Mitigation of Pesticide Toxicity by Food-Grade Lactobacilli

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Abstract

Lactobacilli are Gram-positive bacteria used in fermented foods. Many species are commensal microbiota members that confer host benefits. This thesis investigated lactobacilli mitigation of organophosphate and neonicotinoid pesticide toxicity in mammals and insects, respectively. Lactobacillus rhamnosus GG (LGG) and GR-1 (LGR-1) were found to sequester, but not metabolize, organophosphate pesticides (parathion and chlorpyrifos) in solution. For LGG, this sequestration reduced organophosphate pesticide absorption in a Caco-2 intestinal Transwell model and promoted survival of Drosophila melanogaster lethally exposed to chlorpyrifos. Supplementation of mice with LGR-1 was found to alter host xenobiotic metabolism in the liver, and consequently chlorpyrifos metabolism following acute exposure. Drosophila supplemented with L. plantarum, a species indigenous to the fly, elicited an immune response that was correlated with increased survival following imidaclorpid (neonicotinoid) exposure. Taken together, these experiments suggest that humans and honeybees could benefit from simple and affordable dietary supplementation with Lactobacillus strains to offset pesticide exposure.

Keywords

Probiotics, Lactobacillus, bioremediation, environmental toxicology, pesticide, organophosphate, neonicotinoid, colony collapse disorder, honeybee, IMD/Relish
Co-Authorship Statement

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

**Chapter 2: Reduction of organophosphate pesticide absorption and toxicity by probiotic Lactobacillus rhamnosus.**

Mark Trinder and Gregor Reid conceived the experiments. Mark Trinder performed experiments, collected data, and analyzed data for all experiments with minor exceptions. Tim McDowell and Mark Sumarah designed the high performance-liquid chromatography (HPLC) method and assisted with analytical chemistry data interpretation. Sohrab Ali and Hon Leon provided scientific input and resources for Drosophila experiments.

**Chapter 3: Lactobacillus rhamnosus supplementation alters low dose chlorpyrifos exposure and xenobiotic metabolism in a BALB/c mouse model.**

Mark Trinder, Gregor Reid, and Benoît Foligné conceived experiments. Mark Trinder performed experiments, collected and analyzed data for most experiments with modifications and assistance listed below. Mark Trinder carried out most animal work with assistance from Jordan Bisanz, Benoît Foligné, and Coline Pié. Benoît Foligné and Coline Pié performed and analyzed FITC-dextran permeability and comet assays and performed intestinal histology. Jordan Bisanz analyzed microarray results. Jean Macklaim and Gregory Gloor designed code and had input in 16S rRNA microbiota sequencing analysis. Mark Trinder analyzed the data. Tim McDowell, Mark Sumarah, and Amy McMillan designed liquid chromatography-mass spectrometry (LC-MS) method and assisted with analytical chemistry data interpretation. Mark Trinder prepared and extracted samples. Stephanie Collins and Amy McMillan performed analysis on untargeted liver and plasma metabolomics.

**Chapter 4: Enhancing innate immunity with beneficial microbes reduces pesticide-induced toxicity in Drosophila melanogaster: implications for colony collapse disorder.**

Mark Trinder and Gregor Reid conceived experiments. Mark Trinder performed experiments, collected data, and analyzed data for all experiments with minor exceptions. Josh Dube
assisted with adult *Drosophila* survival experiments. Sohrab Ali and Hon Leon provided scientific input and resources for *Drosophila* experiments. Sohrab Ali and Hon Leon also assisted with *Drosophila* imaging experiments. Tim McDowell and Mark Sumarah designed LC-MS method and assisted with analytical chemistry data interpretation.
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Chapter 1

1 General Introduction

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1.1 Pesticides

Pesticides are heavily used in agriculture to protect crops from damage associated with insects, weeds, plant pathogens, and other microbial infestations. Despite their intended purpose of killing, incapacitating, or repelling organisms deemed pests, pesticides frequently have negative consequences on human and wildlife health. Pesticide pollution is still a major consequence of agricultural practices. However, since many pesticide classes have been designed to be less toxic to vertebrates, prevailing dogma has suggested that at low concentrations these chemicals should have negligible effect on vertebrate species. But, this appears not to be the case. Recent studies in bees (1), birds (2), fish (3), and humans (4) suggest that chronic low dose pesticide exposure can cause long-lasting negative consequences on health, especially neural function. Therefore, humans need to develop improved methods to protect both the environment and themselves from pesticide exposure.

These toxic compounds frequently enter the gastrointestinal tract following oral ingestion of contaminated food or water (5). Gastrointestinal-xenobiotic interactions are complicated by the presence of bacterial communities, collectively known as the microbiota. The gastrointestinal microbiota plays an important role in metabolizing
xenobiotics (6), influencing host xenobiotic metabolism (7), and preventing systemic toxin absorption (8). It is well-established that bacteria can break down pesticides in contaminated soil and water in a process known as bioremediation (9, 10). However, to our knowledge, little work has been carried out to test whether food-grade bacteria can act to reduce the systemic absorption of pesticides in vivo.

1.1.1 Organophosphate pesticides

Due to their toxicity to humans, the physiological effects of first generation organophosphate pesticides are best characterized. There is a wide range of organophosphate pesticides that all possess phosphate ester functional groups (Figure 1-1). Organophosphate pesticides are applied as insecticidal sprays for agriculture and landscaping. These inhibit acetylcholinesterase, an enzyme responsible for metabolizing the neurotransmitter acetylcholine (Figure 1-2). Organophosphate pesticides include: parathion, malathion, methyl parathion, chlorpyrifos, diazinon, dichlorvos, phosmet, fenitrothion, tetrachlorvinphos, azamethiphos, and azinphos methyl. Due to the poor selectivity of these compounds, many countries have restricted or even banned their usage due to their off-target toxicity (5). However, since they are effective at eliminating pests and are affordable, organophosphate pesticides are still used by many businesses, especially in developing countries (11, 12). Current treatment for acute organophosphate poisoning includes pralidoxime and atropine, which bind organophosphates and are muscarinic acetylcholine receptor antagonists, respectively. But these nonspecific treatments do not negate concerns of chronic low dose organophosphate pesticide exposure being implicated in neurodevelopmental disorders (13), diabetes (14), cancer (15), heart disease (16) and several other chronic diseases. Further research into the long-term safety of these compounds is needed with respect to susceptibility to secondary chronic diseases. Notably, the World Health Organization is encouraging greater national regulation of pesticides on a global level, with emphasis on developing countries, in hopes to reduce pesticide use and associated morbidities and mortalities.
Figure 1-1. Chemical structure of organophosphate pesticides.
Figure 1-2. Organophosphate pesticide toxicity.

(A) The currently accepted health risks for pesticide toxicity in humans are neurotoxicity, skin irritation, carcinogenesis, and endocrine system disruption. (B) The mechanism of action of organophosphate pesticides is to inhibit acetylcholinesterase (AChE), leading to an exacerbation of acetylcholine (ACh)-acetylcholine receptor (AChR) signaling in nerve and muscle cells. Images were modified from Servier Medical Art by Servier under the Creative Commons Attribution 3.0 Unported License (http://www.servier.com/Powerpoint-image-bank).
1.1.2 Neonicotinoid pesticides

Neonicotinoid pesticides are classified as neurotoxins (insecticides) that act as an insect-selective nicotinic acetylcholine receptor agonist (17). The use of neonicotinoid pesticides in agriculture has become widespread with almost all US corn and about 1/3 of US soybean crops being planted with neonicotinoid-treated seeds. As of 2008, neonicotinoids made up 80% of seed treatment sales for agricultural crops, but they can also be used with water to irrigate plants. Neonicotinoid pesticides in order of popular usage include: imidacloprid, thiamethoxam, clothianidin, acetamiprid, thiacloprid, dinotefuran, and nitenpyram. Imidacloprid has particularly received attention for its link to undesired toxicity to bees and “colony collapse disorder” (18). Bees experience growth abnormalities (1, 18), motor deficiencies (19, 20), neurologic abnormalities (21), and/or death (19) following imidacloprid exposure. Bees act as critical pollinators for roughly 35% of the global food crop (22). The fact that neonicotinoids also adversely affect insectivorous avian species (2), suggests evidence of bioaccumulation. So far, the literature suggests acute mammalian neonicotinoid pesticide toxicity is remarkably rare and almost negligible at environmentally-relevant levels (23). Thus, the dilemma facing the agriculture industry is how to resolve the issue of preventing bee decline, while at the same time mitigating crop losses associated with pest infestations.

1.2 Bioremediation of pesticides

1.2.1 Bioremediation of organophosphate pesticides

The presence of microorganisms has a direct impact on the stability of organophosphate pesticides in soil (24, 25) and aquatic ecosystems. This suggests that these compounds can act as important carbon, nitrogen, and/or phosphorus sources for microorganisms. Furthermore, there is growing evidence of bacterial strains that encode and demonstrate organophosphate pesticide metabolism. However, identification of individual isolates capable of degrading both parent pesticide and potentially toxic metabolites is rare (26, 27). This has given rise to the potential use of multi-organismal mixtures or genetically-engineered strains capable of completely degrading organophosphate pesticide-contaminated environments. Currently two major families of microbial hydrolases have
been shown to confer organophosphate pesticide metabolism: *opd* phosphotriesterases and *mpd* metallo-β-lactamases (26).

The best classified and most ubiquitous pesticide-metabolizing gene, *opd*, was originally discovered on plasmids harbored by *Sphingobium fuliginis* ATCC 27551 (28) and *Brevundimonas diminuta* GM (29, 30) isolated from organophosphate-treated soils. Enzymes encoded by *opd* display broad temperature and pH enzymatic kinetics and are able to metabolize numerous different organophosphate pesticide structures (26). Interestingly, the *opd* gene from *S. fuliginis* and *B. diminuta* are flanked by an insertion operon associated with a transposase and resolvase (31), which contain multiple *tra* genes to promote conjugation (32), and has an integrase to promote chromosomal incorporation (33). This supports the finding of the *opd* gene and associated homologues spreading to other strains of soil bacteria by lateral gene transfer (34). However, it is still debated whether the *opd* gene originated in response to environmental organophosphate exposure or as a coincidental off target effect. An alternative hypothesis states that the *opd* genes and related homologues encode hydrolytic phosphotriesterases that function primarily in phosphate metabolism (35). It is interesting to speculate on what the potential expansion of microorganisms with *opd*-like genetic potential could be telling us about human interactions with the environment.

The other major gene family associated with organophosphate pesticide metabolism is the methyl parathion degradation (*mpd*) gene family. *Mpd* genes do not display any notable (>20%) homology to *opd* or other genes involved in organophosphate pesticide degradation (36). The *mpd* genes impart an uncommon ability to degrade methyl parathion, methyl paroxon, and chlorpyrifos (26). Although the *mpd* genes are primarily chromosomally encoded, numerous soil bacteria have been both shown and predicted to contain *mpd* genes based on conserved metallo-β-lactamase domains (36). These findings provide evidence for non-organophosphate specific degradative function of *mpd* proteins.

### 1.2.2 Bioremediation of neonicotinoid pesticides

Studies of environmental bioremediation of pesticides has recently expanded beyond the class of organophosphate pesticides. Genera of soil bacteria, such as *Pseudoxanthomonas*
(37), *Ochrobactrum* (38), *Mycobacterium* (39), *Stenotrophomonas* (40), *Ensifer* (41), and *Bacillus* (42) have been shown to have imidacloprid-degrading potential. However, unlike bacterial-mediated organophosphate metabolism, the mechanisms of bacterial neonicotinoid degradation still remain largely unclear.

### 1.3 Introduction to the human microbiota

Humans contain an ‘organ’ composed of 2-6 pounds of bacteria, primarily residing in the gastrointestinal tract, which has gone largely unappreciated until recently. The gastrointestinal microbiome is a dynamic ‘organ’ that performs important physiological functions for its host, and can also be a factor in disease. The human body is initially colonized during vaginal birth by microorganisms from the mother’s genital tract and stool. Subsequent colonization and dynamic changes result due to the newborn’s exposure to breast milk and environmental microbes (43-46). The diverse bacterial communities of young children typically mature to resemble the distinct and stable profiles of an adult by 1-2 years of age (45, 46). Microbes abundantly colonize the human gastrointestinal tract. By adulthood, this region contains 1- to 10-fold more bacteria (approximately 1000 species) than the total human cell count (depending on host body mass) (47) and encodes 100-fold more gene functions (48). Thus, it is not surprising that these bacterial communities, known as microbiotas, play important roles in shaping their host’s physiology. However, until the recent decreased costs and increased throughput of nucleotide sequencing, comprehensive investigations of the gastrointestinal microbiota were not possible. Today, the interest and tools to explore the human microbiome (microbiota and gene functions) have become sufficient to investigate many of the fundamental questions regarding the importance of these host-microbe interactions to human health. The systems biology nature of host-microbiota interactions make questions in these fields of study particularly challenging to answer in humans. However, animal models may provide valuable insights into these processes for the time being. The ability to colonize germ-free animals such as fruit flies, zebrafish, or rodents with whole or selective human microbiota samples is an important study option.
1.3.1 The microbiota and xenobiotics

Xenobiotic metabolism refers to the biochemical processes that convert compounds foreign to an organism, such as drugs and toxins, into alternative forms. Typically, these processes are performed by specialized enzymes that convert lipophilic compounds into more hydrophilic products to enable excretion. In mammals, the predominant organ system involved in xenobiotic metabolism is the smooth endoplasmic reticulum of the liver. The smooth endoplasmic reticulum of epithelial cells present in the gastrointestinal tract, lungs, kidneys, and skin have small roles in localized xenobiotic metabolism. For most orally consumed drugs and toxins, first pass metabolism is an important consideration for evaluating the amount of parent xenobiotic that reaches systemic circulation (Figure 1-3). Since oral drugs must be absorbed in the intestine and pass through the liver via portal circulation prior to reaching systemic circulation, often the amount of parent xenobiotic consumed is much greater than what reaches systemic circulation. Thus, portal circulation is an elegantly designed system that attempts to mitigate the xenobiotic distribution throughout the body. Xenobiotic metabolism can be defined as having two distinct phases: phase I and phase II. It is important to note that xenobiotic metabolism can occur in any order, not all xenobiotics have multi-phase metabolism, and some metabolites are more toxic than the parent compound.
Orally consumed drugs and toxins must be absorbed by gastrointestinal enterocytes before traveling to the liver via portal circulation. The liver often metabolizes these compounds such that the amount of unchanged xenobiotic that reaches systemic circulation is reduced relative to the initial dose. Images were modified from Servier Medical Art by Servier under the Creative Commons Attribution 3.0 Unported License (http://www.servier.com/Powerpoint-image-bank).

**Figure 1-3: First pass metabolism.**
Phase I metabolism of xenobiotics includes oxidation, reduction, and/or hydrolysis reactions that are typically performed by the heme-containing cytochrome P450 (CYP) superfamily. Oxidized CYP isoforms bind xenobiotics and perform biotransformation by coupling CYP reduction to oxygen production, through interactions between nicotinamide adenine dinucleotide phosphate (NADPH) and cytochrome P450 oxidoreductase (POR). The human CYP superfamily contains 57 genes (e.g. CYP3A4) divided into 18 families (e.g. CYP3) and numerous subfamilies (e.g. CYP3A) based on amino acid sequence identity \( \geq 40\% \) or \( \geq 55\% \), respectively (49). However, the CYP isoforms most relevant to human drug metabolism include CYP3A4, CYP2D6, CYCP2C8/9, and CYP1A2 (Figure 1-4). The ability of many CYP450 isozymes to be induced or inhibited by environmental factors make them of clinical significance to pharmacotherapy and toxicology. Other enzymes involved in phase I metabolism include: flavin-containing monooxygenases, alcohol dehydrogenase, aldehyde dehydrogenase, peroxidases, and monoamine oxidase. The remarkable number and complex regulation of phase I metabolic processes demonstrate the importance of these biological processes to organismal homeostasis.
Figure 1-4: Percentage of commonly used drugs metabolized by a given cytochrome P450 isoform (2012).
Phase II metabolism of xenobiotics are conjugation reactions. Often phase I metabolism does not generate compounds polar enough for renal elimination. Phase II metabolism adds endogenous polar substrates such as glucuronic acid, methyl groups, sulfate or sulfuryl groups, glutathione, and/or acetyl groups to a parent or metabolic derivative xenobiotic. Phase II metabolism typically requires phase I metabolism first, since reactive sites (hydroxyl-, carboxyl-, amino, or sulfhydryl) on a xenobiotic are required for conjugation to occur.

The field of pharmacogenomics has demonstrated the importance of dosing many common medications on a personalized basis. This strategy is based on observations that differences in human genotype can have major implications to therapeutic toxicity or efficacy. The implications of how relatively small genetic variations can have a crucial impact on drug dosing are intriguing. An emerging area of research that likely has underappreciated implications to pharmacology and toxicology is the microbiota, or microbial communities (largely bacteria) that reside most profoundly in the gastrointestinal tract (50). This field has been termed “pharmacomicrobiomics” (50). The microbiota has been shown to interact with drugs (51-53) and alter host xenobiotic metabolism (7, 54, 55). These interactions are clinically relevant based on observations of the microbiota conferring protection or toxicity to xenobiotic exposure depending on the nature of the interaction. Studies in germ-free mice have demonstrated that the microbiota is critical for mitigating the absorption and bioaccumulation of cadmium and lead (56). Alternatively, chemical inhibition of the intestinal Escherichia coli β-glucuronidases have been shown to relieve the rate-limiting toxicity of the anticancer drug, camptothecin (57). These studies suggest that microbiome-drug interactions can be targeted, which adds an additional layer of complexity to pharmacology. Given that the gastrointestinal microbiota displays much larger interpatient variation than genetics, the role that the microbiota may play in personalized medicine, at least for orally administered drugs, requires further investigation. A database of the currently known drug-microbiome interactions can be found at http://pharmacomicrobiomics.com. Further study is particularly important since the mechanisms of these interactions remain largely unexplored. Currently it has been shown that the typical lipoteichoic acid receptor, Toll-like receptor 2, is necessary for Cyp1a1 induction (58) and that bacterial metabolites can
modulate the pregnane X receptor, which coordinates detoxification gene upregulation in response to many xenobiotics (59). Thus, it is possible that immunomodulation and/or distant site small molecule signaling are plausible explanations for microbiota regulation of host xenobiotic metabolism. Since organophosphate and neonicotinoid pesticides are substrates for CYP-mediated metabolism by the mammalian liver, the relevance of applying pharmacomicrobiomics principles for the application of limiting pesticide toxicity holds merit.

Given the association of microbiota community profiles with an expanding list of pathologies, it is important to understand how xenobiotics shape the composition. Once mature, the microbiota was believed to be relatively stable in terms of composition (60). However, studies with diet (61), circadian rhythm (62, 63), and, not surprisingly, antibiotics (64) suggests more dynamic status.

Certain microbiota patterns from dietary consumption of artificial sweeteners (65), emulsifiers (66), high fat (67), and red meat (68-70) appear to be affiliated with disease progression. Environmental toxins including pesticides (71) and heavy metals (72) can alter these patterns. This emphasizes that microbes are more sensitive to external factors than originally anticipated.

Current microbiota analyses frequently only take a ‘who is there approach’ in their experimental design. Most studies utilize 16S rRNA gene sequencing to identify a fingerprint of bacterial community composition. The use of other –omics approaches such as metagenomics, metatranscriptomics, and metabolomics/proteomics better assess microbial communities in terms of ‘what is the potential?’, ‘what are they doing?’, and ‘how are they doing it?’, respectively. Notably, just because microbiota abundance profiles do not change does not mean their behaviour is unaltered.

In conclusion, a multi-tool approach is preferable to truly understand systems biology problems associated with the complex interactions between microbial communities and their hosts.
1.3.2  *Drosophila melanogaster* as a simplified model of host-microbiota interactions

1.3.2.1  Overview

One of the biggest assumptions in microbiome research is that both the abundance and identity of microorganisms as a whole have the potential to directly influence host physiology in different ways (73). This is different to the reductionist concept that a single pathogen is responsible for a disease, as proven by Koch’s postulates. In order to test the microbiome concept, germ-free mice are often used and implanted with microbial communities to assess disease causation. Due to the cost and infrastructure challenges of maintaining germ free colonies, an alternative approach is needed to investigate host-bacterial interactions, and by extension to investigate mechanisms of probiotic efficacy in a living system. An ideal approach would include the ability to easily extract material for high throughput screening in an affordable model in combination with a bacterial organism that quickly reproduces, and in which microbial manipulation can be easily performed. *Drosophila melanogaster* is an excellent candidate as it possesses all of these qualities. The tools to investigate host-microbe relationships in *Drosophila* are continuing to evolve, but the species has been extensively used for understanding human stem cell biology, neurobiology, genetics, and innate immunity. It has been hypothesized that 75% of human disease-associated genes have functional homologs in *Drosophila* (74).

*Drosophila melanogaster*, commonly known as the fruit fly, has been used to study bacterial pathogenesis innate immunity. Thus, the idea of using *Drosophila* for developing an understanding of the role that symbiotic bacteria play in the regulation of host physiology has recently been suggested (75-77). The efficient and convenient germ-free *Drosophila* model has yet to be used in probiotic studies. Axenic *Drosophila* colonies can be derived and maintained relatively easily without the requirement of expensive animal facilities. In contrast to the aforementioned complexity of the human microbiota, the *Drosophila* gut microbiota has a low microbial diversity (1-30 species) and is typically dominated by *Lactobacillus* and *Acetobacter* (78-80). This makes it simpler to decipher what each species is doing. Most importantly, *Drosophila*
experimentation is affordable, convenient, timely, and does not require approval by animal ethics review boards. These characteristics make Drosophila the most ideal high-throughput in vivo model for understanding general mechanisms of host-microbiota interactions.

### 1.3.2.2 Drosophila gut anatomy

In comparison to the human gastrointestinal tract, the Drosophila gut has several major differences, but overall structure and function are remarkably similar. The adult gut of Drosophila melanogaster is composed of a tubular structure composed of an epithelial monolayer encircled by visceral muscles, nerves, and tracheae (81). In contrast to humans, gut transit time in adult Drosophila is considerably faster, and has been shown to proceed in less than 1 h (82, 83). The adult gut of Drosophila is sequentially divided into the foregut, midgut, and hindgut. Unlike the critical role of mucous in the mammalian intestine, the adult Drosophila midgut is encircled by a luminal layer of peritrophic matrix (lattice of chitin fibrils and chitin binding proteins) that shields the epithelium from the bacteria-dominant microbiota, which contains approximately $3.5 \times 10^5$ colony forming units (75). The foregut and hindgut are encircled by a luminal layer of impermeable cuticle (82). This division of luminal compartments is important for segregation of midgut digestive enzymes into either the endoperitrophic or ectoperitrophic space (84). The pH of the adult midgut varies with diet but is approximately 7, <4, 7-9, and 5 in the anterior midgut, copper-cell region (midgut region R3), posterior midgut, and hindgut, respectively (82, 85). Acidic conditions of the copper-cell region are analogous to the mammalian stomach and similarly function to denature proteins, enable certain peptidase enzymatic activity, promote metal and lipid absorption, and kill microorganisms (82, 86). Thus, the relatively well-conserved anatomy and signaling pathways that control intestinal development, regeneration, and pathology make Drosophila a useful simplified model that permits a reductionist approach to microbiome research and many other fields (87).

The Drosophila gut physiology is regulated by enteric neurons and endocrine signaling. The neuronal innervation of the Drosophila gut utilizes a variety of neurotransmitters such as serotonin and insulin (82). In contrast to the mammalian intestine that contains
enteric innervation throughout its entirety, the *Drosophila* gut is only innervated at the esophagus-crop-anterior midgut junction, midgut-hindgut junction, and posterior hindgut (88). These innervation locations are predominately associated with muscle valves, which suggests an important role for peristaltic regulation. In addition, some nerve fibers innervate the epithelium (82). Further gut regulation results from secretions from endocrine glands, neuroendocrine organs, and gut resident enteroendocrine cells. Enteroendocrine cells are most prevalent in the midgut and are responsible for multiple peptides relevant to modulating intestinal physiology (82). Taken together, the diverse neuronal and hormonal inputs to the *Drosophila* gut make it a convenient model for the mammalian intestine.

The adult midgut is the best characterized gut region and is the primary site of digestion and absorption of nutrients (Figure 1-5). Epithelial cells of the midgut contain enterocytes or enteroendocrine cells, which are derived from intestinal stem cells. These intestinal stem cells are also found in the foregut and hindgut. The midgut is subdivided into 6 anatomical regions (R0 to R5) based on metabolic and digestive function (82). *Drosophila* contain numerous carbohydrases, proteases, lipases, lysozymes, chitinases, and glucanases. These enzymes degrade macromolecules and microbial cell wall components, which suggest nutrients may also be derived from the bacteria and yeast present on the fly diet of rotting fruit (82). The importance of the microbiota to fruit flies are also supported by studies with germ-free or antibiotic-exposed flies where bacteria are critical regulators of *Drosophila* growth (77), mating (89), and immune regulation (90). Taken together, these observations emphasize the intimate and critical relationship between *Drosophila* and indigenous and environmental microorganisms.
Figure 1-5. 3D model of the *Drosophila melanogaster* midgut.
It is important to note that *Drosophila* feeding behavior and intestinal morphology change throughout development. *Drosophila* larvae utilize mouth hooks to continuously eat solid food in order to sustain rapid growth and development required for metamorphosis (82). In contrast, adult Drosophila feed less and ingest food/liquid through their proboscis. Furthermore, unlike adult flies, larval guts contain 4 gastric ceca in the anterior gut for digestion and nutrient absorption. At each stage of development—larvae, pupae, and adult—*Drosophila* have distinct gastrointestinal tissues (82). The larval and pupal midguts degenerate and are discarded by adult flies following eclosion (82).

### 1.3.2.3 *Drosophila melanogaster* microbe sensing

*Drosophila* microbe-sensing immune responses to pathogen-associated molecular patterns (PAMPs) can largely be separated into two distinct pathways. Pioneering work in *Drosophila* observed rapid activity in either the Toll or immune deficiency (IMD) pathway in response to septic injury. The Toll pathway is activated by lysine (Lys)-type peptidoglycan (Gram-positive bacteria) and β-1,3 glucan (fungi). Alternatively, the IMD pathway is activated by diaminopimelic acid (DAP)-type (Gram-negative and Gram-positive bacilli bacteria) (91). Together the Toll and IMD pathways are critical for upregulating nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) response genes such as antimicrobial peptides drosomycin and diptericin, respectively.

Traditional studies of *Drosophila* systemic immunity have demonstrated that the normally aseptic fat body (analogous to the mammalian liver) is the predominate organ involved in Toll and IMD signaling in response to sepsis. However, the complexity of *Drosophila* mucosal immunity still remains perplexing given the direct contact between host tissue and microorganisms.

Unlike the Toll pathway, the IMD pathway plays a critical role in the *Drosophila* gut for mitigating pathogens and modulating the microbiota (92-95, Figure 1-6). The gut IMD pathway is initiated in response to DAP-type peptidoglycan sensing by the peptidoglycan recognition protein family (PGRP) proteins: membrane-bound PGRP-LC and intracellular PGRP-LE. IMD pathway stimulation results in downstream activation of the
transcription factor, Relish, which initiates transcriptional activation of genes encoding antimicrobial peptides and other effector molecules. Flies deficient in IMD pathway activation are significantly more susceptible to pathogenesis by Gram-negative organisms such as *Serratia marcescens* (96) and *Pseudomonas entomophila* (97). Alternatively, the dominant symbiotic genera of the *Drosophila* microbiota, *Lactobacillus* and *Acetobacter*, contain DAP-type peptidoglycan that initiates a constitutive basal stimulation of the IMD pathway (90, 98). This is unexpected given that constitutive IMD-dependent immune activation would likely have negative consequences to host physiology. Thus, given that *Drosophila* lack adaptive immune systems, these conflicting observations suggest a remarkably complex regulation of optimal IMD signaling in response to beneficial and pathogenic microbes in the *Drosophila* gut. In addition, these observations suggest there are underexplored host physiological benefits of IMD-dependent intestinal activation by the symbiotic microbiota (75).

The *Drosophila* IMD/ NF-κB pathway is complex and contains several negative regulators at different hierarchical levels. PGRP-SC and -LB degrade peptidoglycan in an attempt to prevent overstimulation of the IMD pathway in the gut (99, 100). In addition, Pirk (aka PIMS, Rudra) is a negative feedback loop regulator which is also activated by Relish in response to IMD signaling. Pirk antagonizes the IMD pathway ligand-receptor interaction of DAP-peptidoglycan with PGRP-LC (101-104). Interestingly, despite microbiota dependent activation of Relish, upregulation of downstream antimicrobial genes *dipterisin* and *Cecropin A1* (*CecA1*) is antagonized in a gut-specific manner by Caudal (105). The multiple layers of IMD pathway regulation highlight the essential nature of modulating host-microbe interactions for host physiological homeostasis.
Figure 1-6. IMD pathway regulation in the Drosophila melanogaster midgut.

Images were modified from Servier Medical Art by Servier under the Creative Commons Attribution 3.0 Unported License (http://www.servier.com/Powerpoint-image-bank).
Fundamental questions not addressed regarding gut IMD pathway regulation are: 1) How does constitutive Relish activation by the indigenous microbiota shape host physiology?; 2) Is this pathway targetable? The Relish pathway has been shown to have important roles other than antimicrobial peptide regulation. Notably, Relish promotes host survival in response to noxious stimuli such as radiation (106), and regulates cell death (107). Thus, *Drosophila* may be an excellent model for deciphering the physiological consequences of host-microbiota-immune interactions induced in response to common probiotic *Lactobacillus* species used by humans. Furthermore, honeybees are known to contain functional homologues of the Relish protein. Given that honey bee colony collapse disorder has been attributed to a combination of toxic immunosuppressive pesticides (108) and pathogens, it is interesting to speculate on the potential of using beneficial microbes to augment honeybee survival through a Relish-like pathway.

### 1.4 *Lactobacillus* and *in vivo* bioremediation

#### 1.4.1 Probiotic potential

The importance of the microbiota for numerous aspects of host physiology and health, suggests that its modulation with probiotics, prebiotics, synbiotics (probiotic and prebiotic combination), and/or antibiotics hold therapeutic promise (109). Probiotics have been defined by the Food and Agriculture Organization of the United Nations and the World Health Organization as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (110). Alternatively, prebiotics are non-digestible food supplements that benefit host physiology by selectively stimulating the growth and/or function of a limited number of beneficial bacteria (111). Fermented foods, which frequently contain *Lactobacillus* and/or *Bifidobacterium*, are the best documented for associations with health and safety (112). However, several studies are now utilizing nucleotide sequencing technology to identify candidate probiotics based on organisms present in healthy controls, but absent in diseased counterparts (113).

*Lactobacilli* are Gram-positive commensal bacteria. *Lactobacillus* is a genus used to ferment a number of foods. It is a common constituent of the oral cavity, urogenital tract, and gastrointestinal tract of humans and a wide variety of animals. This has made
*Lactobacillus* a key genus of probiotic organisms (114), with some general and specific strain effects described.

Probiotics have been shown to temporarily alter the function and metabolic read-out of the gastrointestinal microbiota (115) and mucosal immune function (116). This is particularly interesting given that exposure to chlorpyrifos (organophosphate pesticide) can induce dysbiosis of the gut microbiota in rodent and *in vitro* models (71, 117, 118). Thus, there is potential for probiotic effects to help retain homeostasis. This is important since the “healthy” gastrointestinal microbiota plays a role in protecting the human from xenobiotic insults (58, 119). However, this emerging theory needs to be empirically investigated and validated *in vivo* for each toxin and probiotic strain. It is reasonable to suggest that probiotic lactobacilli may be a useful therapeutic against pesticide-induced gut dysbiosis (71, 117, 118) or even pesticide uptake without dysbiosis, and result in reduced pesticide absorption via direct degradation or passive binding and/or modulation of host xenobiotic metabolism.

### 1.4.2 Lactobacilli-mediated protection of gastrointestinal integrity

The tight junctions of the intestinal epithelium form an important barrier against microbes and toxic substances. Rats chronically exposed to organophosphate pesticide demonstrated increased intestinal permeability and both perturbed expression and localization of tight junctions (120). It is possible that perturbed gut barrier function can lead to increased paracellular absorption of toxic compounds through a feed-forward mechanism, and thus exacerbate systemic pesticide exposure. Furthermore, chronic impairment of gut barrier function is hypothesized to contribute to “leaky gut syndrome” (121). Studies are currently investigating the contribution of leaky gut syndrome to the pathogenesis of both intestinal and systemic diseases such as: celiac disease, inflammatory bowel disease, type 1 diabetes, autism, and numerous others. The association of pesticide exposure and diabetes in developed countries raises questions regarding the long-term safety of many pesticide compounds (122). It is interesting to speculate on how chronic low-dose pesticide exposure may influence the development and/or progression of various other diseases.
Numerous studies suggest that lactobacilli have the ability to enhance gut barrier function and thus potentially reduce absorption and secondary damage caused by pesticides. For instance, *Lactobacillus plantarum* MB452 was found to enhance the expression of the tight junction proteins occludin, zona occluden-1 and -2, and cingulin in the Caco-2 intestinal cell-line (121). Strains of lactobacilli have also been shown to maintain gut barrier function when the epithelium has been challenged by bacterial pathogens (123, 124) and excessive unconjugated bilirubin (125). Rats treated with the pesticide, malathion, suffered hepatotoxicity due to excessive inflammation (124). Hence, the finding that probiotic *Lactobacillus casei* DN-114 001 (126) and *Lactobacillus rhamnosus* GG (127) were able to dampen the pro-inflammatory response in a BALB/c mouse model of dextran sodium sulfate-induced colitis, could be relevant to the preventing inflammatory reactions associated with pesticide exposure. Similarly, *Lactobacillus casei* DN-114 001 appears to prevent excessive inflammation by downregulating activity of the NF-κB pathway in the intestinal epithelium and increasing the population of regulatory T-cells in the mesenteric lymph nodes (128). Future studies should investigate if these mechanisms of lactobacilli-dependent reduction of inflammation and immune regulation can be applied to pesticides.

### 1.4.3 Antioxidative potential of lactobacilli

Chronic exposure to organophosphate, neonicotinoids, and glufosinate ammonium pesticides has been associated with increased oxidative stress and associated genotoxicity in humans (11, 12). The use of antioxidant therapies has some effectiveness against organophosphate-induced toxicity in rat models (129). This suggests they either reduce the generation of pesticide-induced reactive oxygen species or enhance host antioxidant enzymatic capacity. There are numerous studies demonstrating that lactobacilli can ameliorate oxidative stress both *in vitro* and *in vivo*. For instance, *Lactobacillus casei* ATCC 334 was able to decrease gastrointestinal DNA damage of rats exposed to the carcinogen 1,2-dimethylhydrazine (130). *Lactobacillus rhamnosus* has also been shown to prevent aflatoxin B1-induced DNA damage and absorption in a Caco-2 Transwell model (131). Thus, lactobacilli appear to be protective against a wide range of toxin-induced oxidative stress and downstream cellular damage, and their use as a probiotic
might succeed through antioxidant activity.

### 1.4.4 Interactions with organophosphate pesticides

Several species of *Lactobacillus* have been shown to remove organophosphate pesticides from dairy products (123, 124), kimchi (132), and wheat. Indirect evidence suggests that lactobacilli are able to metabolize organophosphate pesticides via phosphatase enzymes (133, 134). One study concluded that the organophosphate pesticide degrading ability of *Lactobacillus brevis* WCP902 was associated with functional expression of the organophosphate hydrolase (*OpdB*) gene (134-136). These findings are not surprising; as classical bioremediation has identified strains of bacteria with enhanced ability to degrade pesticide compounds. Genetic engineering approaches have improved the ability of *Escherichia coli* to detoxify organophosphate pesticides by displaying the organophosphate hydrolase enzyme at the cell surface (123, 124). The *organophosphate hydrolase* gene is a phosphotriesterase (aryldialkylphosphatase) that has been well characterized for organophosphate metabolism and is found in several species of *Lactobacillus*.

### 1.5 Rationale and hypotheses

#### 1.5.1 *Lactobacillus* mitigation of organophosphate absorption and toxicity

Despite the support for lactobacilli having the potential to degrade organophosphate pesticides *in vitro* (123, 124, 126, 132), research is needed to validate if these findings can be transferable to reducing systemic absorption of pesticides *in vivo*. Recently, it was demonstrated that consumption of yogurt-supplemented with *Lactobacillus rhamnosus* GR-1 was able to reduce systemic bioaccumulation of mercury and arsenic in Tanzanian pregnant women (8). These studies provide proof-of-concept for the idea of *in vivo* “bioremediation”. Thus, although the chemical structures of heavy metals and pesticides differ drastically, proof-of-principle exists for the consumption of locally produced nutritious yogurt to prevent the absorption of heavy metals and perhaps other environmental toxins such as pesticides in heavily contaminated regions of the
developing world. We hypothesize that the ability of lactobacilli to degrade and/or bind certain pesticides, enhance gut barrier function, modulate host xenobiotic metabolism, and influence gastrointestinal microbiota community structure will reduce the negative consequences of pesticide absorption though the gastrointestinal tract (Figure 1-7).
Figure 1-7. Proposed mechanisms of lactobacilli-mediated reduction of both gastrointestinal pesticide absorption and systemic toxicity.

Certain strains of probiotic lactobacilli can reduce pesticide toxicity directly by either (A) sequestering pesticides from the gastrointestinal environment or (B) metabolizing pesticides into less toxic metabolites (123, 124, 132). (C) Lactobacilli could also reduce the gastrointestinal cytotoxicity caused by pesticide-induced reactive oxygen species (ROS) generation (130, 131). (D) By enhancing tight junction expression, lactobacilli could reduce paracellular absorption of pesticides (131). (E) Direct interactions between lactobacilli and gastrointestinal tract can modulate both the activity and expression of enterocyte and liver (not shown) xenobiotic metabolizing enzymes relevant to host pesticide metabolism (54, 55, 58, 131). (F) Lactobacilli could modulate both the activity and composition of gastrointestinal microbiota (115), which are known to influence numerous components of host physiology and the microbiome that are relevant to reducing pesticide absorption. Images were modified from Servier Medical Art by Servier under the Creative Commons Attribution 3.0 Unported License (http://www.servier.com/Powerpoint-image-bank).
1.5.2 Protective effect of *Lactobacillus* against imidacloprid toxicity in a *Drosophila melanogaster* model of colony collapse disorder

Lactobacilli are commensal bacteria that colonize honeybees (133, 134) and confer beneficial effects to honeybee health and colony size (134-136). Lactobacilli have also been shown to be important stimulators of insect immunity (78, 137, 138). Alternatively, the neonicotinoid, imidacloprid, has been shown to suppress invertebrate immune function and consequently increase these organisms susceptibility to viral infection (139). Thus, the indirect damage of neonicotinoid pesticides to bee health may be more of a problematic issue than anticipated. We hypothesize that probiotic lactobacilli would mitigate pathogen susceptibility and general physiological consequences of imidacloprid-toxicity through immune stimulation in a *Drosophila melanogaster* model of “colony collapse disorder”.

*Drosophila melanogaster* (the common fruit fly) is a convenient insect organism to model the potential for lactobacilli to mitigate pesticide-induced toxicity in bees (139). Both fruit flies and honeybees have a core microbiota composed of *Lactobacillus*, with *Lactobacillus plantarum* being a major species in both hosts (78-80). But more importantly, both species are insects, and are therefore profoundly affected by imidacloprid. We plan to use this pilot study to demonstrate proof-of-principle for mitigating imidacloprid toxicity in an insect model before experimenting in at risk honeybee populations.

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Chapter 2

2 Reduction of organophosphate pesticide absorption and toxicity by probiotic *Lactobacillus rhamnosus*

The material in this chapter is currently under review at Applied and Environmental Microbiology as a full length article and has a content license that can be found in Appendix D.


2.1 Abstract

Organophosphate pesticides, commonly used in agriculture, can pose health risks to humans and wildlife. We hypothesized that dietary supplementation with *Lactobacillus*, a genus of commensal bacteria, could prevent absorption and toxicity of consumed organophosphate pesticides (parathion and chlorpyrifos). Several *Lactobacillus* species were screened for the ability to tolerate 100 ppm of chlorpyrifos (CP) or parathion in MRS broth using 24 h growth curves. Lactobacilli strains were identified that were unable to reach stationary phase culture maxima and displayed abnormal culture morphology in response to pesticide. Further characterization of commonly used, pesticide-tolerant and pesticide-susceptible, probiotic *L. rhamnosus* GG (LGG) and *L. rhamnosus* GR-1 (LGR-1) revealed that both strains could significantly sequester organophosphate pesticides from solution after 24 h co-incubations. This effect was independent of metabolic activity, as no difference in organophosphate sequestration was observed between live and heat-killed strains. Furthermore, LGR-1, and more notably LGG, reduced the absorption of 100 µM parathion or CP in a Caco-2 Transwell model of the small intestinal epithelium. To determine the effect of sequestration on acute toxicity,
newly adult *Drosophila melanogaster* were exposed to food containing 10 µM CP with or without supplementation by live LGG. *Drosophila* supplemented with LGG simultaneously, but not 3 d prophylactically, mitigated CP-induced mortality. In summary, the results suggest that *L. rhamnosus* may be useful for reducing toxic organophosphate pesticide exposure by passive binding. These findings could be transferable to clinical and livestock applications due to the affordable, practical, and convenient ability to supplement products with food grade bacteria.

2.2 Introduction

Organophosphate pesticides are a class of insecticide under scrutiny for being linked to toxic effects in both humans and wildlife (1). However, these compounds are still commonly used in agriculture and pest control programs. Organophosphate pesticides include parathion, malathion, methyl parathion, chlorpyrifos (CP), diazinon, dichlorvos, phosmet, fenitrothion, tetrachlorvinphos, azamethiphos, and azinphos-methyl.

Organophosphate pesticides irreversibly inhibit acetylcholinesterase to induce excessive cholinergic stimulation (2, 3). The potential off-target health consequences of organophosphate pesticide exposure to humans include: neonatal developmental abnormalities (4, 5), endocrine disruption (6), neurodegeneration, cancer (7, 8), metabolic disruption (9), heart disease (10), chronic kidney disease, and other less common pathologies. In addition, organophosphate pesticides are reported to have negative impacts on honey bee colonies, which are critical pollinators for numerous agricultural products (11-13). Although the evidence is mixed and often correlative, the original use of these organophosphates as nerve agents strongly suggests that these pesticides are not aligned with human or wildlife health.

Despite negative consequences, the affordability and need to prevent crop losses associated with insect infestations suggest that organophosphate pesticide use will continue in the near future. One long-considered counter to these effects is the use of microbes to detoxify organophosphate pesticide-contaminated environments (14-20). Classical bioremediation efforts have identified numerous strains of soil bacteria that contain genetically diverse phosphotriesterases capable of organophosphate degradation (15). Moreover, the symbiotic relationship between *Burkholderia* strains and the bean
bug, *Riportus pedestris*, have been shown to confer resistance of these insects to insecticides (21). Taken together, these observations suggest that microorganisms could be employed to reduce the toxic effects of organophosphate insecticides *in vivo*.

There has been development within bioremediation research to investigate the potential of transitory food-grade bacteria to prophylactically prevent the absorption of environmental toxins such as pesticides (22), heavy metals (23), and aflatoxin (24, 25). Many lactobacilli are commonly used in fermented foods such as yogurt, cheese, sauerkraut, pickles, beer, wine, cider, kimchi, cocoa, and kefir (26). Lactobacilli have also been shown to be a natural and beneficial member of numerous organisms relevant to humans such as livestock, honeybees (27), and fish (28). Notably, lactobacilli have been shown to reduce organophosphate pesticide contamination of dairy products (29, 30). The mechanism of action used by lactobacilli against organophosphate pesticides remains unclear and is commonly attributed to phosphatase capabilities. However, one kimchi isolate, *Lactobacillus brevis* WCP902, was shown to contain the *opdB* gene that conferred the active ability to degrade CP (31). Similarly, the common probiotic, *Lactobacillus rhamnosus* GG (LGG), has a predicted hydrolase/phosphotriesterase (*LGG_RS02045*) with high sequence similarity to the experimentally-validated parathion hydrolase (*opd*) found in *Brevundimonas diminuta* (20).

The aim of this study was to better characterize the *in vivo* bioremediation potential of probiotic food-grade bacteria interacting with organophosphate pesticides. We hypothesized that the direct binding or metabolism of organophosphate pesticides by *L. rhamnosus* strains would reduce toxin absorption and downstream organismal toxicity.

### 2.3 Materials and Methods

#### 2.3.1 Chemicals

CP (catalog number: 45395) and parathion (catalog number: 45607) were obtained from Sigma-Aldrich. Stock solutions were prepared at 100 mg/mL dimethyl sulfoxide (Sigma-Aldrich) and stored frozen at 4°C until usage.
2.3.2 Bacterial strains and culture

Lactobacilli strains used were: *L. rhamnosus* GG, *L. rhamnosus* GR-1, *L. casei* ATCC 393, *L. delbrueckii* DSM 20074, *L. plantarum* ATCC 14917, *L. crispatus* ATCC 33820, *L. fermentum* ATCC 11739, *L. johnsonii* DSM 20053, *L. reuteri* ATCC 2773, *L. rhamnosus* ATCC 7469. Lactobacilli were inoculated from Difco lactobacilli de Man, Rogosa and Sharpe (MRS) agar (BD, catalog number: 288130) plates into MRS broth. Inoculated MRS broth cultures were subcultured and incubated overnight (18 h) at 37°C anaerobically and statically for experimental procedures. Bacteria were heat-killed by incubation at 56°C for 90 min when necessary. Bacterial killing was confirmed by spread plating 100 µL of bacterial culture on MRS agar plates and verifying the absence of colony growth following 3 d of anaerobic incubation.

2.3.3 Pesticide tolerance assay

Overnight broth cultures (stationary phase) were subcultured (1:100 dilution) into 96 well plates (Falcon, catalog number: 351177) containing MRS broth with or without the addition of pesticide or vehicle (DMSO). Plates were incubated at 37°C and read every 30 min for 24 h at a wavelength of 600 nm using a Labsystems Multiskan Ascent microplate reader.

2.3.4 RNA extraction, reverse transcription, and qPCR

Overnight subcultures of LGG were pelleted at 5000 g (15 min), washed and resuspended in 50 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES, pH 6.8). LGG was exposed to 100 ppm of CP or parathion or vehicle. LGG-pesticide solutions were incubated protected from light at 37°C with gentle shaking. At 1 and 3 h, 500 µL of culture was mixed with 1 mL of RNAprotect Bacteria Reagent (Qiagen, catalog number: 76506) for 10 min, then pelleted by centrifugation at 5000 g for 5 min. Supernatants were discarded. Pellets were flash frozen with liquid nitrogen and stored at -80°C until RNA extraction.

For RNA extraction, pellets were resuspended in 1 mL TE buffer containing 20 mg/mL lysozyme (Sigma-Aldrich, catalog number: L6876) and 50 U/mL mutanolysin (Sigma-
Aldrich, catalog number: M9901). Samples were incubated at 37°C for 30 min before being pelleted by centrifugation at 5000 g for 5 min at 4°C. Supernatants were discarded and pellets were dissolved in 1 mL of TRIzol (Ambion, catalog number: 15596018) by repeated pipetting. After phase separation with chloroform according to the manufacturer’s protocol, the aqueous phase was combined with equal volume of 100% ethanol. RNA purification was continued using a PureLink RNA Mini Kit (Ambion, catalog number: 12183025) with on-column treatment of PureLink DNase treatment (Ambion, catalog number: 12185010). RNA quantity and purity was accessed with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific). RNA was stored at -80°C until reverse transcription.

cDNA was prepared from 200-2000 ng of purified total RNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, catalog number: 4368813). qPCR was performed with a 7900HT Sequence Detection System (Applied Biosystems) in 20 µL reactions using Power SYBR Green Master Mix (Applied Biosystems, catalog number: 4368702) with 200 µM of appropriate forward and reverse primers. Primers used for qPCR analysis are listed in Supplementary Table 2-1. qPCR results were analyzed using SDS RQ 6.3 manager software (Applied Biosystems) and relative gene expression was calculated using the $2^{-\Delta\Delta ct}$ method.

2.3.5 Pesticide binding and metabolism assay

Overnight bacterial cultures were pelleted at 5000 g, washed and resuspended in 50 mM HEPES (pH 6.8). Bacterial-buffer or buffer-alone solutions were incubated with parathion or CP protected from light at 37°C for 24 h with gentle shaking. Cultures were pelleted and supernatant collected. Pellets were washed in 50 mM HEPES, resuspended in methanol, and sonicated for 15 min. Supernatants were again collected for pellet assessment of organophosphate levels.

Pesticide levels were determined by high performance-liquid chromatography (HPLC) using a Poroshell 120 column (100 × 4.6 mm, 2.7µm; Agilent, Mississauga, Ontario) and
an ultraviolet light detector based on a modified protocol (32). CP and parathion were detected at 288 and 275 nm wavelengths, respectively. Solvent flow rate was 1 mL/min of A (H₂O + 0.1% trifluoroacetic acid) and B (acetonitrile + 0.1% trifluoroacetic acid). Solvent gradient was set to 25, 25, 100, 100, 25, and 25 percent B at 0, 2, 9, 11, 11.5, and 13 min, respectively. Sample peak area units were compared to a linear relationship of appropriate standards of known concentrations.

### 2.3.6 Caco-2 cell culture and Transwell experimentation

Caco-2 cells were obtained as a gift from Dr. Brad Urquhart (Western University, London, Canada). Caco-2 cells were routinely maintained at 37°C in atmospheric conditions with 5% CO₂. Cells were cultured in Dulbecco's Modified Eagle Medium (Gibco, catalog number: 11960044) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 1 mM sodium pyruvate (Gibco catalog number: 11360070), 1% MEM non-essential amino acids (Gibco, catalog number: 11140050), 4 mM L-glutamine (Gibco, catalog number: 25030081), and 100 U/mL penicillin-streptomycin (Gibco, catalog number: 15140122). Cells were used experimentally between passages 35-45.

Cells were seeded onto 12mm Transwell (12-well with 0.4 µm pore polyester membrane insert, Corning, catalog number: 3460) plates at a concentration of 1.5 x 10⁵ cells/insert. Cells were differentiated by culturing cells for 21 d as described (33). Prior to absorption experimentation, apical and basolateral compartments were washed 3x and resuspended with Hank’s balanced salt solution (Gibco, catalog number: 14025092). Cells were treated apically with 100 µM of parathion or CP in the presence or absence of 5 x 10⁸ colony forming units of LGG or LGR-1. Basolateral sampling (100 µL) with replacement was performed at 30 min, 1 h, and 2 h. Following absorption experimentation, Caco-2 monolayer integrity was confirmed by lucifer yellow (lucifer yellow CH lithium salt, Biotium, catalog number: 80015) rejection according to manufacturer’s instructions.

### 2.3.7 Drosophila husbandry

Wild type Canton-S (stock number: 1) stocks were obtained from Bloomington Drosophila Stock Center at Indiana University. *Drosophila* were maintained using media containing 1.5% agar (w/v), 1.73% yeast (w/v), 7.3% cornmeal (w/v), 7.6% corn syrup
(v/v), and 0.58% acid mix (v/v) at 22°C with 12 h light/dark cycles. For experimental procedures, media were supplemented with or without pesticide prior to solidification. Experiments with adult *Drosophila* used newly eclosed flies. All experiments were performed in polypropylene *Drosophila* vials (GEN32-121 and GEN49-102, Diamed Lab Supplies Inc., Mississauga, ON, Canada) containing 10 mL of feed with 15-25 flies/tube.

2.3.8 **Adult *Drosophila* survival assays**

Fifteen to twenty-five newly eclosed *Drosophila* were transferred into standard vials containing media supplemented with vehicle or CP and monitored daily for survival. Media was supplemented with 100 µL (10^9 CFU) of washed and concentrated LGG or phosphate-buffered saline vehicle when experimentally appropriate and allowed to air dry before use.

2.3.9 **Drosophila negative geotaxis assay**

Negative geotaxis assays were performed as previously described (34). Mean distance climbed (after 3 s) of 5 replicates from 3 independent experiments were determined.

2.3.10 **Drosophila microbiota analysis**

Whole *Drosophila* were surface sterilized with 70% ethanol, homogenized with a handheld motorized pestle in 0.01 M phosphate-buffered saline. Serially diluted fly homogenates were spot plated onto MRS agar plates. Plates were incubated anaerobically at 37°C and enumerated after 48 h incubation.

2.3.11 **Statistical analyses**

All statistical analyses were performed in GraphPad Prism 6 using one-way analysis of variance tests with Tukey’s multiple comparisons tests or Kuskal-Wallis test with Dunn’s multiple comparison tests. Alternatively, Mantel-Cox and Gehan-Breslow-Wilcoxon tests were used for *Drosophila* survival analyses.
2.4 Results

2.4.1 Lactobacilli vary in their ability to tolerate high levels of organophosphate pesticides

In an effort to determine a strain of *Lactobacillus* able to tolerate high levels of organophosphate pesticides, lactobacilli were screened for the potential to tolerate 100 ppm of parathion or CP using growth curves. In general, lactobacilli growth was largely unaltered by the presence of parathion or CP relative to the vehicle control. Based on the solubility maxima of parathion and CP at 37°C, pesticide concentrations greater than 100 ppm were not evaluated. However, *L. rhamnosus* GR-1, *L. casei* ATCC 393, and *L. delbrueckii* DSM 20074 demonstrated significantly reduced growth (p < 0.05) in the presence of parathion or CP (Figure 2-1A). Organophosphate pesticide treatment significantly reduced the stationary phase growth maxima of these lactobacilli compared to vehicle control treatment (Figure 2-1B). The organophosphate-induced growth deficiency was associated with abnormal shiny and mucoid culture morphologies for *L. casei* ATCC 393 and *L. delbrueckii* DSM 20074 (Figure 2-1C). It is hypothesized that these strains were more susceptible to organophosphate pesticide-induced insult due to more permeable cell walls or fewer enzymes capable of detoxifying their environment, and thus, consequently formed biofilm-like structures. These biofilm-like phenotypes were not observed in other lactobacilli strains exposed to organophosphate pesticides.
Figure 2-1. Lactobacilli vary in their ability to tolerate high levels of organophosphate pesticides.

(A) Percent maximal growth was calculated from 24 h growth curve of optical density 600 nm using the area under the growth curve of pesticide-treated bacteria relative to vehicle treatment of each bacterial strain. (B) Representative growth curves of *Lactobacillus casei* ATCC 393 and *Lactobacillus delbrueckii* DSM 20074. (C) Representative image of lactobacilli cultures following 24 h treatment with 100 ppm parathion or CP or vehicle. Red box outlines bacteria with differential culture morphology following organophosphate pesticide treatment compared to vehicle. Data are depicted as means ± SEM of 3 independent experiments with triplicate technical replicates. CP = chlorpyrifos, LGG = *Lactobacillus rhamnosus* GG, LGR-1 = *Lactobacillus rhamnosus* GR-1.
2.4.2 Organophosphate hydrolase gene, \textit{LGG\_RS02045}, in \textit{L. rhamnosus} GG was not induced by organophosphate pesticide exposure

The National Center for Biotechnology Information’s BLASTP tool identified WP\_014569076.1 (corresponding gene \textit{LGG\_RS02045} or \textit{php}) as sharing high sequence similarity to the experimentally confirmed parathion hydrolase present in \textit{Brevundimonas diminuta} (20). Protein modelling with Protein Homology/analogY Recognition Engine V 2.0 software best-matched WP\_014569076.1 as a likely member of the metallo-dependent hydrolases and phosphotriesterase-like protein superfamily and family, respectively, with 100\% confidence and 52\% identity. To test for substrate-induced gene expression, relative \textit{php} expression was determined in nutrient exhausted stationary phase LGG cultures that contained parathion or CP as the sole carbon and nitrogen source (in 50 mM HEPES buffer). However, no induction of \textit{php} by 100 ppm of parathion or CP was observed at either 1- or 3 h post-exposure (Figure 2-2). These findings contradict the predicted role of \textit{php} in hydrolysis of organophosphate pesticides.
Figure 2-2. Organophosphate hydrolase gene, *LGG_RS02045*, in *Lactobacillus rhamnosus* GG is not induced by organophosphate pesticide exposure.

Relative gene expression of *php* was determined from stationary phase LGG cultures (10⁹ CFU/mL) following 1 and 3 h exposures to 100 ppm of parathion or CP or vehicle in 50 mM HEPES buffer. Data are expressed as means ± SEM of 3 independent experiments with triplicate technical replicates.
2.4.3 *L. rhamnosus* GG and GR-1 can bind, but not metabolize, organophosphate pesticides

The commonly used commercial probiotics, LGG and LGR-1, were characterized for organophosphate pesticide binding or metabolism since they displayed opposite phenotypes of unimpaired and impaired growth in organophosphate-containing media, respectively. Both LGG and LGR-1 significantly sequestered parathion and CP from solution (p < 0.05); however, this effect was much more pronounced with CP (Figure 2-3A). Given the similar molecular structures of parathion and CP, the enhanced binding of CP by LGG and LGR-1 is likely a result of the 3 chlorine atoms on the benzene ring of CP, opposed to the nitrate group on the benzene ring of parathion. Alternatively, the greater hydrophobicity of CP, compared to parathion, could explain greater sequestration mediated by hydrophobic interactions. Interestingly, organophosphate sequestration was observed by LGG and LGR-1 even after heat-killing and the extent of binding was similar to live bacteria (Figure 2-3A). This finding further supports that the predicted organophosphate hydrolase gene, *php*, from LGG is not involved in organophosphate pesticide metabolism. The binding of organophosphate pesticides by LGG and LGR-1 was further supported by the confirmation of parathion and CP in the bacterial pellets following experimentation (Figure 2-3B). The amount of CP observed in LGG pellets was considerably higher than LGR-1 pellets. Discrepancy between CP sequestered by LGR-1 (Figure 2-3A) and the amount of CP determined in LGR-1 pellets is likely due to weak LGR-1-CP binding interactions being perturbed by washing pellets prior to lysis. This preferential interaction of LGG, rather than LGR-1, to CP was not observed with parathion. Furthermore, disrupting bacterial membranes and surface proteins by using methanol as a solvent for binding experiments prevented any notable observations of bacterial-organophosphate binding (Figure 2-3C).
Figure 2-3. *Lactobacilli rhamnosus* GG and GR-1 bind, but do not metabolize, organophosphate pesticides.

(A) Percent parathion and CP were determined in stationary phase live and heat-killed LGG and LGR-1 cultures relative to pesticide-only controls following 24 h co-incubations in 50 mM HEPES. (B) Percent maximal parathion and CP bound relative to 100 ppm input was determined in bacterial pellets following 24 h pesticide co-incubations 50 mM HEPES. (C) Percent parathion and CP were determined in stationary phase chemically-killed LGG and LGR-1 cultures relative to pesticide-only controls following 24 h co-incubations in methanol. Data are displayed as means ± SD of at least 3 independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001. CP = chlorpyrifos, LGG = *Lactobacillus rhamnosus* GG, LGR-1 = *Lactobacillus rhamnosus* GR-1.
2.4.4  *L. rhamnosus* GG and GR-1 reduced the absorption of organophosphate pesticides in a Caco-2 Transwell model.

The ability of LGG- and LGR-1-mediated organophosphate pesticide sequestration was tested for the potential to reduce intestinal absorption of these compounds using a Caco-2 Transwell model of the small intestine. LGG and LGR-1 reduced the Caco-2 apical—basolateral translocation of both parathion and CP compared to unsupplemented controls (Figure 2-4). Similar to earlier binding experiments, reduction of organophosphate pesticide absorption was more pronounced with LGG compared to LGR-1 and lactobacilli binding was more prominent with CP compared to parathion. Basolateral levels of parathion increased kinetically (30 min > 60 min > 120 min) post-apical exposure for cells exposed to CP with or without supplementation of LGG or LGR-1. However, there was an insignificant (other than CP compared to CP-LGG at 1 h) trend of decreased basolateral parathion levels in LGG or LGR-1 supplemented cells at all time points. Most notably, at 1 h post-apical exposure, CP apical—basolateral absorption was undetectable in CP-LGG and CP-LGR-1 simultaneously-treated cells compared to cells treated with CP alone. However, by 2 h post-apical exposure basolateral CP levels in CP-LGG and CP-LGR-1 treated cells increased notably, but remained significantly less (p <0.05) than cells treated with CP alone. These findings support earlier observations of potentially biologically relevant interactions of probiotic lactobacilli with consumed organophosphate pesticides that vary in effect based on subtle differences in molecular structure.
Figure 2-4. *Lactobacillus rhamnosus* GG and *Lactobacillus rhamnosus* GR-1 reduce the absorption of organophosphate pesticides in a Caco-2 Transwell model.

Caco-2 cells, differentiated into small intestinal-like epithelium on Transwells, were exposed apically to 100 μM parathion or chlorpyrifos with or without simultaneous supplementation with 10⁹ CFU/mL LGG or LGR-1. The basolateral compartment was kinetically analyzed for parathion or CP. Data are displayed as means ± SD from 4 independent experiments. * p < 0.05, ** p <0.01. CP = chlorpyrifos, ND = not detected, LGG = *Lactobacillus rhamnosus* GG, LGR-1 = *Lactobacillus rhamnosus* GR-1.
2.4.5  *L. rhamnosus* GG supplementation reduced the toxicity of chlorpyrifos to *Drosophila*.

Since LGG displayed both the greatest sequestration and reduced *in vitro* intestinal absorption of CP, the ability of oral LGG supplementation to mitigate mortality of *Drosophila melanogaster* lethally exposed to CP-containing food was tested. Adult *Drosophila* were exposed to CP-containing food at various concentrations to determine an optimal CP dosage for intervention testing. *Drosophila* were sensitive to CP-induced mortality in a dose-dependent manner (Figure 2-5A) based on median survival of *Drosophila* determined to be: 1 d for 100 µM CP, 3 d for 10 µM CP, and undeterminable for 1 µM CP and vehicle. Based on these dose-mortality experiments, the 10 µM CP concentration was chosen to investigate the ability of LGG to mitigate acute CP-induced mortality. *Drosophila* that were supplemented with LGG and 10 µM CP had median survival comparable to *Drosophila* receiving just 10 µM CP (both 3 d, Figure 2-5B). However, *Drosophila* simultaneously supplemented with both LGG and 10 µM CP also displayed significantly prolonged overall survival (p < 0.0001) and had fewer early time point deaths (p < 0.01) than those treated with 10 µM CP alone (Figure 2-5B). Notably, at the experimental end point (12 d), 9.901% of *Drosophila* that were supplemented with LGG were still alive; alternatively, 0% of *Drosophila* exposed to 10 µM CP alone survived 10 d post-exposure. These observations suggest that prophylactic priming and continual supplementation of *Drosophila* with LGG may maximize *Drosophila* survival by preventing early time point deaths associated with CP exposure.

Further experimentation attempted to elucidate if the pro-survival effects observed in CP-exposed *Drosophila* supplemented with LGG was due to direct LGG-CP interactions or LGG modifying host responses to pesticide toxicity. *Drosophila* were pretreated with vehicle or LGG for 3 d prior to being transferred to media containing 10 µM CP. No overall (p = 0.7064) or early time point (p = 0.4920) survival benefit was observed in *Drosophila* treated with LGG prior to 10 µM CP exposure (Figure 2-5C). Together these results suggest that LGG likely interacts directly with CP to mitigate CP-induced toxicity.
Figure 2-5. *Lactobacillus rhamnosus GG* supplementation reduces the toxicity of chlorpyrifos to *Drosophila melanogaster*.

(A) Survival curves of adult *Drosophila melanogaster* exposed to media containing 1, 10, or 100 µM CP or vehicle control. (B) Survival curves of adult *Drosophila* exposed to media containing 10 µM CP with or without simultaneous supplementation of live LGG. (C) Survival curves of adult *Drosophila* exposed to LGG or vehicle for 3 d, following subsequent exposure to media containing 10 µM CP. Data are displayed from at least 3 independent experiments. ** p < 0.01. CP = chlorpyrifos, LGG = *Lactobacillus rhamnosus GG*.
The direct effect of LGG supplementation on Drosophila microbiota composition and physiological responses following 3 d exposure to 1 µM CP (not acutely lethal) were further characterized. Since lactobacilli are the dominant member of the Drosophila microbiota, lactobacilli were enumerated from Drosophila supplemented with LGG with/without simultaneous exposure to 1 µM CP. Both female and male Drosophila treated with LGG had significantly higher levels of lactobacilli than Drosophila treated with 1 µM CP alone (Figure 2-6A). Although 1 µM CP-treatment did not significantly reduce Drosophila lactobacilli levels, lactobacilli levels were notably lower in male flies and appeared to display a bimodal response in females (some reduced, some unaffected) exposed to CP compared to vehicle controls. Furthermore, female Drosophila exposed for 3 d to media containing 1 µM CP and LGG were significantly rescued (p < 0.05) from CP-mediated reductions in bodyweight (Figure 2-6B). There was an insignificant trend towards similar bodyweight rescue in male flies. Moreover, since CP impacts insect locomotion due to exacerbated cholinergic stimulation, the ability of LGG supplementation to rescue Drosophila motor deficits following 3 d of 1 µM CP challenge was assessed by negative geotaxis assays. However, no differences in Drosophila locomotion (distance climbed after 3 s) was observed among treatment groups of vehicle, LGG, 1 µM CP, and LGG with 1 µM CP (Figure 2-6C). Together these results suggest that: 1) LGG supplementation can enhance the Drosophila microbiota with lactobacilli, and 2) low dose 1 µM CP may cause developmental, rather than motor deficits, that can be rescued by LGG supplementation.
Figure 2-6. *Lactobacillus rhamnosus* GG promotes body weight gain and lactobacilli enhancement in chlorpyrifos-exposed *Drosophila*, but does not affect locomotion.

Freshly eclosed *Drosophila melanogaster* were treated for 3 d with media containing vehicle, 1 µM CP, LGG, or 1 µM CP and LGG. (A) *Drosophila* bodyweights were determined. (B) Surface sterilized *Drosophila* were homogenized, drop-plated on MRS agar, and enumerated for CFU/*Drosophila*. (C) *Drosophila* locomotion was tested by a negative geotaxis assay in which average fly climbing distance (15-25 flies) of 5 replicates was quantified after 3 s. Data are displayed as means ± SEM from at least 3 independent experiment. * p < 0.05, ** p < 0.01, *** p < 0.001. CP = chlorpyrifos, LGG = *Lactobacillus rhamnosus* GG.
2.5 Discussion

Lactobacilli strains were found to grow in high levels of parathion and CP. Since commonly used probiotic LGG and LGR-1 displayed unaltered growth in the presence of either parathion or CP, we further examined their activity. We showed that the organophosphate hydrolase gene, \textit{LGG\_RS02045}, predicted in LGG, was not likely responsible for LGG-mediated pesticide degradation. Although, genetic cloning was not performed to definitively demonstrate that \textit{LGG\_RS02045} was non-functional, it was not induced by parathion or CP. Furthermore, heat-killed LGG had the same capability to sequester parathion and CP from solution as live LGG. This was also found with LGR-1 and suggests that metabolic activity is not required for bacterial organophosphate sequestration in these strains. Thus, unlike \textit{Cho et al.} (35), we were unable to demonstrate degradation of the organophosphate pesticides, parathion or CP, by LGG or LGR-1. Differences in our experimental approach compared to the use of fermentation in organophosphate-contaminated dairy food products may explain this finding (29, 30). We suspect that the higher temperatures (42°C compared to 37°C) and acidic conditions present in the \textit{in vitro} fermentation conditions of other studies could promote pesticide breakdown more rapidly.

The finding of lactobacilli binding to organophosphate pesticides is congruent with reports of similar interactions with environmental toxins, such as aflatoxin (24, 25, 36), paralytic shellfish toxins (37), and metals (38-41). LGG had a better ability to retain organophosphate sequestration—as measured by CP retained in bacterial pellets—of CP from solution than LGR-1. This was also found in a Caco-2 Transwell model simulating organophosphate absorption in the small intestine. This finding is surprising given the genetic similarity between strains. One major difference between LGG and LGR-1 is that LGR-1 has a unique exopolysaccharide biosynthesis cluster (unpublished). The ability of lactobacilli-derived exopolysaccharide to bind toxins such as lead (41), cadmium (42), and aluminum (42) have been described. Furthermore, sediment microbe-produced exopolysaccharide has been shown to strongly interact with CP (43, 44). It is interesting to speculate that differences in bacterial exopolysaccharide may confer bacterial-organophosphate binding phenotypes in lactobacilli.
Results with *Drosophila* suggest that dietary supplementation of transitory bacteria can be important for conferring host benefits that are not present with the commensal microbiota. Specifically, prolonged interaction between CP and LGG lead to increased survival. Unlike *Drosophila* exposed simultaneously to CP and LGG, those pretreated with LGG prior to pesticide exposure did not receive a survival benefit. These findings further support our hypothesis that LGG supplementation prevents downstream organophosphate pesticide toxicity by preventing absorption. Admittedly, we cannot completely rule out the possibility that LGG exposure results in priming of host detoxification pathways relevant to organophosphate pesticides. Indeed, results from Kamaladevi *et al.* (45) suggest that the survival benefit experienced by CP-challenged *Caenorhabditis elegans* supplemented with *L. casei* may be due to upregulation of host phase-II detoxification genes.

LGG supplementation was able to significantly rescue weight-loss in female flies exposed to 1 µM CP. This trend was also observed in male flies; however, findings were not significant. The outcome corresponded with significantly increased lactobacilli colony forming units per fly, after treatment with LGG relative to CP alone. *Lactobacillus* are the dominant genus in the *Drosophila* microbiota and important for many host physiological processes (46, 47). We speculate that the supplementation of LGG may promote a growth rescue of CP-induced toxicity via stimulation of IMD/Relish signaling pathway (48). The IMD/Relish pathway is a central regulator of mediating microbiota-mediated host responses in *Drosophila* and is required for promoting survival in response to DNA damage (49). This pathway has been implicated in reducing growth to promote maintenance in response to noxious stimuli (49). Alternatively, *Lactobacillus plantarum* isolated from *Drosophila* has been shown to promote host growth in nutrient depleted media via improved protein assimilation (47). Thus, similar to a study by Blum *et al.* (50), we have shown that human probiotic LGG may be capable of conferring beneficial effects to host flies in a similar fashion to the native microbiota.

In summary, this study has shown that the commonly used probiotic organisms LGG and LGR-1 are able to bind, but not metabolize, organophosphate pesticides and reduce intestinal absorption *in vitro*. Furthermore, LGG reduced mortality and growth deficits in
Drosophila exposed lethally and subchronically to CP, respectively. This work expands upon our previous study, which demonstrated L. rhamnosus supplementation could reduce heavy metal bioaccumulation in Tanzanian pregnant women (23). Transitory food grade bacteria have the potential to act like a non-specific “sponge”—absorbing toxins and reducing their uptake by the host. This approach holds promise for supplementing human, livestock, or apiary foods with probiotic microorganisms and reducing downstream toxicity from organophosphate pesticides.

2.6 Acknowledgements

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2.8 References


## 2.9 Supplementary

**Table 2-1. Primers used for qPCR.**

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Chapter 3

3 *Lactobacillus rhamnosus* supplementation alters low dose chlorpyrifos exposure and xenobiotic metabolism in a BALB/c mouse model.


3.1 Abstract

The organophosphate pesticide, chlorpyrifos (CP), used in agricultural practices has been shown to alter the intestinal microbiota, intestinal barrier function, and hepatic xenobiotic metabolism at low doses. We have shown that probiotic *Lactobacillus rhamnosus* GG (LGG) and GR-1 (LGR-1) were able to bind CP and thus reduce CP absorption in a Caco-2 intestinal Transwell model and oral toxicity to *Drosophila melanogaster*. Thus, we hypothesized that LGG or LGR-1 supplementation would mitigate low dose CP-induced early toxicity in a mouse model. Female BALB/c mice were exposed subchronically to 2 mg/L of CP via drinking water with daily oral gavage of LGG, LGR-1, or vehicle. Microbiota analysis by 16S rRNA gene sequencing did not reveal differences in fecal microbiota composition throughout the study. Histological morphology of the intestine, liver, kidneys, and pancreas and gastrointestinal permeability (FITC-dextran assay) were also unperturbed by CP treatment. However, comet assays determined that LGG supplementation of CP-treated mice significantly reduced colonic and hepatic DNA damage in colon and liver. Hepatic RNA expression of toxic insult genes *Gadd45a* and *Atf3* and xenobiotic processing genes *Por*, *Fmo2*, and *Cyp1a1* were significantly altered between treatment conditions. Interestingly, LGR-1, but not LGG, supplementation altered early exposure host metabolism of CP. Liquid-chromatography mass spectrometry determined the CP metabolites 3,5,6-trichloropyridinol (TCP) and CP-oxon were significantly higher and lower in the urine and blood, respectively, after 1 d of oral exposure. To understand LGR-1-mediated alteration of CP metabolism, mice were supplemented with only LGR-1 for 3 d prior to sacrifice. Gene expression analysis of the liver and intestine revealed that hepatic *Por*,
Fmo2, and Fmo3 were significantly upregulated and intestinal Abcb1a expression was altered in LGR-1 treated mice relative to vehicle controls. This study provides preliminary insight into the interactions between food-grade microbes and host xenobiotic metabolism and how microbial supplementation could enable targeted pharmacology and preventative toxicology.

3.2 Introduction

Pesticide usage has been questioned for both environmental and human safeties. The organophosphate insecticide, chlorpyrifos (CP), is commonly used in agriculture for treating fruit and vegetable vineyards and fruit orchards. Consequently, humans and wildlife are often exposed to low oral doses of CP through the consumption of contaminated food and drink. Despite the fact that many toxicology studies use CP levels that far exceed food source contamination, clinical correlations suggest that low dose organophosphate pesticide exposure may induce subtle detrimental changes to human physiology which lead to chronic disease (1-5).

Microorganisms that inhabit the gastrointestinal tract of mammals, commonly referred to as the microbiota, have been shown to be disturbed by conventionally “safe” xenobiotics such as emulsifiers (6), artificial sweeteners (7), antibiotics (8), and pesticides (9). Moreover, evidence from heavy metal exposed germ-free mice suggests the microbiota plays an underappreciated role in mitigating toxic outcomes (10). Culture-based microbial identification demonstrated that CP-induced microbiota shifts in both an in vitro chemostat model of the intestinal tract, in rats (9) and mice (11). Taken together, these studies suggest that understanding the interplay between xenobiotics and the microbiota may improve preventive therapies that target the links between environmental toxin exposure and chronic diseases associated with xenobiotics such as pesticides. It is anticipated that the interplay between the microbiota and pharmacology/toxicology will help future medical professionals improve the morbidity and economic burden of chronic diseases.

Bioremediation has been well-researched for several decades and has shown that bacteria can remove environmental toxins such as organophosphate insecticides from
contaminated environments (12). We have attempted to expand on this concept in vivo by using transitory probiotic microorganisms as sinks for organophosphate pesticides entering into the gastrointestinal tract (13). *Lactobacillus brevis* WCP902 has been shown to contain the organophosphate hydrolase gene, *OpdB*, required for metabolism of organophosphate pesticides (14). However, many strains of *Lactobacillus* have been shown to reduce the levels of organophosphate pesticides in dairy products *ex vivo* (15, 16), suggesting a more generic mechanism of action may also exist. We have shown that the commonly used probiotics *Lactobacillus rhamnosus* GG (LGG) and *Lactobacillus rhamnosus* GR-1 (LGR-1) were able to bind and sequester CP from solution. We further demonstrated that LGG was able to reduce the absorption of CP in a Caco-2 Transwell model of intestinal absorption and prolong the survival of *Drosophila melanogaster* lethally exposed to CP (Trinder et al., submitted 2016). These findings of a binding interaction between probiotic *Lactobacillus* organisms and an environmental toxin are similar to studies of the relationship between lactobacilli and aflatoxin (17), shellfish toxins (18), and metals.

Low-dose oral pesticide exposure is currently an unavoidable consequence of industrialized society and food production requirements to meet the demands of human overpopulation. Interestingly, LGG and LGR-1 are well-documented probiotic organisms that have been shown to oppose many of the CP-induced pathologies such as promoting intestinal health by enhancing intestinal barrier function (19), mitigating microbiota dysbiosis (20), and binding CP and mitigating intestinal absorption and downstream toxicity. Thus, we hypothesized that daily *Lactobacillus rhamnosus* supplementation would mitigate toxic insults to the gastrointestinal tract and associated microbiota of mice exposed sub-chronically to environmentally relevant levels of CP.

### 3.3 Methods

#### 3.3.1 Animals

Six-week-old female BALB/c mice (Charles River Laboratories) were randomly divided into treatment groups and housed in a controlled environment (with a temperature of 22°C, a 12h/12h light/dark cycle and *ad libitum* access to food and water). Animal
experiments were performed in accordance with the guidelines issued by the Animal Use Subcommittee at Western University (protocol number 2015-039) in accordance with the Canadian Council of Animal Care or the Institut Pasteur de Lille’s Animal Care and Use Committee, which are based on the Amsterdam Protocol on Animal Protection and Welfare and Directive 86/609/EEC on the Protection of Animals Used for Experimental and Other Scientific Purposes, updated in the Council of Europe's Appendix A. The animal experiments also complied with French legislation (Government Act 87-848) and the European Communities Amendment of Cruelty to Animals Act 1976. All studies were approved by the local investigational ethics review board (Nord-Pas-de-Calais CREEA 75, Lille, France; protocol numbers 192009R and 042011).

3.3.2 Bacterial culture conditions

LGG and LGR-1 were routinely inoculated from lactobacilli de Man, Rogosa and Sharpe (MRS) agar (BD, catalog number: 288130) plates into MRS broth. Inoculated MRS broth cultures were subcultured (1:100 dilution) and incubated overnight (18 h) at 37° statically and anaerobically. Cultures were pelleted at 5000 g (10 min), washed with sterile normal saline, and concentrated 10-fold in sterile normal saline (10^{10} CFU/mL).

3.3.3 Subchronic experimental design

Mice were randomized into treatment groups that included: vehicle, CP, CP and LGG, or CP and LGR-1. CP (Sigma-Aldrich, 45395) stock solutions were made at 100 mg/mL in dimethyl sulfoxide (Sigma-Aldrich) and stored at -20°C. CP was dissolved into opaque water bottles at a concentration of 2 mg/L of water. Drinking water was replaced every 3 d. Mice received daily oral gavage of normal saline, 10^9 CFU LGG, or 10^9 CFU LGR-1. Whole blood and urine were collected from mice at 1 and 24 d (endpoint) post-CP exposure by retro-orbital bleed and gentle bladder compression, respectively. Fecal samples were collected from mice 1 d prior, 1 d post, and at the endpoint of CP exposure.

Separate mice were randomized into vehicle, CP, CP and LGG and used for the FITC-dextran permeability and comet assays. Mice were euthanized by cervical dislocation and immediately dissected for organ collection. Samples were stored at -80°C until analysis unless otherwise stated.
3.3.4 Acute Lactobacillus rhamnosus GR-1 experimental design

Mice were randomized into treatment groups consisting of daily oral gavage of normal saline or 10^9 CFU LGR-1 for 3 d. On the 4th day, mice were anesthetized by isoflurane inhalation and subsequently euthanized by cervical dislocation. Blood was immediately collected by cardiac puncture and organs were dissected, washed in 0.01 M PBS, and flash frozen with liquid nitrogen. Samples were stored at -80°C until analysis unless otherwise stated.

3.3.5 FITC-dextran gastrointestinal permeability assay

Mice received 12 mg (200 µl of 600 mg/kg) of fluorescein isothiocyanate conjugated dextran (FITC-dextran, average mol wt 3000-5000 Da; Sigma-Aldrich, Saint-Louis, Missouri) by oral gavage. Blood was collected by retro-orbital puncture 4 hours after administration. Blood samples were centrifuged and sera were collected. Concentration of FITC-dextran was determined by spectrophotofluorometry with an excitation wavelength of 485 nm and an emission wavelength of 520 nm.

3.3.6 Comet assay

Comet assays were used to assess DNA damage of tissues. Immediately after dissection, colons and livers were minced using fine scissors and intestinal cells were scrapped, both in cold mincing buffer (Mg²⁺- and Ca²⁺-free Hanks’ balanced salt solution (Gibco, CA, USA) with 20 mM EDTA (Sigma-Aldrich) and 10% DMSO (Sigma-Aldrich)). After centrifugation, cells were counted and cell suspensions (2 x 10⁴ cells) were mixed with 75 µL of 0.5% low-melting point agarose before rapidly transferring onto pre-coated slides (two layers of normal agarose: 1.5% and 0.8% respectively). All of the following steps were sheltered from daylight to prevent the occurrence of additional DNA damage. Slides were immersed overnight at 4°C in cold lysis solution (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris-base, 10% DMSO, 1% Triton-X100). After lysis, slides were placed in electrophoresis solution (1 mM Na₂EDTA, 0.3 M NaOH (pH>13)) for 20 min to allow for DNA unwinding. Subsequently, electrophoresis was conducted at 25 V for 20 min. Slides were immersed in a neutralization solution (0.4 M Tris-base, pH 7.5) for at least 5 min and dehydrated with absolute ethanol for 5 min. Cells were stained with
propidium iodide (20 µg/ml). All slides were independently coded before the microscopic analysis. Comet was observed via fluorescence microscope (Leica Microsystems SAS-DM 2000, Heerbrugg, Switzerland) at magnification of 200x and analyzed by COMET ASSAY IV software (Perceptive Instruments, UK). For each sample, 50 comets/slide were analyzed, with 3 slides scored/sample.

3.3.7 History

Samples for histological analysis were fixed in 4% formaldehyde, dehydrated and embedded in paraffin. Sections (4-7 µm thick) were stained with hematoxylin and eosin or Periodic Acid Schiff with 1% Light Green SF counterstaining.

3.3.8 RNA extraction, reverse transcription, and qPCR

Dissected tissues were stored in RNAlater at -20°C until extraction. Tissues were homogenized in TRizol reagent (Life Technologies) using a Pellet Pestle (Kimble Chase). After phase separation with chloroform according to the manufacturer’s protocols, the aqueous phase was combined with 100% ethanol and purification continued with the PureLink RNA Mini Kit (Ambion, catalog number: 12183025) with on-column treatment of PureLink DNase treatment (Ambion, catalog number: 12185010). RNA quantity and purity was assessed with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific). RNA integrity was confirmed by Bioanalyzer analysis (Agilent Technologies) when required. RNA was stored at -80°C until reverse transcription.

cDNA was prepared from 200-2000 ng of purified total RNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, catalog number: 4368813). qPCR was performed with a 7900HT Sequence Detection System (Applied Biosystems) in 20 µL reactions using Power SYBR Green Master Mix (Applied Biosystems, catalog number: 4368702) with 200 µM of appropriate forward and reverse primers. Primers used for qPCR analysis are listed in Supplementary Table 3-1. qPCR results were analyzed using SDS RQ 6.3 manager software (Applied Biosystems) and relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method. Microarrays were prepared and analyzed as before (21) with the Affymetrix Mouse Gene 2.0 ST array platform.
3.3.9 Microbial DNA extraction, sequencing, and community analysis

Fecal samples were stored in RNAlater at -20°C until DNA extraction was performed with MoBio PowerSoil 96-Well Soil DNA Isolation Kits (Carlsbad, CA) according to the modified Earth Microbiome Project standard protocols. A single fecal pellet (approximately 30 mg) was extracted per sample. The V4/V5 region of the 16S ribosomal RNA gene was PCR amplified with bacterial/archaeal primers 515F and 806R according to previously described methods (22) and modified for the Illumina MiSeq platform.

Amplicons were quantified using Picogreen (Quant-It; Life Technologies, Burlington, ON), pooled at equimolar concentrations before cleanup (QIAquick PCR clean up; Qiagen, Germantown, MD), and sequenced using the MiSeq by Illumina platform, with 2 x 220 paired-end chemistry at the London Regional Genomics Centre at Robarts Research Institute (Western University, London, ON). Reads were quality filtered, overlapped with Pandaseq, and binned into operational taxonomic units (OTUs) using closed-reference OTU picking based on 97% identity using the SILVA reference database.

Open-source QIIME software was used for generation of weighted UniFrac principal coordinate analysis (PCoA) for exploratory data analysis (23). Distance on PCoA plot represents overall dissimilarity between the microbiota profiles of samples using weighted UniFrac phylogenetic distance. Assessment of differentially abundant genera were determined using ALDEEx2 v1.0.0 in R with unpaired Welch's t tests with Benjamini Hochberg's false discovery rate (FDR) method with a q < 0.05 cutoff (24).

3.3.10 Blood and urine sample preparation for targeted liquid-chromatography mass spectrometry

One volume of whole blood was extracted with 2 volumes of ice cold methanol to precipitate proteins. Supernatants were collected following centrifugation at 14 000 g for 15 min. Urine was diluted 10-fold with methanol.
3.3.11 Liver sample preparation for untargeted liquid-chromatography mass spectrometry

Livers samples were thawed on ice. Samples were supplemented with 5 µL and 2 mg of acetonitrile and Ottawa sand (Fisher Scientific) per mg of tissue, respectively. Samples were homogenized for 5 cycles using a FastPrep™ FP120 Cell Disrupter (Thermo Savant) set to speed 4 for 20 s/cycle. Samples were stored on ice for at least 1 min between a series of 5 cycles. Homogenates were centrifuged at 14 000 g for 5 min. Supernatants were dried down and stored at -80°C until analyses. Immediately prior to sample analysis, samples were resuspended with an equal volume of high performance-liquid chromatography (HPLC) grade water containing 0.05 g/mL 6C\textsuperscript{13} phenylalanine internal standard (Cambridge Isotopes, Tewksbury, USA).

3.3.12 CP, CP-oxon and TCP liquid-chromatography mass spectrometry

An Agilent 1290 Infinity HPLC coupled to a Q-Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, USA) with a HESI (heated electrospray ionization) source. Two µL of each sample was injected into a ZORBAX Eclipse plus C18 2.1 x 50mm x 1.6 micron column. Mobile phase (A) consisted of 0.1% formic acid in water and mobile phase (B) consisted of 0.1% formic acid in acetonitrile. The initial composition of 0% (B) was held constant for 1.5mins and decreased to 100% over 3.0 minutes. Mobile phase B was then held at 100% for 1.5 minutes and returned to 0% over 30 seconds, re-equilibrated at 0% (B) for 1 minute for a total run time of 7.5 min.

3.3.13 Mass spectrometry positive mode CP and CP-oxon liquid chromatography-mass spectrometry analyses

The HESI source was operated under the following conditions: nitrogen flow of 20 and 15 arbitrary units for the sheath and auxiliary gas respectively, probe temperature and capillary temperature of 460 °C and 400 °C respectively and spray voltage of 3.25 kV. The S-Lens was set to 60. A top 5 data dependent MS2 (ddMS2) was conducted at a full MS scan between the ranges of m/z 100-1000 in positive mode at 70 000 resolution (AGC target and maximum injection time were 3e6 and 250 ms respectively) and MS/MS at 17,500 resolution, normalized collision energy set to 30 (AGC target and
maximum injection time of 1 x 105 and 100 ms, respectively).

3.3.14 Mass spectrometry negative mode TCP liquid chromatography-mass spectrometry analyses

The HESI settings were optimized for TCP in negative mode with the following values. Nitrogen flow of 20 and 5 arbitrary units for the sheath and auxiliary gas respectively, probe temperature and capillary temperature of 455 °C and 400 °C respectively and spray voltage of 3.50 kV. The S-Lens was set to 40. The ddMS2 experiment was equivalent to the CP with the mass range adjusted to 70–700 m/z.

3.3.15 Statistical analyses

Unless otherwise stated, all statistical analyses were performed in GraphPad Prism 6 using unpaired t or Mann-Whitney tests for comparison of 2 groups. Alternatively, for > 2 groups one-way ANOVA with Tukey’s multiple comparisons test or Kuskal-Wallis test with Dunn’s multiple comparisons test were used.

3.4 Results

3.4.1 Subchronic chlorpyrifos does not alter general mouse behavior or gross intestinal, pancreatic, or kidney architecture

To assure that experimental results obtained were not a result of different pesticide-contaminated water consumption among treatment groups, food and water intake and bodyweight changes were monitored. Throughout the study, treatment groups demonstrated no significant differences in food or water consumption or bodyweight changes (Supplementary, Figure 3-6). Studies have started to report on the toxic effects of conventionally safe xenobiotics such as emulsifiers (6) and artificial sweeteners (7) to the microbiota and gastrointestinal tract. Similarly, it was investigated if CP could induce any gross changes to gastrointestinal morphology. No notable histological abnormalities were observed in the intestine among treatment groups. Duodenal (Supplementary, Figure 3-7), jejunal (Supplementary, Figure 3-8), and colonic (Supplementary, Figure 3-9) mean: villus length, villus width, lamina propria width, mucin+ cells/mm² of villus, inner muscle width, and outer muscle did not differ with CP treatment. These results
suggest that subchronic CP exposure does not alter gross intestinal morphology in mature mice at low doses (2 mg/L).

3.4.2 Chlorpyrifos does not alter intestinal membrane integrity, but does induce colonic DNA damage, which is mitigated by *Lactobacillus rhamnosus* GG

Since CP has been reported to induce gastrointestinal permeability (25) and DNA damage, we sought to evaluate the protective effect of LGG supplementation on these insults. However, with our dosing scheme no difference in gastrointestinal membrane permeability was observed in mice treated with CP or CP with LGG relative to vehicle controls (Figure 3-1A). In contrast, assessment of colonic DNA damage by comet assay, suggested that CP increased intestinal DNA damage (Figure 3-1B). This increase in CP-induced colonic DNA damage was significantly reduced (p < 0.05) by LGG supplementation. These findings suggest that LGG supplementation may protect against innate DNA damage present in colonic tissue in healthy non-toxically-challenged animals.
Figure 3-1. Chlorpyrifos does not alter intestinal membrane integrity, but does induce colonic DNA damage, which is mitigated by *Lactobacillus rhamnosus* GG.

Mouse (A) gastrointestinal membrane integrity and (B) colonic DNA damage were assessed at experimental endpoint of subchronic exposure by FITC-dextran permeability (n = 8) and comet assays (n = 5), respectively. Data are expressed as means ± SD. * p < 0.05. CP = chlorpyrifos, LGG = *Lactobacillus rhamnosus* GG.
3.4.3 Chlorpyrifos exposure does not alter fecal microbiota composition

Studies have shown that commonly consumed xenobiotics such as emulsifiers (6) and artificial sweeteners (7) may be associated with chronic metabolic diseases due to changes to the gastrointestinal microbiota. Other studies have shown that CP can induce community shifts in the gastrointestinal microbiota at relatively high doses (9, 11). This study explored if low dose (non-bolus) sub-chronic CP exposure could alter microbiota profiles in young adult mice. Longitudinal weighted UniFrac analysis of the fecal microbiota composition is depicted as a cross-sectional representation of each time point (Figure 3-2). The lack of any definitive clustering of samples by treatment group at any time point suggests that there is no clear effect of low dose CP exposure on gross fecal microbiota composition. Furthermore, quantification of differentially abundant OTUs between vehicle- and CP-treated mice using AldEx2 demonstrated that no significantly distinct taxonomic differences existed among treatments. These results indicate that unlike a bolus oral dose of pesticide (11, 26), sustained exposure appears to have a less drastic effect on gastrointestinal microbiota composition.
Figure 3-2. Low dose chlorpyrifos exposure does not alter fecal microbiota composition.

3D weighted UniFrac PCoA illustrate the microbiota among the different mouse fecal samples 1 d prior to pesticide exposure (d -1), 1 d post-pesticide initiation (d 1), and at the experimental endpoint (d 24). The following UniFrac PCoA analyses were based on the OTU data, with only the percent variance explained of the first three principal coordinates shown. CP = chlorpyrifos, LGG = *Lactobacillus rhamnosus* GG, LGR-1 = *L. rhamnosus* GR-1.
3.4.4 Hepatic response to low dose chlorpyrifos with and without *Lactobacillus rhamnosus* intervention

CP is rapidly metabolized by the liver into active/toxic (CP-oxon) and non-active secondary metabolites. Given that the liver is the major site of CP metabolism, this study sought to investigate the effect of CP on hepatic morphology and gene expression of enzymes involved in toxin-induced stress and CP metabolism. Gross hepatic histological morphology appeared largely unaltered by CP treatment with or without LGG or LGR-1 supplementation (Figure 3-3A). Alternatively, hepatic DNA damage decreased in order of vehicle > CP > CP + LGG. Hepatic tissues from mice receiving CP and LGG supplementation had significantly (p < 0.01) less DNA damage than vehicle controls (Figure 3-3B). Gene expression of several genes involved in the toxic gene response network to DNA damage were altered by our experimental treatment conditions (27). *Egr1* (insignificant) and *Gadd45a* (p < 0.05) were upregulated in mice treated with CP and LGG or LGR-1, but not CP alone (Figure 303C). Furthermore, *Atf3* was significantly (p <0.05) downregulated in mice treated with CP and LGR-1 relative to mice treated with CP only. Alternatively, the key cytochrome P450 (Cyp) regulator, *Por*, was significantly (p < 0.05) upregulated in CP, CP and LGG, and CP and LGR-1 groups relative to vehicle controls. Furthermore, mice receiving CP with LGG had significantly (p <0.05) upregulated *Fmo2* and *Cyp1a1* expression compared to vehicle- and CP-treated animals, respectively (Figure 3-3D). Together these results suggest that low dose CP exposure can affect early host toxin response genes and hepatic xenobiotic processing, some of which appear to be modified by *L. rhamnosus* supplementation.
Figure 3-3. Hepatic response to chlorpyrifos with/without *Lactobacillus rhamnosus*.

(A) No notable gross histological abnormalities were observed in the liver. Scale bar = 1000 μm. (B) Hepatic DNA damage was assessed by comet assay (n = 5, means ± SD). Relative hepatic gene expression was evaluated for (C) toxic response and (D) xenobiotic metabolism genes. Relative gene expression of target genes was normalized to beta-actin and expressed as means ± SEM (n = 5-8). * p < 0.05, ** p < 0.01, *** p < 0.001. CP = chlorpyrifos, LGG = *Lactobacillus rhamnosus* GG, LGR-1 = *L. rhamnosus* GR-1.
3.4.5 *Lactobacillus rhamnosus* GR-1 supplementation alters host chlorpyrifos metabolism

Previous work demonstrated that LGG and LGR-1 were able to bind and sequester CP from solution, reduce CP absorption in a Caco-2 Transwell model of intestinal absorption, and prolong the survival of *Drosophila melanogaster* lethally exposed to CP (Trinder et al., submitted 2016). Therefore, it was predicted that LGG and LGR-1 supplementation would reduce oral CP absorption in mice. At 1 d post-CP exposure, mice receiving CP with LGR-1 supplementation displayed significantly (p <0.05) lower levels of urine CP-oxon and higher levels of blood TCP, respectively (Figure 3-4). This finding suggests that LGR-1 is priming the murine host’s ability to metabolize initial CP exposures more efficiently or through less toxic metabolic pathways. As a consequence, mice receiving LGR-1 displayed lower excretion of the toxic CP-oxon metabolite and higher blood levels of the end CP metabolite TCP. Alternatively, urine TCP concentration was also observed to be significantly greater (p < 0.01) than CP only treated animals at the d 24 experimental endpoint, but not d1 (Figure 3-4). However, blood TCP concentrations did not differ significantly between treatment groups at the d 24 experimental endpoint (Figure 3-4). This observation is congruent with LGR-1-mediated modifications in host CP metabolism seen at early d 1 post-exposure. It is important to note that the levels of CP-oxon (other than d 1 urine) and CP were not detectable throughout the experiment and makes conclusive interpretation of results challenging. In general, these results suggest that LGR-1, but not LGG, may modify how hosts metabolically respond to low levels of CP.
Figure 3-4. Mice supplemented with *Lactobacillus rhamnosus* GR-1 display altered initial chlorpyrifos metabolism at 1 d post-exposure.

Levels of 3,5,6- trichloropyridinol (TCP) and CP-oxon were quantified in the blood and urine of mice by liquid-chromatography mass spectrometry. Data are expressed as means ± SD (n = 5-8). *p<0.05, **p<0.01. CP = chlorpyrifos, LGG = *Lactobacillus rhamnosus* GG, LGR-1 = *Lactobacillus rhamnosus* GR-1.
3.4.6 *Lactobacillus rhamnosus* GR-1 modulates hepatic defensome transcription

Based on altered CP metabolite profiles in mice supplemented with LGR-1, but not LGG, we further characterized the host xenobiotic transcriptional response in the duodenum, liver, and kidneys of mice receiving acute exposure to LGR-1 by microarrays. The hypothesis was that altered CP metabolism observed in LGR-1-treated mice was a result of modified xenobiotic metabolism in the liver, small intestine, or kidneys of these animals. Genes from microarray analysis that were determined to be significantly differentially expressed (p<0.05, ±2-fold) were subjected to GO enrichment analysis, which revealed that xenobiotic metabolic processes were significantly enriched (p<0.05) in the liver but not the small intestine or kidney. Confirmation of microarray hits in the liver by qPCR, revealed that LGR-1 significantly upregulated (p <0.05) *Por, Fmo2*, and *Fmo3* expression (Figure 3-5A). However, expression of many Cyp enzymes relevant to CP metabolism—*Cyp1a1, Cyp2b10, Cyp3a41b, Cyp27b1*—were found to be unaltered by LGR-1 supplementation. The major drug efflux pump ABCB1A, which is responsible for effluxing CP from the intestine to prevent absorption, was shown to be modulated by LGR-1 in a site-specific manner along the intestinal tract (Figure 3-5B). LGR-1 supplementation enhanced *Abcb1a* gene expression in the duodenum and jejunum, but significantly down regulated colonic expression (p < 0.01). Hence, LGR-1 supplementation appears to modulate transcription of host xenobiotic response processes in the liver and intestine. The consequence of these changes may have far reaching consequences to understanding how microbes can be used to manipulate drug and toxin (such as CP) exposures in humans.

In an effort to explain the mechanism of how LGR-1 modified host xenobiotic metabolism, we cultured lactobacilli from the feces of mice and performed untargeted metabolomics on the liver and plasma of mice at the experimental endpoint. Gene expression changes induced by LGR-1 were correlated with a significant enrichment (p < 0.01) of the culturable fecal microbiota with lactobacilli species that were predominately LGR-1 (Figure 3-5C). However, metabolomic analysis of the liver was unable to identify
a differentially abundant metabolite that could mechanistically explain the altered hepatic xenobiotic metabolism following LGR-1 supplementation (Figure 3-5D). Since the metabolomic analyses were performed 24 h post LGR-1 exposure, it is possible that the metabolite analyses missed the time point in which LGR-1 had notable effects on the host plasma and hepatic metabolome. Future work plans to definitively determine the mechanism and functional consequences of LGR-1-mediated transcriptional changes to host xenobiotic metabolism processes.
Figure 3-5. *Lactobacillus rhamnosus* GR-1 alters hepatic xenobiotic metabolism.

Gene expression in the (A) liver and (B) intestine of mice treated for 3 d with *L. rhamnosus* GR-1 (LGR-1) or vehicle control were evaluated by SYBER Green qPCR. Relative gene expression of target genes were normalized to beta actin and expressed as means ± SEM (n=7). (C) Fecal pellets were collected longitudinally from mice 5 h post-LGR-1 supplementation and plated on MRS agar for enumeration of lactobacilli (n = 9, means ± SEM). (D) Principal component analysis of negative mode liquid chromatography mass spectrometry of the hepatic metabolite abundance data obtained from mice receiving vehicle or LGR-1 treatment (only variance of first two components shown). * p<0.05, ** p < 0.01, *** p < 0.001. LGR-1 = *Lactobacillus rhamnosus* GR-1.
3.5 Discussion

Unlike most other oral CP toxicology studies that utilize daily oral gavage of several mg/kg of CP (26), mice in this study were exposed to chlorpyrifos through ad libitum access to water contaminated with 2 mg/L of CP. Although this approach provides a more environmentally-relevant simulation of human and wildlife CP-exposure, CP has been shown to demonstrate increased degradation in aqueous solution with increasing pH, radiation frequency, water quality, and temperature. However, the half-life of CP at water concentrations of 1-2 mg/L has been reported to be approximately 41 d (28). As an extra precaution to mitigate any potential confounding aqueous CP degradation, mouse water bottles contained neutral pH tap water, water bottles were protected from light, and water bottles were replaced with fresh water every 3 d.

Also, unlike other mouse studies evaluating the toxic properties of CP, we did not observe the drastic CP-induced histological pathology, increased intestinal membrane permeability (25), and/or microbiota disturbances (9, 11, 26). There could be several explanations to these discrepancies such as CP dosage (other studies used daily 1 mg/kg CP bolus gavage versus this studies ad libitum access to contaminated 2 mg/L CP in drinking water), developmental time point of CP exposure, experimental duration, and animal model susceptibility to CP toxicity. In general, neonatal organisms are well-known to be more susceptible to toxic insult. With particular relevance to organophosphate pesticides, adult rats have been shown to be more resistant to parathion and CP toxicity due to higher levels of hepatic aliesterase and Cyp450 dearylation (29). This reasoning could explain why Condette et al. (26) observed intestinal histological and microbiota impairments in rat pups with perinatal exposure to CP, while this study did not. Despite no obvious differences in hepatic histology among treatment groups, this study observed that hepatic DNA damage among treatment groups decreased in order of vehicle > CP > CP with LGG. Furthermore, genes involved in CP xenobiotic metabolism were largely unchanged among treatment groups (30), while the early onset drug-induced toxicity network appeared dysregulated (27). LGG upregulated Egr1 and Gadd45a (p < 0.05) in mice that received CP with LGG or LGR-1, but not CP alone. On the other hand,
hepatic *Atf3* was significantly downregulated (p < 0.05) in mice receiving CP and LGR-1 relative to CP alone. These findings suggest that *Lactobacillus rhamnosus* supplementation may mitigate anti-apoptotic effects that have been reported following low dose CP exposures to human (30). The direct consequences of these results on hepatic function remain unclear, but suggest that *L. rhamnosus* supplementation may improve host pesticide detoxification.

Mice that were exposed to LGR-1 were found to have significant alterations in blood and urine CP metabolites, CP-oxon and TCP, relative to mice exposed to CP alone or CP with LGG. Notably, the metabolite levels were extremely low. Interpretation of these findings is challenged by the fact that the parent compound, CP, was unable to be detected in blood or urine samples. However, this finding is not surprising given low dose exposure and the sensitivity of CP to host xenobiotic metabolism following oral exposure (31). Blood CP levels have been shown to be rapidly metabolized into TCP in rats 3 h after oral gavage (32). Interestingly, CP metabolism was altered in mice that received supplementation with LGR-1, but not LGG. This finding is surprising given the genetic similarity between strains. One major difference between LGG and LGR-1 is that LGR-1 has a unique exopolysaccharide biosynthesis cluster (unpublished). Thus, it is possible that the immune-xenobiotic metabolism axis is differentially regulated in response to strain-specific cell surface moieties (33).

We showed that acute supplementation of BALB/c mice with LGR-1 can alter host hepatic defensome gene expression. Although previous work used germ-free animals to show that the microbiota is critical to host xenobiotic metabolism (8, 34-37), very few studies have characterized the various effects of individual species or strains in a non-sterile host (34, 38). These latter findings have important translational implications to human pharmacology and toxicology. The observations of significantly upregulated hepatic *P450 oxidoreductase* (*Por*), *flavin containing monooxygenase 2* (*Fmo2*), and *Fmo3* is interesting given the context of this study to organophosphate pesticides. Por catalyzes the transfer of electrons to all Cyp450 (Cyp) enzymes, and consequently is important to xenobiotic (39, 40) and toxin (41) metabolism *in vivo*. Rodent homologues for human CYP2C9, CYP2C19, CYP2B6, and CYP3A4 have been shown to be critical
for CP metabolism. In particular, the activities of human CYP2C19 and CYP2B6 have been implicated in CP detoxification and toxicity, respectfully (42). Therefore, it is interesting to speculate on how LGR-1 modulation of Por may alter Cyp-mediated metabolism of not just CP, but other xenobiotics.

The current mechanism of how LGR-1 supplementation modulates host xenobiotic metabolism is unknown. Venkatesh et al. (43) reported that the microbiota can modulate hepatic xenobiotic metabolism via production of small molecules that can interact with the pregnane X receptor, which is a key xenobiotic sensor responsible for Cyp induction. In contrast to the small molecule hypothesis supported by Venkatesh et al. (43), untargeted metabolomics analysis of liver and plasma samples from mice treated with LGR-1 or vehicle failed to identify any small molecules significantly altered in abundance. Alternatively, LGR-1 supplementation could be modulating hepatic activity by microbiota-dependent crosstalk between the immune system and xenobiotic metabolism (44-46). Xenobiotic metabolism regulation has been associated with microbial recognition receptors Toll-like receptor 2 (47) and Toll-like receptor 4 (43). Although studies evaluating the global immune response to LGR-1 supplementation in vivo are limited, LGR-1 interacts with immune cells through the alteration of granulocyte colony-stimulating factor (48, 49). Future work in selectively-colonized germ-free animals would help to better characterize the problems associated with deciphering the effect of exogenous microbial supplementation on the physiology of a non-sterile host.

In summary, this study has shown that sub-chronic exposure to environmentally-relevant levels of CP may not be as toxic as anticipated in non-neonatal mammals. The observed alteration in CP metabolism by LGR-1 supplementation was correlated to enhanced gene expression of Por and Fmo2. These observations highlight the underexplored interaction between consumed microbes and host xenobiotic metabolism. Notably, the findings warrant further functional study and mechanistic characterization. That being stated, this study provides preliminary insight into how targeting the microbiota with probiotics, prebiotics, or synbiotics could be used to optimize pharmacotherapy or mitigate toxin exposures.
3.6 References


28. Jarvinen AW, Tanner DK. 1982. Toxicity of selected controlled release and corresponding unformulated technical grade pesticides to the fathead minnow


3.7 Supplementary

Figure 3-6. Lactobacilli and/or low levels of chlorpyrifos do not alter mouse water or food consumption or bodyweight.

Cumulative (A) water and (B) food consumption and (C) bodyweights were measured in BALB/c mice subchronically orally-exposed to chlorpyrifos (CP). Cumulative water and food consumption were normalized per mouse. Bodyweights are expressed as means ± SD (n = 6-8).
Figure 3-7. Subchronic chlorpyrifos does not alter gross duodenal architecture.

No histological abnormalities were observed in the duodenal tissues (A). Mean duodenal villus length (B), villus width (C), lamina propria width (D), mucin$^+$ cells/mm$^2$ of villus (E), inner muscle width (F), and outer muscle (G) were quantified. Scale bar = 200 µm.
Figure 3-8. Subchronic chlorpyrifos does not alter gross jejunal architecture.

No histological abnormalities were observed in the jejunal tissues (A). Mean duodenal villus length (B), villus width (C), lamina propria width (D), mucin+ cells/mm² of villus (E), inner muscle width (F), and outer muscle (G) were quantified. Scale bar = 200 μm.
Figure 1-9. Subchronic chlorpyrifos does not alter gross colonic architecture.

No notable histological abnormalities were observed in the colonic histology (A). Mean duodenal villus length (B), villus width (C), lamina propria width (D), mucin$^+$ cells/mm$^2$ of villus (E), inner muscle width (F), and outer muscle (G) were quantified. Scale bar = 200 µm.
### Table 3-1. Primers used for qPCR.

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<th>Gene</th>
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<th>Reverse Primer Sequence</th>
<th>Efficiency</th>
<th>R²</th>
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<td>B-actin</td>
<td>GATGTATGAAGGCTTTGGTC</td>
<td>TGTGCACCTTTATTTGGTCTC</td>
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Chapter 4

4 Enhancing innate immunity with beneficial microbes reduces pesticide-induced toxicity in *Drosophila melanogaster*: implications for colony collapse disorder.

Trinder M, McDowell TW, Ali SN, Dube J, Leong HS, Sumarah MW, Reid G.

4.1 Abstract

Honeybees are experiencing massive population declines known as colony collapse disorder in North America, Europe, and Asia due to pathogens, neonicotinoid pesticides, and habitat loss. A link has been established between neonicotinoid-induced honeybee immunosuppression and subsequent increased risk of infections. Thus, we hypothesized that immunomodulation of honeybees with *Lactobacillus plantarum* ATCC 14917 (Lp 39) would promote survival in response to imidacloprid (IMI, neonicotinoid) in *Drosophila melanogaster* model. Preliminary evidence for host-microbe interactions relevant to insect pro-survival in response to pesticides was demonstrated using *Drosophila* lacking the immune deficiency (IMD) pathway (Rel^E20^). Rel^E20^ *Drosophila* demonstrated significantly (p < 0.01) reduced larval eclosion and adult survival in response to both imidacloprid and organophosphate chlorpyrifos exposure. Furthermore, whole fly qPCR and IMD and Toll pathway reporter flies were used to demonstrate that Lp 39 oral supplementation modulates *Drosophila* immunity. Interestingly, simultaneous treatment of adult wild type Canon-S *Drosophila* with 10 μM IMI and vehicle, Lp 39, or a *L. plantarum* strain isolated from *Drosophila* (Lp^iso^) demonstrated that Lp 39, but not Lp^iso^, improved early time point survival. Following 3 d prophylactic treatment with Lp 39, there was significantly improved early (p < 0.05) and overall (p < 0.001) survival of *Drosophila* subsequently exposed to 10 μM IMI relative to vehicle controls. Further clarification that the Lp 39-mediated pro-survival effect was not dependent on a direct interaction between Lp 39 and IMI was confirmed by demonstrating that Lp 39 cannot bind or degrade IMI. Taken together, these findings provide a basis to test supplementation of honeybee colonies with Lp 39 for targeting multiple causes of colony collapse disorder associated with neonicotinoid pesticide exposure.
4.2 Introduction

Apis melifera, commonly known as honeybees, are experiencing massive declines in North America, Europe, and Asia known as colony collapse disorder. This is problematic as honeybees are critical pollinators for roughly 35% of the global food crop (1), and thus, are largely responsible for the world’s food supply and important economically. Colony collapse disorder has been attributed to increased exposure of honeybees to a combination of pesticides (2) and pathogens (3) and increased loss of habitat (4, 5). Neonicotinoid insecticides are a class of pesticide that have been implicated as particularly toxic to bees (2, 6, 7). These compounds act as selective agonists for the nicotinic acetylcholine receptor, which induce bee growth abnormalities (6, 8), motor deficiencies (2, 9), neurologic abnormalities (10), and/or death (2). The commonly used neonicotinoids, imidacloprid (IMI) and clothianidin, have also been shown to suppress immune function and consequently increase susceptibility to viral and fungal infections (11). Thus, the indirect damage to bee health may be more problematic than anticipated due to pesticides dysregulating the honeybee immune system and consequently making them more susceptible to infection. The current dilemma facing the agriculture industry is how to resolve the issue of preventing bee decline, while mitigating crop losses associated with pest infestations. One possible solution to this important economical problem is to fortify honeybee colonies with probiotic organisms (12).

Probiotics are live microorganisms that confer beneficial effects to their hosts when consumed in adequate amounts. Lactobacilli spp. are Gram-positive commensal bacteria that have been shown to colonize honeybees (13, 14) and confer beneficial effects to their health and colony size (14-16). In addition, lactobacilli have been shown to reduce organophosphate pesticide contamination of fermented food products (17, 18) and regulate associated host xenobiotic detoxification pathways (19). We have shown that Lactobacillus species have the ability to sequester organophosphate pesticides from solution and mitigate the absorption and toxicity of these compounds in a Caco-2 intestinal cell-line and Drosophila melanogaster, respectively (Trinder et al., submitted 2016). However, the effect of lactobacilli on neonicotinoid pesticides is largely unknown.
*Drosophila melanogaster* (fruit fly) is a convenient insect organism to model the potential for lactobacilli to mitigate pesticide-induced toxicity in bees (11). Both fruit flies and honeybees have a core microbiota composed of *Lactobacillus*, with *Lactobacillus plantarum* being a major species in both hosts (12, 20, 21). This core microbiota is known to be sufficient for promoting normal fly development (22). As both species are insects, they are therefore profoundly affected by IMI in terms of toxicity and immunosuppressive effects (11, 23). Lactobacilli have been shown to induce positive effects on *Drosophila* (24) and honeybee (25) immune function. In particular, prophylactic lactobacilli supplementation has been shown to mitigate lethal *Serratia marcescens* and *Pseudomonas aeruginosa* infections in *Drosophila* (26). Despite, these observations the mechanisms of action of these microorganisms are underexplored.

The *Drosophila* protein Relish is a key signaling hub for coordinating host physiological responses to bacteria through the immune deficiency/nuclear factor kappa-light-chain-enhancer of activated B cells (IMD/NF-κB) pathway. Relish is best characterized as a downstream transcription factor that elicits antimicrobial peptide immune responses to diaminopimelic acid (DAP)-type peptidoglycan activation of peptidoglycan recognition protein (PGRP)-LE in the midgut and PGRP-LC in the hindgut and foregut (27). *Drosophila* with IMD pathway loss of function phenotypes are significantly more susceptible to Gram-negative bacterial infections (28-30). The indigenous microbiota—*Lactobacillus* and *Acetobacter*—are known to activate Relish in the gut, without downstream antimicrobial peptides due to negative regulation (24, 31). However, the implications of this interaction between intestinal Relish and the *Drosophila* indigenous microbiota is still poorly understood (31). These unanswered questions are interesting since Relish is critical for mediating *Drosophila* pro-survival responses to noxious stimuli (32). We hypothesized that *L. plantarum* supplementation would modulate Relish-dependent immune responses to mitigate IMI-induced *Drosophila melanogaster* mortality and toxicity.
4.3 Methods

4.3.1 Bacterial strains and culture

*Lactobacillus plantarum subsp. plantarum* (14917, Lp 39), was obtained from the American Type Collection Centre. An isolate of *L. plantarum* (Lpiso) was obtained from wild type Canton-S *Drosophila*. Lactobacilli were inoculated from de Man, Rogosa and Sharpe (MRS) agar (BD, catalog number: 288130) or into MRS broth, then subcultured and incubated anaerobically overnight (18 h) at 37°C statically and anaerobically.

4.3.2 *Drosophila* husbandry

Wild type Canton-S (stock number: 1), RelE20 (stock number: 55714), y1 w*; P{UAS-Dpt-cherry}C1 (Dpt-RFP, stock number: 55706), and P{Dipt2.2-lacZ}1, P{Drs-GFP.JM804}1, y1 w* (Drs-GFP, stock number: 55707) stocks were obtained from Bloomington Drosophila Stock Center at Indiana University. *Drosophila* were maintained using media containing 1.5% agar (w/v), 1.73% yeast (w/v), 7.3% cornmeal (w/v), 7.6% corn syrup (v/v), and 0.58% propionic acid (v/v) at 25°C with 12 h light/dark cycles. For experimental procedures media was supplemented with or without pesticide prior to agar solidification. Experiments with adult *Drosophila* used newly eclosed flies. All experiments were performed in polypropylene *Drosophila* vials (GEN32-121 and GEN49-102, Diamed Lab Supplies Inc., Mississauga, ON, Canada).

Media was supplemented with 100 µL (10⁹ CFU) of washed and concentrated lactobacilli or phosphate-buffered saline vehicle when experimentally appropriate and allowed to air dry before use.

4.3.3 Chemicals

IMI (catalog number: 37894) and chlorpyrifos (catalog number: 45395) were obtained from Sigma-Aldrich. Stock solutions were prepared at 10 mg/mL dimethyl sulfoxide (Sigma-Aldrich) and stored frozen at -80°C until usage.
4.3.4 **Adult Drosophila survival assays**

Twenty to twenty-five newly eclosed *Drosophila* were transferred into standard vials containing media supplemented with vehicle or IMI and monitored daily for survival. Media was supplemented with 100 μL (10⁹ CFU) of washed and concentrated Lp 39, Lpiso, or phosphate-buffered saline vehicle when experimentally appropriate and allowed to air dry before use.

4.3.5 **Eclosion assays**

*Drosophila* eggs were collected on grape agar plates. Ten 1st-instar larvae were transferred to *Drosophila* media, incubated at aforementioned conditions, and monitored daily for up to 16 d for eclosion.

4.3.6 **Intravital confocal microscopy**

Dpt-RFP or Drs-GFP *Drosophila* were exposed to cotton gauze soaked in 10⁹ CFU/mL Lp 39 or 5% sucrose (w/v) vehicle for 15 or 24 h, respectively. Wild type Canton-S fed 5% sucrose were used as negative controls. Flies were anesthetized with FlyNap (Carolina Biological Supply Company), immobilized, and imaged using tri-channel laser resonance confocal microscopy (Nikon Inc., Tokyo, Japan). Dpt-RFP *Drosophila* guts were also dissected and counterstained with 162 μM Hoechst 33342, trihydrochloride, tetrahydrate (Molecular Probes, catalog number: H3570). Using 4x and 20x objective lenses, the 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI, 408 nm) channel was utilized to provide background contrast, the Alexa594 (594 nm) channel was used to visualize the RFP expression in dissected guts and the FITC (488 nm) channel was used to visualize GFP expression in the whole fly. Multichannel and z-stack images were taken using the NIS-Elements Advanced software (Nikon Inc., Tokyo, Japan).

4.3.7 **RNA extraction, reverse transcription, and qPCR**

Canton-S *Drosophila* were exposed to cotton gauze soaked in 10⁹ CFU/mL Lp 39 or 5% sucrose (w/v) vehicle for 24 h. Whole flies were flash frozen and stored at -80°C until RNA extraction. Twenty flies were homogenized in 1 mL of TRIzol (Ambion, catalog number: 15596018) using a motorized pestle. After phase separation with chloroform
according to the manufacturer’s protocol, the aqueous phase was combined with equal volume of 100% ethanol. RNA purification was continued using a PureLink RNA Mini Kit (Ambion, catalog number: 12183025) with on-column treatment of PureLink DNase treatment (Ambion, catalog number: 12185010). RNA quantity and purity was assessed with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific). RNA was stored at -80°C until reverse transcription.

cDNA was prepared from 200-2000 ng of purified total RNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, catalog number: 4368813). qPCR was performed with a 7900HT Sequence Detection System (Applied Biosystems) in 20 µL reactions using Power SYBR Green Master Mix (Applied Biosystems, catalog number: 4368702) with 200 µM of appropriate forward and reverse primers. Primers used for qPCR analysis can be found in Supplementary Table 4-1. qPCR results were analyzed using SDS RQ 6.3 manager software (Applied Biosystems) and relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

4.3.8 Pesticide binding and metabolism assay.

Overnight bacterial subcultures were pelleted at 5000 g. Pellets were washed and re-suspended in 50 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES, pH 6.8). Bacterial-buffer or buffer-alone solutions were incubated with IMI protected from light at 37°C for 24 h (unless otherwise stated) with gentle shaking. Cultures were pelleted and supernatant collected then analyzed for IMI levels using liquid-chromatography mass spectrometry.

An Agilent 1290 Infinity high performance-liquid chromatography (HPLC) coupled to a Q-Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, USA) with a HESI (heated electrospray ionization) source. Two µL of each sample was injected into a ZORBAX Eclipse plus C18 2.1 x 50mm x 1.6 µm column. Mobile phase (A) consisted of 0.1% formic acid in water and mobile phase (B) consisted of 0.1% formic acid in acetonitrile. The initial composition of 0% (B) was held constant for 0.5mins and increased to 100% over 3.0 minutes. Mobile phase B was then held at 100% for 1.5 minutes and returned to 0% over 30 seconds, re-equilibrated at 0% (B) for 1 minute for a
total run time of 6.5 min.

The HESI source was operated under the following conditions: nitrogen flow of 20 and 9 arbitrary units for the sheath and auxiliary gas respectively, probe temperature and capillary temperature of 450 °C and 400 °C respectively and spray voltage of 3.9 kV. The S-Lens was set to 65. A full MS with a top 10 data dependent (ddMS2) was conducted at a full MS scan between the ranges of 160-1500 m/z in positive mode at 35,000 resolution (AGC target and maximum injection time were 3e6 and 120 ms respectively) and MS/MS at 17,500 resolution, normalized collision energy set to 27 (AGC target and maximum injection time of 1 x 10^5 and 60ms, respectively).

4.3.9 Pesticide tolerance assay.

Overnight broth cultures (stationary phase) were subcultured (1:100 dilution) into 96 well plates (Falcon, catalog number: 351177) containing MRS broth with or without the addition of IMI or vehicle (DMSO). Plates were incubated at 37°C and read every 30 min for 24 h at a wavelength of 600 nm using a Labsystems Multiskan Ascent microplate reader.

4.3.10 Statistical analyses

All statistical analyses were performed in GraphPad Prism 6 using unpaired t-tests or Mann-Whitney tests, one-way analysis of variance with Tukey’s multiple comparison tests, or two-way analysis of variance tests with Tukey’s multiple comparison tests as appropriate. Alternatively, Mantel-Cox and Gehan-Breslow-Wilcoxon tests were used for *Drosophila* survival analyses.

4.4 Results

4.4.1 Relish is necessary for mitigating pesticide-induced toxicity in *Drosophila*

Relish has been shown to be an important for promoting *Drosophila* survival in response to ultraviolet light-induced stress (32). Similarly, to determine the importance of Relish for mediating *Drosophila* pesticide tolerance, the survivability and development of Relish-knockout (Rel^{E20}) flies were compared to age-matched wild type Canton-S flies.
Adult wild type Canton-S *Drosophila* demonstrated significantly prolonged overall (p < 0.0001) and early (p < 0.0001) time point survival relative to Rel*E20* mutants when lethally exposed to 100 µM IMI or 10 µM CP (Figure 4-1A). Notably, median survival of Rel*E20* and wild type Canton-S *Drosophila* exposed to 100 µM IMI were 1 and 7 d, respectively. This corresponded to all Rel*E20* *Drosophila* succumbing to mortality at 4 d post-100 µM IMI exposure; in contrast, 17.526% of wild type Canton-S *Drosophila* remained alive at the 12 d experimental endpoint following 100 µM IMI exposure. Adult wild type Canton-S and Rel*E20* mortality on vehicle media was negligible (data not shown). Furthermore, eclosion assays confirmed that Rel*E20* larvae were significantly more (p < 0.01) sensitive to imidacloprid- (10 µM) and chlorpyrifos-induced (1 µM) toxicity than Canton-S wild type controls (Figure 4-1B). Together these results suggest that Relish is necessary for promoting *Drosophila* survival in response to toxic pesticide insult.
Figure 4-1. Relish is necessary for mitigating pesticide-induced toxicity in *Drosophila*.

(A) 1st-instar wild type Canon-S or Rel[E20] larvae were monitored for eventual eclosion following incubation on imidacloprid (IMI), chlorpyrifos (CP), or vehicle. Data are expressed as means ± SD from at least 3 independent experiments. (B) Survival curves of newly eclosed wild type Canton-S or Rel[E20] *Drosophila* exposed to media containing 100 µM IMI or 10 µM CP. Data are displayed from at least 3 independent experiments. ** p < 0.01, *** p < 0.001, **** p < 0.0001.
4.4.2 *Lactobacillus plantarum* supplementation alters *Drosophila* immune function through the IMD/Relish, and potentially Toll pathways.

Since the *Drosophila* gastrointestinal microbiota is comprised largely of lactobacilli, which have been shown to induce IMD/Relish signaling (24), it was predicted that Lp 39 supplementation would also activate the IMD/Relish and/or Toll pathways. This hypothesis was assessed by determining the activation of *Dpt* and *Drs*, which encode downstream antimicrobial effectors of the IMD/Relish and Toll pathways, respectively. Adult *Dpt*-RFP *Drosophila* acutely orally exposed (15 h) to Lp 39 demonstrated increased expression of the IMD/Relish downstream antimicrobial peptide gene, *Diptericin (Dpt)*, in the midgut compared to sucrose-treated controls (Figure 4-2A). Interestingly, whole fly gene expression of Canton-S *Drosophila*, determined by qPCR, demonstrated that *Dpt* was significantly repressed (p < 0.05) following 24 h of oral Lp 39 supplementation (Figure 4-2B). However, these differences could potentially be explained by conflicting time points of analysis following Lp 39 exposure. This is supported by observations noting that the activation of *Drosophila* antimicrobial responses has been shown to activate and decline rapidly following microbial exposures (28, 29). Alternatively, newly eclosed *Drs*-GFP *Drosophila* appeared to show increased expression of the Toll downstream response antimicrobial peptide gene, *Drosomycin (Drs)*, following 24 h of oral Lp 39 supplementation (Figure 4-3A). This finding was corroborated with an increase in *Drs* expression, as determined by qPCR of extracted RNA from whole flies following 24 h of oral Lp 39 supplementation (Figure 4-3B). Thus, Lp 39 oral supplementation induces innate immune changes in *Drosophila* that may have implications to promoting *Drosophila* survival and/or resilience to pathogen, toxin, or stress exposures.
Figure 4-2. *Drosophila* show altered *dipteracin* expression in response to Lp 39 oral supplementation.

(A) Newly eclosed Dpt-RFP *Drosophila* and negative control wild type Canton-S were orally supplemented with Lp 39 or vehicle for 15 h. *Drosophila* midguts were dissected, nuclear counterstained with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) and viewed by confocal microscopy. Scale bar = 20 µm (B) *Diptericin* (*Dpt*) gene expression of newly eclosed whole wild type Canton-S *Drosophila* was determined following 24 h oral supplementation with Lp 39 or vehicle (n = 4 independent experiments). Data are expressed as means ± SEM. * p < 0.05.
Figure 4-3. *Drosophila* may show enhanced drosomycin secretion in response to Lp 39 oral supplementation.

(A) Newly eclosed Drs-GFP *Drosophila* and negative control wild type Canton-S were orally supplemented with Lp 39 or vehicle for 24 h. *Drosophila* were anesthetized and viewed by confocal microscopy for intravital imaging. GFP signal represents secreted drosomycin. Scale bar = 500 µm. (B) *Drosomycin* (Drs) gene expression of newly eclosed whole wild type Canton-S *Drosophila* were determined following 24 h oral supplementation with Lp 39 or vehicle (n = 4 independent experiments). Data are expressed as means ± SEM.
4.4.3 Lp 39 promotes point survival of *Drosophila* exposed to imidacloprid

Given that the IMD/Relish pathway is critical for promoting *Drosophila* survival following lethal pesticide challenge and that Lp 39 appears to modulate the IMD/Relish pathway, it was reasoned that Lp 39 could confer resilience to *Drosophila* challenged by pesticide. Adult Canon-S *Drosophila* were found to suffer acute IMI-induced mortality at feed concentrations of 10 μM, but not 1 μM (Figure 4-4A). Therefore, we tested the hypothesis that *Drosophila* exposed to Lp 39 would promote survival with simultaneous 10 μM IMI exposure. This hypothesis was also compared to supplementation with a *L. plantarum* (Lp\textsuperscript{iso}) strain isolated from our wild type Canton-S colony. Canton-S *Drosophila* treated with vehicle had negligible mortality by the experimental endpoint (data not show). Adult Canton-S *Drosophila* treated with Lp 39 were less prone (p = 0.0580) to early time point IMI-induced mortality than the non-supplemented counterparts (Figure 4-4B). However, Lp 39 did not demonstrate appreciable protection against IMI-induced mortality long-term (p = 0.9961). Median survival of flies treated with vehicle, Lp 39, or Lp\textsuperscript{iso} during 10 μM IMI challenge were 10, 8, and 7 d respectively. Interestingly, *Drosophila* treated with Lp\textsuperscript{iso} were significantly more prone to early (p < 0.05) and late (p < 0.01) time point IMI-induced mortality compared to Lp 39 and non-supplemented groups, respectively (Figure 4-4B). These results suggest that prophylactic priming of *Drosophila* with Lp 39 may be required for the observation of long-term survival benefits in IMI-challenged flies. Furthermore, the strain of *Lactobacillus plantarum* administered to *Drosophila* appears to confer different outcomes to IMI-challenged flies. This observation supports the notion of strain-dependent effects of supplementing organisms with beneficial microbes.

It was predicted that prophylactic treatment of *Drosophila* with Lp 39 prior to IMI would prime host tolerance to IMI-induced toxicity. Hence, aforementioned experiments were adapted to provide newly eclosed adult *Drosophila* with 3 d prophylactic exposure to Lp 39 or vehicle prior to 10 μM IMI challenge. Adult Canton-S *Drosophila* that were pretreated with Lp 39 for 3 d were significantly more tolerant to early (p < 0.05) and late (p < 0.001) IMI-induced mortality relative to vehicle treated controls (Figure 4-4C).
Median survival of Drosophila pretreated with vehicle or Lp 39 were 10 and 11 d, respectively. Moreover, *Drosophila* pretreated with Lp 39 had notably greater long term survival (14.811% alive at d 22) compared to vehicle controls (2.139 % at d 22) challenged with 10 μM IMI. These experimental results are interesting given that *Drosophila* were provided only a single Lp 39 inoculum in the feed for 3 d prior to pesticide exposure. Thus, prophylactic exposure to beneficial microbes appears to prime *Drosophila* to cope with toxic-insult.
Figure 4-4. Lp 39 promotes point survival of *Drosophila* exposed to imidacloprid.

(A) Survival curves of newly eclosed wild type Canton-S *Drosophila* were exposed to media containing 1 or 10 µM imidacloprid (IMI) or vehicle control. (B) Survival curves of newly eclosed wild type Canton-S *Drosophila* were exposed to media containing 10 µM CP with or without simultaneous supplementation of live Lp 39 or Lp iso. (C) Survival curves of newly eclosed *Drosophila melanogaster* exposed to Lp 39 or vehicle for 3 d, following subsequent exposure to media containing 10 µM IMI. Data are displayed from at least 3 independent experiments.
4.4.4 Lp 39 can tolerate, but not bind or metabolize, imidacloprid

In order to provide stronger evidence that prophylactic Lp 39 supplementation benefited *Drosophila* subsequently challenged with IMI by a microbe-host interaction rather than microbe-toxin interaction, the interactions between Lp 39 and IMI were determined. It was first assessed if IMI had notable toxic effects on Lp 39, which could alter Lp 39 behavior in culture or infer the potential of IMI to be toxic to members of the *Drosophila* gut microbiota. However, Lp 39 was shown to grow unimpaired at high concentrations of IMI (1 mg/mL ≈ 3911 µM, Figure 4-5A). Alternatively, the ability of Lp 39 was also tested for its ability to promote the degradation of IMI *in vitro*. However, several strains of lactobacilli, including Lp 39, failed to demonstrate any notable metabolism or binding of imidacloprid (0.1 mg/mL, ≈ 391.1 µM) following 24 h co-incubations (Figure 4-5B). These findings suggest that beneficial effects of Lp 39-treatment on *Drosophila* survivability to IMI challenge are not a consequence of direct interactions between Lp 39 and IMI.
Figure 4-5. Lp 39 can tolerate, but not bind or metabolize, imidacloprid.

(A) Percent imidacloprid (IMI) was determined in stationary phase live bacterial cultures relative to pesticide-only controls following 24 h co-incubations in 50 mM HEPES. Data are depicted as means ± SD of 2 independent experiments. (B) Growth curves of Lp 39 in MRS supplemented with vehicle or imidacloprid (IMI). Data are depicted as means ± SEM of 3 independent experiments with triplicate technical replicates.
4.5 Discussion

This study demonstrated that oral Lp 39 supplementation improved survival of *Drosophila* lethally exposed to IMI. There has been considerable investigation into the ability of beneficial microbes to sequester environmental toxins from food products. *Lactobacillus* species have been shown to degrade several organophosphate pesticides from fermented food products (17, 18, 34). However, the bioremediation of neonicotinoid pesticides is less well-understood. In our study, out of the few strains tested, we did not identify any *Lactobacillus* species capable of IMI degradation. Other studies have observed bacteria such as *Pseudoxanthomonas* (35), *Ochrobactrum* (36), *Mycobacterium* (37), *Stenotrophomonas* (38), *Ensifer* (39), and *Bacillus* (40) with IMI-degrading potential. However, the relevance of these organisms to *in vivo* bioremediation applications is unlikely given the observations of notable IMI degradation only after several days following experimentation. Nevertheless, these studies provide promise for engineering gain-of-function beneficial microbes once the genetic details of IMI degradation are better defined. The current benefits of lactobacilli supplementation on insects challenged with neonicotinoid pesticides may largely be a result of immune modulation.

Similar to other studies evaluating *Drosophila* larvae response to ultraviolet light-induced damage (32), this study has shown that the innate immune IMD pathway is necessary for *Drosophila* pro-survival in response to pesticides as both larvae and adults. Although complete understanding of this complex molecular signaling cascade is unclear, negative interactions between NF-κB/Relish and insulin/insulin growth factor signaling (ISS) have been shown to be crucial for growth homeostasis in response to DNA damage (32). The current working hypothesis suggests that systemic Janus tyrosine kinase/signal transducer and activator of transcription (JAK/STAT) pathway activation lead to a hemocyte-dependent repression of ISS in response to damage (32). Recovery of IIS requires Foxo-dependent NF-κB/Relish in the fat body to normalize ISS and maintain organismal homeostasis.

There is conflicting evidence for the duration of exposure and dose required for IMI-induced genotoxicity (41, 42), but it is also possible that pesticide-induced tissue damage
itself requires the IMD pathway for maintenance of organismal physiology to sustain survival (43). These claims are supported by observations of damaged cells releasing JAK/STAT-activating cytokines Unpaired 1–3 (Upd 1–3, 44-46).

Neonicotinoid pesticides have also been shown to downregulate NF-κB insect immune responses at sub-lethal concentrations due to upregulation of the NF-κB negative regulator gene CG1399 present in Drosophila (11). Under normal physiological conditions, NF-κB negative regulator genes require downregulation to initiate immune responses following infection (47). These observations are supported by increased fungal Nosema apis colonization (48) and pathogenicity (49), and deformed wing virus replication (11) in honeybees exposed to neonicotinoid pesticides. Given the importance of the innate immune system for regulating the insect intestinal microbiome (24, 31), the effect of neonicotinoids on microbiota composition remains to be elucidated.

Imidacloprid has also been shown to alter microbial ecosystems in soil (50, 51). Thus, the potential extrapolation of interplay between neonicotinoid pesticides and the gut microbiome requires further study given the gut ecosystem importance for regulating numerous aspects of insect physiology such as growth (22), lifespan (52), and mating behavior (53).

Since acute IMD pathway activation can prime Drosophila to mitigate infections by pathogens (26, 54), we reasoned that targeting this pathway with beneficial microorganisms could also promote survival and rescue immune deficiencies experienced in Drosophila or honeybees during neonicotinoid pesticide challenge. The honeybee has several homologues with high amino acid sequence similarity to the Drosophila Relish protein such as the predicted nuclear factor NFκB p110 subunit isoform X1 (XP_006562282.1), dorsal (NP_001011577.1), and Relish itself (ACT66913.1). These observations combined with the highly conserved nature of innate immunity suggest that translation of these findings will be amendable for future studies in honeybees (55).

Moreover, the use of lactobacilli as honeybee probiotics has been shown to enhance immune responses (25). These findings are analogous to the observations of IMD pathway modulation in Drosophila supplemented with Lp 39 seen in this study. Future work is attempting to definitively demonstrate that the observations of Lp 39-mediated
extension of *Drosophila* survival during IMI exposure is dependent on IMD/Relish pathway modulation. The extension of these findings to honeybees is promising given that colony supplementation with lactobacilli is affordable, feasible, and has already been shown to benefit honeybee colony growth (56), microbiota composition (16, 57), and antimicrobial defense (12, 21, 58).

In summary, this study demonstrated the importance of: 1) the IMD immune pathway for promoting *Drosophila* pro-survival in response to neonicotinoid pesticide challenge, 2) IMD/Relish pathway modulation by oral exposure to exogenous beneficial microbes, and 3) Lp 39-mediated protection of *Drosophila* lethally-exposed to IMI. These results suggest supplementation of honeybee colonies with lactobacilli has the potential to mitigate both direct IMI-induced toxicity and IMI-immunosuppressive effects. These findings support the simple and economically affordable supplementation of honeybee colonies with lactobacilli to mitigate multiple causes of colony collapse disorder.

### 4.6 References


58. **Butler È, Alsterfjord M, Olofsson TC, Karlsson C, Malmström J,**
4.7 Supplementary

Table 4-1. Primers used for qPCR.

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<td>CACCAGCCTTCAGACTGGG</td>
</tr>
</tbody>
</table>
Chapter 5

5 General discussion

5.1 Future directions

5.1.1 Better characterize lactobacilli-organophosphate binding interactions.

Experiments demonstrated that stationary phase cultures \(10^9\) CFU/mL of \textit{Lactobacillus rhamnosus} GG (LGG) and GR-1 (LGR-1) could passively sequester high concentrations of organophosphate pesticide (100 ppm parathion or chlorpyrifos) from 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) solution after 24 h. However, the dose-response relationship, kinetic, potential buffer effect, and mechanism of action still remain unknown.

This will be evaluated for concentration of pesticide and bacteria. Live and heat-killed strains of \(10^9\) CFU/mL LGR-1 and LGG will be incubated with 100, 50, 10, or 1 µM of parathion or chlorpyrifos (CP) for 24 h in 50 mM HEPES. Alternatively, live and heat-killed strains of \(10^9, 10^8, 10^7, \) and \(10^6\) CFU/mL LGR-1 and LGG will be incubated with 100 µM of parathion or CP for 24 h in 50 mM HEPES. Serial supernatant samples will be collected at 0.25, 3, and 24 h. Supernatant samples will be analyzed by high performance liquid chromatography with ultraviolet detection for comparison of pesticide levels relative to untreated samples as previously described in Chapter 2.

Interestingly, in Caco-2 Transwell experiments, the amount of organophosphate pesticide sequestration observed by LGR-1 and LGG was much less than anticipated based on previous binding experiments (data not shown). One major difference between experiments was the usage of 50 mM HEPES and Hank’s balanced salt solution (HBSS) for the binding and Caco-2 Transwell experiments, respectively. It is possible that different buffers alter the proposed binding phenotype initially observed. This phenomenon will be evaluated by repeating the aforementioned experiments using 1x
HBSS as the solvent (instead of 50 mM HEPES). These experiments will hopefully elucidate if solvent conditions play an important role in mediating lactobacilli-organophosphate binding.

Based on initial experiments, the ability of bacteria to sequester organophosphate pesticides from solution appears to be relatively generic. CP levels in the homogenized and methanol-treated LGR-1 and LGG pellets suggests that these similar strains may differ in either affinity or binding potential. There are some unique differences to LGR-1 predicted outer-membrane proteins and an exopolysaccharide biosynthesis cluster (Dr. Jean Macklaim, unpublished poster). Thus, we plan to isolate exopolysaccharide from LGR-1 and LGG to evaluate their organophosphate binding potential. This experiment will attempt to decipher the mechanism of action for LGG and LGR-1 interactions with organophosphate pesticides.

5.1.2 Enhance the ability of Lactobacillus rhamnosus GG and GR-1 to metabolize organophosphate pesticides.

I have designed a gene construct that contains the experimentally confirmed parathion hydrolase gene from Brevundimonas diminuta under the promotion and termination of the lactate dehydrogenase gene found in LGG. Preliminary experiments attempted to insert this construct into LGG and/or LGR-1 using the Escherichia coli-Lactobacillus shuttle pLAB1301 vector. Sequencing and qPCR results suggest this gene failed to be incorporated into the organism despite conferred erythromycin resistance (selectable marker). Swapping gene promoters from lactate dehydrogenase to dlt has been advised as an additional consideration for optimal expression. This strategy is feasible given that the gene construct was designed and synthesized for alteration of promoter, terminator, or gene of interest by restriction enzyme digestion. Reattempting this transformation would allow for assessment of potentially improved LGG or LGR-1-mediated bioremediation potential given that these strains were shown to bind but not degrade CP or parathion in Chapter 2.
5.1.3 Animal studies to investigate the functional effect of *L. rhamnosus* GR-1 on pesticide/drug/toxin metabolism.

Our work has shown that acute supplementation of BALB/c mice with LGR-1 can alter host defensome gene expression in the small intestine, liver, and kidney. Although previous work has used germ-free animals to show that the microbiota is critical to host xenobiotic metabolism (1-5), few studies have characterized the various effects of individual species or strains in a non-sterile host (1, 6). These latter findings have important translational implications to human pharmacology and toxicology. We have shown that hepatic *P450 oxidoreductase* (*Por*), *flavin containing monooxygenase 1* (*Fmo1*), and *Fmo2* are significantly upregulated in mice treated with LGR-1. *Por* catalyzes the transfer of electrons to most cytochrome *P450* (Cyp) enzymes, and consequently is important to xenobiotic (7, 8) and toxin (9) metabolism *in vivo*. Thus, we hypothesize that supplementing the gastrointestinal microbiota with beneficial *Lactobacillus* can prevent the systemic absorption of orally acquired toxic compounds such as the organophosphate pesticide chlorpyrifos through the modulation of xenobiotic metabolism. We plan to further investigate this by mechanistically deciphering the effects of LGR-1 supplementation on the functional activity of mouse CYP homologues relevant to human chlorpyrifos metabolism (CYP2C9, CYP2C19, CYP2B6, and CYP3A4). In particular, the activities of CYP2C19 and CYP2B6 have been implicated in chlorpyrifos detoxification and toxicity, respectfully.

Female BALB/c mice, aged 5-6 weeks, obtained from Charles River, were randomly assigned into saline or LGR-1 treatment conditions. Mice were orally-gavaged vehicle (n=9) $10^9$ CFU of *L. rhamnosus* GR-1 (n=9) daily for 3 d prior to sacrifice. Livers, kidneys, duodenum, and jejunum were flash frozen and stored at -80°C for downstream protein analysis. Blood was collected, separated into plasma and cellular components, aliquoted separately, and stored at -80°C until required. Fecal pellets and small intestinal swabs were flash frozen at sacrifice. Metabolomic analysis of these samples using liquid chromatography-mass spectrometry (LC-MS) will help mechanistically explain the changes in host xenobiotic gene expression we observed previously.
Livers will be homogenized and ultracentrifuged to isolate microsomes for downstream Cyp450 activity assays. Chlorpyrifos will be incubated with hepatic microsomes and plasma from each experimental group. Selective activity of microsomal activity of Cyp3a11 (human Cyp3A4 homolog), Cyp2b10 (human CYP2B6 homolog), and Cyp2c29 (human CYP2C19) will be evaluated between experimental groups in parallel to chlorpyrifos by co-incubating isolated microsomes with probe substrates midazolam, bupropion, and tolbutamide, respectively. Similarly, plasma esterase activity will be assessed by co-incubating isolated plasma with probe substrates propantheline and propoxycaine. Test compounds (1 µM for microsomes, 5 µM for plasma) will be co-incubated with isolated microsomes (0.5 mg/mL) or plasma for 0, 15, 30, 45, and 60 min in triplicate. Samples will be prepared for parent and metabolite compound analysis by liquid chromatography-mass spectrometry. The formation of metabolites will be determined by comparing the peak area of the metabolite compound at each time point to time zero. Each compounds half-life for a given treatment will be estimated from the logarithmic curve of parent compound remaining vs. time given the assumption of first order kinetics. The apparent intrinsic clearance will be calculated from the half-life value for assays with microsomes or plasma.

Forty-eight female BALB/c mice, aged 5-6 weeks, will be obtained from Charles Rivers. Mice will be housed in 4 separate cages (12 mice/cage). Mice from each cage will be used to assess a single pharmacological parameter described below. Mice in each cage will be randomly selected for vehicle or experimental treatment at a ratio of 1:1. Mice will be orally-gavaged vehicle or 10^9 CFU of GR-1 daily for 3 d prior to sacrifice. Plasma parent concentrations, metabolite concentrations, and pharmacokinetic parameters will be determined as the mean from 3 animals (10). All animals will be food-deprived for 16 h prior to experimentation. Mice will be administered an oral or intravenous (tail vein injection) dose of midazolam (Cyp3a11 probe), bupropion (Cyp2b10 probe), or tolbutamide (Cyp2c29 probe). Serial blood samples (20-30 µL) will be collected at 5, 30, 60, 180, and 360 min. Animal work will be performed by an experienced animal technician to ensure experimental accuracy and animal safety. Blood will centrifuged to separate plasma and hematocrit. Samples will be stored at -80°C until analysis by LC-MS. Livers, kidneys, duodenum, and jejunum will be flash frozen and stored at -80°C for
downstream protein analysis. Together these experiments will enable a better understanding of the mechanisms and functional consequences of LGR-1-mediated modulation of host xenobiotic metabolism gene expression. These findings could provide important insight into how fermented food products consumption could influence the clinical variation observed in the efficacy and toxicity of commonly used medications.

5.1.4 Evaluation of *Lactobacillus rhamnosus* GR-1-induced changes to P-glycoprotein activity relevant to intestinal chlorpyrifos absorption

We have shown that duodenal *Abcb1a/P-glycoprotein* expression is altered in mice treated with LGR-1. We will further investigate this result by evaluating intestinal P-glycoprotein protein levels and protein localization in mice from the aforementioned experiments by western blotting and immunofluorescence, respectively. To mechanistically determine if GR-1 can affect P-glycoprotein function, we will perform bidirectional P-glycoprotein transporter assays on differentiated Caco-2 cell monolayers in 12-well, 0.4 µm Corning Transwell Inserts. Cells will be pretreated for 24 h with LGR-1 lysates or vehicle. Transport assays will be performed in HBSS / HEPES solutions containing 10 µM digoxin as a P-glycoprotein probe substrate. Sampling will be taken at 20 µL and 100 µL volumes from the donor and receiving sides, respectively. Donor sampling will be taken at 0 and 80 min. Receiver sampling will be taken at 20, 40, 60, and 80 min. Integrity of Caco-2 monolayers will be confirmed following experimentation by lucifer yellow rejection. Collected samples will be analyzed for digoxin using LC-MS. Two independent experiments with treatments completed containing technical replicates in triplicate will be performed. Digoxin permeability coefficient and efflux ratios will be calculated for experimental conditions as described (11). Follow-up experiments will use the outlined Caco-2 experimental workflow to evaluate chlorpyrifos absorption with or without the use of the P-glycoprotein chemical inhibitor verapamil. The latter experiment could help elucidate if GR-1 can prevent CP absorption by enhancing intestinal P-glycoprotein activity.
5.1.5 Further characterization of Relish and its role in toxicology and potential for targeting by microbes

We have shown that the protein, Relish, is necessary for promoting *Drosophila* survival in response to pesticide (organophosphate or neonicotinoid) challenge (Chapter 4). Relish is most known for its role in mediating antimicrobial peptide responses to Gram-negative bacteria, but has been shown to be important for promoting survival in response to noxious stimuli (12, 13). This is particularly interesting given that this pathway is subject to regulation by indigenous microbiota species, such as *Lactobacillus*, present in the gut. Together these observations suggest that modulation of this pathway with beneficial microbes may promote survival and immune function of important insect species, such as honeybees, that are orally-challenged by pesticides. Future experiments could test the sufficiency of Relish for promoting *Drosophila* survival using both gut localized and constitutive Relish overexpression with the UAS/GAL4 system (14). The work would be complemented with better characterization of how oral supplementation of *Drosophila* with *Lactobacillus* species modulate the intestinal IMD pathway and its relevance to pesticide-induced immune susceptibility. This hypothesis of host-microbe interactions promoting survival following pesticide challenge would be further evaluated using *Drosophila* that have been derived germ-free or subjected to life-long antibiotic exposure (15). Other future experiments could combine IMD pathway reporter *Drosophila* strains, gene expression analysis, and infection models. Hopefully, the resultant data would demonstrate the importance of how directly targeting the IMD pathway with beneficial microbes can mitigate direct and immunosuppressive toxicity of neonicotinoid pesticides to insects.

5.2 Conclusions and implications

Despite the support for lactobacilli being a potential pesticide detoxification mediator *in vitro* and *ex vivo* in food, it was only recently that our lab first demonstrated that chronic consumption of yogurt-supplemented with *Lactobacillus rhamnosus* GR-1 was able to reduce systemic bioaccumulation of mercury and arsenic in Tanzanian pregnant women and children (16). People living around Lake Victoria (Tanzania, Uganda, Kenya) are
exposed to heavy metals, pesticides, and other environmental toxins, particularly through ingestion of fish and water. This issue is also relevant to North Americans, as people living along Lake Erie and Lake Ontario have the potential to be exposed to comparable levels (17, 18). However, North Americans are warned not to consume toxin-laden fish, and have the ability to cope with this resource shortage by accessing alternative foods. In Tanzania and Uganda where lake fish are part of their staple, affordable diet, this is not an option. Thus, the potential to consume locally produced nutritious yogurt, containing bacteria that can sequester toxins, has great appeal to provide major health and longevity benefits to the residents of Lake Victoria. Efforts are now underway to provide people with the opportunity to make such probiotic yogurt containing LGR-1 or LGG (generic strain Yoba).

Although the chemical structures of heavy metals and pesticides differ notably, the present study’s findings provide optimism that lactobacilli may have the ability to act as cost-effective broad-spectrum detoxification. Our lab has proposed clinical field studies to be conducted in Africa using our established Western Heads East and the new International Development Research Centre program. These projects will allow for testing the clinical efficacy of LGR-1 and Yoba in preventing pesticide exposure in humans at high risk. The contents of this thesis have provided a rationale for the anti-pesticide effects.

The ability of probiotic lactobacilli to prevent the systemic absorption of certain pesticides could also be valuable to the agriculture industry. The recent concerns over severe toxicity of neonicotinoid pesticides to honeybees, critically important pollinators, will put pressure on the industry to find other means to kill pests without also killing bees. The colonization of insects by symbiotic bacterial species can confer pesticide resistance (19), and specifically, the ability of lactobacilli to modulate health benefits to honeybee colonies has already been shown to be relevant for gastrointestinal pathogen control (20). Since beekeepers already use ‘pollen patties’ to provide extra nutritional nourishment to bee colonies, there is a possibility that supplementing these patties with probiotic lactobacilli could mitigate neonicotinoid-induced “colony collapse disorder”.
Probiotics already form an important component of the aquaculture industry to promote the growth and reproduction of fish (21-23). Highly adhesive strains of probiotic lactobacilli have been identified for colonization of the fish intestine and improving defenses (24). By protecting fish against pesticides that have leached into soils and subsequently returned to the water supply as a result of runoff, the associated toxicity of pesticide bioaccumulation imposed on humans, via the consumption of these contaminated animals, may also be mitigated.

The consequences of environmental pesticide pollution due to widespread usage in agriculture and soil leaching are starting to become a major societal concern. Although some of the long-term effects of pesticide exposure to humans and wildlife remain unknown, logic suggests these chemicals are not aligned with ecosystem health. Most research focusing on pesticide bioremediation has investigated microbial-pesticide interactions against the organophosphate class of pesticides in vitro or in the environment, rather than directly in humans. Future studies should explore if other strains of lactobacilli have the potential to metabolize widely used classes of pesticide compounds in vivo. Most Lactobacillus species likely share common passive mechanisms for reducing host damage in response to various toxin exposures. However, it is also possible that certain strains have gene suites for active enzymatic degradation or sequestration of various pesticides (25).

In conclusion, probiotic lactobacilli can confer a variety of beneficial affects to humans and animals in the form of nutritious and affordable foods and supplements. Their potential to prevent systemic absorption of pesticides merits further study, especially given the continued widespread use of pesticides and their known potential to cause morbidity and death. More generally, by establishing mechanistic interactions between bacteria and drug metabolism, the present findings could be important for future optimization of medication dosing. It is reasonable to suggest that in the near future many therapeutics and/or diagnostics will target the microbiome.
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2013 - 2014

Frederick W. Luney Entrance Scholarship
2014-2015

Alexander Graham Bell Canada Graduate Scholarship (NSERC-CGS-M)
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