0 Unported License (https://smart.servier.com/).

4.2 Future directions

4.2.1 Proposed mechanism of imidacloprid-induced immunosuppression

While the proposed mechanism is not without flaws, future work would benefit from validating the findings. In particular, improving the specificity of the experiments will allow for a comprehensive understanding of the immune response under the influence of imidacloprid. The current model considers gene expression of whole flies; however, in actuality, these immune responses are localized to specific areas of the fly. The DUOX protein was initially identified in the trachea of flies, but it was later found to be part of the gut epithelial immune response (17). Thus, measuring gut-specific expression of *Duox* would provide a more accurate understanding of immune-related *Duox* expression. Similarly, the whole-body expression of AMPs was considered here, but the model focused on AMP expression in the fat body. Multiple systems make H_2O_2 throughout the body, which means that the analysis of relative H_2O_2 could lack Duox pathway specificity. While the inclusion of relative H_2O_2 levels in *Duox*-RNAi flies is convincing of Duox pathway specificity, these results could be strengthened by measuring hypochlorous acid (HOCl), which is generated by the peroxidase domain of DUOX (18). By improving the specificity of the experiments used to elucidate the proposed mechanism, the accuracy of the proposed mechanisms would be strengthened.

Another limitation of using gene expression data is that they do not always represent what is going on at the protein level—the more functional aspect of physiology. For the Duox pathway, attempts were made to compensate for the limitations of qPCR by looking at H_2O_2. This method could be improved by using western blot of the DUOX protein or for p38 phosphorylation (the activator of *Duox* expression) (19, 20). For AMPs, a more comprehensive analysis could be achieved by looking at fluorescent reports, which provide localization and semi-quantification of proteins (21).

The *Drosophila* microbiota contributes to the innate immune response (22). By using germ-free *D. melanogaster*, the microbiota influence on the immune system would be deleted potentially allowing a better understanding of the host-xenobiotic interactions of

pesticide exposure. Germ-free flies could be exposed to imidacloprid, and gene expression and NO could be quantified to confirm that the overserved changes were a result of imidacloprid and not the microbiota.

Another set of experiments should be aimed at understanding the interaction between imidacloprid and the immune system. Imidacloprid was found to likely acts through the PGRP-LE receptor, although the mechanism is unclear. *In silico* forced modelling analysis could predict potential binding interactions between imidacloprid and PGRP-LE using PyMOL (23). These predictions could be followed up with *in vitro* ligand binding assays (24). After binding is confirmed, a functional analysis would be carried out to determine if the chemical is inhibiting or activating the receptor. While it is likely that imidacloprid is inhibiting PGRP-LE signalling, mutant flies (devoid of other PGRP receptors) would be stimulated with tracheal cytotoxin (which binds and activates PGRP receptors) in the presence or absence of imidacloprid and luciferase activity of AMPs can be quantified (25, 26).

Support for the claim that reduced H_2O_2 contributes to diminished NO signalling and subsequent reduced AMP expression, could be strengthened by using artificial ROS stimulation and mutant *D. melanogaster* fly lines. To validate that specifically Duox pathway H_2O_2 drives NO production, control cross and *Duox*-RNAi flies could be stimulated with uracil to activate Duox pathway H_2O_2 production, and relative NO levels can be quantified using the Griess reagent method (9). It would be expected that *Duox*-RNAi flies have reduced NO generation. Impairments to NO-mediated AMP production could be confirmed by measuring AMP expression of gut-specific *NOS*-RNAi knockdown flies or control cross flies exposed to imidacloprid. These flies can be generated by crossing UAS-*NOS*-RNAi (RRID:BDSC_80469) with a gut-specific GAL4 driver (RRID:BDSC_7098). The expectation is that imidacloprid is only able to decrease AMP expression in control cross flies and not *NOS*-RNAi flies.

Hemocytes are fundamental immune cells in *D. melanogaster* that also function to relay the NO signal to the fat body for distal AMP expression (27). The depletion of hemocytes using the UAS/GAL4 system will allow for testing if hemocytes are required for AMP

expression in the fat body (28, 29). When exposed to imidacloprid, flies deficient of hemocytes would be expected to display no change in AMP expression. Another consideration is that honey bees exposed to imidacloprid display reduced total hemocyte counts (30). Thus, the amount of circulating and resident hemocytes in *D. melanogaster* could be quantified using the established GFP-tagged hemocyte method (31).

Another H_2O_2-producing enzyme in *D. melanogaster* is NADPH oxidase (NOX). While NOX and DUOX both function in the gut to generate ROS, much less is known about the regulation of the Nox pathway. Interestingly, the microbiota has a role in activating NOX-mediated production of H_2O_2, which contributes to epithelial proliferation and immunity (32, 33). Future work should aim to determine if the Nox pathway is affected by neonicotinoid exposure. Initial experiments should quantify the amount of H_2O_2 in control cross and *Nox*-RNAi knockdown flies, to determine if the impairment of the Nox pathway contributes to the observed reduction in H_2O_2 (18). If this is confirmed, experiments should aim to understand the mechanism of how imidacloprid causes this impairment.

Recent work has linked the Duox pathway and other immune pathways to *D. melanogaster* metabolism. In particular, lipid metabolism in *D. melanogaster* enterocytes is found to regulate DUOX protein activity (34). Furthermore, DUOX generation of HOCl binds the TrpA1 receptor to enhances defecation and reduce pathogen load (35). Future experiments could build on the bouncy assay to quantify triacylglycerols using colorimetric assays or use various stains to image tissue samples directly (36, 37). Defecation can be quantified by feeding *D. melanogaster* blue food dye and enumerating the dried defecation spots (35). Additionally, these flies can be imaged to assess intestinal permeability by observing the amount of blue dye that translocates throughout the fly; flies with increased permeability will appear blue, hence the term ‘smurf’ flies (38).

Although *D. melanogaster* provides a channel for a mechanistic understanding of immunosuppression, these results should be tested in honey bees to confirm the overserved effects. Preliminary experiments could test the ability of imidacloprid to reduce the expression of *Duox* in honey bee larvae, which can easily be grafted from a

hive and grown in a laboratory setting (39). Using a data mining approach, data could be collected from the multitude of RNA-seq studies on pesticide exposed honey bees and analyzed for the relationship between imidacloprid exposure and *Duox* expression. This work should expand the tested pesticides to thiamethoxam and clothianidin, which have seen increased use since the restrictions on imidacloprid have been put in place (40).

4.2.2 LX3 combination for immune modulation

Experiments in this thesis demonstrated that three *Lactobacillus* strains, designated LX3, do not mitigate the toxic effects of imidacloprid. However, this probiotic combination does improve immunity in honey bees (41). As demonstrated here, *Lactobacillus rhamnosus* GR-1 (a component of LX3) can mitigate neonicotinoid-induced changes to the immune system; further work should investigate the potential for LX3 to mitigate imidacloprid-induced immunosuppression in honey bees. Preliminary studies can be done using honey bee larvae, which can easily be manipulated in a laboratory setting. These experiments could assess the changes in gene expression, the gut microbiota, and pathogen susceptibility of imidacloprid exposed larvae with or without the LX3 supplementation. Following that, semi-field studies which use large net structures to contain the experiment could be undertaken. These types of studies benefit from maintaining the bee in a relatively natural environment while refraining from transmitting these harmful chemicals into the wild.

Future investigations could also characterize the mechanism of the immune bolstering capabilities of LX3 to improve the understanding of host-microbe interactions. In particular, these experiments could take advantage of the genetic tractability of the established *D. melanogaster* model. Initial experiments should seek to recapitulate the results observed in honey bees, before testing the probiotic combination in flies with genetic knockouts of key immune pathways. Some key flies with mutations in immune receptors such as PGRP or Toll, which are the primary activators of the innate immune response, could be tested. These studies should also look at how probiotics regulate the Duox pathway since this pathway is impaired by imidacloprid exposure.

4.2.3 Pesticide degradation by microbes

The ultimate problem is that honey bees come in contact with pesticides from fields and gardens. While measures can be taken to limit this exposure, or even mitigate the deleterious effects of these chemicals, these remediations are not sufficient. An alternate strategy would be to reduce the absorption of pesticides by supplementing the gut microbiota to block or degrade the chemicals.

A case has been made for the ability of lactobacilli to adsorb organophosphates (42), thereby sequestering the chemicals from the honey bee bolus and allowing the pesticide to be excreted along with the probiotic. This concept has proven effective in humans by reducing the accumulation of heavy metals (43, 44). Certain lactobacilli can even bind aflatoxin to reduce host uptake (45).

In general, lactobacilli are able to bind compounds that are highly aromatic or heterocyclic (46, 47). Many pesticides used today possess these functional groups so even non-viable lactobacilli might sequester the chemicals via their cell walls. The increased peptidoglycan content of these Gram-positive bacteria is the primary binding site (47), but polysaccharides and teichoic acids are also useful mediators (47, 48).

Bacteria used for pesticide sequestration can quickly be narrowed down by screening for favourable cell surface properties. A microbial adhesion to solvents (MATS) assay can be performed using common laboratory solvents (hexadecane, chloroform, and ethyl acetate) to screen for percent hydrophobicity and Lewis acid-base characteristics (49, 50). Bacteria could be directly tested for sequestration ability using HPLC or LC/MS to quantify remaining free pesticide (42).

Bacterial adsorption of xenobiotics benefits from the potential of live or dead microbes successfully reducing the absorption of toxic substances, but non-viable bacteria do not entirely resolve the problem because the pesticides still remain active in the hive environment. Therefore, another option would be to consider using live probiotic bacteria to metabolize pesticides. A good starting point would to examine the native microbiota of honey bees for candidate strains to degrade pesticides. Potentially, these pesticide

degrading bacteria could be stimulated by prebiotics or supplemented into hives that are devoid of them.

Several lactobacilli can metabolize organophosphates (51, 52). One safety consideration is to prevent these probiotics from transferring to pest insects and conferring resistance to the pesticides.

So far, neonicotinoids can only be degraded by specific bacteria that are not optimal for honey bees (53). An alternate option would be to genetically modify honey bee bacteria with the machinery to degrade pesticides. In particular, *Lactobacillus kunkeei* is a prime contended because it is mainly found in bees and is able to harbour transgenic plasmids (54, 55). Also, *L. kunkeei* might colonize the honey bee through beneficial biofilm formation (56). While degradation removes the parent pesticide, consideration of the toxicity of the metabolites is imperative because metabolites could prove more toxic than the parent compound (57).

4.3 Concluding comments

Honey bees are crucial insect pollinators that strongly contribute to the global food supply and agriculture economy. Declining populations of these beneficial insects threaten the agriculture industry and jeopardize food security throughout the world. Unfortunately, the same industry that benefits from honey bee pollination services is also unintentionally contributing to their population decline through the use of pesticides. In particular, neonicotinoid pesticides are a class of insecticide that have been implicated in honey bee population decline. The use of these chemicals is increasing in modern agricultural practices due to their ease of application, superior efficacy against pest insects, and long-lasting systemic activity in plant tissues.

While exposure to high amounts of neonicotinoids is directly lethal to honey bees, exposure to sublethal concentrations of these chemicals threaten multiple aspects of honey bee health. One of the biggest concerns is that neonicotinoids impair honey bee immunity, which increases their susceptibility to infection. Although these observations

have been documented in both field and laboratory settings, limited consideration has gone into identifying the mechanism of immunosuppression.

In this thesis, the neonicotinoid imidacloprid has been shown to impair the immune system of the model organism, *D. melanogaster*, which is characterized by a disruption to the gut microbiota. Imidacloprid interaction through the IMD pathway in the gut, which causes downregulation to the Duox pathway and reduced whole-body basal H_2O_2 levels. The reduction of H_2O_2 depresses the generation of the signalling molecule NO, which decreases distal AMP production in the fat body. Further research should attempt to reproduce these results in honey bees, identifying the direct interaction between the immune system and imidacloprid, and investigating the implications of reduced ROS and AMP expression.

Developing a comprehensive understanding of the problem is the first part of improving the health and productivity of honey bee populations (58). The next logical step is to investigate interventions that can adequately address this concern and fine-tuning them to maximize effectivity. Probiotic supplementation is an intervention potentially able to mitigate the immunosuppression involved with pesticide exposure. Although the strains tested here were not able to eliminate the neonicotinoids and reduce absorption, they showed potential to improve immunity in neonicotinoid-induced immunosuppressed flies. Additional investigations should aim to validate these findings by characterizing the mechanisms by which probiotics can achieve this and verify the outcomes in honey bees.

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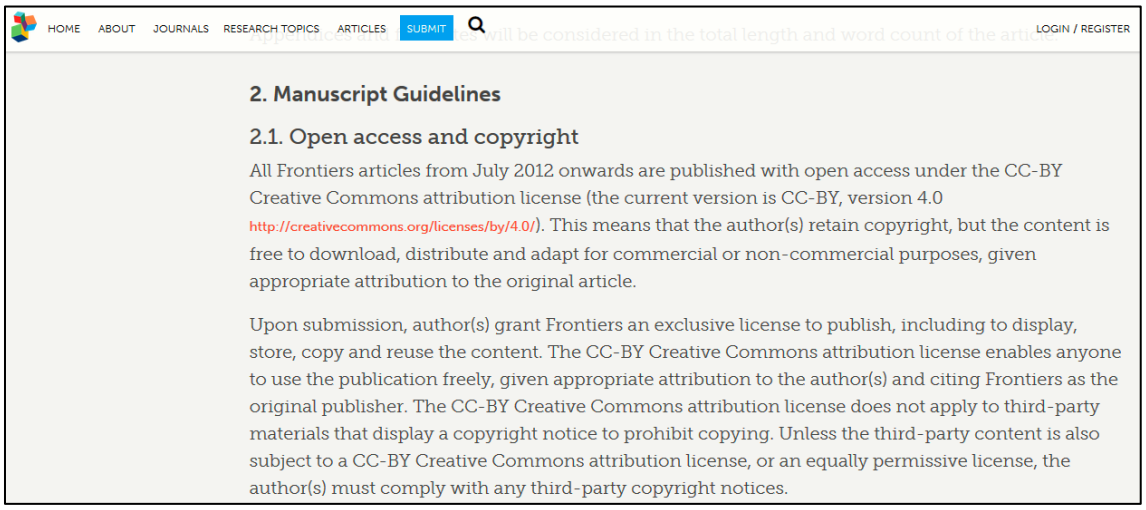
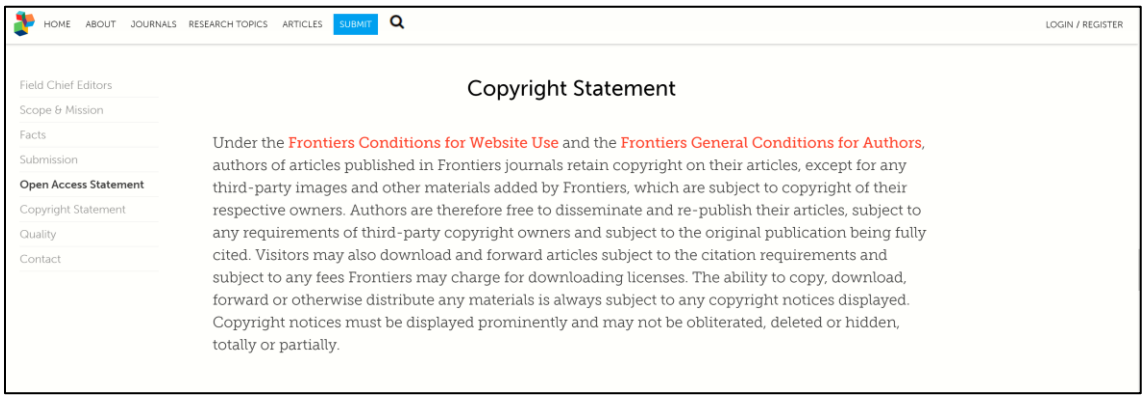
Appendices

Appendix A. Content license from Frontiers in Ecology and Evolution. URL available at: https://www.frontiersin.org/journals/ecology-and-evolution#.





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Curriculum Vitae

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Honours and Awards:	The Western Scholarship of Excellence 2014 Summer Undergraduate Research Fellowship 2016–2017 Dean's Undergraduate Research Opportunities 2018 Regional Winner (US & Canada) in Life Science, The Global Undergraduate Awards 2018 Dr. Fredrick W. Luney Graduate Entrance Scholarship 2018 Alexander Graham Bell Canada Graduate Scholarship (NSERC-CGS-M) 2019–2020
Related Work Experience	Teaching Assistant The University of Western Ontario 2018–2019

Publications:

Chmiel JA, Daisley BA, Pitek AP, Thompson GJ, Reid G. Understanding the effects of sublethal pesticide exposure on honey bees: a role for probiotics as mediators of environmental stress. *Front Ecol Evol*. 2020;8(22):1–19. doi:10.3389/fevo.2020.00022

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Daisley BA, **Chmiel JA**, Pitek AP, Thompson GJ, Reid G. Missing microbes in bees: how systemic depletion of key symbionts erodes immunity. *Trends Microbiol.* 2020.

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Daisley BA, Monachese M, Trinder M, Bisanz JE, **Chmiel JA**, Burton JP, Reid G. Immobilization of cadmium and lead by *Lactobacillus rhamnosus* GR-1 mitigates apical-to-basolateral heavy metal translocation in a Caco-2 model of the intestinal epithelium. *Gut Microbes*. 2018;10(3):321–333. doi:10.1080/19490976.2018.1526581

Submitted:

Daisley BA, Pitek AP, **Chmiel JA**, Gibbons S, Chernyshova AM, Al KF, Faragalla KM, Burton JP, Thompson GJ, Reid G. *Lactobacillus* spp. attenuate antibiotic-induced immune and microbiota dysregulation in honey bees. (Submitted).

Daisley BA, Chanyi RM, Abdur-Rashid K, Al KF, Gibbons S, **Chmiel JA**, Wilcox H, Reid G, Anderson A, Dewar M, Nair SM, Chin J, Burton JP. Hormone-derived oral cancer treatment preferentially enriches for the anticancer gut commensal *Akkermansia muciniphila*. (Submitted).