THE RELATIONSHIP BETWEEN INSECTICIDE SUSCEPTIBILITY AND THE GUT MICROBIOME OF GERMAN COCKROACH (*BLATTELLA GERMANICA* L.)

by

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Dedicated to my Grandma and Poppop – David and Beverly Wiener

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LIST OF ABBREVIATIONS

DAN:	Danville, IL Resistant strain
J-WAX:	S.C. Johnson Wax Susceptible strain
CTRL:	Control treatment
KAN:	Kanamycin/kanamycin treatment
PCR:	Polymerase chain reaction
OTU:	Operational taxonomic unit
CFU:	Colony forming unit

ABSTRACT

The German cockroach (*Blattella germanica*) is a notorious urban pest with exceptional insecticide resistance capabilities at the population level. German cockroaches are widespread in human-dominated urban areas, and are especially impactful in multi-family housing communities. Since German cockroaches host a wide variety of gut microbial species, there is reason to suspect that these gut microbes have an impact on insecticide resistance, tolerance, and/or degradation. The objectives of this dissertation included comparing the whole gut bacterial profiles of insecticide resistant and susceptible *B. germanica* and determining how these profiles, as well as the structure and function of the gut microbiome, change in the presence of an antibiotic. Additional goals were to investigate how antibiotic treatment impacts the toxicity of the bait insecticides fipronil, abamectin and indoxacarb, and to determine how gut bacteria, and specifically the enzymes originating within gut bacteria, metabolize and convert ingested indoxacarb into its toxic metabolite DCJW. Findings show that pre-treatment with the antimicrobial compound kanamycin (KAN) led to reductions in resistance levels for fipronil and abamectin, but also increased basal toxicity levels in both resistant and susceptible strains tested. 16S bacterial sequence surveys revealed that resistant and susceptible cockroach strains were more similar before KAN treatment than after, with a stronger dysbiosis effect in the resistant strain. For the insecticide indoxacarb, regardless of strain, roaches treated with kanamycin-infused water in feeding bioassays were more susceptible compared to the control treatment, but in vial (surface contact) bioassays, only susceptible cockroaches experienced a significant shift in mortality. When the frass of indoxacarb-fed cockroaches was analyzed, fewer molecules of the hydrolytic metabolite DCJW were produced with the introduction of an antibiotic (KAN). This result was further corroborated by esterase activity assays of whole homogenized cockroach guts. All results considered, these findings provide novel evidence of microbe-mediated pro-insecticide activation in the cockroach gut. Overall, the results of this dissertation reveal previously unknown relationships between gut microbiota and their insect hosts. These microbiome relationships exposed important cockroach strain differences which may extend to the host population level. Furthermore, this research has connected a change in enzyme activity in the gut microbiome with indoxacarb, a very important marketplace pro-insecticide.

CHAPTER 1. LITERATURE REVIEW AND DISSERTATION OBJECTIVES

1.1 Literature review

Insecticide-resistant strains of insect pests are more prevalent than ever before. Genetic mutations in insect species allow some insects to resist high concentrations of insecticide [1,2]. Once these insects reproduce, their offspring also contain the mutations that code for resistance. If the same insecticide is consistently applied to the same insect population in the same geographic location, within a small number of generations the majority of the population will express genetic resistance to the specific insecticide. This cycle significantly reduces the lethality of insecticides with each subsequent generation of insecticide-resistant pests. In the United States alone, total pesticide resistance accounts for $1.5 billion in total economic losses each year [3]. It is essential to determine the causes of insecticide resistance to extend the effective useful life of active ingredients and prevent the spread of dangerous, damaging and undesirable insects.

Insecticide resistance is characterized as either a behavioral or a physiological adaptation of an insect species to a respective toxicant. Behavioral insecticide resistance can be characterized by a change in the actions or responses of an insect in the presence of an active ingredient or bait formulation component. For example, cockroach strains that were once attracted to a particular glucose sweetener of a bait matrix now find the ingredient unpalatable; the mutated strains no longer consume the bait and the bait matrix becomes ineffective for pest control [4,5]. Physiological resistance, on the other hand, describes a change in the biochemical composition or microbiome of an insect species. For example, a species may overproduce endogenous detoxification enzymes in the presence of an insecticide. Insect physiology typically influences insect behavior, and likewise insect behavior spurs the development of unique physiological traits [6].

The German cockroach (*Blattella germanica*) is an invasive pest species that has infested houses, apartments, hospitals, schools, and other urban facilities on a worldwide scale [7,8,9]. German cockroaches are widespread in many urban areas, particularly in low-income apartments and housing communities [9,10]. German cockroaches pose a hazard to human health and well-being by carrying pathogens and pathogenic organisms, instigating allergic reactions and

scattering fecal matter and carcasses throughout residences [7,11]. Although it prefers foods rich in carbohydrate compared to foods rich in fat and protein content [12], the German cockroach will eat virtually any type of food substance it encounters [13], allowing it to adapt easily to unkempt areas such as kitchens, bathrooms and pantries. Additionally, German cockroaches forage at random and cannot detect food or water more than a few centimeters away [14], forcing German cockroach colonies to spread out and quickly colonize new territories. This behavior makes infestations worse in high-density housing units meant for transient individuals since people move their furniture and possessions frequently from one building or unit to the next.

B. germanica is highly adaptive to its environment due to its extremely generalist feeding behavior and its ability to withstand nutritional imbalances [15]. German cockroach populations can persist in severely toxic surroundings over time thanks in part to point mutations in their genome. For example, the German cockroach has previously shown physiological knockdown-resistance to pyrethroid insecticides with a single mutation in its voltage-gated sodium channel [16]. The German cockroach has also developed resistance to cyclodiene insecticides, which act by antagonizing GABA action on the GABA receptors in insects [17]. Through a mutational change in the biochemical properties of the target site of the GABA receptor itself, the affinity of the receptor to bind with cyclodienes is reduced significantly, which gives cockroaches up to 100-fold resistance to cyclodiene insecticides [17].

The landmark paper by Kikuchi *et al.* in 2012 [18] revealed that organophosphate-degrading *Burkholderia* can confer resistance to fenitrothion in stinkbug guts. In fact, these fenitrothion-degrading *Burkholderia* can survive in agricultural soils even in the absence of a pest insect so long as trace fenitrothion is present for use as a carbon source. Additionally, these symbionts can be transferred between different pest species (in this case, between bean bugs (*Riptortus pedestris*) and stinkbugs) thereby thriving in a variety of insect guts. These findings are crucial for the development of the hypotheses tested later in this dissertation and for the field of symbiont-mediated insecticide resistance as a whole.

Research using the fall armyworm (*Spodoptera frugiperda*) has shown that some gut bacteria in insect species break down xenobiotics and toxic compounds using a variety of enzymatic mechanisms, facilitating and enhancing an insect's ability to tolerate insecticidal compounds such as deltamethrin, λ-cyhalothrin, chlorpyrifos, spinosad, and lufenuron [19]. The

coffee berry borer (*Hypothenemus hampei*), a devastating pest to coffee plantations across the world, has gut microbes that have developed the ability to degrade the insecticidal compound caffeine [20]. The apple maggot (*Rhagoletis pomonella*) contains a symbiotic bacterium (*Pseudomonas melophthora*) which can degrade up to six different insecticides that would otherwise control the apple maggot [21]. More recently, *Enterococcus* spp. within the guts of the destructive diamondback moth (*Plutella xylostella*) have shown it may help its host degrade chlorpyrifos based on *in vitro* experiments [22].

Dysbiosis is broadly defined as deleterious compositional and functional alterations of the gut microbiome, many of which are thought to contribute to a range of conditions of ill health [23]. The relationship between gut dysbiosis and its host is complicated because of how unique gut microbial species might interact with one another; for instance, dysbiosis does not occur simply because a host loses or acquires a beneficial or deleterious symbiont (respectively) – the holobiont may recover or not be affected at all in this scenario. When an ecosystem (such as the gut microbiome) experiences a major disturbance, organisms that previously had small or minor niches may now have the opportunity to thrive, or compensate for a freshly eliminated competitor.

Thanks in part to the decreasing cost of next-generation sequencing, the field of gut microbiology and immunology has expanded exponentially in the past decade as medical researchers race to find treatments and cures for a myriad of human gastrointestinal disorders like Crohn's disease and irritable bowel syndrome. Dysbiosis in arthropods, however, remains largely unexplored. Investigating the gut microbiome of pest insects would allow insecticide manufacturers to develop dysbiosis-based synergists to increase the effectiveness of other active ingredients. This method would be particularly effective for pest insects which orally feed on bait matrices, such as German cockroaches and other insects in the order Blattodea.

Since the German cockroach is an insect species notorious for its ability to tolerate insecticide applications and is also known to host a plethora of microbial gut symbionts [24,25,26,27], there is reason to suspect that these gut microbes might have an impact on insecticide resistance, tolerance, and/or degradation. Isolating, profiling, and characterizing these microbial species and studying how they react to insecticidal compounds is crucial to determine the mechanisms of insecticide resistance in the German cockroach and in its microbial

symbionts. Learning which bacterial symbionts are present in insecticide-resistant and susceptible cockroaches will give us clues as to which bacterial symbionts might help degrade and detoxify insecticides. Gauging the enzyme activity in cockroach guts with and without an antibiotic treatment would provide us with further information as to which enzymes might be present inside these gut symbionts.

Recent research in German cockroaches has revealed how insecticide resistance can affect gut microbial composition and stability, along with the physiology and life history of the host. Zhang *et al.* [28] observed that beta-cypermethrin-resistant cockroaches exhibited a delayed development period and reduced adult longevity compared with susceptible cockroaches – most importantly, these researchers concluded that the variation in gut microbiota, especially those related to growth and development, was an important influencing factor when comparing resistant and susceptible cockroaches. While this research does not directly link gut microbiota to insecticide metabolism, it is a key study indicating that host fitness costs and physiology can be affected and reflected by the gut microbiome and the species present within.

Additional studies have recorded the impact of various antibiotics on gut microbial communities in German cockroaches. Rosas *et al.* [29] applied rifampicin to German cockroach populations which exerted a drastic effect on gut microbiota composition, although composition recovered in the second generation where antibiotic was not added to the diet. The endosymbiotic *Blattabacterium* population, exclusively found in cockroach fat bodies, remained unaffected by the antibiotic treatment of adults during the first generation but was strongly reduced in the second generation, suggesting that *Blattabacterium* is sensitive to rifampicin only during the infection of mature oocytes, when it is in an extracellular stage. This theme of gut microbial alteration and subsequent reversion was corroborated by two 2020 studies, Dominguez-Santos *et al.* [30] and Li *et al.* [31]. Dominguez-Santos *et al.* found that in an untreated second-generation population that comes from an antibiotic-treated first-generation, the microbiota is not yet stabilized at nymphal stages. However, once feces of a control population were added to the diet, microbiota had fully recovered by the time the second-generation reached adulthood. Li *et al.* treated German cockroach with the antibiotics levofloxacin and gentamicin and found that within 14 days of discontinuing antibiotic treatment, the number of culturable gut bacteria returned to its original level (pre-antibiotic). However, the composition of the new

bacterial community was significantly different from the original community and contained a greater abundance of antibiotic-resistant bacteria.

1.2 Objectives and hypotheses

The objectives of this research include comparing the whole gut bacterial profiles of insecticide resistant and susceptible *B. germanica* and determining how these profiles, as well as the structure and function of the gut microbiome, and associated change in the presence of an antibiotic. Also investigated was the oral toxicity of indoxacarb, abamectin, and fipronil in resistant and susceptible cockroach strains, with and without antibiotic treatment. My hypothesis was that there are differences in gut microbial structure and function between insecticide resistant and susceptible cockroach strains as well as differences in gut microbial structure and function between antibiotic and control-treated cockroaches. An additional objective was to determine how German cockroach gut bacteria, and specifically the enzymes originating within gut bacteria, metabolize and convert ingested indoxacarb into its toxic metabolite DCJW. This concept was tested by performing both surface-contact and feeding single-concentration indoxacarb bioassays. Furthermore, indoxacarb metabolites were extracted from cockroach frass (feces) and hydrolase activity assays performed on whole cockroach guts. The hypothesis for this objective was that hydrolase enzymes within the gut microbiome are metabolizing and toxifying indoxacarb, thus increasing mortality in *B. germanica* when it is exposed to insecticides.

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CHAPTER 2. DIFFERENTIAL MICROBIAL RESPONSES TO ANTIBIOTIC TREATMENTS BY INSECTICIDE-RESISTANT AND SUSCEPTIBLE COCKROACH STRAINS (*BLATTELLA GERMAINCA* L.))[1]

2.1 Abstract

The German cockroach (*Blattella germanica* L.) is a major urban pest worldwide and is known for its ability to resist insecticides. Past research has shown that gut bacteria in other insects can metabolize xenobiotics, allowing the host to develop resistance. The research presented here determined differences in gut microbial composition between insecticide-resistant and susceptible German cockroaches and compared microbiome changes with antibiotic treatment. Cockroaches received either control diet or diet plus kanamycin (KAN) to quantify shifts in microbial composition. Additionally, both resistant and susceptible strains were challenged with diets containing the insecticides abamectin and fipronil in the presence and absence of antibiotic. In both strains, KAN treatment reduced feeding, leading to higher doses of abamectin and fipronil being tolerated. However, LC50 resistance ratios between resistant and susceptible strains decreased by half with KAN treatment, suggesting gut bacteria mediate resistance. Next, whole guts were isolated, bacterial DNA extracted, and 16S MiSeq was performed. Unlike most bacterial taxa, *Stenotrophomonas* increased in abundance in only the kanamycin-treated resistant strain and was the most indicative genera in classifying between control and kanamycin-treated cockroach guts. These findings provide unique insights into how the gut microbiome responds to stress and disturbance, and important new insights into microbiome-mediated insecticide resistance.

2.1.1 Keywords

Blattella germanica, gut microbiome, kanamycin, *Stenotrophomonas*, insecticide resistance, antibiotic resistance.

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2.2 Background

Insecticide-resistant strains of insect pests are more prevalent than ever before. Genetic mutations in insect species allow some insects to resist high concentrations of insecticide [1,2]. Once these insects reproduce, their offspring also contain the mutations that code for resistance. If the same insecticide is consistently applied to the same insect population in the same geographic location, within a small number of generations the majority of the population will express genetic resistance to the specific insecticide. This cycle significantly reduces the lethality of insecticides with each subsequent generation of insecticide-resistant pests. In the United States alone, total pesticide resistance accounts for $1.5 billion in total economic losses each year [3]. It is essential to determine the causes of insecticide resistance to extend the effective useful life of active ingredients and prevent the spread of dangerous, damaging and undesirable insects.

Insecticide resistance is characterized as either a behavioral or a physiological adaptation of an insect species to a respective toxicant. Behavioral insecticide resistance can be characterized by a change in the actions or responses of an insect in the presence of the insecticide or its formulation components. For example, cockroach strains that were once attracted to a particular glucose sweetener of a bait matrix now find the ingredient unpalatable; the mutant strains no longer consume the bait and the bait matrix becomes ineffective for pest control [4,5]. Physiological resistance, on the other hand, describes a change in the biochemical composition or microbiome of an insect species. For example, a species may overproduce endogenous detoxification enzymes in the presence of an insecticide. Insect physiology typically influences insect behavior, and likewise insect behavior leads the way to the development of unique physiological traits [6].

The German cockroach (*Blattella germanica*) is an invasive pest species that has infested houses, apartments, hospitals, schools, and other urban facilities on a worldwide scale [7,8,9]. German cockroaches are widespread in many urban areas, particularly in low-income apartments and housing communities [9,10]. German cockroaches pose a hazard to human health and well-being by carrying pathogens and pathogenic organisms, instigating allergic reactions and scattering fecal matter and carcasses throughout residences [7,11]. Although it prefers foods rich in carbohydrate compared to foods rich in fat and protein content [12], the German cockroach will eat virtually any type of food substance it encounters [13], allowing it to adapt easily to

unkempt areas such as kitchens, bathrooms and pantries. Additionally, German cockroaches forage at random and cannot detect food or water more than a few centimeters away [14], forcing German cockroach colonies to spread out and colonize new areas quickly.

B. germanica is highly adaptive to its environment due to its extremely generalist feeding behavior and its ability to withstand nutritional imbalances [15]. German cockroach populations can persist in severely toxic surroundings over time thanks in part to point mutations in their genome. For example, the German cockroach has previously shown physiological knockdown-resistance to pyrethroid insecticides with a single mutation in its voltage-gated sodium channel [16]. The German cockroach has also developed resistance to cyclodiene insecticides, which act by antagonizing GABA action on the GABA receptors in insects [17]. Through a mutational change in the biochemical properties of the target site of the GABA receptor itself, the affinity of the receptor to bind with cyclodienes is reduced significantly, which gives cockroaches up to 100-fold resistance to cyclodiene insecticides [17].

Research using *Spodoptera frugiperda* has shown that some gut bacteria in insect species break down xenobiotics and toxic compounds, facilitating and enhancing an insect's ability to resist insecticidal compounds [18]. The coffee berry borer (*Hypothenemus hampei*), a devastating pest to coffee plantations across the world, has gut microbes that have developed the ability to degrade the insecticidal compound caffeine [19]. The apple maggot (*Rhagoletis pomonella*) contains a symbiotic bacterium (*Pseudomonas melophthora*) which can degrade up to six different insecticides that would otherwise control the apple maggot [20].

Since the German cockroach is an insect species notorious for its ability to tolerate insecticide applications and is also known to host a plethora of microbial gut symbionts [21,22,23,24], there is reason to suspect that these gut microbes have an impact on insecticide resistance, tolerance, and/or degradation. Isolating these microbial species and studying how they react to insecticidal compounds is crucial to determine the mechanisms of insecticide resistance in the German cockroach and in its microbial symbionts. Learning which bacterial symbionts are present in insecticide-resistant and susceptible cockroaches will give us clues as to which bacterial symbionts might help degrade and detoxify insecticides.

Dysbiosis is broadly defined as deleterious compositional and functional alterations of the gut microbiome, many of which are thought to contribute to a range of conditions of ill health

[25]. Thanks in part to the decreasing cost of next-generation sequencing, this field of research has expanded exponentially in the past decade as medical researchers race to find treatments and cures for a myriad of gastrointestinal disorders like Crohn's disease and irritable bowel syndrome. Dysbiosis in arthropods, however, remains largely unexplored. Investigating the gut microbiome of pest insects would allow insecticide manufacturers to develop dysbiosis-based synergists to increase the effectiveness of other active ingredients. This method would be particularly effective for pest insects which orally feed on bait matrices, such as German cockroaches.

Recent research in German cockroaches has revealed how insecticide resistance can affect gut microbial composition and stability, along with the physiology and life history of the host. Zhang *et al.* [26] observed that beta-cypermethrin-resistant cockroaches exhibited a delayed development period and reduced adult longevity compared with susceptible cockroaches – most importantly, these researchers concluded that variation in gut microbiota, especially those related to growth and development, was an important influencing factor when comparing resistant and susceptible cockroaches. While this research does not directly relate gut microbiota to insecticide metabolism, it is a key study indicating that host fitness costs and physiology can be affected and reflected by the gut microbiome and the species present within.

Additional studies have recorded the impact of antibiotics on gut microbial communities in German cockroaches. Rosas *et al.* [27] applied rifampicin to German cockroach populations which exerted a drastic effect on gut microbiota composition, although composition recovered in the second generation in the case where antibiotic was not added to the diet. The endosymbiotic *Blattabacterium* population, exclusively found in cockroach fat bodies, remained unaffected by the antibiotic treatment of adults during the first generation but was strongly reduced in the second generation, suggesting that *Blattabacterium* is sensitive to rifampicin only during the infection of mature oocytes, when it is in an extracellular stage. This theme of gut microbial alteration and subsequent reversion was corroborated by two 2020 studies, Dominguez-Santos *et al.* [28] and Li *et al.* [29]. Dominguez-Santos *et al.* found that in an untreated second-generation population that comes from an antibiotic-treated first-generation, the microbiota is not yet stabilized at nymphal stages. However, once feces of a control population were added to the diet, microbiota had fully recovered by the time the second-generation reached adulthood. Li *et al.*

treated German cockroach with the antibiotics levofloxacin and gentamicin and found that within 14 days of discontinuing antibiotic treatment, the number of culturable gut bacteria returned to its original level (pre-antibiotic). However, the composition of the new bacterial community with greater abundance of antibiotic-resistant bacteria was significantly different from the original community.

The objective of this research was to compare the whole gut bacterial profiles of insecticide resistant and susceptible *B. germanica* and determine how these profiles, as well as the structure and function of the gut microbiome, change in the presence of an antibiotic. In parallel, we also investigated oral toxicity of the two insecticide bait active ingredients abamectin and fipronil in resistant and susceptible cockroach strains, with and without antibiotic treatment. We hypothesized that there would be differences in gut microbial structure and function between insecticide resistant and susceptible cockroach strains as well as differences in gut microbial structure and function between antibiotic and control-treated cockroaches. Our findings show antibiotic-induced dysbiosis in only the resistant strain, as well as possible roles for gut microbiota in insecticide resistance and in facilitating insecticide toxicity under basal conditions.

2.3 Results

2.3.1 Insecticide Bioassays and Antibiotic Synergism

Probit calculations followed Finney [30]. Under basal conditions, the Danville resistant (R) strain showed significant resistance to both abamectin and fipronil upon ingestion, with LC50 resistance ratios relative to the susceptible J-wax (S) strain being 4.844 and 7.882, respectively (Table 2.1). In both strains and with both insecticides, KAN treatment led to higher doses being required to cause median mortality. However, resistance ratios between the resistant and susceptible strains decreased by approximately half with KAN treatment, suggesting potential roles for gut bacteria in mediating resistance. Parallel investigations into feeding effects of KAN treatment revealed that food consumption decreases with KAN treatment, but feeding amounts were identical between R and S strains (Fig. 2.1). Thus, the decrease in resistance ratios after KAN treatment suggest a significant influence of gut microbiome on resistance.

2.3.2 16S Sequencing: Alpha diversity

Antibiotic treatment had a significant effect on microbial diversity in both Danville (R) and J-wax (S) guts (p-values: Shannon: 0.000135, inverse Simpson: 0.0107). However, there were not significant differences in gut microbial diversity between the Danville (R) and J-wax (S) cockroach strains to the genus level when KAN treatment was not considered (p-values: Shannon: 0.411204, inverse Simpson: 0.8528). The p-values for combined Treatment:Strain interaction were 0.058173 and 0.5006 for Shannon and inverse Simpson's diversity, respectively. Alpha diversity metrics extend just beyond the $p < 0.05$ statistical significance threshold, however differences in diversity can still be observed (Fig. 2.2).

2.3.3 16S Sequencing: Beta diversity

Bacterial communities were unique to each treatment type in terms of their taxonomic diversity (Fig. 3). Kanamycin-treated samples were clustered less densely compared to their control counterparts, indicating the kanamycin treatment had slightly unique and different effects on each sample.

2.3.4 Differential bacterial abundance by treatment and strain

When the Danville strain was fed antibiotics, *Stenotrophomonas* spp. was substantially greater in relative abundance than all other genera combined (Fig. 2.4). In addition to an increase in *Stenotrophomonas*, kanamycin exposure effectively decreased the relative quantities of all other bacterial genera except for *Dysgonomonas*, *Alistipes* and a select group of unclassified *Bacteriodales* spp. While relative quantities of each genus might vary by treatment type and even by replication within the same treatment type, most taxa were retained between each strain (Fig. 2.5).

2.3.5 Differential bacterial abundance using DESeq2

Figure 2.6 indicates the differential abundance using DESeq between treatment types (control vs kanamycin) colored by phylum and labeled by genus [31]. A select group of genera belonging to the *Proteobacteria* phylum (including *Stenotrophomonas* spp.), *Dysgonomonas*, *Alistipes* and some unknown *Bacteroidota* taxa increased in relative quantity once the

microbiome was exposed to kanamycin. Most other bacterial taxa decreased in relative quantity after kanamycin exposure.

2.3.6 LEfSe (Linear discriminant analysis Effect Size)

Significant differences in OTUs between strains and treatment types were identified by LEfSe analysis [32]. LDA scores are shown in figures 2.7A-D. LEfSe analysis confirmed the same taxa as the prior differential abundance analyses. *Alistipes* was more likely to be present in Danville (R) roaches compared to J-wax (S) roaches, meanwhile unidentified species from the order *Bacteroidales* and the very diverse class *Gammaproteobacteria* were more likely to be present in J-wax (S) roaches. The majority of bacteria associated with KAN treatment are previously unidentified or unknown species.

2.4 Discussion

This study investigated microbiome differences between insecticide-resistant and susceptible cockroach strains; specifically, resistance to the bait insecticide active ingredients fipronil and abamectin. We found that pre-treatment with the antimicrobial compound kanamycin (KAN) led to reductions in resistance levels and increased basal toxicity levels in both resistant and susceptible strains tested. 16S bacterial sequence surveys revealed a wide variety of undescribed bacterial taxa, but also both strains were more similar before KAN treatment than after, with a stronger dysbiosis effect in the resistant strain. The discovery of such a wide variety of undescribed bacterial taxa identified in this study is of significant interest; it is possible that these unique bacteria might provide niche benefits to the cockroach host or other gut symbionts, especially in terms of xenobiotic detoxification.

2.4.1 Insecticide bioassays and implications

The abamectin and fipronil challenges reveal that KAN treatment resulted in higher insecticide tolerance in both the R and S strains tested. However, KAN treatment also decreased resistance ratios by approximately half for both insecticides, suggesting that gut microbiota increase resistance for both abamectin and fipronil. This resistance could be explained by either feeding behavior or by the activation of insecticidal compounds by microbial enzymes –

particularly in the case of fipronil, which has two active forms (one being the parent compound itself, and also the sulfone metabolite) which are both toxic to cockroaches [33,34]. There is yet to be a documented case of microbial insecticide activation or detoxification in German cockroach, however, comprehensive research on the gut microbiome of German cockroach has just begun. More studies on host feeding, metabolism and degradation are needed before we can determine specific relationships these microbes might have with their host, or perhaps each other.

2.4.2 Microbial diversity

Cockroach guts treated with kanamycin were less diverse than cockroach guts in the control group, suggesting that kanamycin eliminated a wide variety of bacterial taxa from the whole gut during the 72-hour treatment window before gut extraction. Antibiotic treatment had a significant effect on alpha diversity in both the Danville and J-wax population. The Danville and J-wax cockroach strains do not have significant differences in gut bacterial taxa when treatment is not considered, at least to the genus level, while combined treatment and strain interaction yielded a significance value of 0.058173 and 0.5006 for Shannon and inverse Simpson's diversity, respectively. Based on significance at the 90% confidence level (which accounts for type II error), the combined effects of treatment and strain were indicative of how microbiota shift in the gut when challenged with an antibiotic.

Our findings suggest the Danville (R) strain has a gut physiology which allows for a unique dysbiosis effect in the presence of kanamycin, while the J-Wax (S) strain's physiological shift is less pronounced. Whether or not this dysbiosis is related to insecticide resistance at a host population level is yet to be confirmed, but the bacterial taxonomic differences between strains are considerable. Further investigating the metabolomic functions performed by these microbes will help reveal the relationships between bacterial species and the structure and function of the host gut microbiome.

2.4.3 Abundance and taxa of interest

While the presence of *Dysgonomonas* and *Alistipes* spp. were higher in the Danville strain, they are present to a reduced extent in the guts of J-wax roaches as well. It is possible that

there are further differences between the two strains at the species level. *Dysgonomonas* has been previously isolated from the guts of the subterranean termite *Reticulitermes speratus* and researchers suggest this genus requires heme to grow [35,36]. *Dysgonomonas* has not been well-studied, especially outside of human guts, so it would be inappropriate to draw conclusions on insecticide resistance based on its presence in a resistant cockroach strain. *Alistipes* is a nascent sub-branch genus of the *Bacteroidetes* phylum which are commonly associated with chronic intestinal inflammation in humans [37] and was first discovered in samples of children with appendicitis [38]. *Alistipes* has one of the highest numbers of putrefaction pathways amongst human gut commensal bacteria. Putrefaction is the fermentation of undigested proteins in the GI tract which typically leads to bacterial production of harmful (or occasionally helpful) metabolites [39,40]. Similar to *Dysgonomonas*, the authors cannot presently draw conclusions about the contributions of *Alistipes* spp. in relation to insecticide resistance and degradation, and these genera are likely (but not conclusively) naturally present in different relative abundances between Danville (R) and J-wax (S).

Stenotrophomonas spp. are present in every sequence sample to a relative extent, but no more so than in the Danville-Resistant cockroaches that were fed kanamycin. *Stenotrophomonas* is a genus known for its role in the nitrogen and sulfur cycles in the soils of various ecosystems; it has the ability to detoxify xenobiotics and break down complex organic molecules [41], which might allow a strain of insecticide resistant German cockroaches to tolerate higher doses of insecticides. Also, many *Stenotrophomonas* spp. have a high level of intrinsic resistance to antibiotics [41] which could also explain why it was able to overwhelmingly colonize the gut microbiome once kanamycin was introduced; kanamycin was clearly less effective at eliminating *Stenotrophomonas* compared to other bacterial genera. Introducing a disturbance (in this case, an antibiotic) to the microbiome most likely allowed for substantially tolerant *Stenotrophomonas* bacteria to take advantage of resources in the gut without competition from other microorganisms. Since *Stenotrophomonas* can effectively decompose organic compounds, perhaps this genus consumed dead or dying bacteria in the gut (a result of kanamycin treatment) and grew in quantity over 72 hours as a result. Alternatively, *Stenotrophomonas* could be filling niches leftover from other dead or dying bacteria, explaining the growth after 72 hours.

Firmicutes was among the phyla most sensitive to kanamycin exposure. Firmicutes is widely diverse and has been studied in both human and animal gut microbiology, especially in its links to obesity [42,43]. Many of these Firmicutes are in class Clostridia, a common digestive tract bacterium consisting of only anaerobes [44]. Research on the Turkestan cockroach (*Shelfordella lateralis*) suggests that both gut tissue and microbiota contribute to oxygen consumption and suggest that oxygen status in the gut influences microbial colonization success [45]. This same principle could hold true of German cockroach gut microbiota as well; if so, we could expect to see variable microbial alpha or beta diversity metrics based on oxygen consumption or concentration in host tissue. Oxygen consumption was not measured in this experiment, but we recommend follow-up research to determine how oxygen presence (and concentration) might affect the gut microbiome (and coinciding potential insecticide resistance and susceptibility) in other cockroach species.

The family *Lachnospiraceae* (phylum *Firmicutes*, class *Clostridia*) contains anaerobic bacteria that are routinely isolated from the gastrointestinal tract of animals [46]. These bacteria are motile, curved rods, and usually stain Gram negative or weakly Gram positive [46]. *Lachnospiraceae* has mostly been found in mammalian digestive tracts; its main function is to digest complex plant polysaccharides via hydrolysis [47]. Members of *Lachnospiraceae* have been linked to obesity and protection from colon cancer in humans, mainly due to the association of many species with the production of butyric acid, a substance that is important for both microbial and host epithelial cell growth [48]. *Lachnospiraceae* likely did not play a role in insecticide degradation in this experiment, although more studies should be implemented to determine how this family might degrade a pro-insecticide prone to hydrolysis (i.e., indoxacarb).

Blattabacterium spp present in the sequence survey likely came from fat bodies outside of the digestive tract [49,50] and thus it is a possible contaminant to our whole gut sample. For this reason, *Blattabacterium* spp were eliminated from downstream diversity analyses.

2.4.4 Comparison to previous studies

Pérez-Cobas *et al.* [51] pyrosequenced the hypervariable regions V1–V3 of the 16S rRNA gene of the whole bacterial community of German cockroach when exposed to different diets. Three diets differing in protein were tested at two time points in lab-reared individuals. In

addition, the gut microbiota of wild adult cockroaches was also analyzed. The most abundant families sequenced were *Porphyromonadaceae* (Bacteroidetes), *Ruminococcaceae* (Firmicutes), *Rikenellaceae* (Bacteroidetes), *Lachnospiraceae* (Firmicutes), *Desulfovibrionaceae* (Proteobacteria) and *Bacteroidaceae* (Bacteroidetes) [34].

Pietri *et al.* [23] investigated whole guts from untreated German cockroaches, or cockroaches continuously exposed to 0.5% doxycycline (another antibiotic) for 4 days before dissecting guts and surveying bacterial 16S rRNA genes. Sequence results showed taxa consisting primarily of *Proteobacteria*, *Bacteroidia*, *Firmicutes* and *Fusobacteria* [23]. These researchers also successfully demonstrated that gut microbiota can differ between insecticide-resistant, antibiotic-treated, and insecticide-susceptible German cockroaches [23].

Kakumanu et al. [22] reported on the microbiota from whole body, whole guts and feces of German cockroaches. The overall mean microbial compositions of all the replicates of lab-reared and field-collected cockroaches were remarkably similar at the phylum level, dominated by *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* [22]. However, Kakumanu *et al.* also observed considerable variation in microbial compositions between samples at different locations, as well as differences among individual cockroaches of opposite sexes from the same location [22].

The prior research noted above corroborates our findings that the oral administration of an antibiotic effectively reduces bacterial species diversity in German cockroaches. Additionally, these researchers found that relative abundances of bacterial taxa in the gut can vary drastically from individual to individual, location to location, and even among individuals in a laboratory environment kept under different dietary regimes [22,23,49]. We used the same primers to amplify the V4 region as Kakumanu *et al.* [22] and observed some of the same families. While previous literature supports many of our observations, especially in terms of species observed at the phylum level, there is not complete agreement. For instance, Pérez-Cobas *et al.* [40] used pyrosequencing to sequence the V1-V3 region instead of MiSeq to sequence the V4 region, as Illumina's platforms were not as frequently used during the time of publication, although both studies produced similar results in terms of species abundance. Additionally, a different antibiotic was used (doxycycline) and species observed differ slightly when comparing Pietri et al's [23] DE (Destin, FL – Resistant) and ORL (Orlando, FL – Susceptible) to our Dan (Danville, IL – Resistant) and J-wax (Susceptible) strains. Unfortunately, there is also no information on

how gut microbiota shift once the ORL – Susceptible cockroaches had been fed antibiotics. The largest limitation of our current research is that many of our reads yielded undescribed species, which reduces our ability to compare our research with past studies and sequences.

2.5 Conclusions

Information obtained from sequencing German cockroach gut microbiota can be used to develop specialized microbial control strategies for German cockroaches and potentially other insects. By exposing vulnerabilities in the gut microbiome, researchers can develop products that attack beneficial microbes or augment gut diversity in a deleterious manner. Alternatively, combining an antibiotic – or perhaps another antimicrobial agent – with an active ingredient in a pesticide formulation may have unintended consequences. Ramifications include (but are not limited to) gut bacterial antibiotic tolerance, decreased insecticide efficacy through reduced bioactivation of pro-insecticidal compounds, or overall reduction in bait consumption due to dysbiosis . All of these possibilities should be important considerations when developing pesticides that act through microbial inhibition.

Contrary to our original hypothesis, the gut microbiomes of Danville (R) and J-wax (S) German cockroaches are not significantly different on their own, but the introduction of orally ingested kanamycin eliminated certain taxa while increasing the relative abundance of others. This shift and apparent dysbiosis revealed important cockroach strain differences which may extend to the host population level. *Stenotrophomonas* spp. can colonize a gut microbiome with limited other symbionts in the presence of kanamycin. The antibiotic-induced dysbiosis and insecticide tolerance that occurred in the resistant strain suggest new, exciting mutualistic relationships between gut microbiota and their insect hosts. These microbes may have a role in modulating insecticide toxicity or changing feeding behavior, whether to the benefit or detriment of the host. The mechanisms of antibiotic resistance, as well as potential insecticide degradation and metabolism should be investigated further in *Stenotrophomonas*. More research is needed to determine the specific phylogenetic classifications of many undescribed species discovered in the experiment, as well as their functions, structures, and relationships to the German cockroach host. Once these relationships have been explored more extensively, researchers will have a better understanding of how to develop products aimed at controlling German cockroach by

engineering dysbiosis or by building stronger levels of insecticide selectivity and safety. The research presented here is an important initial step towards developing more effective products that can better manage this important public health pest.

2.6 Materials and Methods

2.6.1 Insects

Both insecticide-resistant and insecticide-susceptible strains of male German cockroaches were obtained and tested for their ability to resist and detoxify insecticides. The insecticide-resistant strain of *B. germanica* was originally obtained from Danville, IL (Danville-R) and has shown field resistance to Indoxacarb, Abamectin and Fipronil [9]. The insecticide-susceptible strain known as S.C. Johnson Wax susceptible (J-wax-S) is a standard susceptible lab strain that has been in culture for over 70 years with no previous exposure to Abamectin, Fipronil or any other insecticides [9].

2.6.2 Rearing and preparation of traditionally raised insects

Methods for rearing were obtained from Gondhalekar and Scharf [52]. Rearing was conducted in 3.8 liter plastic containers which were held in a reach-in environmental chamber at 25 ± 1°C temperature and 12:12 hour light:dark photoperiod. The inner top portions of the rearing units were lightly coated with a mixture of petroleum jelly and mineral oil (2:3) to prevent the cockroaches from escaping. Each rearing unit contained corrugated cardboard harborages, a water source, and rodent diet (No. 8604; Harlan Teklad, Madison, WI).

2.6.3 Treatment and subsequent gut extractions

Adult male cockroaches were separated into four treatment groups: Danville (insecticide-resistant) roaches treated with/without antibiotics and J-wax (insecticide-susceptible) roaches treated with/without antibiotics (Table 2.2). Treatments were held in groups of ten male adult cockroaches per petri dish (each dish containing a single pellet (approx. 1g) of Purina kitten chow (number 100137; Nestlé Purina, Neenah, WI) along with 1.5 mL of either NanoPure water or kanamycin-infused NanoPure water) for 72 hours before the gut extraction was conducted.

Kanamycin sulfate (CAS 25389-94-0; Acros Organics/Thermo Fisher Scientific, Fair Lawn, NJ) was dissolved in 1.5 mL NanoPure water at 50.0 µg/mL (5% w/v). This concentration was chosen as it was determined to be the highest concentration of kanamycin that could be fed to the cockroaches over 72 hours without causing mortality higher than the control treatment. The control group received only 1.5 mL NanoPure water. The whole gut, including the bacteria inside of the gut, of these cockroaches was extracted and homogenized in PBS (Phosphate Buffered Saline). DNA was isolated from the homogenization of the guts using a BDC 2010 homogenizer at 70 rpm (Caframo, Georgian Bluffs, ON, Canada) (10 ups and downs).

2.6.4 Insecticide Bioassays

Kanamycin was the antibiotic used in the main experiment, as it is a broad-spectrum antibiotic shown to reduce the microbial community inside insect guts [53]. Kanamycin was applied at 50.0 µg/mL (5% w/v) and dissolved in NanoPure water. Cockroaches did not receive food or water for 24 hours prior to exposure to the food pellet (consists of a kitten diet pellet plus insecticide diluted in acetone). Roaches were held with food pellet for 72 hours before final mortality was assessed. Treatments were evaluated for average percentage mortality every 24 hours until the 72-hour holding period is complete – the 72-hour mortality score is used when calculating the LC50 measurement for data analysis. An additional experiment was conducted with the same bioassay setup (with no insecticide on the food pellet – only an acetone blank) to control for how much food and water were consumed once each strain was treated with kanamycin. Food and water were measured at both the beginning and end (72 hours) of the feeding bioassay.

Insecticides were purchased either from Sigma-Aldrich (St. Louis, MO) or from Thermo Fisher Scientific (Waltham, MA). Insecticides for the bioassay were chosen based on resistance assays performed in Fardisi *et al.* 2017 [9] and were serially diluted in 2-fold steps with acetone. Treatments contained ten roaches per replicate and were categorized based on insecticide resistance capability, insecticide type, and applied insecticide concentration. A series of 8-9 serial dilutions plus acetone controls were prepared. Abamectin serial dilutions ranged from 25.6 to 0.2 µg/per food pellet, whereas Fipronil serial dilutions ranged from 0.32 to 0.0025 µg/food

pellet. Different concentration ranges were tested under different experimental conditions as follows:

Danville + Abamectin [25.6, 12.8, 6.4, 3.2, 1.6, 0.8, 0.4, 0.2 µg/per food pellet],
Danville + Abamectin + Kanamycin [25.6 ,12.8, 6.4, 3.2, 1.6, 0.8, 0.4, 0.2 µg/per food pellet],
J-wax + Abamectin [25.6 ,12.8, 6.4, 3.2, 1.6, 0.8, 0.4, 0.2 µg/per food pellet],
J-wax + Abamectin + Kanamycin [25.6 ,12.8, 6.4, 3.2, 1.6, 0.8, 0.4, 0.2 µg/per food pellet],
Danville + Fipronil [0.32, 0.16, 0.08, 0.06, 0.04, 0.02, 0.01, 0.005, 0.0025 µg/food pellet],
Danville + Fipronil + Kanamycin [0.32, 0.16, 0.08, 0.06, 0.04, 0.02, 0.01, 0.005, 0.0025 µg/food pellet],
J-wax + Fipronil [0.32, 0.16, 0.08, 0.06, 0.04, 0.02, 0.01, 0.005, 0.0025 µg/food pellet],
J-wax + Fipronil + Kanamycin [0.32, 0.16, 0.08, 0.06, 0.04, 0.02, 0.01, 0.005, 0.0025 µg/food pellet].

Treatments were replicated three times each.

2.6.5 PCR and sequencing

DNA was isolated from the homogenization of the gut using the QIAGEN DNeasy kit (QIAGEN, Hilden, Germany) and replicated using PCR. For this experiment, incubation time was increased to 16 hours (overnight) with a reduced temperature of 37 ˚C instead of 4 hours at 56 ˚C. This modification increased the quantity of nucleic acids released from gut bacteria which may have been hidden in thick folds of cockroach gut tissue. The gut, including the bacteria inside of the gut, of five roaches of each treatment type were extracted and homogenized in 1.5 mL PBS as detailed above for gut extractions. Bacterial 16S rDNA was PCR-amplified using the previously published primers 338F (ACTCCTACGGGAGGCAGCAG) and 518R (ATTACCGCGGCTGCTGG) [54]. PCR was carried out in a total volume of 15 µl. Each reaction contained 7.5 µl of the Ssofast evagreen supermix reagent (Bio-Rad, Hercules, CA), 0.5 µl of each of the forward and reverse primers (stock 10 µM), 3 ng of template DNA, and nuclease-free water up to 15 µl. The Bio-Rad MyCycler thermocycler reaction conditions were:

initial denaturation at 95 ˚C for 3 min; 30 cycles of denaturation at 95 ˚C for 15 s, annealing at 55 ˚C for 15 s, and elongation at 72 ˚C for 30 s; and a final elongation at 72 ˚C for 5 min. An additional 5 cycle PCR (with the same conditions) was performed to add barcodes to the resulting 30-cycle PCR product. To avoid PCR bias, the lowest DNA template quantity and the fewest possible PCR amplification cycles were chosen. The integrity and quantity of the amplicons were verified by agarose gel (2%) electrophoresis. DNA concentration was quantified on a nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Samples were sequenced using Illumina Mi-Seq at the Purdue Genomics Core Facility (Purdue University, West Lafayette, IN). The sample pool was titered using a KAPA Library Quantification Kit (Roche, Basel, Switzerland) and run as 5% of a MiSeq 500 cycle kit run (Illumina, San Diego, CA). Each strain of cockroaches (insecticide-resistant and susceptible) and each treatment type (with and without antibiotic treatment) was replicated 3 times, for a total of 12 biological replications each containing 5 whole homogenized guts (Table 2.1). The results of the sequence will determine the relative abundance of different bacterial taxa between insecticide-resistant and susceptible strains of *B. germanica*.

2.6.6 Sequence filtering

The sequences were processed using Cui *et al.* [55] and Mothur v.1.39.3 [56] following the MiSeq standard operating procedure (SOP) proposed by Kozich *et al*. [57]. Low-quality sequences were removed from the analysis if they contained ambiguous characters or were over 325 bp. After merging any duplicates, the pre-cluster method was applied to further reduce the sequencing errors produced by the MiSeq Illumina sequencing platform. Chimeras were identified and removed using chimera.vsearch and remove.seqs, respectively. The Silva database (version 138) was used to align and classify the sequences. The sequences were clustered into OTUs at a distance threshold of 0.03 using the average neighbor method. The sequences were sampled to a depth of 24390.

2.6.7 Statistical analysis

The sequences were subsampled to a depth of 24390 as this was the number of sequences in the sample with the fewest sequences present. Alpha-diversity and species evenness were

estimated using the Shannon diversity index and the inverse of Simpson's evenness index, respectively. All diversity indices were calculated with Mothur v. 1.39.3 [56]. The differences in indices among bacteria present in Danville, J-Wax, kanamycin-treated and control samples were analyzed by one-way ANOVA followed by Tukey's test. NMDS and perMANOVA were performed using the Vegan package in R [58] to compare and evaluate differences between bacterial communities in the two strains and two treatment types. Barplots of phylum and genera present in each sample were constructed, along with a heatmap containing the 20 most abundant genera in each sample to compare how the bacterial community varies between treatments. *Blattabacterium* were pruned from the downstream analyses as they are present only in cockroach fat bodies and would represent contamination in the context of this sequence.

2.7 Tables and Figures

Table 2.1. Probit analysis of bioassay results after 72 hours of abamectin and fipronil treatments. Separated by strain and kanamycin exposure (sample size = 10). Kan +/- Ratio = LC50 of KAN-treated / LC50 of untreated. Resistance Ratio = LC50 of Danville (R) / LC50 of J-wax (S). Chi-squared values are within acceptable range for conducting probit with the exception of abamectin (both J-wax treatments) and fipronil (J-wax untreated)

Insecticide	Strain	KAN	N	Slope	ChiSq-test (χ2) Sig	LC50	95 % CI	95 % CI	Kan +/- Ratio	Resistance Ratio
						(µg/dish)	(lower)	(upper)		
Abamectin	Jwax (S)	+	429	0.839 ± 0.180	0.99	5.191	2.309	11.672	2.800	
		-	424	1.25 ± 0.127	0.89	1.854	1.045	3.288		
	Danville (R)	+	432	1.50 ± 0.121	0.00	10.951	6.334	18.933	1.219	2.109
		-	429	1.88 ± 0.102	0.00	8.981	5.657	14.258		4.844
Fipronil	Jwax (S)	+	465	1.02 ± 0.152	0.00	0.180	0.091	0.359	7.911	
		-	469	1.46 ± 0.106	0.06	0.023	0.014	0.037		
	Danville (R)	+	463	0.888 ± 0.203	0.00	0.680	0.272	1.701	3.781	3.767
		-	473	1.24 ± 0.133	0.00	0.180	0.099	0.327		7.882

Table 2.2. Summary of 12 experimental groups sequenced. Groups are categorized by strain and treatment, with three replications per strain and treatment combination (sample size = 5 guts)

Dan-Ctrl (5 guts)	Dan-Kan (5 guts)	J-wax-Ctrl (5 guts)	J-wax-Kan (5 guts)
Dan-Ctrl (5 guts)	Dan-Kan (5 guts)	J-wax-Ctrl (5 guts)	J-wax-Kan (5 guts)
Dan-Ctrl (5 guts)	Dan-Kan (5 guts)	J-wax-Ctrl (5 guts)	J-wax-Kan (5 guts)

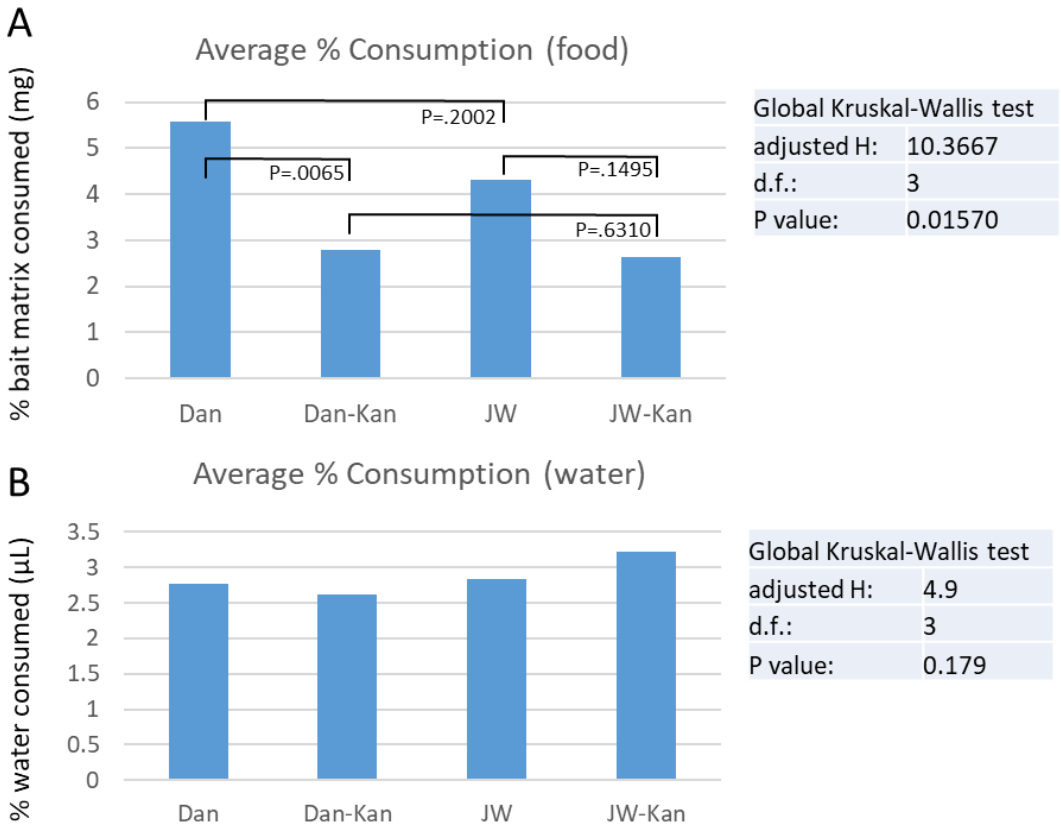
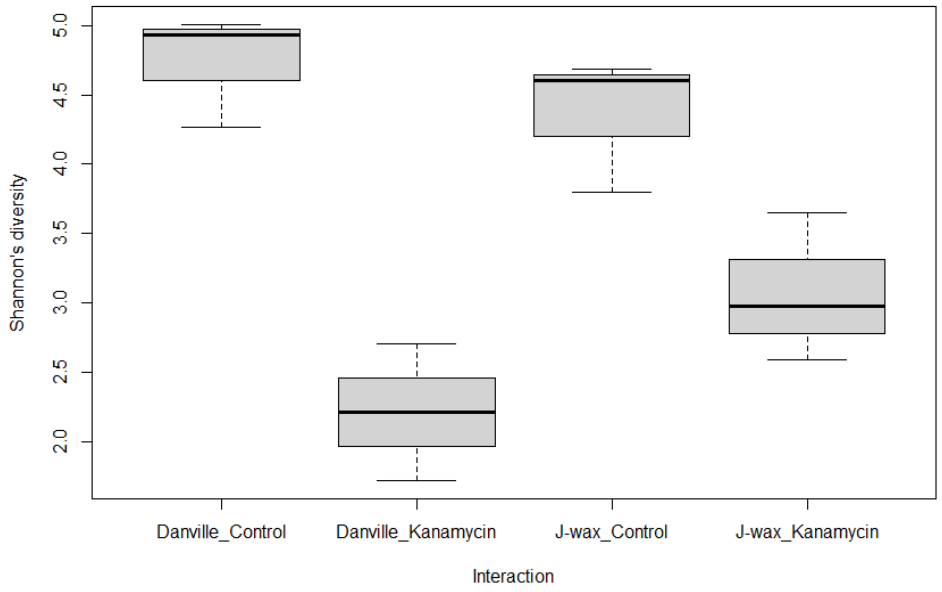
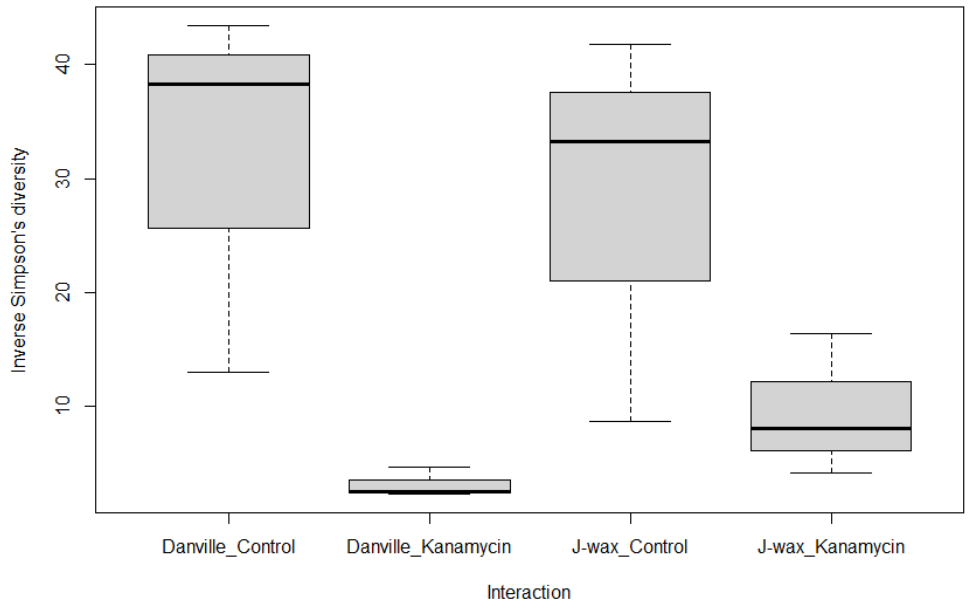


Figure 2.1A & 2.1B. Figure 2.1A represents the average percent of bait matrix consumed per cockroach, per treatment type, per strain. Danville (R) cockroaches consumed significantly less bait when treated with kanamycin, meanwhile J-wax (S) cockroaches did not consume significantly different quantities of bait. P-values for differences in treatment types = 0.0065 (Danville), 0.1495 (J-wax), p-values for differences in strains = 0.2002 (control), 0.6310 (Kan). Figure 2.1B represents the average percent of liquid (NanoPure water or kanamycin-infused NanoPure water) consumed per cockroach, per treatment type, per strain. There were not significant differences in liquid consumed and/or evaporated between all strains and treatment types

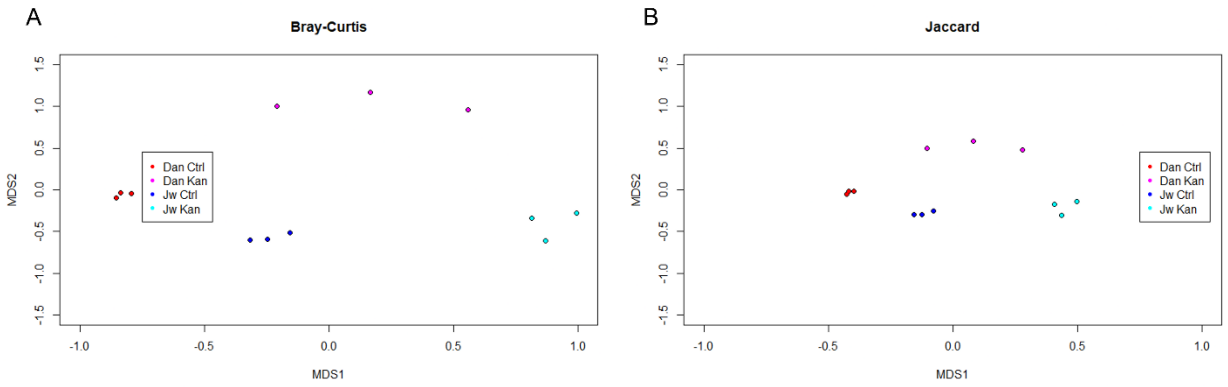
A



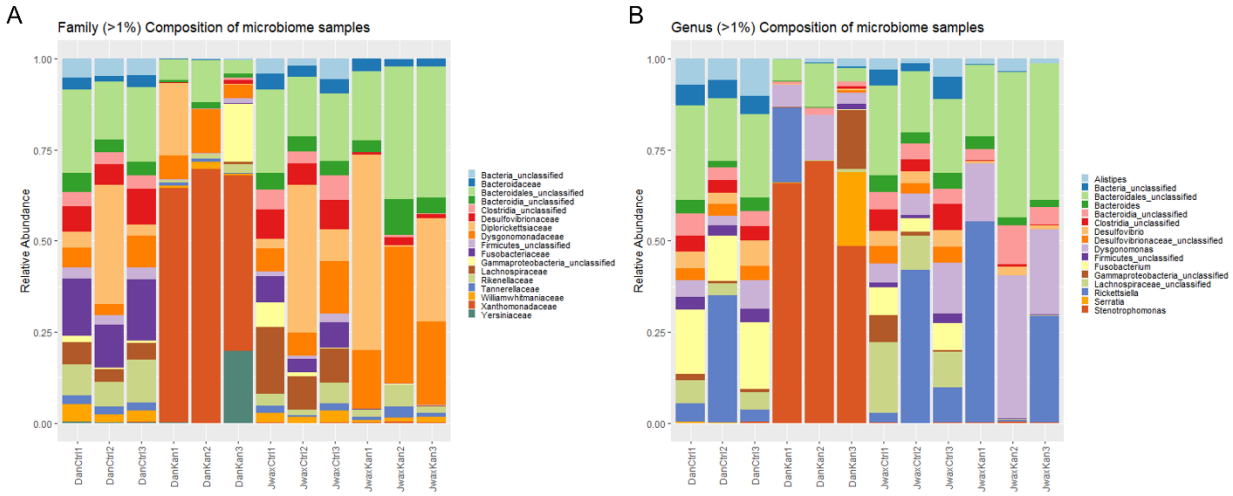
B



Figures 2.2A & 2.2B. Boxplots showing the median (horizontal line in the box), interquartile range (IQR, the box), minimum and maximum (lines below and above the box, respectively) of alpha diversity (top: Shannon (2.2A), bottom: Inverse Simpson (2.2B)) categorized by treatment P-values for global Kruskal-Wallis comparisons between strain and treatment combinations are 0.02607 (Shannon) and 0.05222 (inverse Simpson)



Figures 2.3A & 2.3B. NMDS of beta-diversity (left: Bray-Curtis (2.3A), right: Jaccard (2.3B)) categorized by treatment and strain combined. Overall significance of the models as determined by PERMANOVA: Bray-Curtis Shannon (p = 0.000999), inverse Simpson (p = 0.001998), Jaccard Shannon (p = 0.000999), inverse Simpson (p = 0.000999)



Figures 2.4A & 2.4B. Relative abundance of families (2.4A) and genera (2.4B) with over 1% composition throughout the entire sequence categorized by treatment and replication. Bars are colored by family (2.4A) and genus (2.4B). Group is categorized by strain, treatment and replication (sample size = 10)

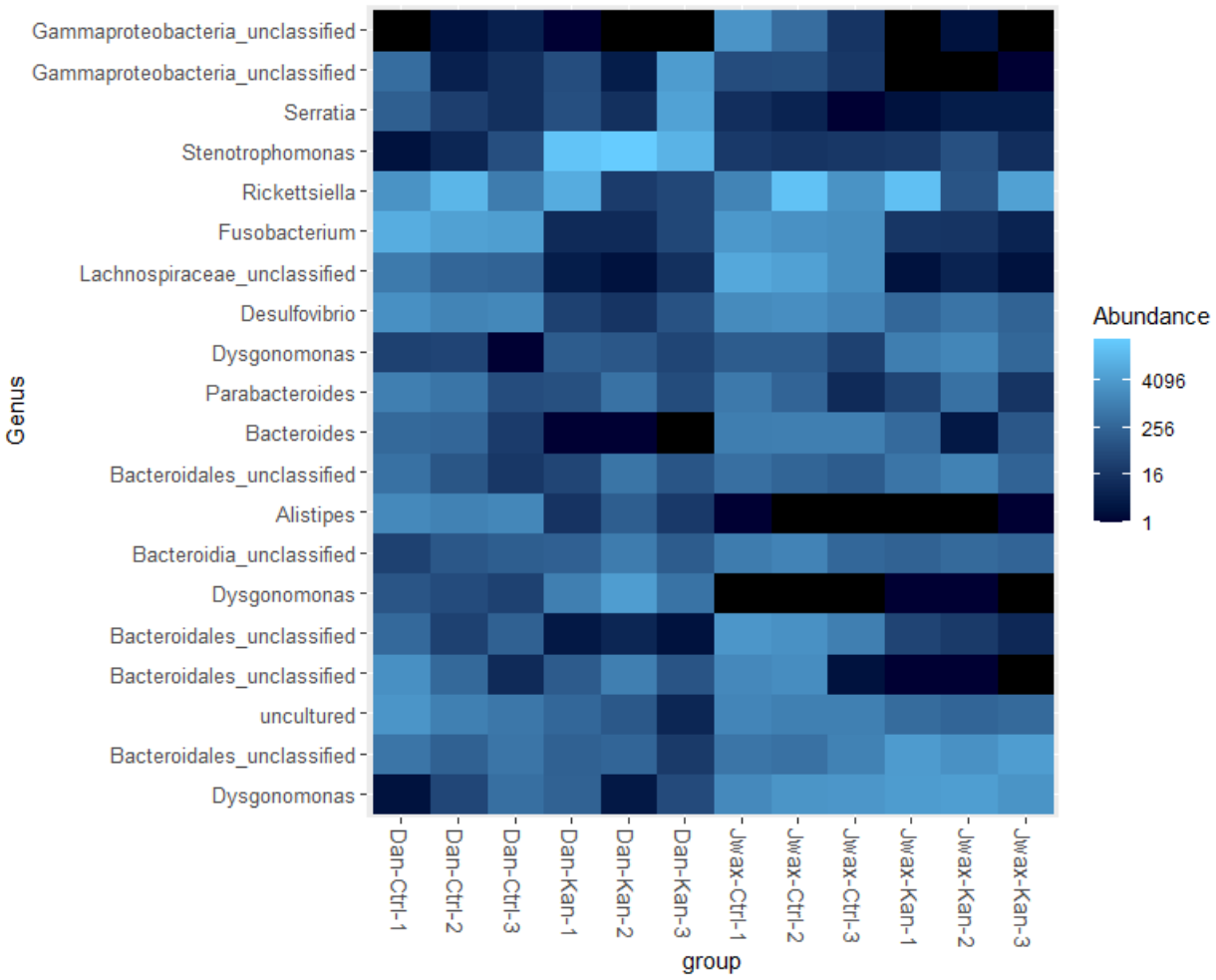


Figure 2.5. Heat map of top 20 genera throughout the entire 16S MiSeq. Figure includes uncultured and unclassified bacterial genera. Group is categorized by strain, treatment and replication. Black = absent

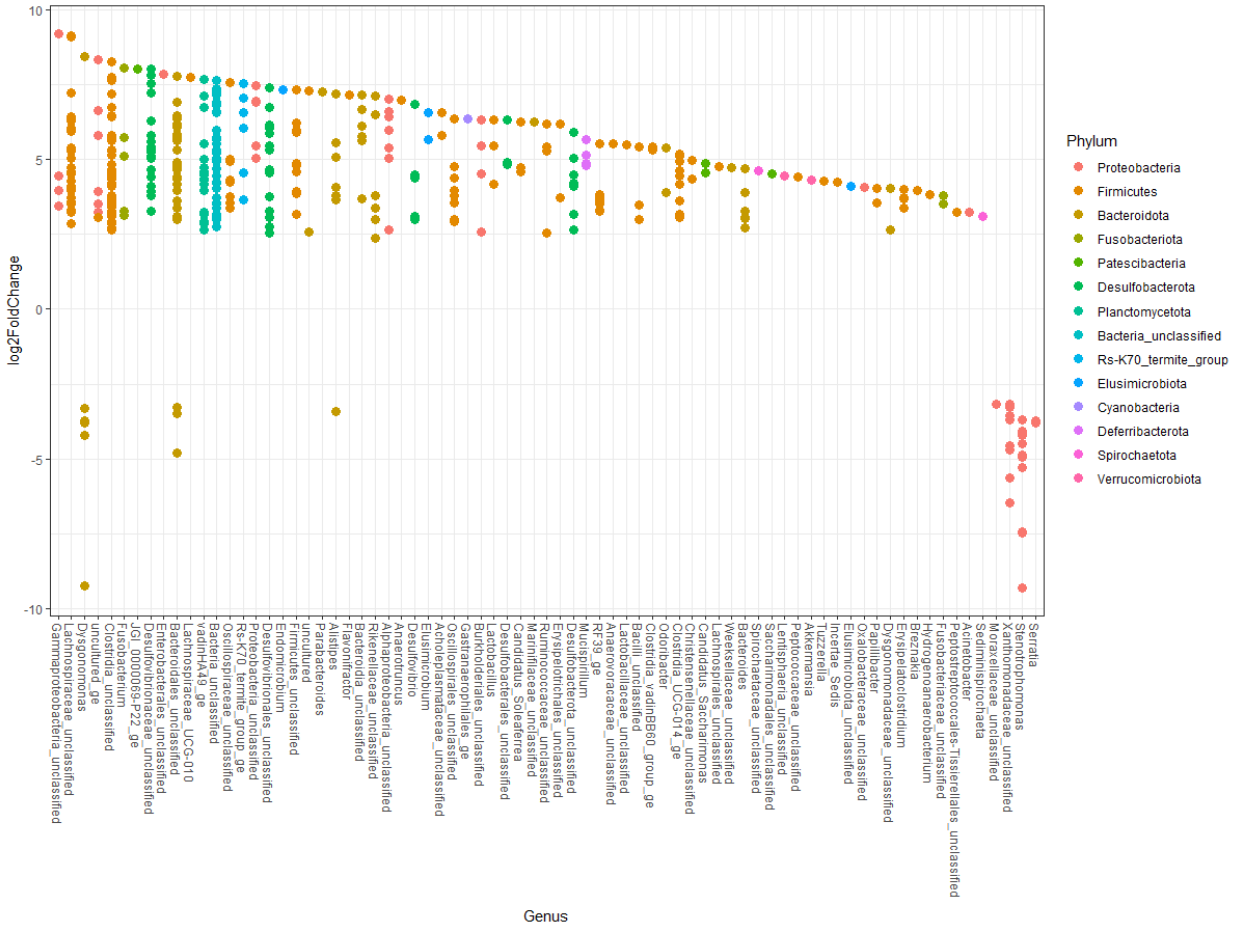
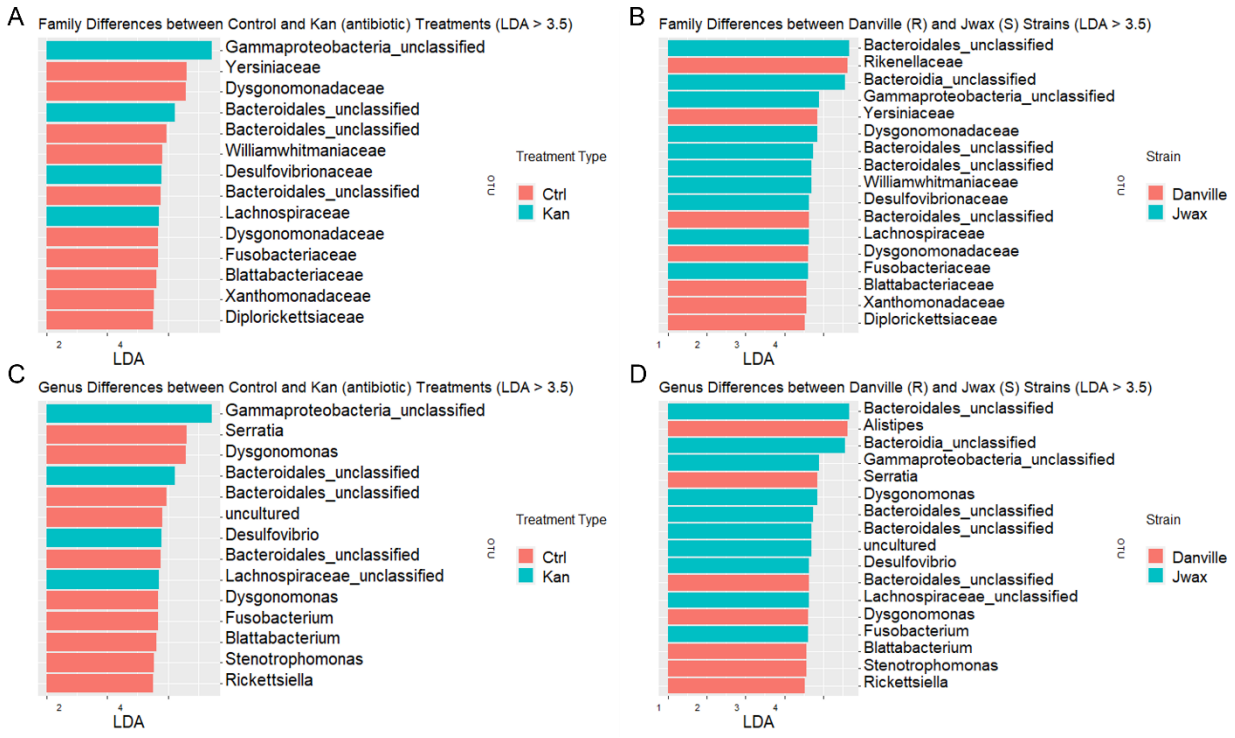


Figure 2.6. Differential abundance using DESeq between treatment types (control vs kanamycin). Values are colored by phylum and labeled on the x-axis by genus. Positive log2 FoldChange values indicate the presence of a genus is more indicative of a control treatment, whereas negative log2FoldChange values indicate the presence of a genus is more indicative of a kanamycin (antibiotic) treatment



Figures 2.7A, 2.7B, 2.7C, 2.7D. LEfSe (Linear discriminant analysis Effect Size). Differences are measured by Linear Discriminant Analysis (LDA). The graphs represent the families (2.7A) and genera (2.7C) most likely to differ between treatment types, as well as the families (2.7B) and genera (2.7D) most likely to differ between strains. *Alistipes* was more likely to be present in Danville (R) roaches compared to J-wax (S) roaches, meanwhile unidentified species from the order *Bacteroidales* and the diverse class *Gammaproteobacteria* were more likely to be present in J-wax (S) roaches

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CHAPTER 3. MICROBE-MEDIATED ACTIVATION OF INDOXACARB IN GERMAN COCKROACH (*BLATTELLA GERMANICA* L.)

3.1 Abstract

The German cockroach (*Blattella germanica* L.) is a major urban pest worldwide and is notorious for its ability to resist and detoxify insecticides. German cockroaches have generalist feeding habits that expose them to a range of potential hazardous substances and host a wide variety of unique microbial species, which may potentially facilitate unique detoxification capabilities. Since field German cockroach populations are routinely exposed to both bait and spray insecticide treatments, it is reasonable to ponder whether these unique gut microbes play a role in resistance capabilities of the host insect. The goals of this research were to understand the metabolic processes inside the German cockroach gut after exposure to kanamycin (KAN), a broad-ranging antibiotic, and indoxacarb, an important oxadiazine pro-insecticide used in cockroach bait products. In these experiments, two resistant cockroach strains were obtained from field populations in Danville, IL and compared to a laboratory strain that was completely susceptible and had no previous exposure to insecticides (J-wax). Roaches treated with kanamycin-infused water had lower median mortality to indoxacarb compared to the control treatment in feeding bioassays regardless of strain, but in vial (surface contact) bioassays, only susceptible cockroaches experienced a shift in mortality. When frass extracts of indoxacarb-fed cockroaches were analyzed, fewer molecules of DCJW were produced with the introduction of an antibiotic (KAN). This result was further corroborated by esterase activity assays of whole homogenized cockroach guts. Taken together these results provide novel evidence of microbe-mediated pro-insecticide activation in the cockroach gut.

3.2 Introduction

The German cockroach (*Blattella germanica*) is an invasive pest species that has infested houses, hospitals, schools, pig and chicken farms, and other urban facilities on a worldwide scale [1,2,3]. German cockroaches are widespread in many cities and urban areas, particularly in low-income apartments and housing communities [3,4]. German cockroaches pose a hazard to human

health and well-being by carrying pathogens, instigating allergic reactions and scattering feces and carcasses throughout residences [1,5]. Although it prefers foods rich in carbohydrate compared to foods rich in fat and protein content [6], German cockroaches will eat virtually any type of food substance they encounter [7], allowing them to adapt easily to human-occupied habitats. Additionally, German cockroaches forage at random and cannot detect food or water more than a few centimeters away [8], forcing roaches from infested residences to spread out and populate new areas quickly. This behavior makes infestations more severe in high-density housing units since people move their furniture and possessions frequently from one building or unit to the next.

B. germanica is highly adaptive to its environment due to its extremely generalist feeding behavior and its ability to withstand nutritional imbalances [9]. German cockroaches can survive and thrive for long periods of time despite ingesting severely toxic compounds in part due to point mutations in their genome. For example, the German cockroach has previously shown physiological knockdown-resistance to pyrethroid insecticides with a single mutation in its voltage-gated sodium channel [10]. The German cockroach has also developed resistance to cyclodiene insecticides, which act by antagonizing GABA action on the GABA receptors in insects [11]. Thanks to a mutation in the biochemical properties of the target site of the GABA receptor itself, the affinity of the receptor to bind with cyclodienes is reduced significantly, giving cockroaches up to 100-fold resistance to cyclodiene insecticides [11].

Many cases of resistance can also be caused by cytochrome P450 enzymes, a diverse category of detoxification enzymes which are present in many organisms [12,13]. Cytochrome P450s, or CYPs, catalyze the oxidation of drugs, toxins, or other xenobiotics that enter the host body [12,13]. Since this family of enzymes is large and organisms host upwards of dozens of unique CYP proteins (*B. germanica* has 158 CYP genes in its genome; [14]), xenobiotics may follow a number of detoxification pathways, especially in the case of organic insecticides [15,16].

Indoxacarb is an important marketplace pro-insecticide for controlling German cockroach [17,18]. Indoxacarb is converted to the metabolite DCJW after hydrolytic activation [18], and it is DCJW which is the true sodium channel blocker causing toxicity in the insect body. Gondhalekar *et al.* in 2016 [18] demonstrated the various pathways that indoxacarb can be

metabolized through, and not all pathways lead directly to DCJW or a similarly toxic metabolite – especially when indoxacarb encounters cytochrome P450 enzymes. Gut bacteria in other species are known to possess enzymes that aid their host in digestion, detoxification, and disease prevention and mitigation [19,20,21]. Because unknown bacterial species may contain a variety of xenobiotic metabolizing enzymes like cytochrome P450s, hydrolases and esterases (or others), it is critical to understand the metabolic pathways within the gut microbiome in relation to indoxacarb and other insecticides. As noted above, there are 158 CYP P450 genes in the *B. germanica* genome and in addition, and 62 esterase-type hydrolases of the type expected to make the indoxacarb-DCJW conversion [14]. However, the specific enzymes involved in indoxacarb hydrolytic activation remain unknown.

Scharf *et al.* in 2022 [22] reported on the relationship between host fitness, indoxacarb resistance and internal parasite/pathogen levels in German cockroach. In this prior study it was observed that high-level indoxacarb resistance apparently resulted from a dual process whereby the host tolerated indoxacarb through a number of potential mechanisms, and also host fitness was further increased as the cockroach body was cleared of parasites and pathogens. In short, there was a decrease in parasitic and microbial transcripts after indoxacarb metabolism became more efficient, or vice-versa, during the resistance evolution process. This could have resulted from either direct antimicrobial effects of indoxacarb, the indirect effects of antimicrobial compounds included in the indoxacarb bait matrix used, or a co-selection for dual detoxification and immune pathways in the Arbor Park-FL (R) strain.

The overall objective of this research was to determine how German cockroach gut bacteria, and specifically the enzymes originating within gut bacteria, might metabolize and convert ingested indoxacarb into its toxic metabolite DCJW. The hypothesis for this objective is that hydrolase enzymes within the gut environment are metabolizing and toxifying indoxacarb, thus increasing mortality in *B. germanica* when it is exposed to indoxacarb. To meet the overall objective and test the above hypothesis, the following experiments were conducted on cockroaches with or without antibiotic treatments: (1) conducting a series of indoxacarb bioassays involving exposure via feeding and surface contact, (2) collecting cockroach frass after indoxacarb ingestion and performing metabolomics analyses on frass extracts to identify

indoxacarb metabolites, and (3) performing hydrolase enzyme activity assays on protein extracts obtained from whole cockroach guts.

3.3 Materials and Methods

3.3.1 Cockroach strains

The insecticide-resistant strain of *B. germanica* "Danville Normal" was originally obtained from a multifamily housing site in Danville, IL and has shown resistance to a wide range of insecticides as described in Fardisi *et al.* 2017 [3]. A second strain from Danville IL is the "single bait" strain that was treated with and abamectin bait product (Vendetta^{TM}) every month for 5 months before survivors were collected post-abamectin treatment as reported in 2016 by Fardisi *et al.* in 2019 [23]. The Danville Single Bait strain additionally received periodic abamectin selections in the laboratory in the months prior to the current study. The insecticide-susceptible strain known as "S.C. Johnson Wax susceptible" (J-wax-S) is a standard susceptible lab strain and has had no previous exposure to insecticides [3].

3.3.2 Kanamycin treatments

Kanamycin was the antibiotic used throughout this study. It is a broad-spectrum antibiotic shown to reduce the microbial community inside insect guts [24; also see Chapter 2]. Kanamycin was delivered at 50.0 µg/mL (5% w/v) dissolved in NanoPure water in a 1.5 mL centrifuge tube.

3.3.3 Indoxacarb bioassay #1 (dose-response feeding)

For dose-response feeding bioassays, cockroaches with and without kanamycin pre-exposure, received a range of indoxacarb doses in treated food pellets – the base of the food pellet consisted of ~1g of Purina Kitten Chow (number 100137; Nestlé Purina, Neenah, WI). Cockroaches did not receive food or water for 24 hours prior to exposure to the food pellet (consisting of a kitten diet pellet plus insecticide diluted in acetone). Roaches were held with treated food pellets for 72 hours before mortality was assessed and used for LD probit analysis.

Indoxacarb was purchased from Sigma-Aldrich (99.5% purity; St. Louis, MO) and serially diluted in 2-fold steps with acetone. Treatments contained ten roaches per replicate and were categorized based on insecticide resistance capability, insecticide type, and applied insecticide concentration. A series of 6 serial dilutions plus acetone controls were prepared. Serial dilutions ranged from 1.5 to 48 µg/per food pellet. Different concentration ranges were tested under different experimental conditions as follows:

Danville + Indoxacarb [48, 24, 12, 6, 3, 1.5 µg/per food pellet],
Danville + Indoxacarb + Kanamycin [48, 24, 12, 6, 3, 1.5 µg/per food pellet],
J-wax + Indoxacarb [48, 24, 12, 6, 3, 1.5 µg/per food pellet],
J-wax + Indoxacarb + Kanamycin [48, 24, 12, 6, 3, 1.5 µg/per food pellet].

Treatments were replicated four times each.

3.3.4 Indoxacarb bioassay #2 (single-dose feeding)

A follow-up feeding bioassay was performed using indoxacarb against three strains: Danville Normal, Danville Single Bait and J-wax-S. Kanamycin was the antibiotic used as in the preceding experiment at 50.0 µg/mL (5% w/v) in NanoPure water. Cockroaches did not receive food or water for 72 hours prior to exposure to an indoxacarb-treated food pellet (consisting of a kitten diet pellet plus insecticide diluted in acetone). This bioassay contained a single dose of indoxacarb (5.47 µg) and used five replicates for the three separate strains. Roaches were held with food pellets for 72 hours before final mortality was assessed. An additional experiment was conducted with the same bioassay setup (with no insecticide on the food pellet – only an acetone blank) to control for how much food and water were consumed once each strain received kanamycin. Food and water were measured at both the beginning and end (120 hours) of the feeding bioassay.

3.3.5 Indoxacarb bioassay #3 (single-concentration vial)

An additional vial bioassay was also performed. The vial bioassay was necessary as a complement to the feeding bioassay to determine if the variability in mortality in indoxacarb could be explained through altered feeding behavior (due to kanamycin treatment) rather than underlying gut bacterial metabolism. The vial bioassay contained a single concentration of

indoxacarb per vial (5.47 µg) and used nine replicates for three separate strains: J-wax, Danville Normal, and Danville-single bait. Roaches were held with an untreated food pellet for 72 hours before the bioassay began to ensure ingestion of kanamycin, which was fed to the roaches in a 1.5 mL centrifuge tube at 50.0 µg/mL (5% w/v) in NanoPure water. Treatments were evaluated for average percentage mortality every 24 hours until the 72-hour holding period was complete. The 72-hour mortality score was used when calculating mortality for data analysis.

3.3.6 Metabolite profiling and analysis (method based on Gondhalekar et al. 2016)

A bioassay with a single dose of indoxacarb (~3 µg, weight-adjusted per cockroach) was set up to perform metabolite analysis. Indoxacarb was diluted in acetone and provided to roaches on a 1g pellet of kitten diet. Kanamycin dissolved in NanoPure water (or NanoPure water alone in the case of the control treatment) was also provided to the roaches in a 1.5mL centrifuge tube. Cockroaches also did not receive food for 24 hours prior to being exposed to the bait matrix (containing the ~3 µg of indoxacarb) to ensure kanamycin ingestion as well as complete feeding and receipt of a full indoxacarb dose. Control treatments consisted of (1) only indoxacarb and a cat food pellet, and no cockroaches [negative control], (2) roaches fed only water and a cat food pellet with no acetone or indoxacarb, (3) roaches fed only Kanamycin-infused water and a cat food pellet with no acetone or indoxacarb and (4) roaches fed water and a cat food pellet with only acetone. Bioassays were carried out in glass petri dishes to allow for the extraction of contaminant-free metabolites from frass. Individual replicate petri dishes contained ten adult male roaches and each treatment was replicated 3x. Roaches were held with diet for 72 hours before frass extraction was conducted.

Frass was extracted from each replicate petri dish individually by rinsing with 0.5 mL of acetone. The acetone rinsate was pooled in 3 mL of hexane (Hex) and purified using a hexane-preconditioned solid phase extraction (SPE) column (glass Pasteur pipette plugged with glass wool and filled with 2g silica gel). These extracts, which contained indoxacarb and its metabolites [18], were pooled (final volume 12 mL), evaporated under nitrogen, and dissolved in 1 mL acetone for HPLC analysis.

The HPLC system used was a Waters AcquityTM UPLC, and the MS system was Quattro Premier XE Tandem Mass Spectrometry (Waters Corp., USA). It was equipped with a

Turbo IonSpray interface operating at an ionization voltage of +0.5 kV and a source temperature of 450°C, the nebulizer gas was nitrogen, and the collision was Argon. The entrance and cone potentials were set at 4 and 30 V. The collision energy was set at 16 and 13 V for different daughter ions 249.1 and 293.1, respectively. The multiple reaction monitoring (MRM) was selected to be scan mode. The analyte was chromatographed using an Acquity UPLC BEH C18 (55 mm × 2.1 mm, 1.7 μm particle sizes) reversed-phase column. The mobile phase was a mixture of 0.1% formic acid solution in water and acetonitrile (30:70, v/v) at 0.3 mL/ min. The sample volume injected was 10 μL, and the temperature was set at 35°C [25].

An Agilent 1260 Rapid Resolution liquid chromatography (LC) system coupled to an Agilent 6470 series QQQ mass spectrometer (MS/MS) was used to analyze indoxacarb (CAS# 144171-61-9) and its metabolite "DCJW" in each sample (Agilent Technologies, Santa Clara, CA). The methods are similar to Dun-Ming *et al.* 2008 (see above). The internal standard for the assay was d3-Indoxacarb (Catalog # I654003 Toronto Research Chemicals, Toronto, ON). Each sample was spiked with 50 ng of the internal standard prior to analysis. A Water's Xbridge C18 2.1 mm x 100 mm, 3.5 μm column (Water's Corp. Milford, MA) was used for LC separation. The buffers were (A) water + 0.1 % formic acid and (B) acetonitrile + 0.1% formic acid. The linear LC gradient was as follows: time 0 minutes, 10 % B; time 2.0 minutes, 10 % B; time 8 minutes, 100 % B; time 12 minutes, 100 % B; time 12.1 minutes, 10 % B; time 16 minutes, 10 % B. The flow rate was 0.3 mL/min. Indoxacarb was eluted at 8.1 minutes and the metabolite at 8.3 minutes. Multiple reaction monitoring was used for MS analysis. The data were acquired in positive electrospray ionization (ESI) mode according to Table 2. The jet stream ESI interface had a gas temperature of 325°C, gas flow rate of 7 L/minute, nebulizer pressure of 45 psi, sheath gas temperature of 250°C, sheath gas flow rate of 7 L/minute, capillary voltage of 4000 V in positive mode, and nozzle voltage of 1000 V. The ΔEMV voltage was 400 V. Agilent Masshunter Quantitative Analysis software was used for data analysis (version 10.1). For quantitation of indoxacarb/d3-Indoxacarb, the transition 528.5 → 218.2/531.5 → 221.2 was used. For DCJW, the transition 470.4 → 267.2 was used.

3.3.7 Gut protein isolation and hydrolase activity assay

A bioassay with no insecticide treatment (kanamycin or acetone only) was set up prior to conducting protein isolations and esterase assays. The goal of these assays was to determine if DCJW metabolite formation is related to changes in gut biochemistry or antibiotic-induced changes in feeding behavior. After the completion of the 72-hour bioassay, the full guts of 5 cockroaches per treatment type (3 biological replicates per treatment type/strain combination) were extracted and homogenized in 3 mL PBS at 70 rpm (Caframo, Georgian Bluffs, ON, Canada) (10 ups and downs). The homogenate was then transferred to a 1.5 mL tube and centrifuged at 4 °C for 15 minutes at 3220 g. The resulting supernatant was filtered through cheesecloth into a 15 mL tube and placed on ice. Total protein was determined from protein supernatants using a commercial Bradford assay [26] (Thermo Fisher Scientific, Waltham, MA) and by reading at 595 nm in a microplate reader, alongside a bovine serum albumin standard curve. Assays were conducted in clear 96-well microplates (Corning Inc., Corning, NY). A reaction buffer was set up to determine esterase activity; 50 µL pNPA (p-nitrophenyl acetate) (Sigma-Aldrich, St. Louis, MO) stock was added to acetonitrile (at 0.2 M), then the mixture was dissolved in 10 mL sodium phosphate buffer (100 mM, pH 7.5) (Thermo Fisher Scientific, Waltham, MA), after which 5 µL of protein homogenate was immediately combined with the reaction buffer in a microplate well. Blank reactions contained pNPA, acetonitrile and sodium phosphate buffer but no cockroach gut homogenate proteins. Esterase activity was monitored at 405 nm every 20 seconds for 5 minutes in a PowerWave 340 spectrophotometer (BioTek/Agilent Technologies (Winooski, VT). Specific activity was calculated using the spectrophotometer software. The extinction coefficient for the end product p-nitrophenol (6.53 $mM^{-1}cm^{-1}$) was used for calculating specific activity, which was expressed as nmol/min/mg protein of each well. All enzyme and protein assays were performed in triplicate and averaged for final extinction coefficient measurements. Significance was determined by Mann-Whitney U-test between treatment types within each strain.

3.3.8 Statistical analysis

Probit calculations for dose-response curve followed Finney [27]. Global Kruskal-Wallis analysis of variance was performed on both the indoxacarb metabolism and esterase activity

results. Global Kruskal-Wallis analysis of variance was also performed on both the feeding and vial bioassays. Values of $P < 0.05$ were determined to be significant when calculating concentrations and mortalities relative to one another, both within each strain and between strains. Standard error was used in each graph to assess the variation between mortality results.

3.4 Results

3.4.1 Indoxacarb bioassay #1 (serially diluted dose-response)

Roaches receiving kanamycin-infused water were less likely to die from an equivalent indoxacarb dose as the no-KAN control treatment, especially as the dose increased (Fig. 1, Fig. 2). LC50 values for the Danville (R) strain were 46.132 µg/dish and 9.967 µg/dish for kanamycin and control treatments respectively (Table 3.1), leading to a 4.629-fold increase in tolerance with kanamycin treatment. The corresponding LC50 values for the J-wax (S) strain were 28.332 µg/dish and 6.589 µg/dish respectively, leading to a similar 4.3-fold increase in tolerance with kanamycin treatment. Resistance ratios between Danville (R) and J-wax (S) were relatively unaffected by KAN treatment (1.6x and 1.5x with and without KAN treatment) (Table 3.1). These results support that cockroach gut microbiota play a role in indoxacarb activation but not in the slight indoxacarb resistance shown by the Danville strain.

3.4.2 Indoxacarb bioassay #2 & 3 (single-dose feeding and vial)

These experiments were conducted to directly compare kanamycin effects on indoxacarb toxicity between feeding and surface-contact (vial) bioassays. The trends present in the single-dose feeding bioassay reflected those above in the dose-response feeding bioassay, i.e., KAN treatment had the counter-intuitive effect of decreasing indoxacarb toxicity in all three strains (Fig. 3.3). In vial assays, KAN treatment had no effect on the Danville (R) strains and only the J-wax (S) strain showed reduced susceptibility (Fig. 3.4). The discrepancy in mortalities between the feeding bioassay and vial bioassays supports the idea that gut bacteria as impacted specifically through indoxacarb feeding might be responsible for the toxic conversion of indoxacarb to DCJW. Alternatively, feeding measurements before and after a 120-hour period in the single-dose feeding bioassay reveal that KAN treatment had a significant impact on food

pellet consumption in all strains (Fig. 3.5). This shift in feeding behavior affected tolerance to indoxacarb at a replicate (n = 10 roaches) level.

3.4.3 Metabolomic analysis

LC-MS analysis of fecal extracts revealed that relative to the parent molecule indoxacarb, fewer molecules of DCJW were produced when antibiotic (KAN) was present, which agrees with above results showing overall toxicity is reduced with KAN treatment. When analyzing chromatograms produced from LC analysis, there are more DCJW peaks in the kanamycin-treated gut samples than in the untreated samples, although the area underneath each peak for control samples in both strains is much smaller overall (Figs. 3.6, 3.7). This means that there were much higher concentrations of DCJW in our untreated gut samples and perhaps a greater variety of unique DCJW metabolites or isomers in our kanamycin-treated samples (Fig. 3.6). This analysis provides insights as to the types and quantities of enzymes that might be present in gut bacteria within these German cockroach strains. Both Danville and J-wax roaches produced fewer DCJW metabolites when antibiotics were introduced compared to their parent indoxacarb counterpart, supporting lower hydrolytic activity in cockroach guts once most of the bacteria were removed (Fig. 3.7). This result corroborates with the above feeding bioassays in which fewer cockroaches died in the presence of indoxacarb + kanamycin (Figs. 3.1, 3.2, 3.3).

3.4.4 Esterase (hydrolase) activity assay:

Based on the above results showing reduced formation of the hydrolytic metabolite DCJW, hydrolase activity was investigated in closer detail using the model colorimetric substrate p-nitrophenyl acetate (pNPA). The results of these assays show significant decreases in specific hydrolase activity with kanamycin treatment for both the Danville (R) and J-wax (S) strains (Fig. 3.8). This means that, if kanamycin was effective at clearing bacteria out of the gut, then a significant proportion of hydrolase activity must be bacterial in nature. This corroborates further with the above bioassays and metabolic profiles of the same treatment types (Figs. 3.6 & 3.7).

3.5 Discussion

The experiments performed in this study investigated the differences in microbial metabolism of indoxacarb, a marketplace pro-insecticide widely used in bait products for German cockroach control. A series of indoxacarb bioassays were performed to gauge resistance levels as well as the effect of antibiotics (KAN) on resistance levels between strains. Metabolic profiling was performed on the frass of cockroaches receiving antibiotic (KAN) + indoxacarb, or indoxacarb alone, in order to determine the effect that gut microbes had on metabolizing or activating indoxacarb into its bioactive metabolite DCJW. Additionally, a follow-up hydrolase assay was performed on whole homogenized cockroach guts, with and without antibiotic (KAN) treatment, to estimate the quantity of hydrolase activity that might be derived from gut microbes in relation to the cockroach host gut. By considering these results together, we are able to estimate the impact of the gut microbiome on insecticide metabolism of pro-insecticides such as indoxacarb. This is the first documented example of microbe-mediated insecticide activation in insects.

3.5.1 Contrasting bioassay results and secondary mortality

A key difference between the results of the vial and feeding bioassays is that, while there may exist an element of secondary kill in the single-concentration vial bioassay, it would be more pronounced in the single-dose feeding bioassay. Secondary kill can be defined as mortality that would result from individuals not being directly exposed to a bait product via feeding, but rather would result from "secondary" contact with contaminated feces or corpses [28]. There is much more indoxacarb parent compound available in vial bioassays that cockroaches and their microbes might be processing in many ways (via feeding, surface contact, etc.); whereas, in the feeding bioassay there are higher levels of active metabolite that the roaches can acquire through secondary routes. Combined bioassay and metabolomics results from the present study show clearly how mortality can increase because of the phenomenon of secondary mortality via horizontal transfer.

3.5.2 Metabolomics reveal microbial DCJW conversion with complex metabolism pathways

Metabolomic analysis of frass revealed reduced DCJW production in cockroach frass (feces) in association with microbiota shifts that occur after antibiotic feeding [24; Chapter 2]. These results also support that there is secondary toxicity being caused by frass that cannot be accounted for in vial bioassays alone. Gondhalekar *et al.* in 2016 showed that general indoxacarb metabolism pathways are complex and involve a variety of enzymatic pathways including hydrolysis and P450-based activation [18], which we believe occurred in the present study as well (Fig. 3.9). However, formation of the bioactive metabolite DCJW is not as complex and results from an apparent 2-step hydrolytic reaction [18,29,30]. Because there were multiple isomers of DCJW present in LC-MS analysis, and because we know from previous literature [18] that some of these isomers are derived specifically from the action of host P450s (particularly in the case of ring-open DCJW), it is likely that multiple pathways, both dependent on gut bacteria and the cockroach host (especially in the absence of bacterial hydrolase enzymes), are influencing indoxacarb biotransformation in the gut. The contrasting significance between our different bioassay formats also suggest a variety of pathways could be in play that affect indoxacarb toxicity through different exposure routes. The concept of secondary toxicity is particularly important in the German cockroach, which has been shown to feed on the frass of its community [31,32]. The current study provides important new information regarding the influence of gut bacteria on DCJW formation in cockroaches and its links to secondary toxicity.

3.5.3 Correlation of general hydrolase activity

There are many classes of enzymes that could impact insecticide metabolism as a whole, including but not limited to hydrolases, which include esterases, proteases and other enzyme families [33,34,35]. However, because indoxacarb contains an ester group, and DCJW does not contain said ester group, we believe that the pNPA hydrolase assay performed here qualitatively gauges how gut bacterial enzymes might activate indoxacarb and convert it into DCJW. Since significantly lower pNPA hydrolysis activity was present in antibiotic treated cockroach guts, it is reasonable to conclude that much of the observed hydrolase activity is microbial in nature. Another possibility is that antibiotic treatment directly affected metabolism of indoxacarb at the host level; however, this is not likely based on the known mode of action of kanamycin [36] and

based on the notable impacts of KAN on cockroach gut microbe composition [37, Chapter 2]. Esterases are only one type of hydrolase, but pNPA represents a great gauge for determining total hydrolase activity in the gut as a whole. The reduced hydrolase activity observed after antibiotic treatment agrees well with the finding that fewer indoxacarb molecules were converted to DCJW, which in fact can lead to increased tolerance of indoxacarb. This finding also agrees with the lack of impacts on indoxacarb toxicity seen previously by the general insect esterase inhibitor DEF [18].

3.5.4 Associations to the microbiome

The gut microbiome is its own unique ecosystem within its host, and has inputs and outputs depending on host behavior, physiology, and environmental conditions. When exposed to insecticides, microorganisms living within the microbiome may experience stress, mortality, or perhaps even a change in their own physiology or transcriptome composition [19,20,21,22,38,39]. This change in gut microbial physiology may also affect host physiology, whether in the gut or at the whole-organism level [38,39]. Developers of insecticide and insecticide bait products should be wary of directly including antibiotics in formulations, however, as this may lead to gut microbial antibiotic resistance. A wiser action may include using RNAi-based solutions targeted at either beneficial or detoxifying gut microbes, or perhaps targeted at the host itself in an attempt to disrupt the highly intricate gut microbiome. Additionally, understanding the underlying resistance mechanisms – and whether they stem from the host or the microbial species within the host – can allow manufacturers to better target insecticide resistant pests. While this concept is particularly important for the control of cockroaches, for which feeding and bait products are usually standard in field settings, this also has implications when considering insecticide use on any pest species. Microbes do not exclusively live in the gut, and as pesticides become more complex in their application methods and activation pathways, it will be necessary to consider their toxicological limitations beyond host physiology itself. We still do not know the precise pathways nor the specific microbes that cause the apparent activation of indoxacarb, and further investigation is required to determine the origins of this phenomenon.

3.5.5 Comparison to previous studies

Cytochrome P450s (CYPs, for short) can detoxify many different classes of insecticide, including indoxacarb [12,13,18]. Cytochrome P450s are thought to be the most common class of enzyme that catalyzes insecticides and xenobiotics, likely due to the diverse nature of the P450 superfamily, the large diversity of CYPs typically present in the host, and the variety of compounds CYPs can oxidize [12,13,18,40,41]. It is likely – but not confirmed by the results of this study – that gut microbes contain CYPs which may or may not detoxify xenobiotics for their cockroach host [42,43]. However, Gondhalekar *et al.* 2016 [18] also concluded that NADPH/P450-dependent indoxacarb metabolism is confined almost entirely to the microsomal membrane fraction. If it can be assumed that bacterial P450s are cytosolic, and thus not likely involved in the documented P450-based oxidations of indoxacarb – the pathway(s) includes host P450s in this scenario. Even if said microbes are not directly involved in CYP-mediated insecticide degradation or activation, they likely benefit host physiology in one form or another since they have thrived inside the host over millennia.

Pietri *et al.* in 2018 [44] performed topical bioassays of indoxacarb with and without antibiotics (doxycycline) on cockroach strains of varying resistance capabilities. Their findings indicated three key observations: (1) microbial regulation of resistance to indoxacarb is specific to select, field-derived laboratory cockroach colonies, (2) microbial regulation of resistance applies exclusively to orally administered insecticide, and (3) only a small fraction of indoxacarb resistance is microbially mediated in the resistant strain. The first two of these concepts are corroborated in our findings by the difference in mortalities between feeding and vial-surface contact indoxacarb bioassays. Petri *et al.* also suggested it is highly probable that the microbial community in the cockroach gut is involved in regulating metabolic rate and perhaps the growth and life history of cockroach populations. These researchers, however, could not abolish resistance completely using antibiotics, which also agrees with the findings of the present study documenting involvement of the gut microbiome in indoxacarb activation.

3.6 Conclusions

In conclusion, this chapter summarizes studies into German cockroach gut microbial metabolism and enzyme activity and shows the highly novel finding of microbial activation of

indoxacarb, a predominant marketplace pro-insecticide, in the cockroach gut. These findings are the first of their kind and distinctly significant in terms of highlighting the importance of gut bacteria in cockroach bait insecticide efficacy. The importance of these experiments is that we have connected a change in enzyme activity in the gut microbiome with a prominent marketplace pro-insecticide (as opposed to possible antibiotic impacts on reduced feeding and indoxacarb intake). This chapter builds on the preceding (chapter 2) by further characterizing and distinguishing the enzymes present in German cockroach gut bacteria that were previously identified to genus level.

A relationship exists between indoxacarb, antibiotics, and the activity of hydrolases in the cockroach gut. Antibiotic (KAN) treatment reduced both hydrolase activity as well as the formation DCJW metabolites from its parent molecule indoxacarb within the cockroach gut. It should also be noted that internal organosoluble metabolites within the cockroach body were not sampled, and thus, information on the impacts of antibiotic treatment on the fate of indoxacarb and metabolites within the cockroach body remain unknown. However, multiple pathways, both dependent on gut bacteria and their cockroach host (especially in the absence of said bacteria), are influencing indoxacarb biotransformation in the gut. Combining the results of these assays with the three indoxacarb bioassays performed in this chapter, we can conclude that gut microbial hydrolase enzymes can increase the overall toxicity of pro-insecticides. This increased toxicity may lead to decreased tolerance at the host level in both resistant and susceptible cockroach strains. Additionally, the indoxacarb bioassays performed here show mechanistically how mortality can increase due to the phenomenon of secondary mortality via horizontal transfer in cockroach feces.

3.7 Tables and Figures

Table 3.1. Probit analysis of indoxacarb dose-response feeding bioassay results after 72 hours of treatment. Separated by strain and kanamycin exposure (sample size = 10). Kan +/- Ratio = LC50 of KAN-treated / LC50 of untreated. Resistance Ratio = LC50 of Danville (R) / LC50 of J-wax (S). Chi-squared values are within acceptable range for conducting probit

Insecticide	Strain	KAN	Slope	Chi-test (χ2) Sig	LC50	95 % CI	95 % CI	Kan +/- Ratio	Resistance Ratio
					(µg/dish)	(lower)	(upper)		
Indoxacarb	Jwax (S)	+	1.271	0.017	28.332	14.658	54.761	4.300	
		-	1.546	0.976	6.589	3.886	11.171		
	Danville (R)	+	2.391	0.000	46.132	28.810	73.867	4.629	1.628
		-	1.819	0.039	9.967	6.261	15.865		1.513

Table 3.2. Multiple reaction monitoring table for data acquisition of indoxacarb and DCJW. For analysis and quantitation of indoxacarb/d3-Indoxacarb, the transition 528.5 → 218.2/531.5 → 221.2 was used. For DCJW, the transition 470.4 → 267.2 was used

Compound Name	Precursor Ion (m/z)	Product Ion (m/z)	Collision Energy (V)
Indoxacarb	528.5	249.3	15
Indoxacarb	**528.5**	**218.2**	**25**
Indoxacarb-d3	531.5	249.3	15
Indoxacarb-d3	**531.5**	**221.2**	**25**
DCJW-theoretical	**470.4**	**267.2**	**10**
DCJW-theoretical	470.4	223.2	30
DCJW-theoretical	470.4	207.2	30

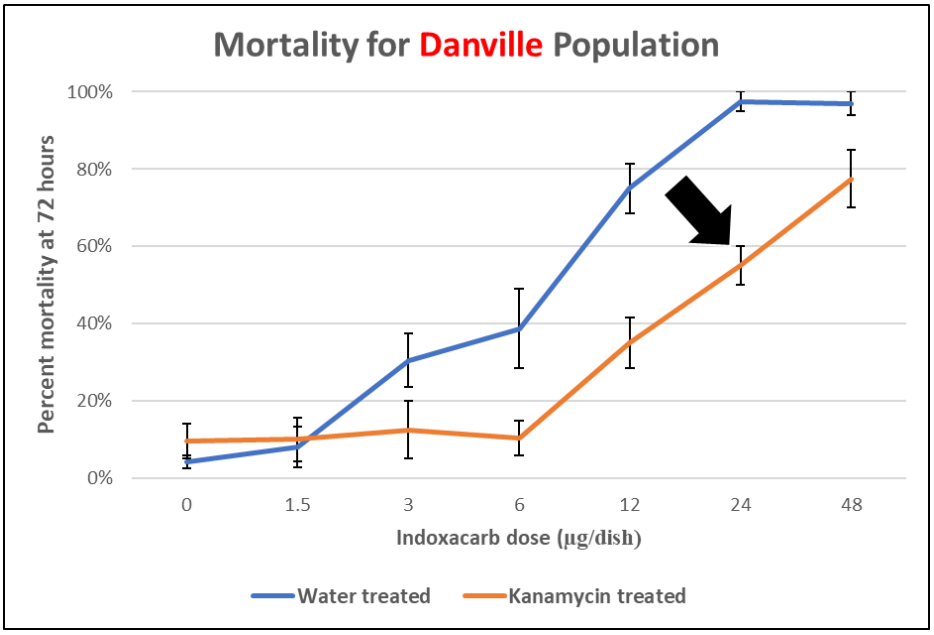


Figure 3.1. Indoxacarb dose-response feeding bioassay results for Danville-IL (R) cockroaches. See Table 1 for full probit analysis results

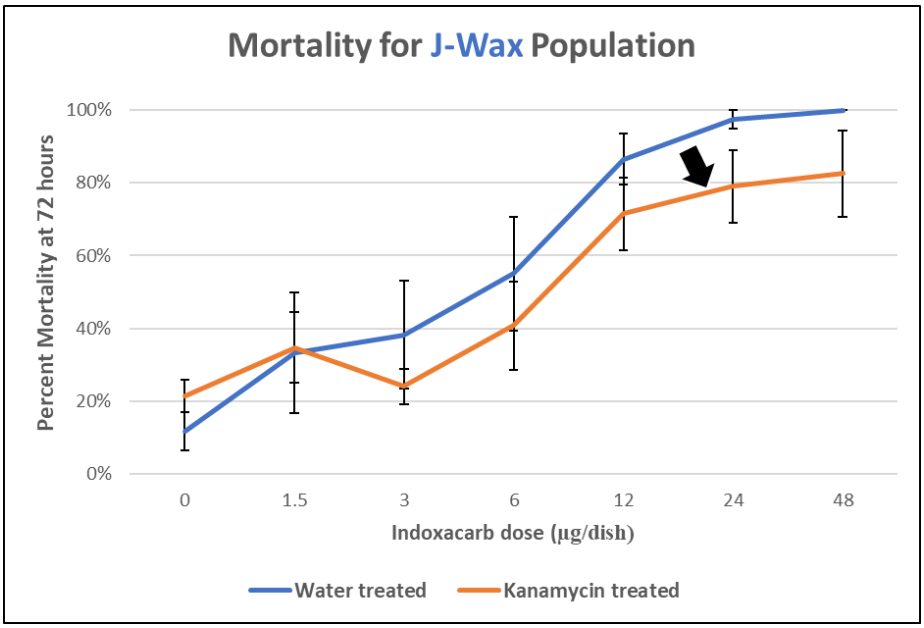


Figure 3.2. Indoxacarb dose-response feeding bioassay results for J-wax (S) cockroaches. See Table 1 for full probit analysis results

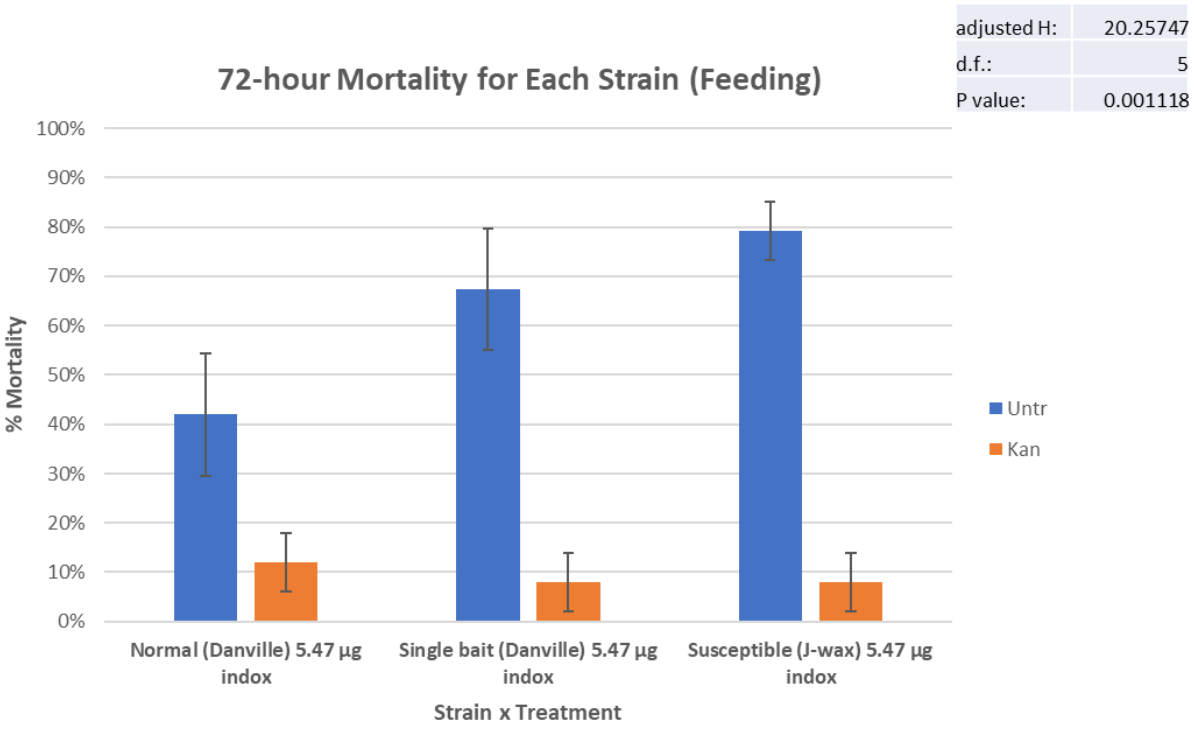


Figure 3.3. Comprehensive indoxacarb single-dose feeding bioassay results for all cockroach strains. Untr = Untreated, Kan = Kanamycin-treated. Global Kruskal-Wallis values indicate significance (p = 0.001118) between all strains and local values indicate significance between both treatment types within all three strains. P-values = 0.021 (Normal (Danville) Untr vs Kan), 0.028 (Single bait (Danville) Untr vs Kan), and 0.074 (J-wax Untr vs Kan)

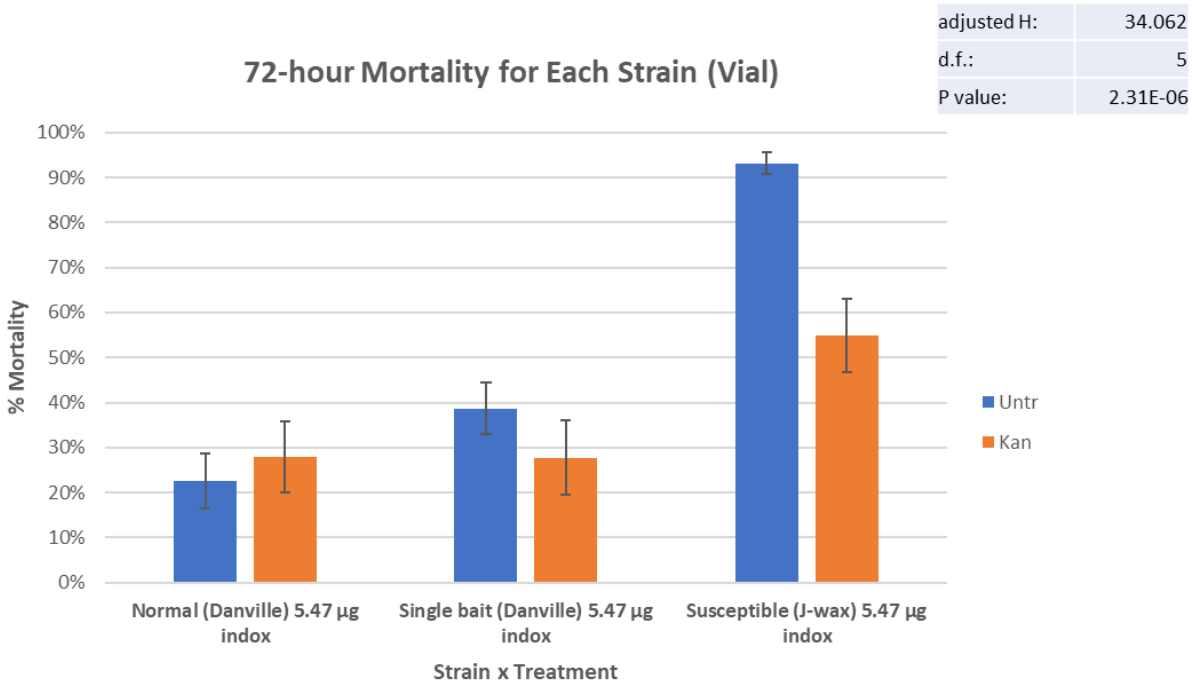


Figure 3.4. Comprehensive indoxacarb single-concentration vial (surface contact) bioassay results for all cockroach strains. Untr = Untreated, Kan = Kanamycin-treated. Global Kruskal-Wallis values indicate significance between all strains (P = 2.31E-06) but only indicate significance within J-wax (S) cockroaches when calculating between treatment types. P-values = 0.922 (Normal (Danville) Untr vs Kan), 0.209 (Single bait (Danville) Untr vs Kan), and 0.001286 (J-wax Untr vs Kan)

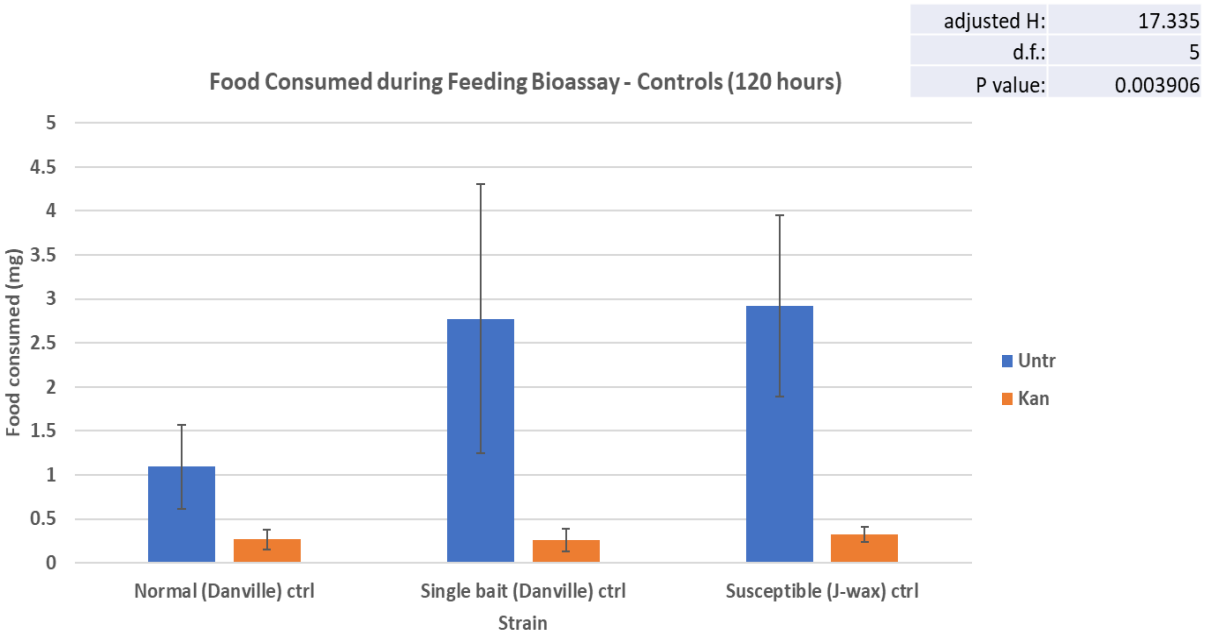
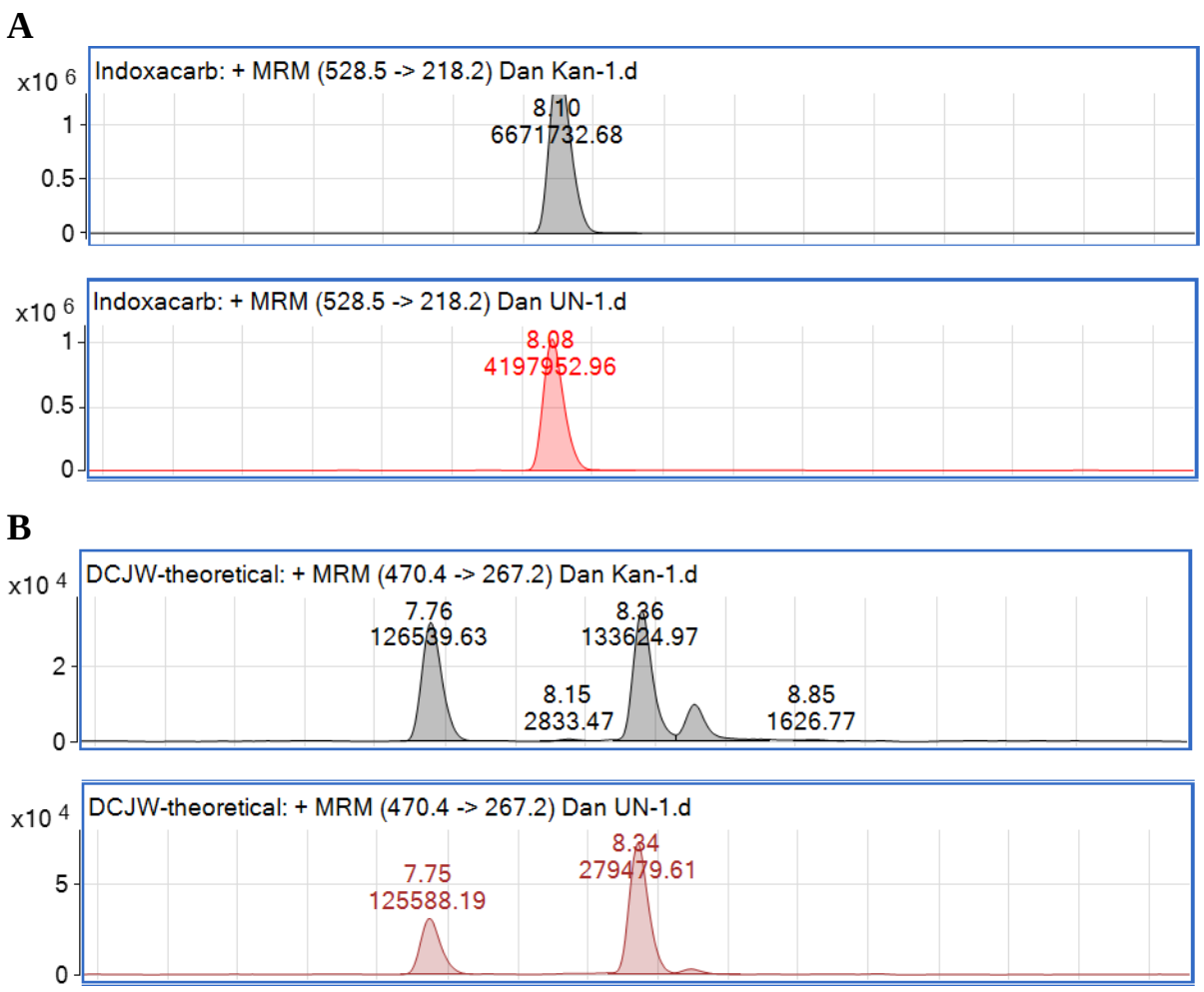


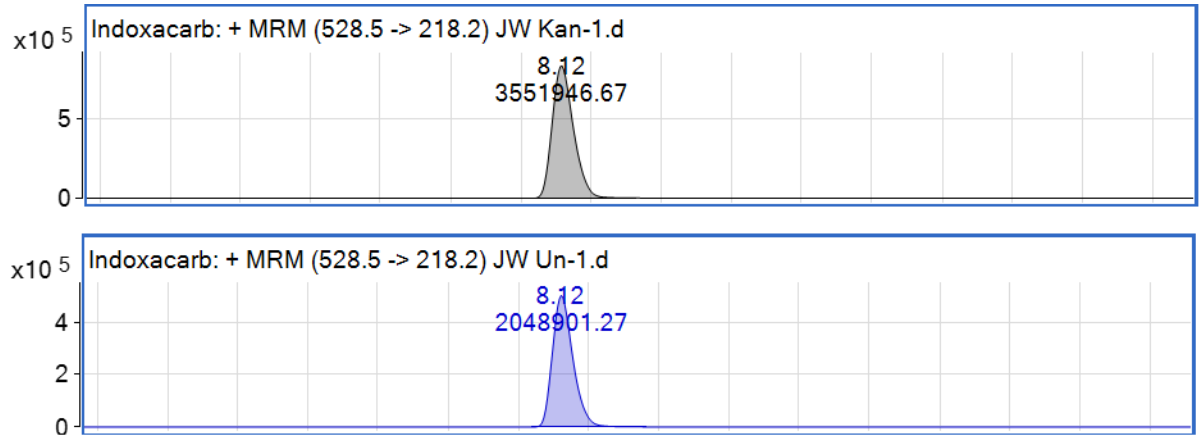
Figure 3.5. Comprehensive feeding results for all cockroach strains after 120 hours. Untr = Untreated, Kan = Kanamycin-treated. Global Kruskal-Wallis values indicate significance (p = 0.003906) between all strains and local values indicate significance between both treatment types within all three strains. P-values = 0.043 (Normal (Danville) Untr vs Kan), 0.021 (Single bait (Danville) Untr vs Kan), and 0.02 (J-wax Untr vs Kan)



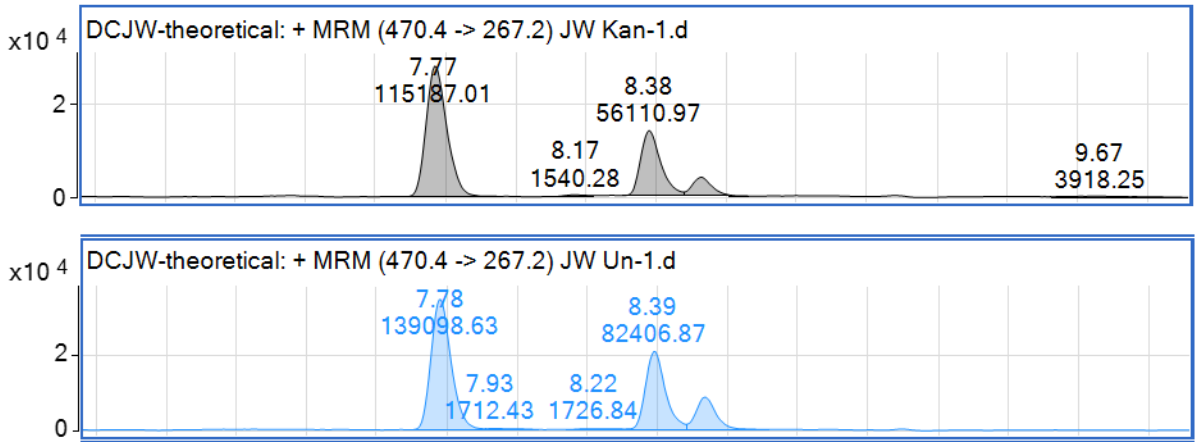
Figures 3.6A, B, C, D. Chromatograms of indoxacarb and its DCJW metabolites derived from cockroach frass by treatment type (1 replicate shown per treatment x strain combination). A = Danville (R) indoxacarb peak, B = Danville (R) DCJW peak(s), C = J-wax (S) indoxacarb peak, D = J-wax DCJW peak(s). Un = untreated, Kan = Kanamycin treated. Average retention time(s) = 8.088 (Danville-untreated-indoxacarb), 8.098 (Danville-Kan-indoxacarb), 8.121 (J-wax-untreated-indoxacarb), 8.120 (J-wax-Kan-indoxacarb), 8.348 (Danville-untreated-DCJW), 8.361 (Danville-Kan-DCJW), 8.391 (J-wax-untreated-DCJW), 8.386 (J-wax-Kan-DCJW)

Figure 3.6 continued

C



D



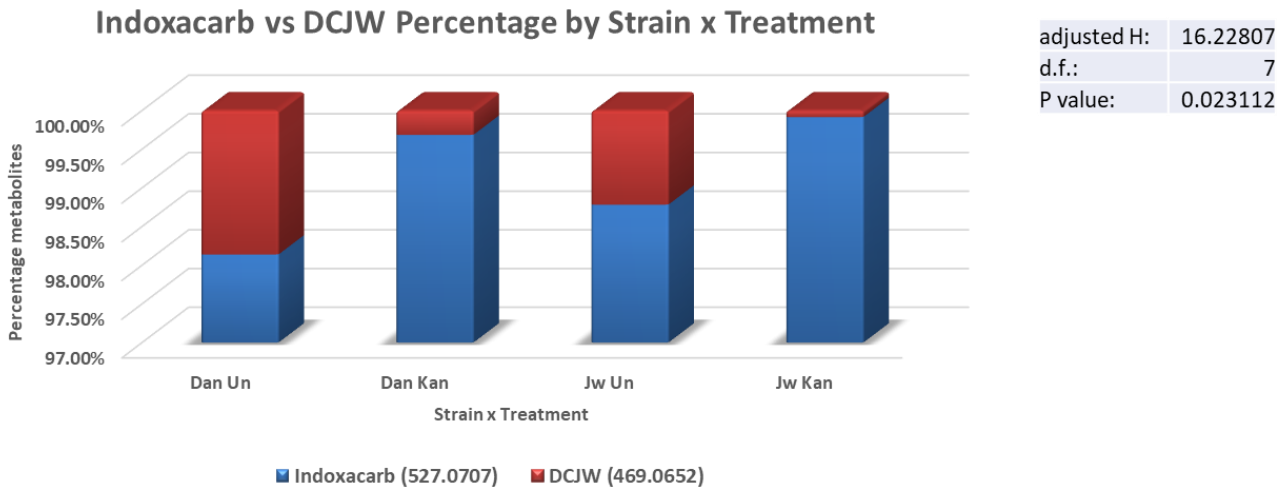


Figure 3.7. Combined indoxacarb and DCJW metabolite percentages extracted from cockroach frass by strain and treatment type (Un = control treatment, Kan = Kanamycin treatment). P-value = 0.023

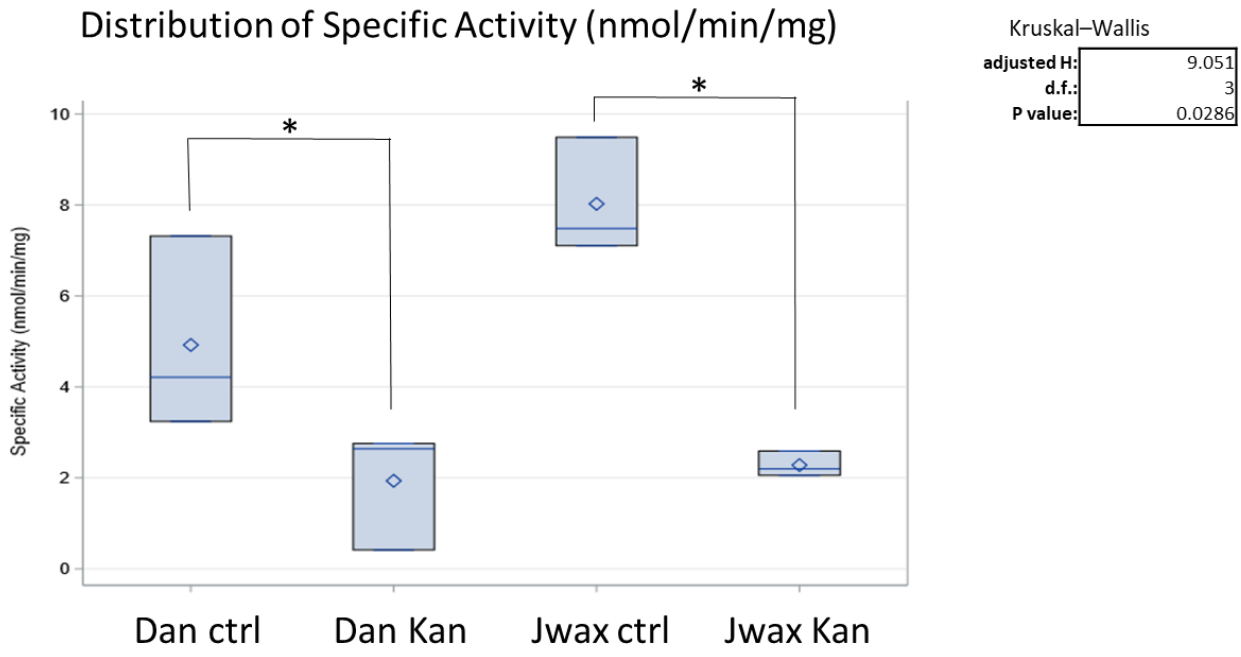


Figure 3.8. Specific hydrolase activity separated by treatment type (Ctrl = control treatment, Kan = Kanamycin treatment). Global Kruskal-Wallis values indicate significance between all strains (P = 0.0286) P-values for individual strains = 0.0495 (Danville) and 0.0495 (J-wax)

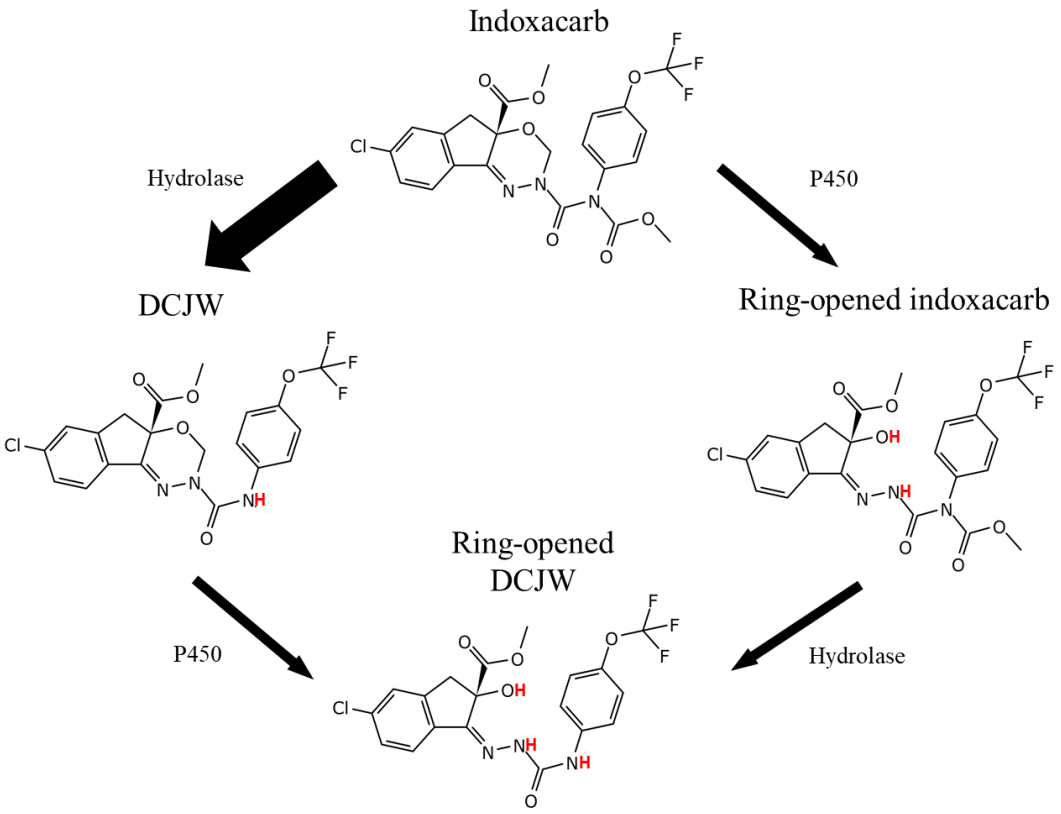


Figure 3.9. Conversion of parent indoxacarb to DCJW (via hydrolysis), oxadiazine ring-opened indoxacarb (via P450 oxidation), and oxadiazine ring-opened DCJW (via hydrolysis and P450 oxidation)

3.8 Acknowledgements

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CHAPTER 4. CONCLUSIONS

4.1 Conclusions and implication of results

The research contained in this dissertation was undertaken to compare the whole gut bacterial profiles of insecticide resistant and susceptible *B. germanica* and determine how microbial profiles change in the presence of an antibiotic. Additionally, I wanted to investigate how antibiotic treatment impacts the toxicity of the bait insecticides fipronil, abamectin, and indoxacarb, and to determine how gut bacteria, and specifically the enzymes originating within gut bacteria, metabolize and convert ingested indoxacarb into its toxic metabolite DCJW.

The objectives of chapter 2 included comparing the whole gut bacterial profiles of insecticide resistant and susceptible *B. germanica* and determining how these profiles, as well as the structure and function of the gut microbiome, change in the presence of an antibiotic. Also investigated was oral toxicity of the insecticides indoxacarb, abamectin, and fipronil in resistant and susceptible cockroach strains, with and without antibiotic treatment. It was hypothesized that there are differences in gut microbial structure and function between insecticide resistant and susceptible cockroach strains, as well as differences in gut microbial structure and function between antibiotic and control-treated cockroaches. The objective of chapter 3 was to determine how German cockroach gut bacteria, and specifically the enzymes originating within gut bacteria, metabolize and convert ingested indoxacarb into its toxic metabolite DCJW. The hypothesis for this objective was that hydrolase enzymes within the gut microbiome are metabolizing and toxifying indoxacarb, thus increasing mortality in *B. germanica* when it is exposed to insecticides. In chapter 2 I discovered that the gut microbiomes of Danville (R) and J-wax (S) German cockroaches are not significantly different on their own. However, the respective shifts of each strains' gut microbiome were unique once kanamycin was introduced. This shift and apparent dysbiosis revealed important cockroach strain differences which may extend to the host population level. The antibiotic-induced dysbiosis and insecticide tolerance to abamectin and fipronil that occurred in the Danville (R) strain suggest new, potentially exciting mutualistic relationships between gut microbiota and their insect hosts. As introduced in chapter 1, dysbiosis and the changes in gut microbiome composition, especially those related to growth and development, could be important influencing factors when comparing resistant and

susceptible cockroaches. These microbes may have a role in modulating insecticide toxicity or changing feeding behavior, whether to the benefit or detriment of the host.

The findings of chapter 3 revealed that DCJW formation decreased when kanamycin was introduced, meaning the percentage of active toxic compounds also decreased with kanamycin treatment [1]. From Chapter 2 it was also observed that *Stenotrophomonas* spp. can dominate a gut microbiome (with limited other symbionts) in the presence of kanamycin. If *Stenotrophomonas* increased but DCJW formation decreased, then this genus must not account for activation of indoxacarb. There are likely other genera, or a combination of genera, which activate indoxacarb into DCJW in the untreated cockroach gut. Such associations cannot be determined by 16S rRNA sequencing alone, therefore more functional and translational research must be performed to enable broad conclusions about enzymatic differences between taxonomies. More research is also needed to determine the specific phylogenetic classifications of many undescribed species discovered in this experiment, as well as their functions (especially as they relate to enzymology) and relationships to the German cockroach host. Once these relationships have been explored more extensively, researchers will have a better understanding of how to develop products aimed at controlling German cockroach by engineering dysbiosis or by building stronger levels of insecticide selectivity and safety.

Chapter 3 also showed a relationship exists between indoxacarb, antibiotics, and the activity of hydrolases in the cockroach gut. The goal of the chapter 3 enzyme assays was to determine if DCJW metabolite formation is related to changes in gut biochemistry. The importance of performing this experiment is that it is now possible to connect a change in enzyme activity in the gut microbiome with a prominent marketplace pro-insecticide. Additionally, abamectin and fipronil bioassays (Chapter 2) revealed that kanamycin treatment resulted in higher insecticide tolerance in both resistant and susceptible strains. This change in tolerance can be partially explained by changes in enzyme activity noted in Chapter 3 and extrapolates to provide insights into the development of field tolerance to these insecticides. Indoxacarb challenges and the affiliated metabolic profiles also reveal the importance of secondary mortality from pro-insecticides via horizontal transfer in cockroach feces.

A future direction of this research is to create a “germ-free” cockroach colony on which to perform similar insecticide bioassays. Testing insecticides on this new strain would allow me

to determine the true impact of gut bacteria (or the lack thereof) on host tolerance and detoxification. An attempt to create such a strain was made as part of this dissertation but ultimately failed. More information can be found about the creation of the germ-free strain in appendix A.

Overall, this dissertation shows that while there were not initial differences in microbial composition between strains, antibiotic (KAN) challenges induced major differences in bacterial phylum and genus composition between the Danville (R) and J-wax (S) strains. These differences may influence insecticide activation or detoxification at the host level and change host tolerance to abamectin and fipronil, especially when considering that selection for higher-level resistance may also be associated with elimination of commensal, pathogenic and/or parasitic microbes [2]. The hydrolase activity assays and metabolomic profiling experiments revealed that the conversion pathway of indoxacarb to DCJW is more complex than anticipated and, alongside the indoxacarb bioassays performed in chapter 3, reveal that host tolerance to indoxacarb is indeed influenced by gut microbial enzymes. Overall, the results of this dissertation show how antibiotic treatment affects the gut microbiome and insecticide metabolism, which can lead more broadly to host tolerance and/or susceptibility to bait insecticides.

4.2 References

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APPENDIX. CLEAN STRAIN CREATION AND WIDESEQ

Introduction

This appendix outlines the attempted creation of a "clean" – or germ-free – German cockroach strain. An effort to create a clean cockroach strain failed due to lack of gnotobiotic rearing materials and storage facilities (e.g., glovebox, gnotobiotic hood, germ-free diets and harborages). While microorganisms that are vertically transferred from the mother would still be present in the cockroach colony, an attempt was made to control external microorganisms that are acquired after hatching since they are acquired through the feces of the colony [1]. Once the clean strain had been established, an insecticide assay would have been performed on clean strain adult males identical to the insecticide assays performed on traditionally-raised field and lab resistant and susceptible strains as described in chapter 3. Unfortunately, many clean strain nymphs died before adulthood and many adults did not thrive in their germ-free habitat.

Materials and Methods

Creation of clean strain

Ootheca were manually detached from pregnant *B. germanica* females and placed in a sterile centrifuge tube. 0.5% sodium hypochlorite was added to the tube and the egg cases were rinsed by pipetting up and down for 1 min. 0.5% sodium hypochlorite was removed from the tube and discarded. 70% ethanol was added to the tube and the egg cases were rinsed again by pipetting up and down gently for 1-2 min. 70% ethanol was removed from the tube and discarded. The egg cases were then rinsed at least 3 times with NanoPure water by gently pipetting for at least 1 min each wash. After each wash, the water was removed and discarded. Once surface sterilized, the egg cases were handled with sterile forceps. Validation of sterility was executed by trying to culture microbes in brain heart infusion agar from the hatched egg case – cockroach nymphs were allowed to feed on a petri dish filled with brain heart infusion agar for 72 hours. These roaches excreted feces on the BHI (source info for BHI agar) plate, meaning if there were in fact aerobic bacteria present in feces, they would have grown on the BHI plate

during the 72 hour period (Figure A.1). Therefore, roaches thriving on petri dishes containing no bacterial CFUs (colony forming units) were considered “clean” and transferred to an unused Ziploc bin with autoclaved food, water and carboard harborages. These materials never came in contact with traditionally-raised German cockroach colonies.

WideSeq

For each biological replicate, 5 whole guts were extracted from each Danville strain: 5 from Normal and 5 from Clean. There were 3 biological replicates per strain. The 5-gut sample was homogenized in 1.5 mL PBS 70 rpm (Caframo, Georgian Bluffs, ON, Canada) (10 ups and downs). 10 µL of gut homogenate was then plated on a petri dish containing solidified BHI agar. Plates were incubated at 37 ºC for 24 hours. A new set of BHI agar plates was generated (autoclaved as directed). After another 24-hour incubation period, a wire loop was used to scoop each unique CFU (Colony Forming Unit) found on each plate. Totals were 3 unique CFUs across 2 Clean plates and 4 unique CFUs across 2 Normal plates. The wire loop was cleaned with 70% ethanol + flame before each scoop. After the wire loop scoop, the wire loop was dipped (with the CFU) into 40 mL BHI broth in a falcon tube. The falcon tube was incubated at 37 ºC for 48 hours. After the 48-hour incubation period, the broth + bacteria was centrifuged in a falcon tube to form a pellet at the bottom of the tube. Liquid agar was drained, and a subsequent DNA extraction was performed on the pellet using the Qiagen DNEasy kit. 16S PCR was performed on each DNA extraction sample as described in chapter 2. PCR products were submitted to the Purdue Genomics Core (West Lafayette, IN) for WideSeq. *Blattabacterium* spp. (NCBI txid331104) were used as a reference sequence for submitting WideSeq products.

Plating verification and CFU counting

For each biological replicate, 5 whole guts were extracted from each Danville strain: 5 from Normal and 5 from Clean. There were 3 biological replicates per strain. The 5-gut sample was homogenized at 70 rpm (Caframo, Georgian Bluffs, ON, Canada) (10 ups and downs) and serially diluted in 10, 20, and 40 mL PBS. 10 µL of gut homogenate was then plated on a petri dish containing solidified BHI agar and incubated at 37 ºC for 24 hours – this allowed me to verify sterility of adults.

Results

WideSeq

Results of the WideSeq are available in Table A.1. Out of the 3 Danville-clean unique CFUs processed via WideSeq, the CFUs returned Firmicutes (1, 2, 3) matches at the phylum level and Bacilli (1), Bacilli and Clostridia (2), and Bacilli (3) at the class level. Out of the 4 unique Danville-normal CFUs processed via WideSeq, the CFUs returned Firmicutes (1, 2, 3, 4) matches at the phylum level and Bacilli (1, 2, 3, 4) at the class level. *Blattabacterium* were not present in WideSeq results. Based on WideSeq results alone, there is no conclusive likelihood that CFUs present in the clean strain are qualitatively different from those present in the normal strain (Table A.1).

Plating

At the 10 µL dilution (in PBS), > 300 CFUs were counted on the Danville-normal plate (after 24 hours) while only 242 CFUs were counted on the Danville-clean plate (Fig. A.2). At the 20 µL dilution (in PBS), > 300 CFUs were counted on the Danville-normal plate (after 24 hours) while only 80 CFUs were counted on the Danville-clean plate (Fig. A.2). At the 40 µL dilution, Danville-normal plates still yielded > 300 CFUs while the Danville-clean plate yielded 48 CFUs, extending just above the minimum acceptable threshold of 30 CFUs per plate (Fig. A.2). Total CFUs on each plate were divided by 5 when plotting to represent gut equivalents (5 guts per sample). Since there was no dilution range in which both Danville-normal and Danville-clean plates had between 30 and 300 CFUs, I can conclude that there were quantitative differences in gut bacterial loads between the strains but cannot assign a specific numerical value to said difference.

Discussion

While I believe the method for testing sterility of 1st instar cockroach nymphs was valid, there were few valid options to measure sterility after the cockroaches had molted into successive instars. Cockroach guts were extracted and plated from adult males, but this method

fails to account for when and how any contamination could have been exposed to the nymphs. While performing verification, there were observable differences between Danville-normal and Danville-clean strains in gut bacterial CFUs on BHI plates, but realistically the clean strain needed to have 0 CFUs to truly be considered statistically "germ-free." Additionally, while there were differences in species level-results processed via WideSeq, the results were not definitive or consistent enough to determine species differences in CFUs as a whole, and results did not differ at the phylum or class level of bacterial taxonomy. In the future, I would recommend that clean or germ-free cockroaches should only be hatched and raised in a single container (theoretically a sterile glass vial or plastic isolating chamber) with no external inputs until food and water (theoretically dissolved in BHI agar) have been completely depleted. A similar method was used by Benschoter and Wrenn [2], but researchers in this study only measured developmental rates, reproduction, and longevity of cockroaches with no plans to handle the roaches outside of the rearing environment or to pursue insecticide challenges. More research should be performed to determine the longevity, the life span, and ultimately the insecticide tolerance capabilities of *B. germanica* when its gut bacteria are absent at all life stages.

Tables and Figures

Table A.1. Most likely observed species from each CFU processed via WideSeq. Unique CFU = Distinctive CFU present on respective strain plate. Count = total number of processes returning the most likely hit. Percent of Reads = percent of reads that match the most likely hit (relative to other detected hits in the sample)

Unique CFU	Count (phylum level)	Percent of Reads (phylum level)	Phylum	Count (class level)	Percent of Reads (class level)	Class	Count (species level)	Percent of Reads (species level)	Species
Clean 1	40,051	88.3%	Firmicutes	40,015	88.2%	Bacilli	6,109	13.5%	Staphylococcus xylosus
Clean 2	24,156	93.3%	Firmicutes	13,256	51.2%	Bacilli	2,167	8.4%	Clostridium baratii
Clean 3	43,197	92.9%	Firmicutes	43,140	92.8%	Bacilli	12,233	26.3%	Uncultured Bacillus
Normal 1	14,071	94.7%	Firmicutes	14,063	94.7%	Bacilli	3,101	20.9%	Uncultured Enterococcus
Normal 2	45,344	90.7%	Firmicutes	45,310	90.6%	Bacilli	13,229	26.5%	Uncultured Enterococcus
Normal 3	42,111	93.6%	Firmicutes	42,059	93.5%	Bacilli	5,864	13.0%	Uncultured Enterococcus
Normal 4	43,496	92.7%	Firmicutes	43,461	92.6%	Bacilli	9,869	21.0%	Uncultured Enterococcus

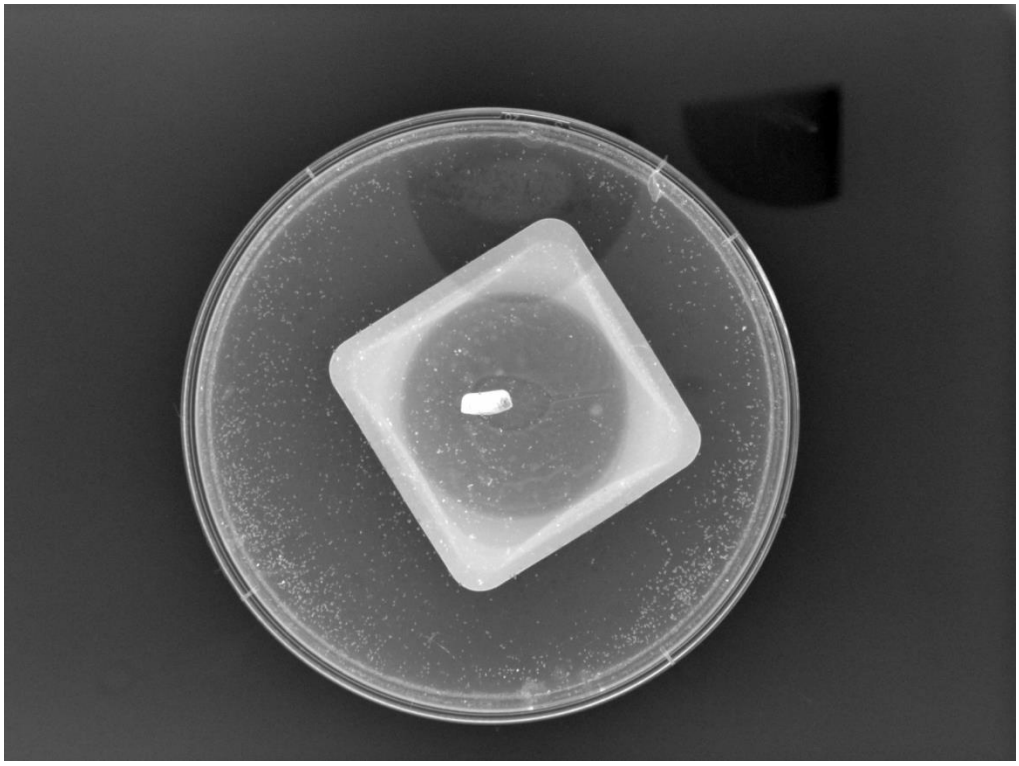

Figure A.1. Example of bacteria-free BHI agar plate used to verify sterility of clean cockroach nymphs. The CFU count to determine any verified plates was 0. A weigh boat was used to hold the cleaned ootheca in the center of the plate. 1st instar roaches fed on BHI agar for 72 hours. Small dots represent chewing marks and bacteria-free feces

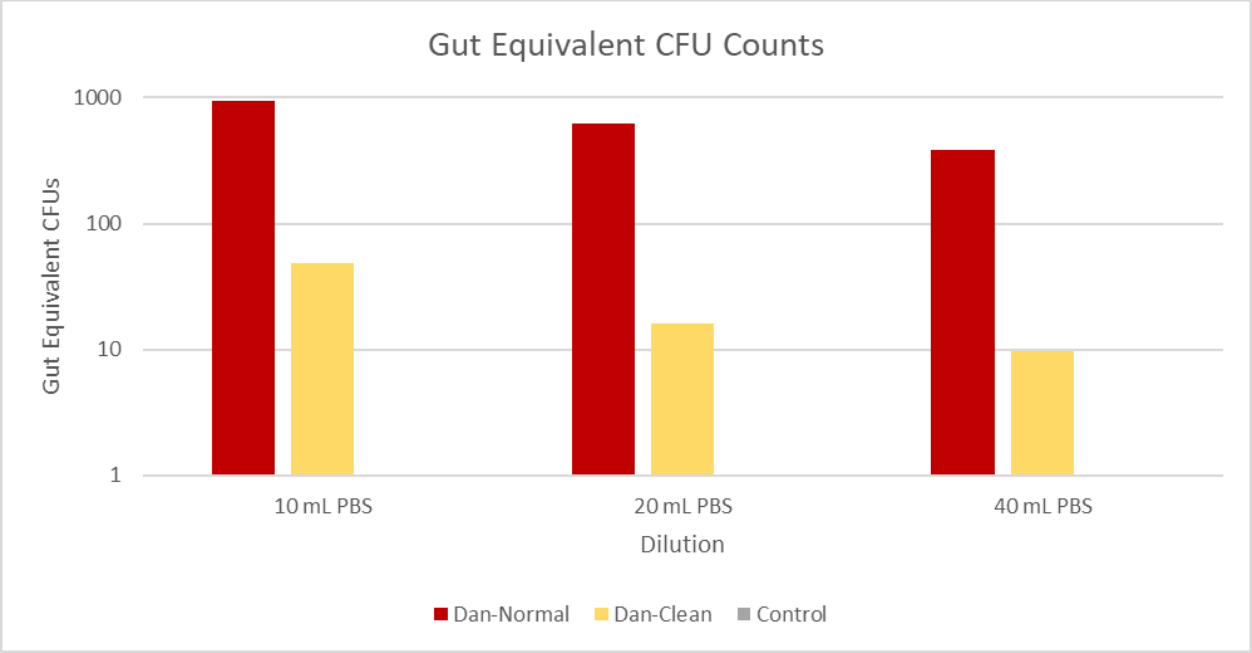

Figure A.2. Gut equivalent CFU counts for both Danville-normal and Danville-clean guts. P-values are undeterminable due to lack of proper replication

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