Investigating antimicrobial resistance in the gut bacteria of insects feeding on plants

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This thesis has been submitted in partial fulfilment of the requirements of the degree of Doctor of Philosophy at the University of East Anglia

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John Innes Centre
Norwich
June 2016

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Statement

The work submitted within this thesis is entirely my own, except where due reference has been paid, and has not be submitted to this or any other university as part of any degree.

I'm very highly educated. I know words, I have the best words.

D. J. T.

Abstract

It has been previously described that antibiotic-resistant bacteria can be found in the guts of insects feeding on a variety of plants and not exposed to significant levels of antibiotics. Such naturally-occurring resistance has implications for clinically-relevant antibiotic resistance, which is a worldwide problem, and for using plants as a source of potential novel antibiotics. We investigated this phenomenon further. Firstly, we searched for antibiotic resistance in different insects' guts and explored its origin, using two lepidopteran hosts feeding on artificial food containing either ciprofloxacin or oxytetracycline. We discovered that these antibiotics have a diverse impact on the insect gut microbiome, beyond simply inducing antibiotic resistance. Secondly, we used the insect gut bacteria to identify plant extracts with antibacterial activity, and purified their active fractions. We found that vindoline, from leaf extract, and serpentine, from root extract, are the most abundant metabolites in active fractions of Madagascar periwinkle extracts. Finally, we developed one of the insect species we used, *Galleria mellonella*, into a laboratory model for antibiotic efficacy testing, toxicity testing and as a model for human baby gut. In summary, in this project we explored different aspects of the antibiotic resistance that can be found in the insect gut and used it to guide us towards plant metabolites with antibacterial properties.

Acknowledgements

I would like to thank my supervisor Prof. Tony Maxwell for his support and encouragement. If I could start my project all over again, I would change a lot of things, but definitely not my supervisor. I would also like to thank my supervisory committee: Dr Janneke Balk, Dr Nicolas Burton, Dr Martin Rejzek and Dr Martin Stocks, for their advice and constructive criticism.

I would like to thank the members of the JIC Entomology Facility: Dr Ian Bedford, Anna Jordan, Gavin Hatt and Jake Stone, and Metabolite Services: Dr Lionel Hill, Paul Brett and Baldeep Kular. Their assistance was irreplaceable and advice priceless.

I would like to thank Lesley Mitchenall and other members of the Maxwell lab for such a positive working environment. Even though I was the odd one out not working on topoisomerases, they made me feel like a true member of the group. Additionally, I would like to thank the undergraduate students I have supervised: Aristide Lebreton, Ryan Hull and George Newell, who I hope learned as much from me as I have learned from them.

On a more personal note, I would like to thank my parents, Ewa and Kazimierz Ignasiak, and my wonderful twin Marta Ignasiak. They keep motivating me and support me through anything, even creating radioactive cauliflowers, which they thought I worked on.

I wouldn’t have worked as hard without the encouragement from my husband Sigurjón Ingólfsson. His long working hours and lack of work/life balance made working towards a PhD feel like a holiday. I am grateful to have him by my (left) side, even when he is 5,000 miles away.

Finally, I would like to thank all my friends, especially the ones I have met in Norwich: Jenny, Emma, Farzana, Inga, Tash, James, Andrew and Jorge. They all smell and it was a great pleasure to be mean to them.

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1 Introduction

In this project I investigated the gut microbiota of insects feeding on plants. We explored the origin of antibiotic resistance in the insect gut, examined the antibiotic properties of plant secondary metabolites and developed greater wax moth larvae into a laboratory animal model.

I will open this chapter with a description of plant secondary metabolites, their uses and environmental roles. I will follow with an introduction about insects, their relationship with plants and the bacterial communities associated with them. The following section will focus on antibiotics, antibiotic resistance and the current crisis in antibiotic discovery and will lead to a part about using insects for research purposes. I will summarize with presenting the aims and objectives of each part of the project.

1.1 Plant secondary metabolites

1.1.1 Classes of plant secondary metabolites

Plant secondary metabolites (also referred to as phytochemicals) are compounds that are not directly involved in the normal growth, development, or reproduction of the plant and their absence does not result in immediate death. They used to be considered by-products of plant metabolism with no role in the plant fitness [1]. More recently secondary metabolites have been indicated in plant defence (against herbivores, microbial pathogens, and other plants), signalling and attraction (for pollination, seed dispersal, and symbiosis), and abiotic stress response. Secondary metabolites can be specific to plant families or species. For example flavonoids from *Ginkgo biloba*, ginkgolides A, B, C and J, commonly used to treat impaired cerebral circulation, have not been found in any other living species [2]. Phytochemicals have beneficial role to the plant under stress conditions, but their production is costly and needs to be tightly regulated.

Secondary metabolites derive from primary metabolism and can be divided into four

classes: 1) phenolics and polyphenols, 2) alkaloids, 3) terpenoids and essential oils, and 4) lectins with polypeptides.

Firstly, phenolics and polyphenols are a diverse class, consisting of aromatic compounds, including flavonoids, quinones, tannins, and coumarins. Phenolics and polyphenols are characterised by the presence of phenol groups and their derivatives, when the double bonds of the phenol groups are rearranged.

Polyphenols are often considered the component responsible for the health benefits of fruit and vegetables. Pomegranate (*Punica granatum*), which was used in traditional medicine for the treatment of cough and sore throat, diarrhea, dysentery, dental plaque, bleeding noses and ulcers (see review by Ismail et al., 2012 [3]), contains high amounts of polyphenols. Punicalagin (Figure 1-1a) is one of the most abundant, reaching levels of 11–20 g/kg in the peel powder. Punicalagin is a reactive oxygen species scavenger and a metal chelator and was described to have antioxidant, antiproliferative, apoptotic, anti-inflammatory, and hepatoprotective properties (see review Faria and Calhau, 2011 [4]). Similarly, tea (*Camelia sinensis*) polyphenols are considered beneficial for a number of conditions. Epigallocatechin-3-gallate (Figure 1-1b) is an abundant tea polyphenol, constituting up to 7% dried tea leaves. Epigallocatechin-3-gallate has antiviral [5], anticancer [6], and neuroprotective activities [7], but also suffers from poor bioavailability with relatively high doses leading to mild side effects [8].

The second class, alkaloids, are a group of heterocyclic nitrogen-containing compounds. These compounds are derived from amino acids. The nitrogen-containing groups in alkaloids often carry positive charge, but can also carry neutral or negative charge.

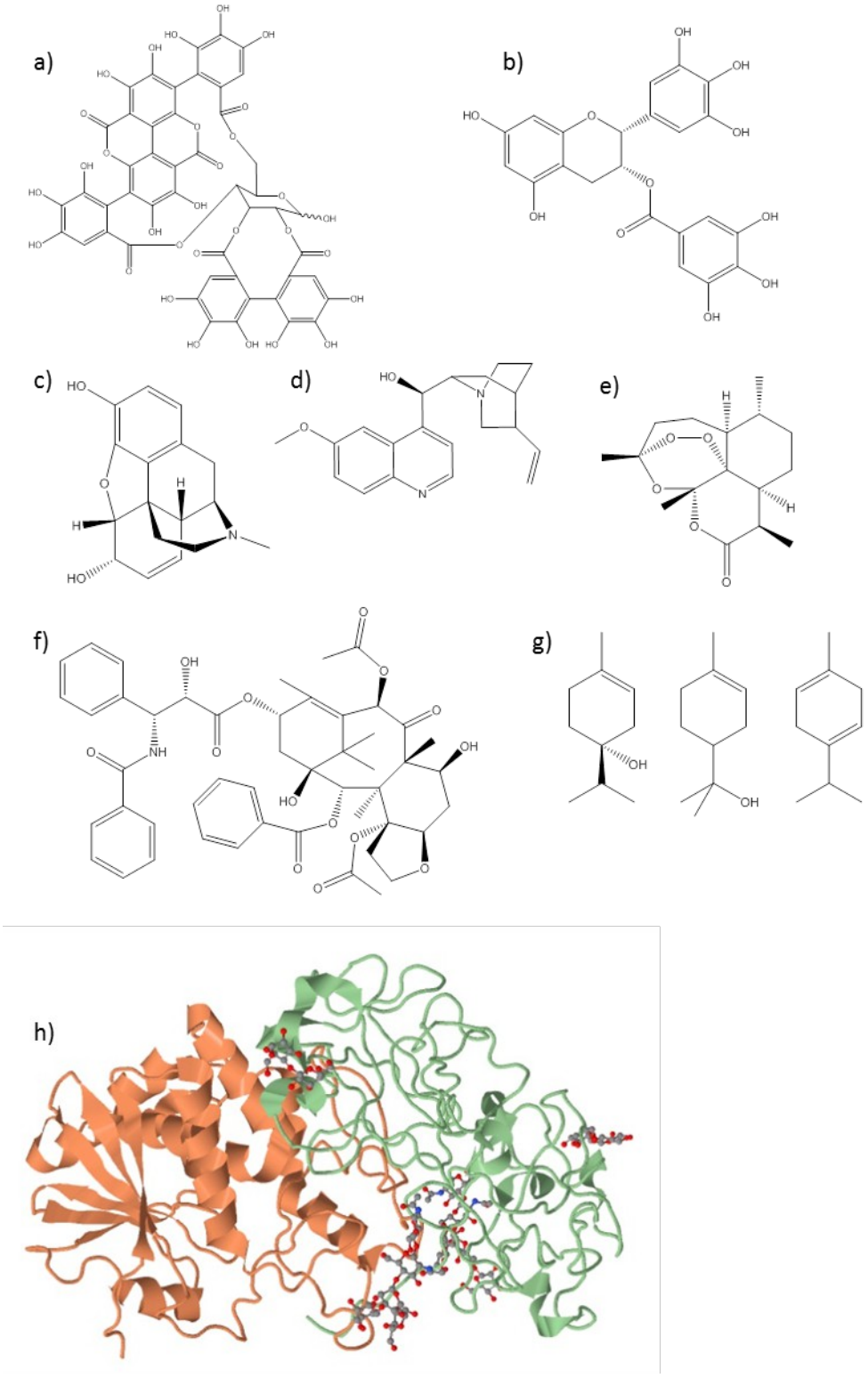


Figure 1.1: Examples of phytochemicals with medicinal properties. Punicalagin (a) from pomegranate and epigallocatechin-3-gallate (b) from tea are examples of polyphenols. Morphine (c) and quinine (d) are alkaloids with high economic value. Artemisinin (e) and taxol (f) are economically and medically important terpenes. Components of tea tree oil γ-terpinene, α-terpined and terpinen-4-ol (g) are examples of terpenes in essential oils used for medicinal purposes. h) Tertiary structure of ricin. Subunit A is shown in orange and subunit B in green (PDB file 2AAI).

Alkaloids, like painkiller morphine and antimalarial drug quinine, have been used in both traditional and modern medicine and are often of high commercial value. Morphine (Figure 1-1c), isolated from the straw of opium poppy (*Papaver somniferum*), is an analgesic (pain-relieving compound) used to treat mild to severe cancer pain. Morphine and its metabolites, morphine-3-glucuronide and morphine-6-glucuronide, bind with different affinities to subclasses of opioid receptors in the central nervous system to exert their analgesic action [9]. Another high value alkaloid is antimalarial quinine (Figure 1-1d) isolated from the bark of *Cinchona* trees. The extracts of *Cinchona* bark have been used since the 17th century to treat malaria, but since 2006 the active component of the extracts, the drug quinine, is no longer recommended as first-line treatment due to drug resistance issues. Quinine is toxic to *Plasmodium falciparum* parasite by interfering with the haemoglobin metabolism, but the mechanism of action is not well understood [10].

The third class, terpenes and essential oils, consist of five-carbon isoprene structural units linked in various configurations. Terpenes and essential oils are derived from the hydrocarbon metabolism.

Artemisinin (Figure 1-1e) is one of the most recognizable terpenoids, with the 2015 Nobel Prize in Physiology or Medicine being awarded for its discovery [11]. Artemisinin can be isolated from *Artemisia annua* and kills *Plasmodium falciparum* parasites by inducing reactive oxygen species formation in their mitochondria [12]. Another notable example of plant-produced terpene is the anticancer drug taxol (Figure 1-1f). Taxol, isolated from the bark of the Pacific yew *Taxus brevifolia*, stabilizes microtubule filaments, preventing their disassembly, blocking progression through cell cycle and leading to cell death. Examples of bioactive essential oils are clove oil and tea tree oil. The active ingredient of clove oil is eugenol, which is used in dentistry as an antiseptic and anaesthetic, but in large doses is cytotoxic [13]. Tea tree oil, containing γ-terpinene, α-terpined and terpinen-4-ol (Figure 1-1g), has been used as an antimicrobial and anti-inflammatory agent in traditional and complementary medicine [14].

Finally, lectins and polypeptides are amino acid chains. Unlike alkaloids, which are

derived from amino acids and are often heavily modified, the general structure of the amino acid units in lectins and polypeptides is preserved.

Ricin (Figure 1-1h) is an example of plant-produced lectin. It occurs naturally in castor oil plant (*Ricinus communis*). Ricin is extremely toxic with LD_{50} of 22 µg/kg body weight by inhalation or injection. Ricin's extreme cytotoxicity is due to the inactivation of the eukaryotic ribosome by catalytic cleavage of N-glycosidic bond of adenine 4324 of 28S rRNA within the ribosomal 60S subunit [15].

These examples highlight how plants and their extracts have been used for medical purposes for centuries. Ayurveda, traditional Chinese and African medicines depend on plants with medicinal properties as sources of treatments.

1.1.2 The use of plants in traditional medicine

Traditional medicine is defined by the WHO as the knowledge, skills, and practices based on the theories, beliefs, and experiences indigenous to different cultures used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness [16]. Adopting these practices by modern societies outside of their traditional context is complimentary, non-conventional or alternative medicine. Before the rise of modern, evidence-based medicine, traditional medicine was the only option offering treatment for disease. Many empirically-tested traditional treatments are still in use today and in some African countries up to 80% of the population depend on traditional medicine for their primary healthcare, while 50 – 90% Europeans declare having used complimentary or alternative treatments at least once [16].

Plant extracts are used for numerous purposes, compounds are sometimes crudely purified and often complex mixtures are used. Some mixtures are surprisingly complex considering their narrow specificity. A medieval eye infection remedy composed of garlic, oxgall, wine, leek or onion, and copper salts was shown to be active against methicillin-resistant *S. aureus* (MRSA) but not vancomycin-resistant enterobacteria (VREs), *Acinetobacter baumanni* or *Pseudomonas aeruginosa* [17].

Traditional medicine treatments are sometimes criticised over the insufficient knowledge of their composition and the lack of data on their efficacy and safety. Normally the long history of popular use is accepted as a proof that a treatment is safe to use. Even though there is no scientific basis to the belief that plant-based treatments are by their nature beneficial and safe, they require fewer clinical trials before being accepted for use that conventional medicines, and are commonly used even in the absence of data suggesting superiority over placebo in clinical trials (see review by Moreira et al., 2014 [18]). Moreover, for many traditional treatments the only toxicity data are from lack of observed adverse effects during short-term use, sometimes leading to the use of formulations that are carcinogenic, hepatotoxic, nephrotoxic or teratogenic in long term.

Additionally, misidentification and mislabelling of traditional treatments is a common issue. A genetic survey of 15 traditional Chinese medicines revealed some preparations were lacking the main ingredient listed on the ingredient list, but also the presence of ingredients from vulnerable and endangered plant and animal species [19]. More importantly, sometimes misidentification leads to serious toxicity issues. Chinese star anise (*Illicium verum*) is used not only for culinary purposes but also as an antiviral [20], as it contains high amounts of shikimic acid. Shikimic acid is a precursor in the production of oseltamivir phosphate, known better under its commercial name Tamiflu, used as a treatment for influenza virus A [21]. Chinese star anise can be easily mistaken with a similarly looking poisonous Japanese star anise (*Illicium anisatum*) [22] causing neurological symptoms in infants treated with star anise tea for infant colic.

Interestingly, some herbal treatments used in self-medication can gain popularity even when they are ineffective [23]. Such treatments often lack clinical data on safety and efficacy, and have no history of use in traditional medicine. Their ineffectiveness leads to longer use by the original users, who demonstrate the treatment and spread it to a larger number of converts. Even though the treatment is abandoned by the original users due to the lack of efficacy, the large number of converts keeps spreading the use of such ineffective treatments.

1.1.3 The biological roles of plant secondary metabolites

Plants and their preparations are often toxic to humans, but limited data are available about the biological roles of many plant metabolites. The non-essential function of plant secondary metabolites makes them difficult to study in the native context. Some compounds are reported to be poisonous, but the testing is often conducted above physiologically relevant concentrations.

For example, when bees feed on caffeine-rich nectar of *Citrus* species, the bitter caffeine can act as a feeding deterrent. However, it is not detectable by bees at the levels present in nectar and instead it enhances learning and memory [24]. The caffeine acts on adenosine receptors of Kenyon cells in bee brains to form a "memory trace" for the scent associated with reward during and after the conditioning. A number of studies automatically propose anti-herbivory role for secondary metabolites without strict testing of the hypothesis, while mediating plant insect interactions can be more nuanced.

1.2 Insects

1.2.1 Insect anatomy

Insects are a class of invertebrates characterized by three part body consisting of head, thorax and abdomen, a periodically-molted exoskeleton composed of α-chitin, three pairs of legs with joints, compound eyes and antennae. Apart from primitive wingless insect orders (Archaeognatha – bristletails and Thysanura – silverfish), insects have wings. Insects are extremely diverse and numerous group of animals with over a 1.5 million already described species, out of a predicted 5 to 30 million [25].

Generally speaking, the external anatomy of an insect consists of three body parts (Figure 1-2a): head, thorax and abdomen. Internally, nearly the entire body cavity (haemocoel) is filled with the digestive tract (Figure 1-2b). The digestive tract can be divided into foregut, midgut and hindgut, all composed of a single epithelial cell layer surrounded by muscles. The midgut is of endodermal origin, while foregut and hindgut

are of ectodermal origin and are therefore lined with cuticle that molts. The haemocoel is filled with hemolymph, a fluid analogous to blood in vertebrates. The nervous system can be located either dorsally or ventrally depending on the insect species. Other important organs are the malphigian tubule system, responsible for excretion, and fatbody, biosynthetic and storage organ analogous to vertebrate liver.

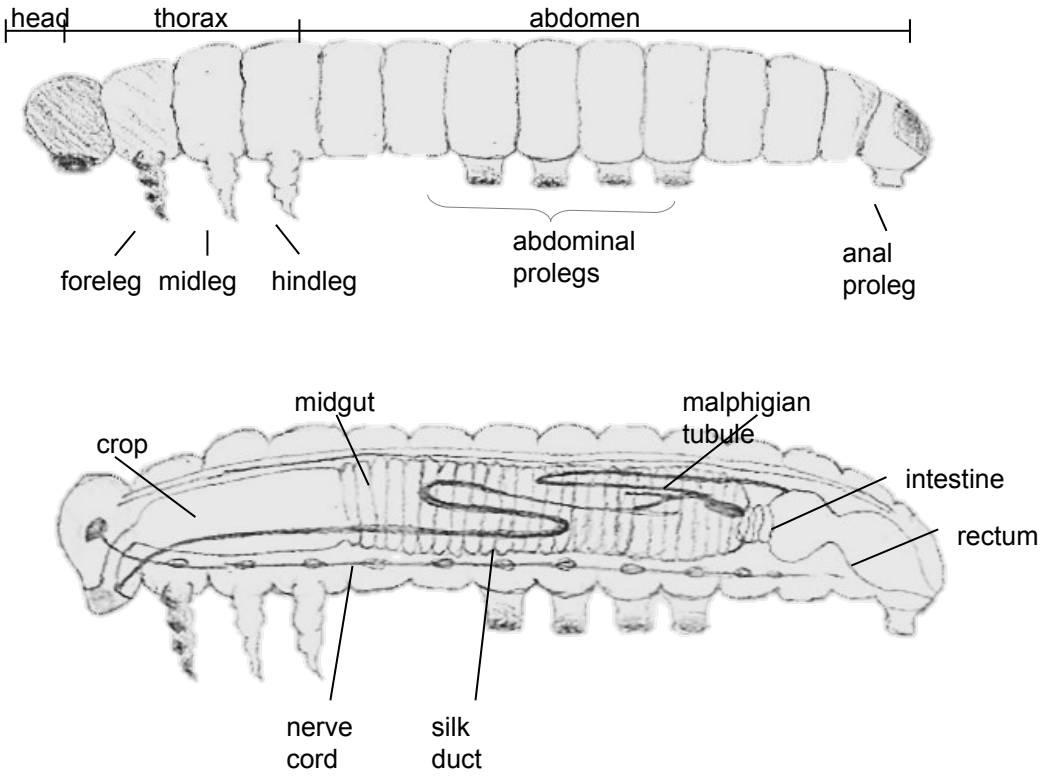
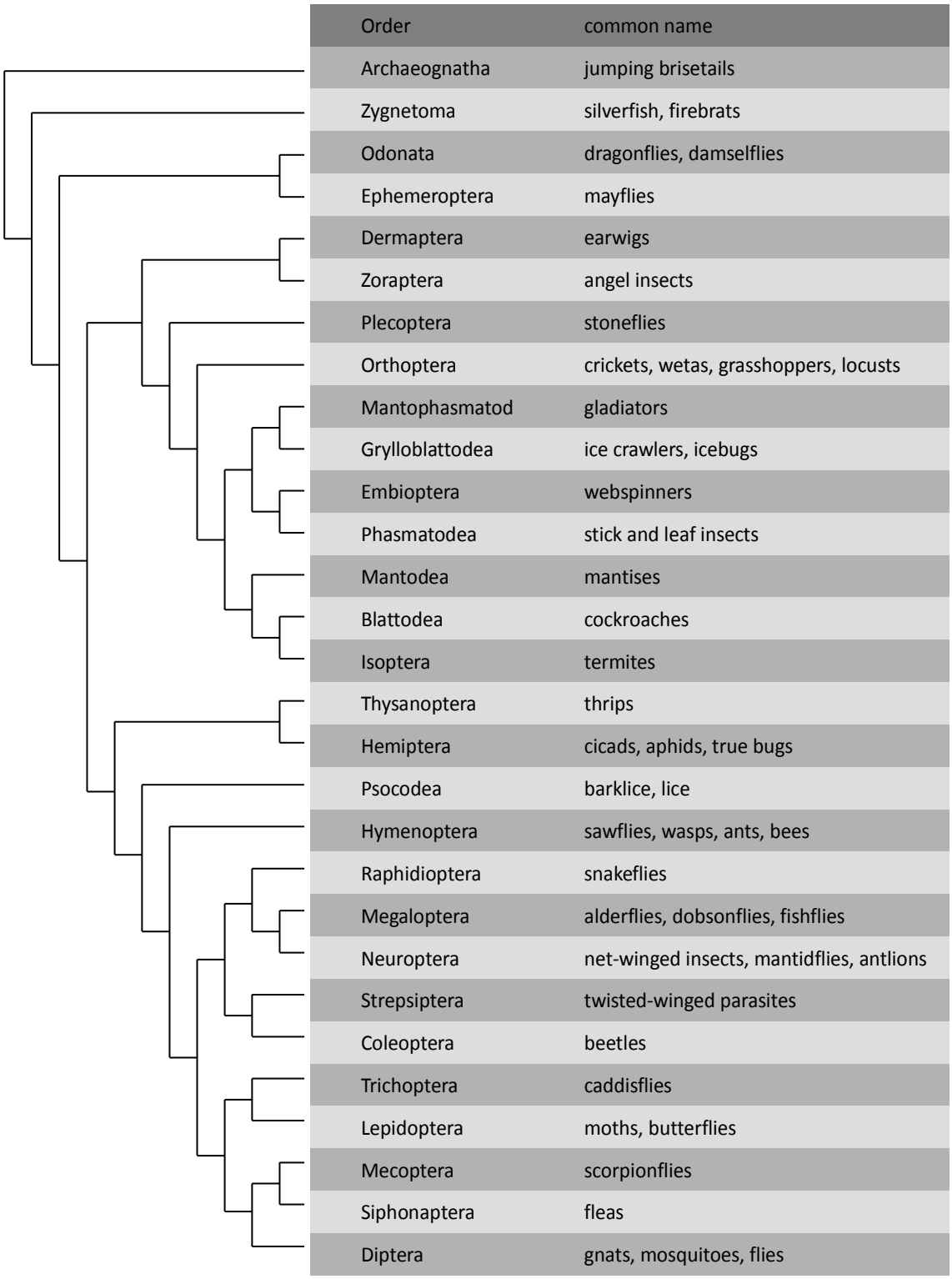


Figure 1.2: The generalized anatomy of an insect on an example of a larva.

1.2.2 The role of insects

Insects have important ecological roles, including herbivory, predation, pollination and nutrient recycling. In tropical forests ants are not only a major herbivore, with herbivorous ants consuming 12-15% total leaf area, but also an important carnivore, with carnivorous ants making up to 25% total animal biomass in some areas [25].

Insects are responsible for the pollination of most flowering plants. Importantly, insects have an economic role, as domesticated pollinators bees alone contribute between $1.6 and $5.7 billion to US agriculture alone [26].



Order	common name
Archaeognatha	jumping brisetails
Zygnetoma	silverfish, firebrats
Odonata	dragonflies, damselflies
Ephemeroptera	mayflies
Dermaptera	earwigs
Zoraptera	angel insects
Plecoptera	stoneflies
Orthoptera	crickets, wetas, grasshoppers, locusts
Mantophasmatod	gladiators
Grylloblattodea	ice crawlers, icebugs
Embioptera	webspinners
Phasmatodea	stick and leaf insects
Mantodea	mantises
Blattodea	cockroaches
Isoptera	termites
Thysanoptera	thrips
Hemiptera	cicads, aphids, true bugs
Psocodea	barklice, lice
Hymenoptera	sawflies, wasps, ants, bees
Raphidioptera	snakeflies
Megaloptera	alderflies, dobsonflies, fishflies
Neuroptera	net-winged insects, mantidflies, antlions
Strepsiptera	twisted-winged parasites
Coleoptera	beetles
Trichoptera	caddisflies
Lepidoptera	moths, butterflies
Mecoptera	scorpionflies
Siphonaptera	fleas
Diptera	gnats, mosquitoes, flies

Figure 1.3: An overview of insect phylogeny (adapted from Misof et al., 2014 [27]).

1.2.3 Insect diversity

Insects are extremely diverse group, with 0.2 mm long parasitic wasps *Alaptus magnanimus* and 35 cm long stick insects *Phobaeticus chani*, but are most likely a monophyletic group [25]. Even though fossil record suggests insects originated in Late Silurian, phylogenomic data suggest Early Ordovician [27]. The clade is divided into 27 orders, with Coleoptera (beetles), Diptera (gnats, mosquitoes and flies), Hymenoptera (sawflies, wasps, ants and bees) and Lepidoptera (moths and butterflies) being the largest groups [27]. Phylogenetic relationships between different insect groups were only clarified by an extensive survey of 1478 single-copy nuclear genes from different insect taxa and other arthropods in combination with validated set of fossils. The phylogenetic relationships resulting from this analysis is presented in Figure 1-3.

1.2.4 Insect immunity

Insect immune system consists of cellular response and systemic (humoral) response [25]. Specialized cells, hemocytes, are the components of cellular response system and include cells capable of phagocytosis, encapsulation of pathogens, and storing of antimicrobial peptides [28, 29]. These cells circulate in the hemolymph of a healthy insect. Systemic immune response is activated in response to wounding or infection and involves the fat body and gut epithelium producing large amounts of antimicrobial peptides [30].

Cellular responses are mediated by six types of hemocytes: adipohemocytes, granulocytes, oenocytoids, plasmatocytes, prohemocytes, and spherule cells [25]. Adipohemocytes are tissue-bound and have a secretory function. Granulocytes are involved in the early stages of capsule formation around pathogens and attract phagocytic plasmatocytes to the infection site. Spherule cells are involved in encapsulation of pathogens that are too big to be neutralised by phagocytosis. Oenocytoids have a role in melanisation associated with wound healing and encapsulation. Prohemocytes are considered hemocyte stem cells, they divide and differentiate into other hemocyte types. Generally, these six types are the most

common, but additional hemocyte types exits in some insects [29]. The number and types of hemocytes vary between different developmental stages.

Systemic or humoral responses are induced by the detection of a pathogen [30, 31]. The response is mediated by soluble effector molecules: antimicrobial peptides, melanin, and proteolytic cascades. Antimicrobial peptides kill bacteria and fungi by forming pores and depolarising their cell membrane [32]. Melanin is produced and deposited around the invading pathogen as a part of the encapsulation process [33]. Proteolytic cascades are induced by the pathogen recognition and they initiate encapsulation and melanisation processes, as well as other cellular responses [31].

The insect immune responses are not only inducible but also specific against the type of invading pathogen [32]. The genes encoding receptors responsible for the pathogen recognition can be alternatively spliced into a large variety of isoforms. Depending on pathogen recognition different proteolytic cascades activate a different arsenal of immune defences.

1.2.5 Similarities between insect and human immunity

Innate, not inducible, immune response is the more ancient part of immune system. Many parts of innate immune responses are conserved between different animals, for example insects and humans. Both structural and functional similarities can be found.

Often the receptors and pathways from different animals carry conserved molecular patterns. For example, there is structural homology between *Drosophila* Toll receptors and human TLR4 (Toll-like receptor 4) [34]. The family of receptors was originally discovered in insects and only later the structural homology between the insect Toll receptors and human interleukin 1 receptors was discovered.

Insects and mammals are distantly related and structural homology is rare, but functional homology is more common. The most striking similarities between insect and human immunity are between their cellular responses [29]. The central role is played by phagocytic cells, which use lectin-mediated phagocytosis. Lectins on the surface of phagocytic cells directly recognize pathogen-associated molecular patterns

on the surface of the bacteria or fungi and that interaction initiates phagocytosis. For both insects and mammals the ingested pathogen cells are neutralised by the same reactive oxygens species: O_2^-, H_2O_2, NO^-. Degranulation, the release of antimicrobial molecules from the granules in immune cells, and certain antimicrobial peptides (peroxynectin, transferrin, lysozyme, defensin) are shared between insects and mammals. These similarities between insect and human immunity makes insects a suitable model used in immunology research, virulence and antibiotic efficacy studies.

1.3 Insects in research context

1.3.1 Insect species used in immunity studies

The majority of studies in insect immunity have been conducted in drosophila. More recently, some studies on insect immunity have been conducted in greater wax moth, *Galleria mellonella*. For example, the inducible immune response was described in *G. mellonella* larvae. Inducible immune responses are not constitutively expressed and were thought to be absent in insects. The pre-exposure of *G. mellonella* larvae to non-lethal doses of *Aspergillus fumigatus* conidia increased resistance to a second, lethal dose [35]. The immune response comprised of increased hemocyte and antimicrobial peptide concentration in the hemolymph 24 hours after the initial exposure.

Apart from providing insight into the functional basis of animal immunity, the biggest impact of insect immunity studies have been on understanding the colony collapse disorder in bees (*Apis mellifera*). Colony collapse disorder occurs when the majority of worker bees disappear from the beehive leaving behind the food, immature bees, queen and only a few nurse bees to take care of them. No single factor has been found to be solely responsible for the disorder, but rather an interaction between pathogens and other stress factors. More prone colonies have higher pathogen loads, are more likely to be co-infected with different pathogens and have accumulation of coumaphos, a pesticide used for mite control [36].

A likely factor in colony collapse disorder is the extensive use of antibiotics in apiculture. Bee colonies in the US have been prophylactically treated with

oxytetracycline for control of larval foulbrood pathogens *Melissococcus pluton* and *Paenibacillus larvae* for the last 50 years. Those colonies have accumulated antibiotic resistance genes in their gut microbiomes at high frequencies [37, 38]. Eight tetracycline/oxytetracycline resistance loci, conferring high levels of resistance, were identified: efflux pump genes tetB, tetC, tetD, tetH, tetL, and tetY, and ribosome protection genes tetM and tetW. The genes were rarely present in wild bee colonies and colonies not exposed to antibiotics for at least 25 years. This demonstrates the impact of long-term antibiotic exposure on an organism with highly stable, well-defined microbiota.

1.3.2 Using insects as scientific tools

Bees are not the only insect species exposed to antibiotics over prolonged periods of time. The impact of dietary antibiotics on insect survival and development has been investigated in the greater wax moth (*Galleria mellonella*) [35], as antibiotics are routinely added to insect culture media. Diets with penicillin, streptomycin, fluconazole and griseofluvin decreased larval survival and increased development time between 50% and 80%. Interestingly, high concentrations of penicillin and low concentrations of streptomycin, fluconazole and griseofluvin increased the wet weight of the larvae, either by promoting growth or increasing water retention. The authors did not investigate the impact of the dietary antibiotics on the wax moth gut microbiota, but nonetheless they demonstrated a significant effect of these compounds on insect health.

Insects are interesting not only in their ecological context and through their role in agriculture but also as models for more complex organisms. Because of their small size and high reproductive rate, insects have been used in many molecular, cellular, ecological and evolutionary studies. The fruit fly *Drosophila melanogaster* has been an irreplaceable model in genetics (see review by Bier, 2005 [39]). Silkworm (*Bombyx mori*) and greater wax moth (*Galleria mellonella*) have been used for acute toxicity testing *in vivo* [40, 41]. Greater wax moth is also commonly used in virulence [42] and antimicrobial efficacy studies [43]. The main benefit of using insects in place of other

laboratory animals is the reduced cost and relatively uncomplicated procedures. In many cases using insects is a more ethical way of obtaining larger datasets.

1.3.3 Interactions between plants and insects

Herbivorous insects have a close ecological relationship with their plant-food. Food plants can drive speciation of insect species. For example two host plants of the stick insect *Timema cristinae* have repeatedly driven speciation between the insect ecotypes [44]. The insects are endemic to California and different ecotypes are adapted to feeding on either *Adenostoma fasciculatum* or *Ceanothus spinosus.* Genetic analysis of the stick insects' genome revealed repeated evolution of partial reproductive isolation between different ecotype pairs from the same geographical locations. Plants can also drive insect extinction events, especially through biodiversity loss [45]. Habitat loss has been driving insect extinction in biodiversity hotspots around the world, especially that conservation efforts are rarely aimed at preservation of insect species.

As mentioned before, insects have important ecological roles. These roles are sometimes mediated by microbes and include aiding digestion and providing essential nutrients, detoxification of food, use of novel hosts, pathogen resistance, mediating fertility and temperature tolerance, invasiveness and adaptive body coloration (see review by Feldhaar, 2011 [46]).

1.3.4 Bacteria can mediate interactions between insects and plants

The relationship between herbivores and their food plants cannot be considered without the digestive tract of microbial symbionts. The symbionts are often the component responsible for the digestion of the complex plant material. Cellulose-degrading microbes are responsible for 75% of the cellulose digestion in lower termites such as *Mastotermes darwiniensis* and *Coptotermes lacteus* [47]. The vertically-transmitted bacteriocyte symbiont *Buchnera aphidicola* of sap-feeding aphids increases the nitrogen utilization from the insect food by synthesizing essential amino acids and riboflavin [25].

Interestingly, bacteria can drive insect behaviour. The life cycle of Aster Yellows phytoplasma strain Witches' Broom depends on the transmission between two of its hosts: the leafhopper *Macrosteles quadrilineatus* and the plant *Arabidopsis thaliana*. The pathogen modifies the plant development, inducing conversion of flowers into leaf-like structures [48]. This conversion attracts insect colonisation. The insects feed on plant sap, become infected with the phytoplasma and transmit the pathogen to the next plant they feed on. It has been demonstrated that one of phytoplasma's virulence proteins SAP54 selectively destabilizes transcription factors of the MADS-domain family which regulate flower development [49]. Disturbing the plant development the pathogen creates sterile plants and that increases insect feeding.

Other roles of insect microbiota include protection from potential pathogens. Fungiculture-practicing ants *Acromyrmex octospinosus* carry an antibiotic-producing actinomycete on their cuticle [50]. The actinomycete specifically supports the growth of the fungal garden, while inhibiting the growth of fungus *Escovopsis*, a specialized parasite of the fungus/ant symbiosis. Interestingly, fungiculture-practicing ants, beetles and termites from different habitats and continents share similar microbiota [51]. Culture-independent bacteria identification techniques demonstrated that microbiota from these insects converge not only functionally, but also structurally. The microbiomes are primarily compromised of genera *Enterobacter*, *Rahnella* and *Pseudomonas* and the species within them exhibit high degree of whole-genome similarity. Such investigations into bacterial communities are only possible thanks to recent developments in culture-independent methods in microbiology.

1.4 Microbiome diversity

1.4.1 Studying bacteria: from culturing single isolates to population studies

Microbiology was developed as a scientific discipline due to the possibility to cultivate bacteria, which biased the studies towards culturable isolates of organisms capable of causing disease. Only later did it become apparent that bacteria behave differently *in*

vitro and in their natural habitats, where different species and ecotypes co-exist and the cell density is much lower [52]. An interesting example is bacteria growing in biofilms – genetically antibiotic-susceptible cells are often antibiotic-resistant when growing in a biofilm [53]. The extracellular matrix secreted by the bacteria forming the biofilm protects the cells from toxic concentrations of antibiotics, the host immune response and other external factors.

Developments in molecular biology techniques and sequencing technology allowed for detailed studies of bacterial populations as they occur in nature, without the need to culture these organisms. These developments allowed for a better understanding of the microbiota as the interactive community of microorganisms sharing an ecological niche.

1.4.2 Microbiome composition and function

Cheaper sequencing and more robust data analysis allows the microbiome research to move from simple composition studies to analysing functional data. Even though most microbiomes are complex and spatially and temporally variable, some organisms, such as honey bees, host sets of bacteria that are small and consistent across different populations.

Worker honey bee gut hosts nine core phylotypes (defined as strains with ≥97% sequence identity in 16S gene): two Gammaproteobacteria (Gamma-1, also known as *Gilliamella apicola,* and Gamma-2), one Betaproteobacterium (*Snodgrassella alvi*), two Firmicutes, one Bifidobacterium, two Alphaproteobacteria, and one Bacteroidetes (*Apibacter adventoris*) [54, 55]. Genes from these core species constitute between 95% and 99% of all reads in the bee gut, but the frequencies of each phylotype in an individual gut vary to a large extent. On an individual gut level most communities are dominated by the two Firmicutes phylotypes, constituting over a half of reads in 80% of the guts. Each gut also contains Gamma-1 and *Snodgrassella alvi* at frequencies of up to 40%. The occurrence of Gamma-2, Alpha-2 and Bifidobacterium is high, but their frequency is low. Alpha-1 is present in 35% guts at low frequencies. *Apibacter adventoris* is also present rarely at low frequencies.

Such highly defined and conserved composition of a bacterial community suggests functional specialization of the microbiota. It was demonstrated that normal microbiota correlates with the protection against bacterial [56] and protozoan pathogens [57]. Shotgun metagenomic analyses of the bee microbiome revealed that the community is enriched in carbohydrate-related functions (such as sugar-uptake systems), fitting to the carbohydrate-rich diet of nectar [58]. Mannose uptake systems (mannose phosphotransferase systems) were abundant, even though mannose is present in trace amounts in nectar. Mannose is toxic to bees suggesting a detoxifying role of microbiota. An arabinose efflux permease gene family was also enriched and the genes were diverse. Some of the enriched genes have homology to known drug efflux pumps, including close homologs to tetracycline efflux pumps. *Gilliamella apicola* (Gamma-1 phylotype) was indicated in pectin metabolism [58]. Pectin is a component of the pollen primary cell wall and bees lack enzymes capable of digesting it. The metagenomics study predicted *G. apicola* to be the only isolate in the bee gut capable of digesting pectin, however *in vitro* experiments showed that not all *G. apicola* clades are capable of digesting pectin. PCR screening of the *G. apicola* isolates revealed that clades lacking pectinase genes also lack the ability to degrade pectin, and that the isolates belong to two separate phylogenetic clades.

Research into the function of non-core bee microbiota revealed that *Fructobacillus* and *Lactobacillus* species that colonize beehives (brood cells, bee bread, and nectar), but not bees, facilitate the colonization of bee gut by core microbiota, namely Firmicutes and Bifidobacteria [58]. In co-culture assays *Fructobacillus* isolates facilitated the growth of core phylotypes above the expected cell density. Cell-free supernatant of the *Fructobacillus* isolates had the same effect, suggesting that the metabolic by-products or excretions of the culture were responsible for the effect, but the chemical nature of the signal is not yet known.

1.4.3 Functional verification of the roles of microbiome

Metagenomic analyses allow some insight into the role of the microbiome for host health. The genomic approach has been applied to the study of microbial communities

in insects but the interactions between the members have rarely been validated by other methods. To confirm such functional data an *in vivo* mechanism of the interaction has to be shown. Even in simple communities the number of interactions between the host and members of the microbial community and within the community itself is large.

An example of the role of bacteria in host health is *Wolbachia* infection of the fruitfly *Drosophila melanogaster* [59]. *Wolbachia* are endosymbiotic bacteria that reduce aggressive behaviour in male fruitflies, without affecting the ability to fly or fight. Transcriptional analysis revealed that the bacteria downregulate the octopamine biosynthesis pathway. Octopamine is a neurotransmitter responsible for energy-expensive behaviours, such as flying, egg-laying and jumping, and has been previously shown to be able to alone regulate aggressive behaviour in drosophila [60]. As well as different levels of transcripts of octopamine pathway biosynthesis genes, different levels of the neurotransmitter were detected in the insect heads by chromatographic methods [59]. However to fully explain how the bacteria control insect gene expression, a molecule secreted by *Wolbachia* would have to be identified and its interaction with host would have to be shown. Transcriptomic data alone only imply correlation of *Wolbachia* infection and male fruitfly aggression.

Similarly, the speciation of a parasitic wasp genus *Nasonia* was thought to be driven by genetic loci within the wasp genome, associated with hybrid lethality. It was demonstrated that the hybrids between different *Nasonia* species have higher lethality when their microbiome is not similar to either of the parent species [61]. The lethal phenotype was rescued when the wasps were bred in germ-free conditions and transcriptomic data showed that nearly 40% of the immune genes were underexpressed in these germ-free hybrids. This suggests hybrid lethality might be caused by the breakdown of host-symbiont network.

These examples demonstrate how bacteria play an important role in insect life, but the exact mechanisms mediating the interactions are rarely known. Microbial communities are an interesting subject to study from the ecological point, especially that bacteria have been mostly studied as pathogens.

1.5 Microbiology

1.5.1 Antibiotics and antibiotic resistance

1.5.1.1 The history of antibiotics

Bacteria used to be studied primarily as the causative agent of human disease. Before the discovery of antibiotics, bacterial diseases used to be the major cause of death, with pneumonia, tuberculosis and enteritis responsible for nearly 40% of deaths [62]. In the 19th century infectious diseases, both bacterial and viral, used to kill 800 people out of 100,000 per year. Advances in microbiology, especially Pasteur's invalidation of the theory of spontaneous generation [63] and Koch's germ theory of disease [64], allowed for a better understanding of the spread of infections. The introduction of better hygiene, especially in hospitals, lowered the incidence of infections. Joseph Lister pioneered hospital hygiene and antiseptic technique, using solutions of phenol for sterilisation, in Glasgow Royal Infirmary in 1867 [65]. However, only the development of arsphenamine in 1909, the discovery of penicillin in 1928 and development of sulpha drugs in 1932 gave doctors tools to clear bacterial infections.

These advances paved the way to what is now known as the golden era of antibiotic discovery. Penicillin was introduced to the market in 1940s and many more antibiotics were to follow (Figure 1-4). All currently used broad-spectrum antibiotic classes in primary healthcare were first brought to the market between 1940s and 1970s [66]. Importantly, most antibiotic classes that were brought to the market recently were discovered before the 1970s. An example of an antibiotic that was recently approved for treatment but has been discovered in the last century is fidaxomycin. Fidaxomycin, which is a macrocyclic antibiotic used to treat *Clostridium difficile* infections, was discovered in 1948 and introduced to the market in 2011 [67].

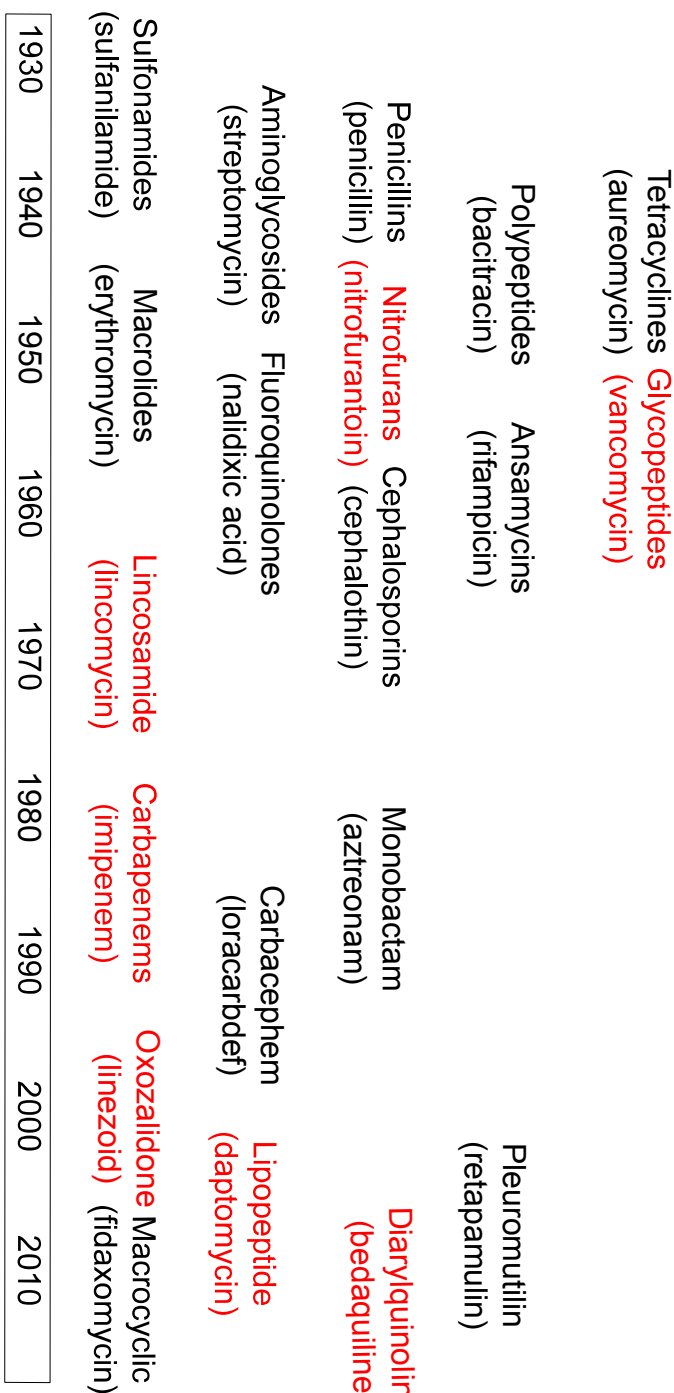


Figure 1.4: Timeline of antibiotic discovery. Antibiotic classes and an example of each class are shown on a timeline. Most antibiotic classes were discovered between 1940s and 1960s. Antibiotic classes highlighted in red are ones reserved for second line therapies against resistant bacteria.

Recently developed antibiotics are either narrow spectrum topical treatments like retapamulin, indicated for the treatment of impetigo caused by *Staphylococcus aureus*, or are not available in primary health care, reserved for the treatment of the most resistant infections, like daptomycin and linezolid. Both antibiotics are indicated for the treatment of infections caused by multi-drug resistant Gram-positive organisms. Interestingly, both daptomycin and linezolid are also examples of antibiotics that were approved for treatment much later than they were discovered. Daptomycin was discovered in 1986 and first marketed in 2003 and linezolid was discovered in 1955 and first marketed in 2000 [67].

1.5.1.2 The emergence of antibiotic resistance

Whenever a new antibiotic was discovered, resistance to it would be observed soon after [68]. Resistance to penicillin, introduced for clinical use in 1943, was already observed in 1946. Antibiotic resistance arises even to antibiotics like vancomycin that were shown not to cause any significant resistance *in vitro*. Vancomycin resistance of clinical significance was observed 31 years after the introduction of the antibiotic to the market [69].

Antibacterial resistance is manifested by the ability of bacteria to grow and multiply in the presence of an antibiotic that would normally kill them or limit their growth. It can be caused by three different mechanisms: a target mutation where the antibiotic target changes so that it is no longer sensitive to the compound, efflux pump which removes the antibiotic or toxin from the bacterial cell or an antibiotic-modifying enzyme where a separate protein capable of deactivating the antibiotic is present. Resistance by target mutation requires point mutations to arise spontaneously, while efflux pumps and modifying enzymes are normally acquired via horizontal gene transfer.

The sequencing of bacterial genomes revealed features of bacteria that readily allow the acquisition and spread of resistance [70]. The genomes of MRSA and VRSA revealed antibiotic resistance gene clusters on pathogenicity islands and mobile genetic elements, indicating readiness to pick up resistance elements from the environment. On the other hand *P. aerugionosa* PA01 genome revealed many two-component

regulatory systems, numerous porins, and efflux pumps, suggesting extreme plasticity of environmental responses.

1.5.1.3 Factors contributing to antibiotic resistance

Antibiotic resistance genes are often associated with mobile genetic elements. The fitness cost of resistance genes is often surpassed by the host organism and without that cost the antibiotic-resistant phenotype readily replaces the susceptible phenotype (see review by Aminov and Mackie, 2007 [71]). In cases like that the bottleneck for antibiotic resistance spread is the original release of the resistance genes which is triggered by a selection by a high use of antibiotics. Therefore environments with historically low use of antibiotics have relatively low level of antibiotic resistance.

Apart from the intrinsic ability of antibiotic resistance genes to spread, the main factors contributing to the rise in antibiotic resistance are overprescription by doctors combined with misuse by patients, and antibiotic use in agriculture, horticulture and animal husbandry. Antibiotic misuse in healthcare is often a result of using antibiotics to treat viral infections that present themselves with similar symptoms to bacterial disease [70]. In case of viral infections, a response to antibiotic is not mechanistically possible. The only outcome of such treatment is deterioration of normal host microbiota. Doctors are sometimes prone to sub-optimal dosing and patients are prone to not using prescribed antibiotics correctly, for example by not following the dosing regimen or terminating the treatment early [70]. Furthermore some official medical practice guidelines recommend treatments that have been shown to promote bacterial resistance. Persistent urinary tract infections are treated with sub-inhibitory doses of ciprofloxacin over 7 to 9 days, even though it has been demonstrated such treatment is not effective and it selects for antibiotic-resistant populations [72]. Similarly, cephalosporins are frequently administered to patients before surgeries for antibacterial prophylaxis, resulting in some patients acquiring coagulase-negative staphylococci within hours of admission to the hospital [73].

In animal husbandry antibiotics are used to raise the weight and fat content of farm animals and prevent infections, but they contribute to the spread of microbes with

antibiotic resistance [70]. In many countries the use of antibiotics in animal husbandry continues even after an extensive body of evidence confirmed a link between antibiotic use and the spread of antibiotic resistance from animal farms to clinical settings [74]. In 1994 in Denmark 1000 times less vancomycin was used as a human therapeutic than avoparcin, a vancomycin analog used in animal feed [70]. Even though the use of antibiotics for growth promotion was banned in the European Union in 2006 [75], other countries still allow antibiotic use for non-medical purposes. In the US, out of 14.9 million kilograms of antibiotic sold for animal use, only 28% were used solely for therapeutic indications [76].

1.5.2 Antibiotic resistance in the ecological context

1.5.2.1 The origin of antibiotic resistance

Interestingly, antibiotics and antibiotic resistance are not phenomena specific to the 20th and 21st centuries and human antibiotic use. Antibiotic resistance pre-dates modern antibiotic era. Tetracycline resistance genes were present in 2% of enterobacteria before the widespread clinical use of tetracycline [77]. It demonstrates how antibiotic resistance was present in bacteria before the strong selective pressure imposed by human antibiotic use was present, but also how the level was low and only became of significance due to human activity. The primary role of antibiotic resistance is the protection of the producing organism. Modifications of the antibiotic target or increased efflux are necessary for the survival of the antibiotic producing organism [78]. Without the protection from the antibiotic action, the host bacteria would be killed.

Modern day antibiotic resistance genes have been shown to originate from ancient genes with similar function [71, 79]. The diversity found in the gene pool originates from an ancient diversification event. The genes only spread from the environmental antibiotic-producing bacteria to pathogens recently in the evolutionary history. Diverse resistance genes conferring resistance to β-lactams, tetracyclines and glycopeptide antibiotics have been found in 30,000 years old permafrost sediments [80]. Studies on

a vancomycin resistance gene from the sediment indicated a high level of similarity to modern variants, suggesting that rapid emergence of clinical antibiotic resistance after the discovery of antibiotics was caused by the selection for pre-existing genes.

1.5.2.2 The role of antibiotic resistance in ecosystems

In bacterial communities antibiotics are defence compounds and signalling molecules. It has been shown that in Streptomyces antibiotics have a role mediating interactions between competing strains [81]. Antibiotics are present in the environment at low concentrations, below their minimal inhibitory concentrations, at which they induce transcriptional changes, such as increased virulence and biofilm formation. In response to cues from their competitors, Streptomyces activate or increase their antibiotic production and supress the antibiotic production in their competitors. Such behaviour prevents resource-expensive strategy of constitutive antibiotic production while minimising the threat from the competitors.

Another possible origin of antibiotic resistance genes is the co-selection for antibiotic resistant phenotypes by other factors such as the presence of heavy metals. An example of an antibiotic resistance gene associated with a gene conferring resistance to heavy metals is genetic element *Tn21* (see review by Liebert et al., 1999 [82]). The element consists of mercury-resistance operon, genes conferring resistance to streptomycin, spectinomycin and sulphonamides, and is transposable. Interestingly it was found in Japan in enterobacteria isolated in the 1950s.

Resistance phenotypes can be pleiotrophic – naturally-occurring amino acid variation in the GyrA subunit of gyrase selected for by fluoroquinolone exposure confers the ability to use different carbon, nitrogen and phosphate sources for growth [83]. Gyrase is responsible for maintaining the topology of bacterial chromosome and mutations in gyrase genes have been shown to affect transcription levels of supercoiling-sensitive genes, many of which are stress-response genes. Specifically a substitution at position 87 of *Salmonella enterica* GyrA lowers antibiotic susceptibility to quinolones, β-lactams, folate synthesis inhibitors and aminoglycosides [84]. The mutation alters global supercoiling levels, resulting in changes in transcription of stress-responsive

sigma factors *rpoS*, *rpoE* and *rpoN*. Such response explains why mutants are more evolutionarily successful, because the mutation is beneficial under a range of conditions.

1.5.3 Antibiotic resistance in the context of human antibiotic use

1.5.3.1 Significance of antibiotic resistance

As the antibiotic development pipeline dries out, antibiotic resistance becomes more widespread. Antimicrobial resistance is a burden both in clinical settings and in everyday life. Overall first line (initial) antibiotic treatment failure in primary healthcare increased by 12% between 1991 and 2012 [85]. Antibiotic treatment failure can be defined as inefficacy of the antibiotic against infections it is indicated for. While treatment failure stays under 20% for most commonly prescribed antibiotics, the current failure rates for trimethoprim, ciprofloxacin and cefalexin are 70.1%, 30.8% and 30.8% respectively. The economic burden (defined as a cost to the health service, the labour market and the individual) of infectious disease in England is estimated at £30 billion each year [86].

1.5.3.2 Antibiotic discovery gap

The effectiveness of antibiotic treatment of bacterial disease diminishes as antibiotic resistance is on the rise both in incidence and spectrum and the number of new antibiotics in the discovery pipeline declines. The last time a broad spectrum drug against a novel target was introduced was in 1986 when daptomycin was shown to depolarize the cell membrane by inserting itself into the cell membrane, altering the cell surface curvature and creating leaks. Daptomycin was only brought to market in 2003 [67]. More recently, teixobactin has been shown to inhibit cell wall synthesis by binding to precursors of teichoic acid, which is a novel target in antibiotic discovery [87]. However, teixobactin has not been approved for clinical use.

This decline is sometimes attributed to the shortage of commercial funding as big pharmaceutical companies close down their antibiotic development programs [88, 89].

The investment in antibiotic development is rarely economically viable without private-public partnerships and financial incentives from national governments, especially for smaller biotechnology companies. Apart from promoting and funding collaborations, governments encourage re-investment in antibiotic discovery by extending patent lifetimes and guaranteeing purchase of a defined stockpile of the ready antibiotic, as is the case with some vaccines.

Economic incentives might not be enough to stimulate re-investment in antibiotic discovery. The antibiotic development programs were often closed as a result of a shortage of hits from the discovery platforms over decades of research. The golden era of antibiotic discovery was a consequence of the discovery of common classes of broad-spectrum antibiotics that are often found in large quantities in the producer organism [67]. Such compounds are frequently re-discovered in high-throughput screens of bacterial strain libraries and novel chemical scaffolds are scarcely discovered. As the antibiotic discovery platforms stopped delivering new hits, the programs were deemed unsuccessful and closed down. Hardly any information about the closed down programs is publicly available and no definite conclusions can easily be drawn from them. Current antibiotic development programs require more innovative approaches and are significantly more difficult [90]. Most common strategies involve screening large collections of bacterial strains and screening chemical libraries.

1.5.3.3 Reasons behind the discovery void

Antibiotics have a finite therapeutic lifetime due to antibiotic resistance potential present in bacteria. Antibiotic production and the development of antibiotic resistance are parts of evolutionary arms race between bacteria. Both antibiotic production and resistance are under constant evolutionary pressure and lead to an effect known as “The red queen effect” [91]. This phenomenon can be observed when an organism needs to continually evolve just to keep pace with its evolving competitors, even though on average their fitness remains the same [92]. The antibiotic resistance genes evolve not only in response to changes in antibiotic production genes in other bacteria,

but also in response to human antibiotic use. There is a constant need for discovery and development cycles of new antibiotics if human antibiotic use is to keep pace with bacteria and antibiotic resistance.

Not only have few new antibiotic classes been brought to the market since the 1970s, but the number of companies researching them steadily declined [88]. At the moment there are only a handful of large pharmaceutical companies researching antibiotics: GlaxoSmithKline, Novartis, Allergan/AstraZeneca, and Merck. Fewer resources are being devoted to antibiotic research and development because they are not as profitable as other medicines.

Antibacterials are as expensive as other drugs to develop, yet their clinical use is discouraged to aid antibiotic efficacy conservation. Especially in developing countries antibiotics have to affordable. Novel drugs are often much more expensive than old antibiotics. A single course of fidaxomicin, approved for treatment of *Clostridium difficile*, costs over £1350 while a more standard treatment with vancomycin costs about £35 [93]. The decrease in resources devoted to antibiotic discovery and the pressure to keep the price of new drugs low make for an extremely unattractive economic model for antibiotic discovery programs.

Apart from the economic reasons the discovery void arises from screening libraries that fail to bring the expected hits and leads [94], especially against Gram-negative pathogens [95]. Standard chemical libraries, most commonly used for screening against human targets, are rarely optimised for antibiotics – they often follow the Lipinski's rule of five [96]. The rule is a set of guidelines for pharmacologically-active molecules that are suitable for oral treatment in humans. The rule states a compound should have a molecular mass of less than 500 Da, fewer than 5 hydrogen bond donors (the total number of nitrogen–hydrogen and oxygen–hydrogen bonds), fewer than 10 hydrogen bond acceptors (all nitrogen or oxygen atoms) and the log P value (octanol-water partition coefficient; an indication of the hydrophobicity of the compound) not greater than 5. Antibiotics often do not fit within the rule. For example aminoglycoside streptomycin is 581 Da, has 16 hydrogen donor bonds and 18 hydrogen acceptor bonds. Even though streptomycin is readily water-soluble, it is relatively toxic at 430

mg/kg .

It was also suggested that not enough chemical space is probed to discover novel molecules with antibacterial activity [97]. When compared to other drugs, for example used to treat diseases of the central nervous system, antibiotics have relatively similar structures and properties. In theory it should be possible to identify novel chemical scaffolds, dissimilar to currently used ones, with antibiotic properties. However, chemical libraries with novel scaffolds that have not been screened for antibacterial activity are scarce. To yield useful new leads, chemical libraries need to mimic the variety found in natural product libraries and natural product libraries need to be fused with combinatorial chemistry to accommodate the human drug requirements (see Lipinski rule discussed above).

Another important aspect of the development gap in antibiotic discovery is the lack of novel targets and mechanisms of action [70]. Only a few processes in bacterial physiology are utilised as drug targets: inhibition of cell wall synthesis and inhibition of folate synthesis in Gram-positive bacteria, and in Gram-negative bacteria inhibition of protein synthesis and inhibition of DNA or RNA synthesis.

Historically cell wall synthesis inhibition and protein synthesis inhibition were the most common strategies for antibiotic development, because they are multi-step processes and offer many opportunities for disrupting bacterial cells. Physiological processes can be targeted in various ways, for example protein synthesis can be interfered with at initiation step by linezolid or at elongation step by disruption of either the 50S complex (by macrolides, chloramphenicol and clindamycin) or the 30S complex (by aminoglycosides and tetracyclines) [70]. Each of the classes mentioned works by a different mechanism. For example macrolides inhibit peptidyl transferase by sterically blocking the elongation of the polypeptide chain, while chloramphenicol inhibits peptidyl transferase by binding to it and preventing substrate binding.

1.5.3.4 Potential solutions

The scale of the resistance crisis has prompted World Health Organization (WHO) and

local governments to action. WHO released a report in 2012 that outlined options for action against antibiotic resistance [96]: improved surveillance of antimicrobial resistance, measures to ensure better antibiotic stewardship, reducing antibiotic use in animal husbandry, infection prevention and fostering innovation in antibiotic discovery. Another WHO report on global resistance surveillance in 2014 [97] summarized available data on the prevalence of antibiotic resistance and its health and economic burden, identifying gaps in data from low income countries. In the UK the Chief Medical Officer made antibiotic resistance the focal point of the annual report in 2011 [86]. A number of recommendations for policy makers have been made, summarised in four areas: prioritising antimicrobial resistance as a national security risk, improving education and awareness of appropriate antibiotic use, developing surveillance systems for monitoring infections and antibiotic stewardship, and developing better diagnostic technologies for rapid identification of the pathogen allowing narrow-spectrum treatment.

A number of consortia have been set up to tackle the problem on antibiotic resistance. Europe's largest public-private partnership the Innovative Medicines Initiative, a joint project between European Union and the European Federation of Pharmaceutical Industries and Associations, funded a project aiming to tackle the antibiotic resistance problem. The project is called New Drugs for Bad Bugs (http://www.imi.europa.eu/content/nd4bb) and has a number of sub-sections spanning a number of subjects: COMBACTE (Combatting Bacterial Resistance in Europe) (https://www.imi.europa.eu/content/combacte), DRIVE-AB (Driving re-investment in research and development and responsible antibiotic use) (https://www.imi.europa.eu/content/drive-ab), ENABLE (European Gram-negative Antibacterial Engine) (https://www.imi.europa.eu/content/enable), and TRANSLOCATION (Molecular basis of the bacterial cell wall permeability) (https://www.imi.europa.eu/content/translocation).

Another initiative is University of Queensland's Community of Open Antimicrobial Drug Discovery (CO-ADD) which specializes in screening of compounds that would not normally be screened for antimicrobial activity. The initiative is funded by the

Wellcome Trust and free of change for academic groups. Importantly, the results of all screens are open-access and the ownership of the compound screened remains with the original owner of the compound.

The efforts to develop more antibiotics are not limited to screening for novel molecules. A number of alternative therapies and antibiotic resistance breakers are being researched, but so far there has been little success. An interesting approach has been targeting bacterial virulence as a way to target a process necessary for pathogenicity but non-essential to survival. To some extent the combination therapy of clindamycin and penicillin in the treatment of streptococcal shock syndrome is an example of such treatment [98] – penicillin kills the cells by inhibiting cell wall synthesis and clindamycin prevents bacterial virulence by inhibiting protein synthesis and toxin production.

1.5.3.5 Rational approach to plant screening for antibiotic discovery

Plants have been proposed as a potential source of novel antibiotics [99]. A number of commercial high-throughput screens have failed to identify potent, non-toxic, broad spectrum antimicrobials from plants [100]. They were undertaken by pharmaceutical companies Merck and Pfizer, and by two biotech firms Phytera and Shaman Pharmaceuticals. Unfortunately the outcomes of the screens have not been published.

Only a few reports have been published as a result of medicinal plant screens. Over 60 Indian medicinal plants were screened for antibacterial activity against four bacterial strains (*E. coli*, *B. subtilis*, *P. aeruginosa* and MRSA) with a method employing the dye resazurin as an indicator of bacterial growth [101]. Two plant species, *Celastrus paniculatus* and *Lobelia nicotianifolia*, were identified by the authors as having antimicrobial activity at concentrations similar to the reference antibiotics: streptomycin and tetracycline (10-25 µg/ml), but a closer look at the data reveals that the plant extracts were at least an order of magnitude less active than the reference antibiotics. In another screen 1,220 Brazilian plant extracts have been tested against *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa* and *Escherichia coli* [102]. 50 plant extracts showed antibacterial activity against one or

more bacteria at the initial concentration of 100 μg/mL and those extracts have been fractionated. A follow-up screen identified three anti-*E. fecalis* compounds lupeol, α-amyrin and 3β-hydroxyglutin-5-ene from *Symphonia globulifera* [103].

The scarcity of data from past screens and especially the reasons for lack of commercial success of plant-derived compounds makes it difficult to rationally design new screens.

1.5.3.6 Successful plant-derived medicines

Almost a half of drugs approved for human use between 1994 and 2007 were plant-derived [104], yet there are no plant-derived antibiotics in clinical use. An example of a plant-derived medicine is camptothecin, a potent inhibitor of human topoisomerase I, used in anticancer therapy. Camptothecin is a quinoline alkaloid isolated from the stem tissue and bark of *Camptotheca acuminata*, happy tree [105]. The compound binds to topoisomerase I and DNA complex, stabilising it and leading to the formation of single strand DNA breaks [106]. Camptothecin induces cell death when the single strand DNA breaks are converted into lethal double strand DNA breaks as the transcription machinery collides with the topoisomerase I covalently bound to the nicked DNA [107].

Many plants are reported to have antibacterial activity but only in some cases the compound responsible for the activity has been identified, and even less frequently a specific mechanism of action has been identified. Garlic has antimicrobial activity and the component responsible for the activity is allicin (diallylthiosulfinate) [108] which reacts with thiol groups of various enzymes in bacterial, protozoan, plant and mammalian cells. Allicin is reactive sulphur species and it unspecifically reacts with glutathione and cysteine residues in proteins, disrupting the cell's redox state. Its lack of specificity makes it unsuitable as a drug and any of the health claims are difficult to prove.

A number of phytochemicals reported to have antibiotic activity are compounds known as pan assay interference compounds (PAINs) [109]. They are artefacts of the screening procedure and their activity is due to unspecific interaction between the molecule and proteins. PAINs can interfere with the redox state of the cell, covalently modify the

target protein, disrupt cell membranes, sequester metal ions from target proteins, or be chemically unstable themselves. An example is resveratrol which is listed to have a wide impact on human health: having anticancer [110, 111] and antidiabetic properties [112], suppressing angiogenesis [113], improving memory [114], protecting against coronary heart disease [115] and Alzheimer's disease [116], but also has been described as a PAIN [117].

The lack of plant-derived antibiotics is not due to plants lacking antimicrobial compounds. An example of a successful antimicrobial drug in clinical use is the antimalarial artemisinin is isolated from *Artemisia annua* herb. The example of arteminisin illustrates a number of issues in drug discovery from plant sources. Firstly phytochemicals are notoriously difficult to purify. The purification of the active component from the infusion of *A. annua*, used in traditional medicine for the treatment of malarial fever, was not possible. Only after ethyl ether extraction was utilised, the component was purified, but even then it was not identified until seven years later [118]. The compounds are present in small quantities, often in mixtures with other similar compounds. A hectare of *A. annua* plants produces between 3.39 and 24.39 kg artemisinin [119]. Plant extracts are much more difficult to prepare and purify on high throughput platforms, because of the small proportion of active ingredient.

Plants grow much slower than bacteria and their genomes are less dynamic. However, plant genomes are extremely large with a proportion devoted to fast-evolving secondary metabolism. In a study of the natural variation of *Arabidopsis thaliana* metabolome only 13.4% metabolites were shared between different accessions [120] revealing a diversity of phytochemicals even within single plant species. Such diversity makes the metabolome an ideal resource for novel chemical scaffolds.

1.6 What can we learn from the insect gut bacteria?

Interestingly, insects feeding on plants can harbour antibiotic resistant bacteria in their guts [121]. The authors developed a method to identify novel antibiotic resistance elements by screening the metagenomic sequencing library from gypsy moth larvae

(Lepidoptera, *Lymantia diaspar*) for resistance to antibiotics when plated on solid media. Normally genes are identified *in silico* by homology to known genes and novel genes, but such approach undersamples novel or unusual genes.

The functional metagenomics of cultured bacteria from the gypsy moth larvae revealed a reservoir of antibiotic resistant genes. Six different isolates were selected: two were resistant to penicillin-type β-lactam antibiotics, another two were moderately resistant to erythromycin and the last two were moderately resistant to a panel of antibiotics (erythromycin, carbenicillin and chloramphenicol). The resistance was produced by varied mechanisms: efflux pump, extended-spectrum β-lactamase, and a transcriptional factor. Worryingly the levels of antibiotic resistance in the gypsy moth guts were clinically relevant and readily transferable.

The authors hypothesised that the resistance in the insect guts could have been selected for by the toxins in the insects' food. The insect diets compromised a variety of plant species: larch, white oak, willow, and aspen. The phenomena described in this paper inspired us to investigate further the insect gut. Among our aims, stemming out of this research, was to test if the antibiotic resistance observed in the gypsy moth guts is a universal property and to test if it is correlated or linked to phytotoxins present in the insect food.

1.7 Summary and aims

In summary, this chapter introduced the diversity of plant secondary metabolites, insects and their microbiomes. I closed the chapter introducing antibiotics and antibiotic resistance. It is intended as a background for three following results chapters: "The impact of antibiotics on the gut microbiota of two lepidopteran hosts: *Plutella xylostella* and *Galleria mellonella*", "Investigating antibiotic resistance in the insect gut bacteria: the search for novel plant-derived antibacterials" and "Developing *Galleria mellonella* (Greater wax moth) larvae into a laboratory model: acute toxicity trials, antibiotic susceptibility testing and replacement of native gut bacteria with human faecal microbiota". In these chapters I will describe the experimental work of the project.

The main aim of the first results chapter was to test whether antibiotics in the lepidopteran host's diet cause the gut bacteria to acquire antibiotic resistance. Specifically I tested whether the insect gut bacteria acquire single-antibiotic and multi-antibiotic resistance after exposure to either ciprofloxacin or oxytetracycline. In the second results chapter I wanted to test if the antibiotic resistance present in the insect gut can be induced by the plant-derived toxins present in the insect food and if we can utilize the resistance to these toxins in assay-guided purification of the plant extracts. Finally the aim of the last chapter was to assess the usefulness of *G. mellonella* in toxicity testing and antibiotic efficacy assays.

2 Materials and methods

2.1 Media and solutions

2.1.1 Solutions

Alkaline PEG reagent: Mix 60 g poly(ethylene glycol) 200 (Sigma-Aldrich), 0.93 mL 2 M KOH, and 39 mL distilled water. Adjust pH to 13.3 – 13.5 with KOH. Store at -4°C.

PBS buffer: Add 8.0 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4 and 0.24 g/L KH_2PO_4 to MiliQ water (Merck). The ready solution should have a pH of 7.4 when the correct ratios of phosphates are used. If necessary, carefully adjust pH to 7.4 with KOH. Sterilize by autoclaving for 20 minutes at 121°C.

PBS/tergitol: Prepare PBS as described above. Sterilize by autoclaving for 20 minutes at 121°C. Add 0.5% (w/v) tergitol and sterilize the solution by filtration through Minisart syringe filters (16532-K, Sartorius Stedim).

TAE agarose: Use 50× TAE base (Formedium; final concentrations: 40 mM TRIS base, 20 mM acetic acid, 1 mM Na_2EDTA) with 1.0 or 1.8% agarose (w/v). 1.0% agarose gels were used for 16S PCR products and 1.8% agarose gels were used for tet-resistance PCR products.

2.1.2 Media

Lysogeny Broth (LB) medium: Use LB base (LMM0202, Formedium; final concentrations: 1% (w/v) tryptone, 0.05% (w/v) yeast extract, 1% (w/v) NaCl). Adjust pH to 7.0 with HCl. For solid media add 1% agar (AGA03, Formedium). Sterilize by autoclaving for 20 minutes at 121°C.

LBG: Supplement LB medium with glucose at 20 g/L (G8270, Sigma Chemicals). Sterilize by autoclaving for 20 minutes at 121°C.

MacConkey Agar: Use MacConkey Agar base (212123, Difco; final concentrations: 17.0 g/L pancreatic digest of gelatin, 3.0 g/L peptone from meat and casein, 10.0 g/L lactose, 1.5 g/L bile salts no. 3, 5.0 g NaCl, 13.5 g/L agar, 0.03 g/L neutral red, 1.0 mg/L crystal violet) according to the manufacturer's instructions. Adjust pH to 7.1 with HCl. Sterilize by autoclaving for 20 minutes at 121°C.

Mueller-Hinton Agar: Use Mueller-Hinton Agar base (105437, Merck; final concentrations: 2.0 g/L meat infusion, 17.5 g/L casein hydrolysate, 1.5 g/L starch, 13.0 agar) according to the manufacturer's instructions. Adjust pH to 7.2 - 7.6 with HCl. Sterilize by autoclaving for 20 minutes at 121°C.

Reinforced Clostridia Agar (RCA): Use Reinforced Clostridia Broth base (27546, Sigma Chemicals; final concentrations: 3.0 g/L yeast extract, 10.0 g/L Lab-Lemco powder, 10.0 g/L peptone, 5.0 g/L glucose, 1.0 g/L soluble starch, 5.0 g/L NaCl, 3.0 g/L sodium acetate, 0.5 g/L cysteine hydrochloride) according to the manufacturer's instructions. Add 1% (w/v) agar (AGA03, Formedium). Adjust pH to 6.8 with HCl. Sterilize by autoclaving for 20 minutes at 121°C.

Tryptic Soy Agar (TSA): Mix 17.0 g/L peptone from casein, 3.0 g/L peptone from soymeal, 2.5 g/L glucose, 5.0 g/L NaCl, 2.5 g/L K_2HPO_4, and 10 g/L agar in MiliQ water (Merck). Adjust pH to 7.3 with HCl. Sterilize by autoclaving for 20 minutes at 121°C.

TY agar: Add 6 g/L tryptone, 3 g/L yeast extract, 0.38 g/L $CaCl_2$, and 10 g/L agar to MiliQ water (Merck). Adjust pH to 7.0 with HCl. Sterilize by autoclaving for 20 minutes at 121°C.

2.1.3 Test compounds

Toxicity testing: Adenosine triphosphate, carbenicillin, chloramphenicol, chloroquine, ciprofloxacin, etoposide, glucose, glutamic acid, novobiocin, sodium chloride, streptomycin S6501, and tetracycline were supplied by Inspiralis Ltd. at 25 mg/ml in 50% DMSO in water (Table 2-1). Due to insolubility issues, amsacrine was supplied at 8 mg/ml in 50% DMSO in water, kanamycin was supplied at 25 mg/ml in 25% DMSO in

water, and norfloxacin was supplied at 25 mg/ml in 50% DMSO in water with 50 mM KOH in the final concentration. Doxurubicin was only available as a liquid and was supplied at 5.5 mg/ml in 50% DMSO in water. A tube of pure 50% DMSO in water was also supplied. All compounds were supplied in numbered tubes without compound names to avoid bias in the experiment. The identity of the compounds was assigned only when the testing procedure was finished and the data from *Galleria mellonella* was compared to material safety data sheet (MSDS) pages available with the compounds.

Table 2.1: The identity of compounds in the trial. The compounds were made at 25 mg/mL in 50% DMSO in water unless indicated otherwise.

Compound name	Stock solution concentration [mg/mL]	Notes
Adenosine triphosphate	25	
Amsacrine	8	Made at 8 mg/ml as precipitated at 25 mg/ml
Blank: 50% DMSO in water	0	
Carbenicillin	25	
Chloramphenicol	25	
Chloroquine	25	
Doxurubicin	5.5	Only liquid available, made at 5.5 mg/ml
Etoposide	25	
Glucose	25	
Glutamic acid	25	
Kanamycin	25	Dissolved in 25% DMSO in water as precipitated in 50%
Norfloxacin	25	Made in 50% DMSO in water with 50 mM KOH
Novobiocin	25	
Sodium chloride	25	
Streptomycin S6501	25	Dissolved in 25% DMSO in water as precipitated in 50%
Tetracycline	25	

Antibiotic efficacy testing: Ampicillin, ciprofloxacin, rifampicin and tetracycline for efficacy testing were obtained from Sigma Chemicals.

Determination of minimal inhibitory concentration: Vindoline, loganin, ajmalicine and catharanthine were obtained from Sigma Chemicals and resuspended in ethanol at 5 mg/mL.

2.2 Insect methods

2.2.1 Insect husbandry

Plutella xylostella and *Galleria mellonella* were the only insects reared in the lab by us. All other insects were collected and dissected on the same day or reared in the JIC Insectary by the Insectary staff.

***Plutella xylostella*:** Second to fourth instar *P. xylostella* larvae were obtained from the JIC Insectary. The larvae were kept at room temperature in standard Petri dishes (90 mm, Sterilin) with four 5×5×5 mm cubes of artificial food. The food was replaced daily.

Galleria mellonella: A colony of *G. mellonella* was obtained from the JIC Insectary. The colony was kept in the dark at 30°C for antibiotic feeding procedure (see Chapter 3) and toxicity testing, and at 37°C for antibiotic efficacy testing (see Chapter 5). The larvae were kept in large Petri dishes (140 mm, Sterilin) filled with artificial food to burrow through. The food was replaced at least once per week, unless not enough was left for the larvae to feed on, in which case more food was added to the containers.

2.2.2 Artificial food

***Plutella xylostella* food:** General Purpose Diet for Lepidoptera (soy and wheat germ based; F9772, Frontier Agricultural Services) was prepared according to the manufacturer's instructions. Antibiotics were added to the agar base of the diet before mixing with the rest of ingredients. Ciprofloxacin was added at a range of concentrations: 1 µg/mL final food volume, 3 µg/mL, 10 µg/mL and 30 µg/mL, and oxytetracycline was added at a single concentration 3 µg/mL. The food was set in square petri dishes (100 mm, Sterilin), cut to 5×5×5 mm cubes, and stored in sealed containers at 4°C when not used.

***Galleria mellonella* food**: The artificial food was composed of 300 mL honey (Sainsbury's Honey, Clear), 400 mL glycerol (G5516, Sigma Chemicals), 200 g milk powder (Dried Skimmed Milk Powder, Marvel), 200 g wholemeal flour (Strong

Stoneground 100% Wholemeal Flour, Sainsbury's), 100 g yeast powder (103753, Merck), 100 g wheat germ (Neal's Yard Wholefoods Natural Wheatgerm) and 400 g bran (Neal's Yard Wholefoods Natural Wheat Bran). First, the dry and wet ingredients were mixed separately, and then the mixtures were mixed together. The diet was mixed with beeswax pellets at a 2:1 ratio. Unused food was stored at 4°C. Oxytetracycline (OTC) was added to the glycerol batches at 1 mg/100 g diet dry weight, 10 mg/100 g diet dry weight, 100 mg/100g diet dry weight, 400 mg/100 g diet dry weight and 1000 mg/100 g diet dry weight. The food was prepared fresh at least once per week.

2.2.3 Collection of insects

These insects were collected as plant-insect pairs (see Chapter 4). Two adult giant lime green stick insects (*Diapherodes gigantea*) were collected from the JIC Insectary after they had died of old age. The insects were immediately frozen and stored at -20°C until dissection. Five *Plutella xylostella* larvae, ten death's head hawkmoth larvae (*Acherontia atropos*) and ten beet army worm (*Spodoptera exigua*) larvae were obtained from the JIC Insectary. Eleven cinnabar moth larvae (*Tyria jacobaeae*) were collected from the playing fields of University of East Anglia. Ten rosemary beetles (*Chrysolina americana*) were collected from lavender plants on the JIC site. Where possible the insects were starved for two hours before dissection to enrich the gut contents in bacteria. The insects were flash frozen in liquid nitrogen and surface sterilized in 70% ethanol with a subsequent rinse in distilled water.

2.2.4 Insect dissection

***Galleria mellonella* dissections**: All steps of the procedure were carried out on ice and in a biological safety cabinet. A sterile Petri dish was used as a dissecting surface. One larva was dissected at a time, to prevent defrosting of the samples. The head of a larva was cut off, while the insect was stabilized with forceps. While still stabilizing the insect, an incision was made down the abdomen on the ventral side of the body using a sterile razor (Figure 2-1). The gut contents, which are brittle when frozen, were

carefully picked out using fresh sterile forceps and placed in a sterile, pre-weighed 2 mL tube. Three guts per tube were collected.

Rosemary beetle (*Chrysolina americana*) gut sample preparation: The rigid exoskeleton of the beetles prevented accurate dissection of the gut contents. Instead whole insects were homogenized with sterile micropestles in 1.5 mL Eppendorf tubes with 200 µL PBS buffer.

Other insect dissections: All steps of the dissection procedure were carried out in the biological safety cabinet. A sterile Petri dish was used as a dissecting surface. The general procedure followed is shown in Figure 2-1. Using a sterile razor and stabilizing the insect using sterile forceps the head of the insect was cut off. Still stabilizing the insect body, an incision was made on the ventral side starting at the previous cut and running down the length of the body. The gut was identified as a large tubular structure running along the body. Where possible the gut tissue was cut open and the frozen gut contents were collected. In other cases the entire gut was removed. Care was taken not to confuse the insect gut with other internal body structures, such as the Malphigian tubes, which can resemble the mammalian gut.

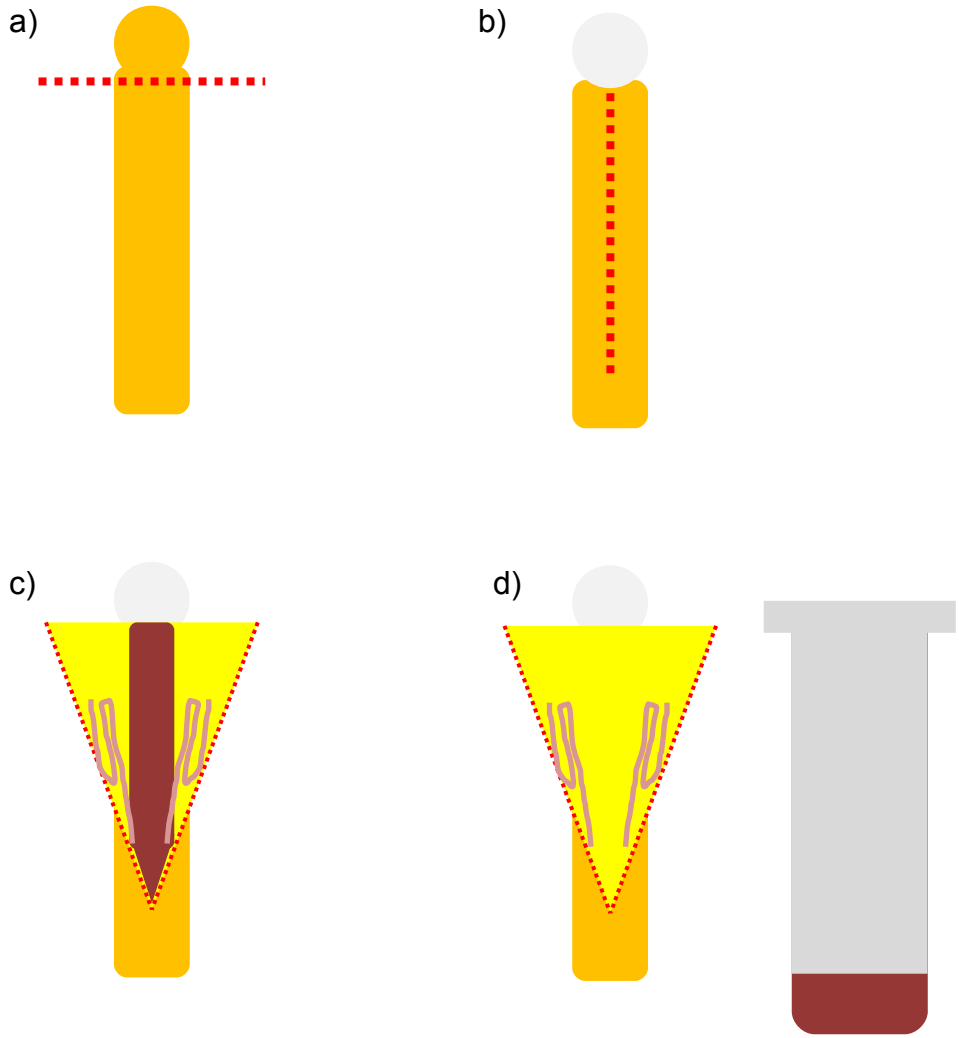


Figure 2.1: A schematic of general dissection procedure. Prior to dissection, the insects were euthanized by immersion in liquid nitrogen. All procedures were performed on ice and under sterile conditions. a) Decapitate the insect. b) Make an incision down the abdomen of the insect. c) Open the body cavity separating the insect tissues (yellow) from the gut contents (brown). d) pick the gut contents with sterile forceps and place in an Eppendorf tube.

2.2.5 Experimental procedures

***Plutella xylostella* feeding procedure:** Third and fourth instar *P. xylostella* larvae feeding on cabbage were collected from the JIC insectary. The larvae were divided in groups of five and each group was supplied with different food: artificial food with no antibiotic or artificial food with a dose of an antibiotic. The antibiotic doses were: 1

µg/mL ciprofloxacin, 3 µg/mL ciprofloxacin, 10 µg/mL ciprofloxacin, 30 µg/mL ciprofloxacin, and 3 µg/mL oxytetracycline. Fresh artificial food was supplied daily for three days. After three days the larvae had their guts dissected.

***Galleria mellonella* feeding procedure.** The colony was divided into six groups, each group feeding on a different diet. Ten fifth instar larvae were collected for gut dissection as described above. The remaining ones were moved to larger containers to pupate. The pupae were moved to clean containers to pupate and the moths were supplied with honey solution. The moths laid eggs along the edges of the lining of the container. Eggs were collected and placed on fresh food with or without antibiotics. This procedure was followed for five generations. Larval mortality was high in the groups feeding on 10 mg OTC/100 g diet or more, leading to cases when no individuals feeding on those oxytetracycline concentrations were left. In such cases, a new colony was established from antibiotic-free larvae on the appropriate antibiotic diet.

***Galleria mellonella* injection procedure**: Five to ten larvae (250-320 mg each) were selected at random for each step in the procedure. Any larva with darkening of the cuticle was discarded. The test compounds were injected into the hemocoel in DMSO or PBS buffer through the last left proleg (Hamilton syringe 701N, volume 10 µL, needle size 26s, cone tip) unless stated otherwise. The larvae were placed on medical tissues (Kimtech) to stop hemolymph leaking. The larvae were incubated in the dark for 5 days and mortality was recorded daily.

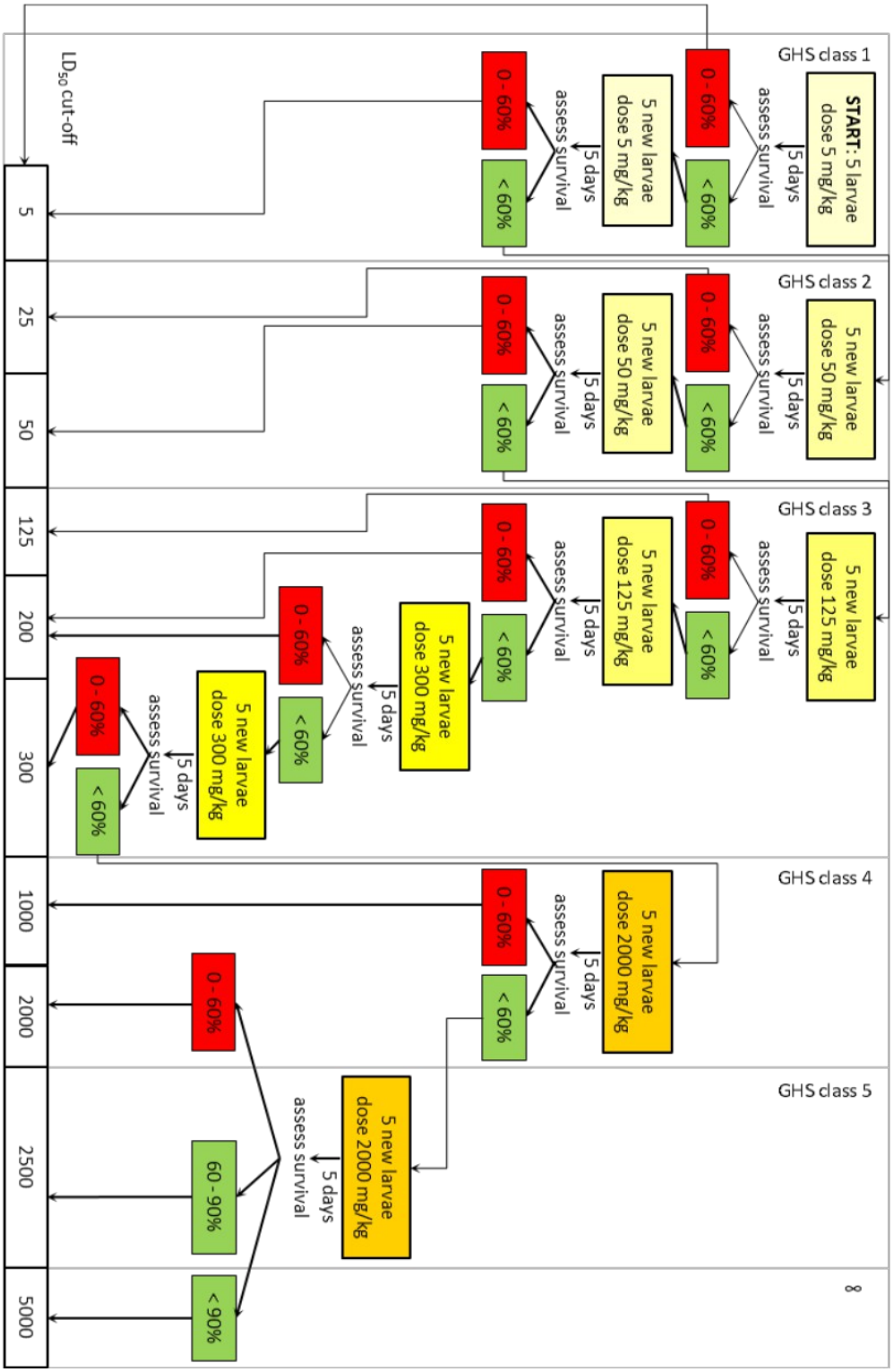


Figure 2.2: A flowchart representing consecutive steps in the acute toxicity test. A starting dose of 5 mg/kg body weight was administered and the insects were scored for mortality. If the mortality was over 40%, the compound is assigned the highest toxicity class. If the mortality is below 40%, the dose was re-tested and the testing continued until a toxic dose was established.

100% DMSO toxicity testing procedure: A flowchart, adapted from the OECD guidelines for acute toxicity [122], was used to select the toxic dose of test compounds (Figure2- 2). The acute toxicity testing was started by injecting five larvae with the initial dose of a compound (5 mg/kg body weight). Larvae mortality was recorded daily. If three or more larvae died, the compound was assigned the highest toxicity class (GHS 1). If three or more larvae survived for five days, the toxicity testing was continued by re-testing the initial dose (5 mg/kg body weight) on a new cohort of larvae. If three or more larvae of the second cohort survived, a higher dose (25 mg/kg body weight) was tested in five fresh larvae. The experiment was continued until a toxic dose was established. If a compound was not toxic at the highest tested dose (2000 mg/kg body weight), the compound was classified as non-toxic. The obtained toxic dose was compared to a dose reported in Material Safety Data Sheet (MSDS) page for the compound. Where possible the reported value used for the comparison was from mouse or rat via an intraperitoneal injection, when such data were absent the data from oral toxicity tests in a mammalian system were used. Each step in the procedure included three control groups: untreated control, traumatized control (cuticle was pierced with a needle) and buffer-injected control.

Low-DMSO toxicity testing procedure: A second round of testing was conducted to control for DMSO toxicity. Ciprofloxacin, sodium chloride, tetracycline, glucose and streptomycin solutions were prepared with PBS buffer and tested by an intraperitoneal injection on *G. mellonella* at 300 and 2000 mg/kg body weight. Five larvae were used per test group. The test doses of the compounds in PBS buffer were injected into the hemocoel of the larvae as described before. The mortality was recorded daily for five days until a toxic dose was established.

Determination of the infective dose of bacteria to *Galleria mellonella*: An infective dose of bacteria was determined by injecting groups of five larvae with bacterial suspension at 5×10^4 colony forming units (cfu) per injection, 5×10^5 cfu, 5×10^6 cfu and 5×10^7 cfu. The larvae were incubated for five days. An infective dose was one that caused an immune response, recognizable by the darkening of the cuticle. In *G.*

mellonella larvae immune response leads to the formation of melanin plaques around bacteria immobilized by the cells of immune system. These plaques appear dark through the cuticle. An infective dose of bacteria was one that caused 60-80% lethality within 48 h, but not 100% lethality within 24 h. The larvae were incubated at 37°C as bacterial virulence changes with temperature and the experiment was designed to mimic infection in humans.

Antibiotic efficacy testing in *Galleria mellonella*: A flowchart was used to assign an antibiotic therapeutic dose against a panel of bacteria (Figure 2-3). For the antibiotic efficacy experiment five larvae were injected into the last left proleg with a pre-determined infective dose of bacteria and incubated for two hours at 37°C. After the incubation the larvae were injected in the last right proleg with the lowest dose of antibiotic (5 mg/kg body weight) and returned to incubation at 37°C. The mortality was recorded daily for five days. If three or more larvae survived, the lowest dose was re-tested and the lowest antibiotic dose was assigned as therapeutic. If three or more larvae died, the antibiotic was tested against the same infective dose of bacteria at a higher dose (25 mg/kg body weight). The experiment was continued until a therapeutic dose was assigned or an antibiotic was ineffective against the infection. The values obtained were compared to values recommended for human use [123]: ampicillin – 50-200 mg/kg body weight/day, ciprofloxacin – 10-15 mg/kg body weight/day, tetracycline – 25-50 mg/kg body weight/day and rifampicin – 10-20 mg/kg body weight/day. Each step in the procedure included three control groups: untreated control, traumatized control (cuticle was pierced with a needle) and buffer-injected control.

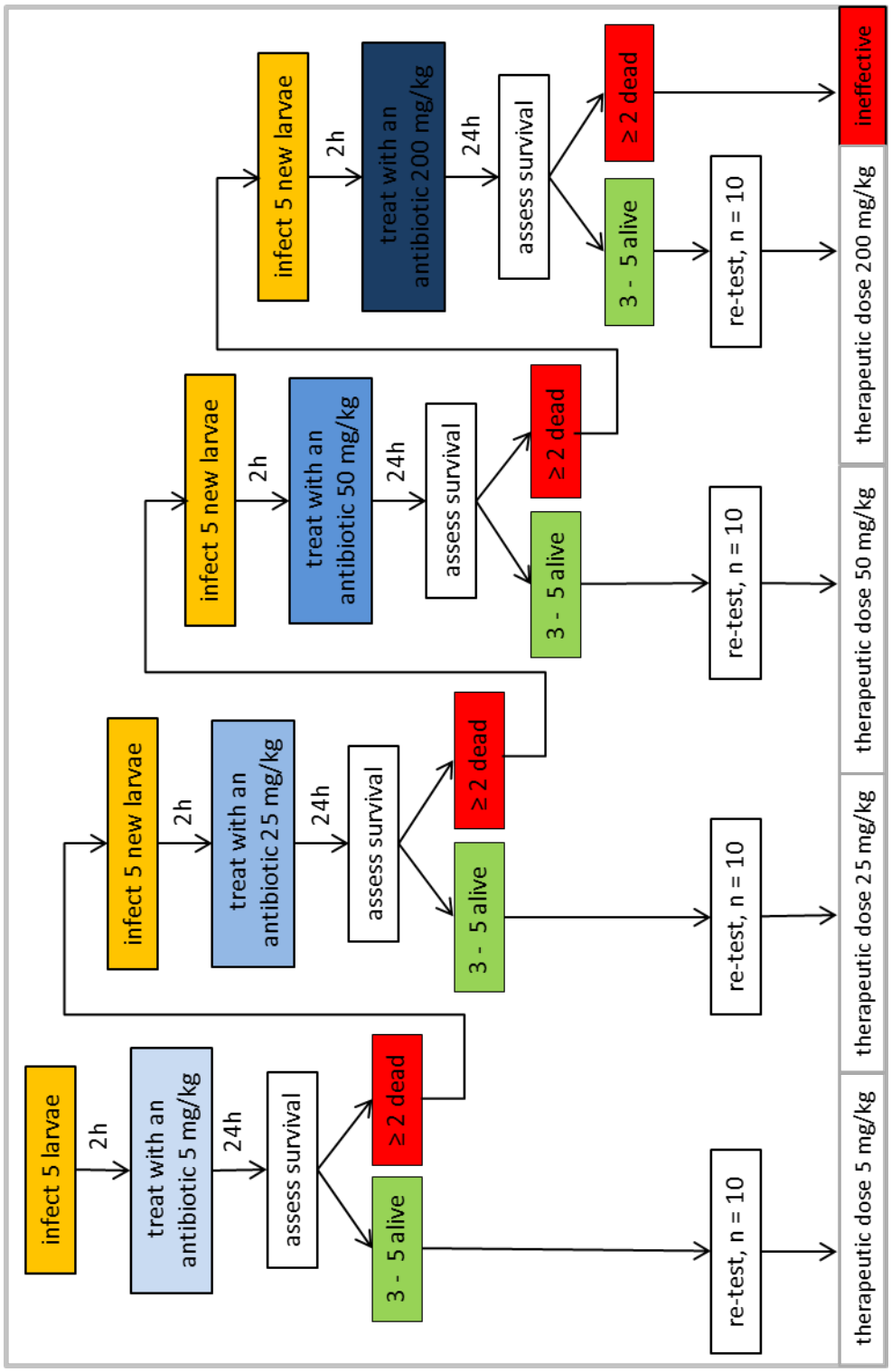


Figure 2.3: A flowchart representing consecutive steps in the antibiotic efficacy test. A flowchart representing consecutive steps in the antibiotic efficacy test. A starting dose of 5 mg/kg body weight was administered and the insects were scored for survival. If the mortality was under 40%, the compound was assigned the lowest therapeutic dose. If the mortality was over 40%, a higher dose was tested until a therapeutic dose was established.

Artificial food for replacing native microbiota with baby gut bacteria: Faecal slurry (gift from Lindsay Hall, Institute of Food Research) was prepared by homogenizing of 6.3 g baby V3 faeces and 18 mL of sterile PBS with sterile glass beads. The suspension was filtered through 40 µm filters and 3 mL solution was mixed with 15 g insect food. The food was replaced at least every 2-3 days. When not used the faecal slurry was stored at -80°C.

Procedure for replacing native microbiota with baby gut bacteria: Two groups of 36 third instar larvae were obtained from the JIC Insectary: a group feeding on artificial food with slurry and a control group. Nine larvae were collected from each group before the beginning of the feeding procedure. The larvae were supplied with artificial food and artificial food with faecal slurry. After three days nine larvae were collected from each group and fresh artificial food with and without faecal slurry was supplied to the remaining insects. After one more day a further nine larvae were collected from each group and fresh artificial food was supplied again. After seven days from the beginning of the experiment the remaining larvae were dissected.

2.3 Microbiology

2.3.1 General procedure for identifying insect gut bacteria

Culturing of insect gut bacteria: The gut contents were suspended in 200 µL PBS buffer and diluted 10, 100 and 1000 times. 50 µL of each dilution were plated on LB, LBG and TSA media. The plates were incubated for one to three days at 30°C in aerobic conditions. The isolates were streaked out on fresh agar plates and incubated as before. This ensured the purity of the bacterial isolates before identification.

Identification of bacteria: Each isolate was identified using 16S PCR with alkaline PEG reagent using 63f and 1389r primers (5' CAGGCCTAACACATGCAAGTG 3' and 5' ACGGGCGGTGTGTACAAG 3') and *Taq* DNA polymerase (28104, Quiagen). Single colonies were picked from an agar plates and resuspended in 500 µl distilled water. The

samples were spun down at 6000 rpm for 4 minutes. Without disturbing the pellets, 490 µl sample was removed and 100 µl alkaline PEG reagent was added. The samples were mixed well by pipetting and incubated for 15 minutes at room temperature. 1 µl was added to the *Taq* PCR mix, prepared according to the manufacturer's instructions. Reactions were carried out in a PTC-200 Thermo Cycler (MJ Research). The initial denaturation was carried out for 10 minutes at 95°C, followed by 30 cycles of denaturation (95°C for 1 minute), annealing (57°C for 1 minute), and extension (72°C for 2 minutes). The final extension was carried out at 72°C for 10 minutes. The PCR products were soaked at 10°C until further use. The PCR products were separated on 1% agarose TAE gels and purified using QIAquick PCR Purification Kit (28104, Qiagen). DNA was sequenced using a BigDye v3.1 kit (Applied Bioscience) in a 10 µL reaction volume. Reactions contained BigDye 3.1 mix, 1x reaction buffer, 50-100 ng DNA template and 20 µM sequencing primer. Reactions were carried out in a PTC-200 Thermo Cycler (MJ Research). The initial denaturation was carried out for 1 minute at 95°C, followed by 30 cycles of denaturation (95°C for 30 seconds), annealing (45°C for 15 seconds), and extension (60°C for 4 minutes). The final extension was carried out at 72°C for 10 minutes. The PCR products were soaked at 10°C until further use. The samples were sent to The Genome Analysis Centre for processing. Sequencing data were returned in the form of .txt and .abi chromatogram trace files. The sequences were trimmed to remove poorly recognized bases and run through the blastn algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against "Nucleotide collection (nr/nt)" database. Bacteria were identified if the sequence was ≥97% similar to 16S RNA gene in the database and had an e-value close or equal to 0.

Table 2.2: Origin and summary of the type strains used. Pathogenicity group 1 are not a hazard to human health, but pathogenicity group 2 bacteria are opportunistic human pathogens.

Species name	Collection number	Collection	source	pathogenicity group	Gram stain
Bacillus amyloliquefaciens	ATCC 23350	CRBIP	soil	1	positive

Species name	Collection number	Collection	source	pathogenicity group	Gram stain
Bacillus aquimaris	DSM 16205	DSMZ	sea water of a tidal flat	1	positive
Bacillus licheniformis	ATCC 14580	DSMZ	soil	1	positive
Bacillus subtilis	ATCC 6051	HPA	boiled hay infusion	1	positive
Bacillus vietnamensis	DSM 18898	DSMZ	vietnamese fish sauce	1	positive
Burkholderia fungorum	CIP 107096T	CRBIP	fungus	2	negative
Enterobacter amnigenus	ATCC 33072	HPA	soil	2	negative
Enterobacter asburiae	ATCC 35953	DSMZ	lochia	2	negative
Escherichia coli	ATCC 25922	HPA	human	2	negative
Kocuria rhizophila	ATCC BAA-50	CRBIP	rhizosphere of *Typha angustiflora*	1	positive
Microbacterium foliorum	DSM 12966	CRBIP	phyllosphere of grasses	1	positive
Microbacterium gubbeenense	DSM 15944	DSMZ	surface of a smear-ripened cheese	1	positive
Microbacterium oxydans	DSM 20578	CRBIP	air	1	positive
Microbacterium paraoxydans	DSM 15019	DSMZ	human blood	2	positive
Pantoea agglomerans	ATCC 27155	DSMZ	knee laceration	2	negative
Pseudomonas putida	ATCC 12633	DSMZ	soil	2	negative
Raoultella terrigena	ATCC 33257	CRBIP	drinking water	1	negative
Rhizobium pusense	DSM 22668	DSMZ	rhizosphere soil of chickpea	1	negative

Species name	Collection number	Collection	source	pathogenicity group	Gram stain
Rhodococcus erythropolis	ATCC 4277	CRBIP	soil	1	positive
Sanguibacter keddieii	ATCC 51767	CRBIP	bovine blood	1	positive
Sphingobacterium multivorum	ATCC 33613	CRBIP	human spleen	2	negative
Staphylococcus epidermidis	ATCC 14990	CRBIP	human nose	2	positive
Staphylococcus warneri	ATCC 27836	CRBIP	human skin	1	positive

Type strains. For each identified gut-isolated strain a corresponding type strain was obtained from one of the following culture collections: Centre de Resources Biologiques de l'Institut Pasteur (Paris), Health Protection Agency (Salisbury) or Deutsche Sammlung von Mikroorganismen und Zellkulturen (Brunswick). The strains obtained were: *Bacillus amyloliquefaciens* ATCC 23350, *Bacillus aquimaris* DSM 16205, *Bacillus licheniformis* ATCC 14580, *Bacillus subtilis* ATCC 6051, *Bacillus vietnamensis* DSM 18898, *Burkholderia fungorum* CIP 107096T, *Enterobacter amnigenus* ATCC 33072, *Enterobacter asburiae* ATCC 35953, *Escherichia coli* ATCC 25922, *Kocuria rhizophila* ATCC BAA-50, *Microbacterium foliorum* DSM 12966, *Microbacterium gubbeenense* DSM 15944, *Microbacterium oxydans* DSM 20578, *Microbacterium paraoxydans* DSM 15019, *Pantoea agglomerans* ATCC 27155, *Pseudomonas putida* ATCC 12633, *Raoultella terrigena* ATCC 33257, *Rhizobium pusense* DSM 22668, *Rhodococcus erythropolis* ATCC 4277, *Sanguibacter keddieii* ATCC 51767, *Sphingobacterium multivorum* ATCC 33613, *Staphylococcus epidermidis* ATCC 14990, and *Staphylococcus warneri* ATCC 27836. The origin of each strain is given in Table 2-2. Strains classified as type 2 pathogens were handled according to JIC Standard Operating Procedure “Culturing Hazard Group 2 bacteria from insect guts”.

2.3.2 Methods used for *Galleria mellonella*

Culturing of gut bacteria: 250 µl PBS/tergitol was added to each tube. Each tube was disrupted in a FastPrep FP120 instrument (Qbiogene) for 45 s, five times. 50 µl of neat suspension, 1:200 dilution and 1:4000 dilution were plated on LB, RCA and MacConkey media. The plates were incubated for 24-48 h at 30°C in aerobic (LB and MacConkey media) and anaerobic (RCA) conditions.

Culturing of bacteria for antibiotic efficacy testing: *Escherichia coli* (ATCC 25922), *Mycobacterium smegmatis* (ATCC 700084), *Pseudomonas aeruginosa* (ATCC 15692) and *Staphylococcus aureus* (ATCC 29213) were grown from glycerol stocks in LB broth at 37°C.

Identification of tetracycline resistance elements. The gut samples with large gDNA fragments were subjected to diagnostic PCRs to establish if tetracycline genes are present. Ex Taq DNA polymerase (Clontech-Takara) was used in a 25 µL reaction volume, containing 10 ng template DNA. All reactions were carried out in a Mastercycler nexus X2 (Eppendorf). The initial denaturation was carried out at 94°C for 10 minutes, followed by 35 cycles of denaturation (94°C for 30 seconds), annealing (variable temperature for 1 minute), and extension (72°C for 2 minutes). The final extension was carried out at 72°C for 10 minutes. The PCR products were soaked at 10°C until further use. The primers used are summarised in Table 2-3.

Table 2.3: A list of primers used to identify tet-resistance genes

	Primer	*Target gene*	*Sequence (5'-3')*	*size (bp)*	*Reference*
1	63f	16S rRNA	CAGGCCTAACACATGCAAGTG	~500	Weisburg 1991[124]
	1389r		ACGGGCGGTGTGTACAAG		
2	TetA-F	tetA	GCTACATCCTGCTTGCCTTC	210	Fan et al. 2007 [125]
	TetA-R		CATAGATCGCCGTGAAGAGG		
3	TetB-F	tetB	TTGGTTAGGGGCAAGTTTTG	659	Fan et al. 2007 [125]
	TetB-R		GTAATGGGCCAATAACACCG		

	Primer	*Target gene*	*Sequence (5'-3')*	*size (bp)*	*Reference*
4	TetC-F	tetC	CTTGAGAGCCTTCAACCCAG	418	Fan et al. 2007 [125]
	TetC-R		ATGGTCGTCATCTACCTGCC		
5	TetD-F	tetD	GAATGCCTGCACCTTTCTGATG	346	Fan et al. 2007 [125]
	TetD-R		GGCAATAAATCCGGCGAAAA		
6	TetE-F	tetE	TCGGGATTGTTAGTTGTCTTTTTC	549	Fan et al. 2007 [125]
	TetE-R		GTGGATTACCCTACCTGGATGGA		
7	TetG-F	tetG	GCTCGGTGGTATCTCTGCTC	468	Fan et al. 2007 [125]
	TetG-R		AGCAACAGAATCGGGAACAC		
8	TetH-F	tetH	GTGATGTGACTCCCGCTAAAAAT	407	Fan et al. 2007 [125]
	TetH-R		CCAGAACCGCCAAAGACATACC		
9	TetJ-F	tetJ	ACAGACTCGCCAATCATTACGGTA	300	Aminov et al. 2002, 2004 [126, 127]
	TetJ-R		GCACCACCCAAAAAACCGAAAT		
10	tetL_121F	tetL	CCGGCGAGTACAAACTGGGTGA	215	Tian et al., 2012 [37]
	tetL_335R		GCAGCTGCACCAGCTCCTTGAAT		
11	tetL_363F	tetL	GCGCCTCTGCGAAAGGTACGC	631	Tian et al., 2012 [37]
	tetL_994R		GCGCGCAACTACAACCATCACGAG		
12	TetY-F	tetY	ATTTGTACCGGCAGAGCAAAC	181	Aminov et al. 2002, 2004 [126, 127]
	TetY-R		GGCGCTGCCGCCATTATGC		
13	TetZ-F	tetZ	CCTTCTCGACCAGGTCGG	204	Aminov et al. 2002, 2004 [126, 127]
	TetZ-R		ACCCACAGCGTGTCCGTC		
14	Tet30-F	tet30	CATCTTGGTCGAGGTGACTGG	210	Aminov et al. 2002, 2004 [126, 127]
	Tet30-R		ACGAGCACCCAGCCGAGC		
15	TetBP-F	tetBP	AAAACTTATTATATTATAGTG	169	Aminov et al. 2001, 2004 [126, 127]
	TetBP-R		TGGAGTATCAATAATATTCAC		
16	TetM-F	tetM	ACAGAAAGCTTATTATATAAC	171	Aminov et al. 2001, 2004 [126, 127]
	TetM-R		TGGCGTGTCTATGATGTTCAC		
17	TetO-F	tetO	ACGGARAGTTTATTGTATACC	171	Aminov et al. 2001, 2004 [126, 127]
	TetO-R		TGGCGTATCTATAATGTTGAC		
18	TetQ-F	tetQ	AGAATCTGCTGTTTGCCAGTG	169	Aminov et al. 2001, 2004 [126, 127]
	TetQ-R		CGGAGTGTCAATGATATTGCA		

	Primer	*Target gene*	*Sequence (5'-3')*	*size (bp)*	*Reference*
19	TetS-F	tetS	GAAAGCTTACTATACAGTAGC	169	Aminov et al. 2001, 2004 [126, 127]
	TetS-R		AGGAGTATCTACAATATTTAC		
20	TetT-F	tetT	AAGGTTTATTATATAAAAGTG	169	Aminov et al. 2001, 2004 [126, 127]
	TetT-R		AGGTGTATCTATGATATTTAC		
21	TetW-F	tetW	GAGAGCCTGCTATATGCCAGC	168	Aminov et al. 2001, 2004 [126, 127]
	TetW-R		GGGCGTATCCACAATGTTAAC		
22	Tet32-F	tet32	TCGACCTACAGCGTGTTTACC	277	Aminov et al. 2001, 2004 [126, 127]
	Tet32-R		CTAATAGTTCATCGCTTCCGG		
23	Tet34-F	tet34	TGCTGAAAAACAGATGCCAG	212	Szczepanowski et al., 2009 [128]
	Tet34-R		TAACCTTCGCCATCACCTTC		
24	tetX-1	tetX, tetX2	TTAGCCTTACCAATGGGTGT	242	Bartha et al., 2011 [129]
	tetX-2		CAAATCTGCTGTTTCACTCG		
25	tetX1-1	tetX	TCAGGACAAGAAGCAATGAA	149	Bartha et al., 2011 [129]
	tetX1-2		TATTTCGGGGTTGTCAAACT		

2.3.3 Metagenomic analyses

gDNA isolation. The genomic DNA (gDNA) was isolated from the lysed larval gut samples using FastDNA SPIN Kit for Soil (MP Biomedicals). 50 µL larval gut sample was added to the lysing matrix tube with 978 µL PBS buffer and 122 µL MT buffer. The samples were homogenized in FastPrep instrument for 3 minutes at setting 6. The tubes were then centrifuged at 13,000 rpm for 15 minutes to pellet the cell wall debris. The supernatant was transferred to clean 2.0 mL microcentrifuge tubes and 250 µL Protein Precipitation Solution was added. The solutions were mixed by shaking the tube by hand 10 times. The samples were centrifuged at 13,000 rpm for 10 minutes to pellet the precipitated proteins. The supernatant was transferred to 15 mL tubes and mixed with 1 mL resuspended Binding Matrix. The tubes were inverted by hand for 2 minutes to allow binding of DNA and then placed in a rack for 3 minutes to allow

settling of the silica matrix. 500 µL of supernatant was discarded without disturbing the settled Binding Matrix. The settled Binding Matrix was resuspended in the remaining supernatant and transferred to the SPIN™ Filter tubes. The SPIN™ Filter tubes were centrifuged at 13,000 rpm for 1 minute. 500 µL SEWS-M buffer was added to the filter tubes and the Binding Matrix was resuspended gently, before centrifugation at 13,000 rpm for 1 minute. The centrifugation was repeated to dry the filters of residual wash solution. The spin filters were dried for 10 minutes at room temperature and for 5 minutes at 37°C. The Binding Martix was resuspended in 100 µL DNase/Pyrogen-Free Water and centrifuged at 13,000 rpm for 1 minute to elute the DNA. The samples were separated by electrophoresis on 1% agarose TAE gel to confirm the presence of large gDNA fragments.

Metagenomics. The gDNA isolated from the larval guts was sequenced at ChunLab (Seoul, South Korea) using MiSeq Nano platform. The sequencing workflow started with incorporating barcodes into each sample. Many samples can be sequenced during one run and barcodes are used to tell them apart after the sequencing reactions are performed. After barcoding the samples were pyrosequenced, which is a method of sequencing dependent on the synthesis of a DNA strand complimentary to the strand being sequenced. The activity of the DNA polymerase incorporating one of four DNA bases is then detected indirectly with other chemiluminescent enzymes. The sequenced region was the V3-V4 region of the 16S gene, which codes for one of the variable regions of the 16S RNA and is frequently used in metagenomic analyses. After the sequencing reactions, the resulting reads had their barcode labels and sequencing primer sequences trimmed. These processed reads were then filtered and low-quality sequences were discarded. The filtered sequences were assembled into contigs, continuous overlapping assemblies representing consensus DNA regions. These contigs were then classified into operational taxonomic units based on sequence similarity between them. Taxonomic classification was assigned to each operational taxonomic unit at the species level using the ChunLab's EzTaxon-e database and blastn algorithm [130]. Chimeric sequences, which are contaminants originating from two separate DNA

sequences, were filtered out using UCHIME program [131]. The sequencing reads were supplied in .clc format. The sequencing results analysed and visualised with CLcommunity software supplied by ChunLab.

2.3.4 Antibiotic susceptibility testing

Assay: The minimal inhibitory concentrations (MIC) for ampicillin, chloramphenicol, ciprofloxacin, kanamycin, rifampicin and tetracycline were determined by broth microdilution [132] for each gut-isolated strain and type strain pair. Briefly, 96-well plates with serial dilutions of antibiotics were inoculated with bacterial suspension and incubated for 24 h at 30°C. The MIC was assigned when instead of a suspension of bacterial growth, a well with a clear broth was present. To confirm lack of bacterial growth, the OD was measured at 600 nm in a CLARIOstar plate reader (BMG Labtech). The control organism was *E. coli* ATCC 25922.

Modification: The MICs for catharanthine, vindoline, loganin, loganic acid, and ajmalicine were determined by broth microdilution method [134] for the gut-isolated strains from the beet armyworm, matching type strains, *Escherichia coli* (ATCC 25922), *Mycobacterium smegmatis* (ATCC 700084), *Pseudomonas aeruginosa* (ATCC 15692) and *Staphylococcus aureus* (ATCC 29213). 96-well plates with two-fold dilutions of the compounds were inoculated with bacterial suspension at OD_{600} = 0.08-0.11 and incubated for 24 h at 30°C. The MIC was assigned when instead of a suspension of bacterial growth, a well with a clear broth was present. To confirm lack of bacterial growth, the OD was measured at 600 nm in a CLARIOstar plate reader (BMG Labtech).

2.4 Plant extracts

2.4.1 Plant extract preparation

The plant extracts were prepared by homogenizing dried leaves of eucalyptus (*Eucalyptus dalrympleana*), cabbage leaves (*Brassica rapa*), lavender leaves and flowers

(*Lavendula angustifolia*), ragwort leaves (*Jacobaea vulgaris*), potato leaves (*Solanum tuberosum*), and Madagascar periwinkle leaves and roots (*Catharantus roseus*) with methanol. 100 g dried plant material was ground to fine powder either with a pestle and mortar or using an electric coffee grinder (Andrew James Stainless Steel Wet and Dry Coffee, Nut and Spice Grinder). The plant powders were soaked overnight in 300 ml methanol, filtered and soaked again twice in 300 ml methanol. The plant extract had deep green, nearly opaque colour. The plant material was soaked until it was no longer green. The three methanolic extracts were filtered, pooled and concentrated in an EZ-2 Elite evaporator (GeneVac). The extracts were de-fatted by liquid-liquid fractionation with petroleum ether in a 1:1 ratio. Only the methanol fraction was used in subsequent purifications.

2.4.2 Antibacterial activity testing

To standardize the different plant extracts, the crude extracts were dried to powder in an evaporator (DNA SpeedVac, Savant) and resuspended in methanol at 100 µg/mL. Lawns of bacteria were prepared by overlaying TY agar plates with a mixture of 3 mL overnight bacterial culture at OD 0.08 – 0.11 and 3 mL molten and cooled TY agar. Paper discs (Whatman, 5 mm) were infiltrated ten times with 10 µL aliquots of the extract, dried to remove methanol, and placed on the bacterial lawns. After 24 h incubation at 30°C the clear zones were measured with a ruler.

2.4.3 Activity-guided fractionation

Solid-phase extraction: Plant extracts with activity against bacteria tested were purified on solid-phase extraction (SPE) weak anion exchange columns (Oasis WAX 6 cc cartridge, Waters) according to the manufacturer's instructions. Briefly, each column was primed with 6 mL methanol and calibrated with 6 mL 2% formic acid in MiliQ water (Merck) before loading no more than 5 mg sample resuspended in methanol. The columns were then washed with 6 mL 2% formic acid, 6 mL methanol and 6 mL 5% ammonium hydroxide in methanol. The flow through was collected, concentrated and

assayed for antibiotic activity as described before.

High-pressure liquid chromatography: The active SPE fractions of plant extract were subjected to reverse-phase high-pressure liquid chromatography (HPLC). Non-concentrated samples were used for the fractionation to avoid loss of accuracy when too much sample is loaded. Before the fractionation the samples were centrifuged for 10 min. When the samples were too concentrated for the analysis, they were serially diluted at 1:10, 1:100 and 1:1000 ratios. The samples were run on a Liquid Chromatograph Mass Spectrometer LCMS-2020 (Single-quad, Shimadzu). Separation was on an analytical 2×100 mm 3 µm Luna C18(2) column (Phenomenex) and semi-preparative 10×250 mm 5 µm Luna C18(2) column (Phenomenex), run at 0.3 mL/min. for the analytical columns and 3 mL/min. for the semi-preparative columns. All separations were run at 40°C. We used a gradient of methanol and 0.1% formic acid in MiliQ water (Merck). A general HPLC method consisted of a 10 – 100% methanol gradient over six column volumes followed by a 100% methanol was over two column volumes and 10% methanol wash over two column volumes. UV/visible spectra (190-600 nm) and UV chromatograms (260 nm) were collected at 6.25 Hz with a time-constant of 0.16 sec. using the on-line detector between the HPLC column and the fraction collector. When fractions were collected, the fraction collector was set up to collect fractions of 2 mL throughout the duration of the method. After the fractionation all fractions were dried in an evaporator and assayed for antibacterial activity as described before.

2.4.4 Mass spectrometry

The Liquid Chromatograph Mass Spectrometer LCMS-2020 (Single-quad, Shimadzu) is a mass spectrometer, but the instrument is most functional when used in high-pressure liquid chromatography. The instrument can reach high pressures (above 6000 psi), which means it is capable of separating peaks that elute close together. The mass spectrometer part of the instrument is an excellent feature for mass-guided fractionation, but is not accurate enough for precise identification of compounds. To

precisely identify masses of compounds we used LCMS-IT-ToF Mass Spectrometer (Shimadzu).

To investigate the composition of some fractions, they were analysed on LCMS-IT-ToF Mass Spectrometer. The samples used in the analysis were not concentrated. They were collected before the solvent was evaporated for antibiotic activity testing. The samples were mixed with 20% methanol in 1:4 ratio and centrifuged for 10 min. The supernatant was transferred to small glass inserts for analysis. Serial dilutions (1:10, 1:100 and 1:1000) of a sample were prepared when the original sample was too concentrated and reached above the limit of detection of the instrument. The samples were run on a Prominence/Nexera UHPLC system attached to an ion-trap ToF mass spectrometer (IT-ToF, Shimadzu). Separation was on a 100×2.1 mm 2.6 µm Kinetex EVO C18 column (Phenomenex) using a gradient of acetonitrile versus 0.1% formic acid in water, run at 0.6mL/min. at 40°C. In contrast with previous LC method, this method was developed and optimised specifically for compounds in periwinkle leaf extract and adapted for other plant extracts (Tatsis, personal correspondence). The gradient consisted of a 10 – 100% acetonitrile gradient over six column volumes followed by a 100% acetonitrile wash over two column volumes and 10% acetonitrile wash over two column volumes. Detection was by UV/visible absorbance and positive electrospray MS. UV/visible data were collected from 200-600 nm at 6.25 Hz with a time-constant of 0.16 sec. Full MS data were collected from m/z 150-2000 with a maximum ion accumulation time of 20 msec., and automatic sensitivity control set to a target of 70% of optimum base peak intensity. The instrument also collected automatic MS2 data of the most abundant precursor ions at an isolation width of m/z 3.0, 50% collision energy, and 50% collision gas, and 15 msec. ion accumulation time. The instrument was set up to collect two successive spectra of each precursor ion that was selected, and then ignore that precursor for 2 sec in favour of the next most abundant precursor. Spray chamber conditions were 1.5 L/min. nebulizer gas, 250°C curved desorbation line, 300°C heat block, and drying gas “on”. The instrument was calibrated immediately before use, using sodium trifluoroacetate cluster ions according to the manufacturer’s

instructions. The results were analysed with LabSolutions software (Shimadzu) and Profiling Solution software (Shimadzu).

3 The impact of antibiotics on the gut microbiota of two lepidopteran hosts: *Plutella xylostella* and *Galleria mellonella*

Undergraduate project student Aristide Lebreton helped with some of the experiments described in this chapter as part of his project. He helped set up and dissect P. xylostella larvae feeding on artificial food containing oxytetracycline and isolated and identified the gut bacteria from the larvae.

All data in this chapter was generated by me and I produced all figures and tables on my own.

3.1 Abstract

Clinically-relevant multidrug resistance is sometimes present in bacteria not exposed to human-made antibiotics, in environments without a high concentration of these compounds, such as the insect gut [121]. In this chapter we investigate the impact of antibiotics on the insect gut microbiome by identifying antibiotic resistance in the gut bacteria of the larvae of two lepidopteran hosts: Plutella xylostella and Galleria mellonella, feeding on artificial food containing the antibiotics ciprofloxacin and oxytetracycline. For P. xylostella larvae the resistance to a panel of antibiotics was assessed and it was discovered that antibiotic-resistant bacteria are present not only in the guts of insects feeding on antibiotics, but also in guts of larvae feeding on artificial food without antibiotics. In a follow-up experiment, five generations of G. mellonella larvae were fed on artificial food containing a range of oxytetracycline doses. While oxytetracycline-resistant strains were isolated from the gut of G. mellonella larvae feeding on antibiotics, more genetically diverse resistance elements were present in samples from antibiotic-free larval guts. Our results indicate that antibiotics have diverse impact on the insect gut microbiome, beyond only inducing antibiotic resistance. Our findings highlight the significance of antibiotics in natural environments and clinical settings.

3.2 Introduction

3.2.1 Antibiotic resistance is ancient

Widespread antibiotic resistance in pathogenic bacteria is a global problem, posing a significant threat to public health. However, antibiotic resistance is not a modern phenomenon. Antibiotic resistance is ancient and originates from the environment [133]. For example antibiotic resistance genes have been found in permafrost samples dating from the Late Pleistocene period [80]. The sediment samples contained genes conferring resistance to β-lactams, tetracyclines and glycopeptide antibiotics, illustrating that antibiotic resistance genes pre-date human antibiotic use. Phylogenetic analyses of genetic sequences and protein structures of resistance elements also reveal their ancient origin. β-lactamases are a diverse group of proteins with ancient origins [134] and within that group OXA class β-lactamases have been predicted to have been mobilized to a plasmid twice in the evolutionary history, at 116 and 42 million years ago [135]. These findings suggest that antibiotic resistance genes have a role in the fitness of some bacteria, either by providing protection against naturally-occurring antibiotics or serving a different ancient metabolic role.

Antibiotic resistance has been shown to be a trait often accompanied by a high fitness cost (see review by MacLean et al., 2010 [136]). Resistance elements are normally lost in the absence of selective pressure unless accompanied by rare mutations compensating the fitness cost. However, antibiotic resistance can be found in environments that do not contain high levels of antibiotic resistance, indicating that the genes can persist without direct selective pressure [121, 137]. Some antibiotic resistance genes isolated from natural environments share a high level of similarity to genes from human pathogens.

3.2.2 Antibiotic resistance is widespread

Antibiotic resistance genes are present in environments seemingly not affected by human factors, such as glaciers in Central Asia, North and South America, Greenland and Africa [138]. 48 different resistance genes were identified from 54 samples collected from glaciers. The most frequently recovered genes were two aminoglycoside

acetyl transferases of a clinical origin (24/54 sites and 18/54 sites), streptomycin-resistance gene *strA* with homology to genes of agricultural origin (21/54 sites), and a metallo-β-lactamase bla_{IMP} gene conferring resistance to carbapanem antibiotics (20/54 sites). Similarly, antibiotic resistance genes with homology to genes from human pathogens are present in marine environments. Plasmids from fish farm sediment bacteria carried multiple antibiotic resistance elements, enriched in tetracycline resistance genes [139]. A total of 652 plasmids have been isolated, two thirds of them carrying genes with less than 40% homology to known resistance elements. Six genes had over 90% identity to known resistance elements from human pathogens, two of them conferred resistance to macrolides, and the remaining four to aminoglycosides, fluoroquinolones, chloramphenicol and tetracycline. However, as with OXA β-lactamases discussed earlier, it is not possible to easily distinguish whether the genes have the same role in the natural environment as in clinical setting or confer a novel beneficial phenotype.

3.2.3 Antibiotic resistance is present in the insect gut

Another reservoir of antibiotic resistance is the insect gut. Insects not exposed directly to antibiotics can have antibiotic-resistant microbiota. Laboratory-reared Mexican fruit flies, *Anastrepha ludens*, harbour predominantly bacteria of the genus *Listeria*, *Enterobacter*, *Providencia*, *Serratia*, and *Staphylococcus* [140]. The bacteria test positive for ampicillin and penicillin resistance, even though their wheat germ- and yeast extract-based diet is antibiotic-free. A more striking example is the oil fly (*Helaeomyia petrolei*) larvae, which swim in asphalt seeps and feed on dead insects trapped in the petroleum pools. Their gut microbiome is dominated by *Providencia rettgeri* [141], which is resistant to a range of antibiotics (tetracycline, vancomycin, bacitracin, erythromycin, novobiocin, polymyxin, colistin, and nitrofurantoin) and organic solvents (overlays with cyclohexane, xylene, benzene, toluene, and xylene-cyclohexane). However, in the case of oil fly larvae, whose guts are exposed to crude petroleum, the microbiota is under high selective pressure to develop tolerance or resistance to a variety of compounds.

Insects associated with human habitats are often under high selective pressure for

antibiotic-resistant microbiomes. For example bedbugs (*Cimex lectularius*) from hospital patients have been found to carry vancomycin-resistant *Enterococcus faecium* and methicillin-resistant *Staphylococcus aureus* [100]. The bacteria were also resistant to other antibiotics: *E. faecium* to ampicillin, teicoplanin, and aminoglycosides, and *S. aureus* to erythromycin. Similarly, two Indian cockroach species *Periplaneta americana* and *Blattella germanica*, collected from households and food-handling establishments, carried a variety of pathogenic bacterial species, including *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Escherichia coli* [142]. Each of the strains was resistant to ampicillin, tetracycline and sulfamethoxazole/trimethoprim, with additional resistance of *P.aerugionsa* to cephalothin, chloramphenicol and gentamycin, *K. pneumoniae* to gentamycin, and *E. coli* to piperacillin, cephalothin, chloramphenicol and ceftazidime.

3.2.4 Insects as vectors mediating the spread of antibiotic resistance

Apart from being a reservoir of antibiotic resistance genes, insects can be vectors of bacterial diseases, spreading antibiotic-resistant strains. Antibiotic-resistant bacteria, present in the faeces of farm animals fed antibiotics for growth promotion, are picked up by flies (Diptera) and cockroaches (Dictyoptera) and spread between farms and surrounding urban environments (see review by Zurek and Ghosh, 2014 [74]). A body of evidence from different studies demonstrates a link between antibiotic use in animal husbandry, bacterial resistance in livestock, and resistance in human pathogens, transmitted directly by insect vectors.

Antibiotic use in agriculture has a profound effect on bacterial populations associated with insects. Oxytetracycline is the most commonly used antibiotic in apiculture and the long term exposure of bees to this antibiotic has led to abundance of tetracycline resistance genes in the bee gut microbiota [37]. Oxytetracycline is used in the control of European foulbrood, a disease of honeybees caused by *Melissococcus plutonius*. The antibiotic is applied mixed with sugar or water and reaches minimal inhibitory concentrations in the larval guts within 24 h post treatment, but declines rapidly to a level under MIC within two to five days [37, 143]. In the US bees have been exposed to prophylactic oxytetracycline use for over 50 years and high levels of tetracycline resistance can be detected in the bee guts [37]. In contrast, such genes are rare and

only present at low frequencies in bees not exposed to antibiotics (from countries where antibiotics are not permitted in apiculture) and wild bumblebees. Together these studies suggest that the exposure of insect microbiota to antibiotics leads to an accumulation of antibiotic resistance.

3.2.5 Implications of the presence of antibiotics in insect diets

Antibiotic-induced insect mortality is a significant issue, as insects are already facing high extinction rates. Over 40% invertebrate pollinator species are estimated to be facing extinction, threatening global agricultural production [144]. Declining pollinator numbers affect food and biofuel supply, as well as medicines, fibres, forage for livestock and construction materials. Importantly crops depend not only on managed pollinators such as bees, but also on a wide variety of wild pollinators. The presence of a variety of pollinators contributes to higher crop yields.

In natural environments antibiotics can be contaminants contributing to ecotoxicity. Ecotoxicity can be defined as the effects of pollutants, such as human-made antibiotics, on ecosystems as a whole. Microbiomes are integral parts of all higher organisms and changes to the community composition or bacterial metabolism affect the host organisms. Even low concentrations of antibiotics in the environment can trigger transcriptional changes in the microbiomes (see review by Martinez, 2009 [145]). The impact of such changes on the ecosystems and human health is currently difficult to assess.

3.2.6 Objectives

The hypothesis tested was that insects feeding on compounds with antibacterial activity acquire antibiotic-resistant microbiota. This hypothesis was part of a proof of concept for the experiments in the following chapter (see Chapter 4. Investigating antibiotic resistance in the insect gut bacteria: the search for novel plant-derived antibacterials), in which we look for evidence of multi-antibiotic resistance in the insect gut as an indication of antibacterials in the plant diet.

3.3 Results

The aim of this project was to demonstrate the correlation between the antimicrobial compounds in the insect food and the presence of antibiotic resistance elements in the insect gut. The data collected demonstrate that antibiotics have a pleiotropic effect on insects, beyond inducing antibiotic resistance in the gut microbiome.

3.3.1 *Plutella xylostella* feeding on artificial food with ciprofloxacin

3.3.1.1 Diamondback moth (Plutella xylostella)

The objective of this project was to demonstrate that compounds with antimicrobial activity in the insect food lead to the accumulation of antibiotic resistance genes in the gut microbiome. The initial experiments were conducted on diamondback moth larvae (*Plutella xylostella*), which were feeding for three days on artificial food containing either ciprofloxacin or oxytetracycline. *P. xylostella* is a lepidopteran pest of cruciferous plants, with a wingspan of 15 mm and a body length of 6 mm. The pale green larvae have four instars, the first instar larvae burrow in the plant leaves, emerging after the first moult to feed on the bottom leaf surface.

P. xylostella larvae are a notoriously destructive pest of cruciferous plants [146]. The insects are often insecticide-resistant, but the mechanism is most likely not microbe-mediated. An investigation of radish-fed groups of *P. xylostella* larvae, revealed presence of a wide range of bacterial species present at low abundance [147]. 342 different operational taxonomic units, all below 2% abundance, were identified between all samples collected. The guts were dominated by Proteobacteria, especially of the order Enterobacteriales. The authors determined that the differences in gut microbiota composition were independent of the insecticide exposure of the sampled larvae.

3.3.1.2 Gut bacteria isolated from P. xylostella exposed to ciprofloxacin

Cabbage-reared *P.xylostella* larvae were shifted to an agar-based artificial food spiked with a range of ciprofloxacin concentrations (Figure 3-1). Ciprofloxacin was the antibiotic of choice, because the resistance it normally induces is well-studied and

involves a point mutation in the DNA gyrase gene [148].

The doses of antibiotics were selected to be similar to physiologically-relevant human doses. An estimate was calculated of how the antibiotic doses in artificial food correlate to the doses recommended for human use. The recommended human dose for ciprofloxacin is 25 mg/kg body weight/day [123]. Five larvae (approximate weight 20 mg each) fed on four 5×5×5 mm cubes of artificial food per day. Assuming the ciprofloxacin dose was 3 µg/ml, the larvae consumed 1.5 µg drug/100 mg body weight/day, which equals to 15 mg/kg body weight/day ciprofloxacin. The other ciprofloxacin doses are summarised in Table 3-1.

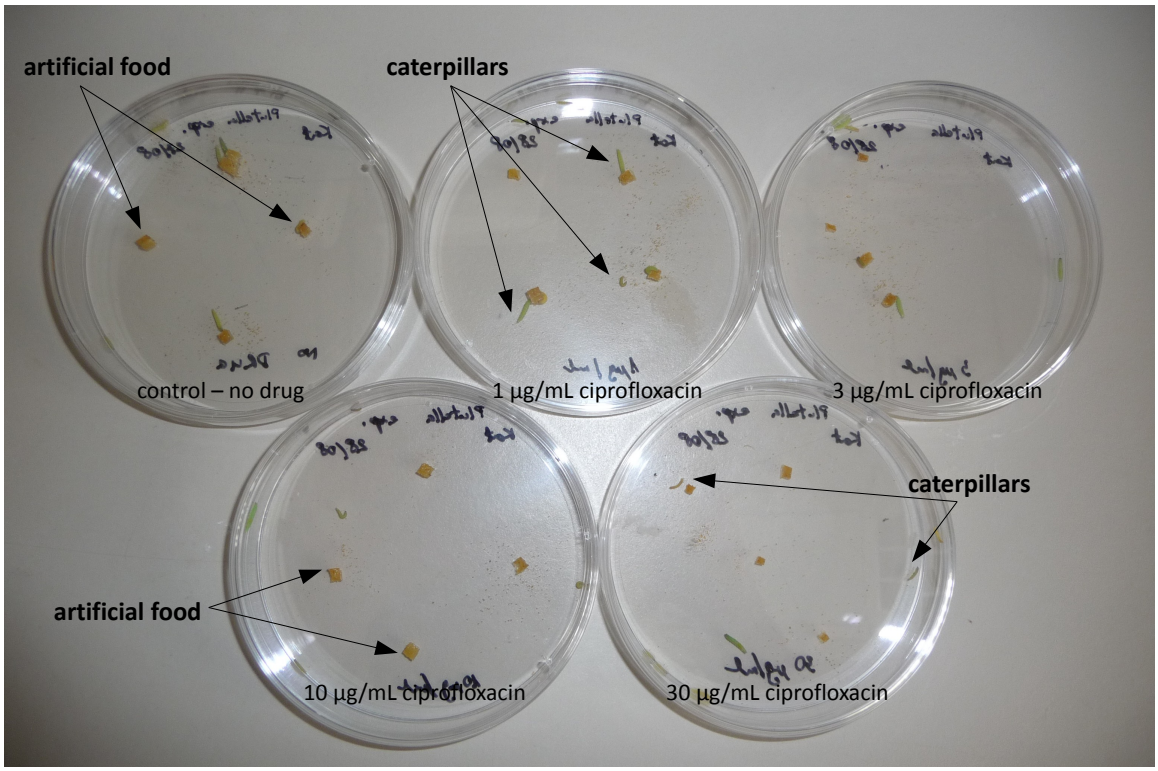


Figure 3.1: Plutella xylostella larvae feeding on artificial food containing ciprofloxacin. Light green larvae at third and fourth instar eating cubes of agar-based artificial food containing no drug, 1 µg/mL ciprofloxacin, 3 µg/mL ciprofloxacin, 10 µg/mL ciprofloxacin and 30 µg/mL ciprofloxacin.

Table 3.1: Antibiotic doses used in P. xylostella feeding study were selected to reflect a range of physiologically relevant human doses. Ciprofloxacin is recommended for human use at 25 mg/kg body weight/day. The lowest dose is representative of a sub-inhibitory antibiotic dose, the two middle doses represent a low and high limit of the recommended human dose. The highest dose is above the dose recommended for human dose, but is not high enough to have toxic effects on the larvae.

ciprofloxacin dose in the artificial food	ciprofloxacin dose per body weight
1 µg/mL	5
3 µg/mL	15
10 µg/mL	50
30 µg/mL	150

After three days the *P. xylostella* guts were dissected and their gut contents were plated. Seven different bacterial species were isolated from larvae exposed to ciprofloxacin: *Bacillus subtilis*, two species of *Sanguibacter*, three species of Enterobacteria, including *Enterobacter amnigenus*, and a species that was not identified with the methods used (Table 3-2).

Only *Bacillus subtilis* and *Enterobacter amnigenus* were used in following experiments. Further work, requiring the use of type strains from a culture collection, was carried out only with the strains identified to species level (*B. subtilis* and *E. amnigenus*). The strains were used to assay for antibiotic susceptibility differences between the gut-isolated strains and the type strains, therefore only the isolates identified to species level were used.

	gut bacteria identified in the guts of *Plutella xylostella* feeding on artificial food with ciprofloxacin						
ciprofloxacin dose in the insect food [µg/mL]	*Bacillus subtilis*	*Enterobacter amnigenus*	*Enterobacter* species 1	*Enterobacter* species 2	*Sanguibacter* species 1	*Sanguibacter* species 2	unidentified species
no drug	+	-	+	+	+	+	+
1	+	-	-	-	+	+	-
3	-	+	-	-	+	+	-
10	+	+	-	-	+	+	-
30	+	+	-	+	-	+	+

Table 3.2: An overview of bacteria isolated from the guts of diamondback moth larvae feeding on artificial food containing a range of ciprofloxacin doses. The experiment was repeated twice, each time the dissections included at least five larvae.

3.3.1.3 Antibiotic resistance in the gut bacteria of P. xylostella larvae

At the next stage of this experiment, the strains identified were assayed for antibiotic resistance in a broth microdilution assay [132]. The resistance profiles for the strains exposed to antibiotics in the insect gut were compared to type strains obtained from bacterial culture collections (Table 3-3). There are three possible outcomes of such

comparison: 1) no difference between the strains tested, 2) gut-isolated strain is more resistant than the type strain, 3) gut-isolated strain is more susceptible than the type strain.

In most cases there was no difference in antibiotic susceptibility between the type strain and the gut-isolated strain. In four cases differences in antibiotic susceptibility were observed. *B. subtilis* isolated from the *P. xylostella* gut was more resistant to ampicillin and chloramphenicol than the type strain and similarly *E. amnigenus* from the diamondback moth larvae gut was more resistant to tetracycline than the type strain. *B. subtilis* from the insect gut was less resistant to ciprofloxacin, which it was exposed to, than the type strain. We expected to see some differences in susceptibility where the type strain is more antibiotic resistant than the gut strain. However it was surprising that the *B. subtilis* type strain was more ciprofloxacin-resistant than the gut-isolated strain, which was exposed to ciprofloxacin.

Table 3.3: Antibiotic susceptibility profiles of the gut bacteria from P. xylostella feeding on artificial food with ciprofloxacin. The antibiotic susceptibility of the gut-isolated strains and the type strains was assessed in a broth microdilution assay. Differences in antibiotic-resistance levels between the strains have been highlighted in green when the gut-isolated strain was more resistant than the type strain and in red when the type strain was more resistant than the gut-isolated strain. $p < 0.001$.

	Minimal inhibitory concentrations of antibiotics in broth dilution assay [µg/mL]			
	Bacillus subtilis		*Enterobacter amnigenus*	
	gut strain	type strain	gut strain	type strain
ampicillin	>64	2	>64	>64
chloramphenicol	16	2	2	2
ciprofloxacin	<0.01	0.02	<0.01	<0.01
kanamycin	1	1	2	2
rifampicin	2	2	2	2
tetracycline	1	1	0.03	0.02

3.3.2 *Plutella xylostella* feeding on artificial food with oxytetracycline

3.3.2.1 Re-design of the experimental procedure

To further test the hypothesis the experiment was re-designed based on a study by Tian et al. [37]. In this study the impact of oxytetracycline use in beehives was investigated. Over 50 years oxytetracycline was heavily used in apiculture, leading to a significant increase in tetracycline resistance elements in the guts of bees treated with the antibiotic compared with the wild bee populations. Based on these data, the *Plutella* experiment was re-designed: the artificial food was spiked with oxytetracycline and the feeding procedure was extended to span a number of generations. Oxytetracycline was added to the artificial food at 3 µg/ml, which is equivalent to the recommended human dose for oxytetracycline (15 mg/kg body weight/day) (Table 3-4). The feeding procedure was extended to prolong the exposure of the larvae to the antibiotic and to sample antibiotic-fed insects over generations.

Table 3.4: The antibiotic dose used in P. xylostella feeding study was selected to reflect a physiologically-relevant human dose. Oxytetracycline is recommended for human use at 15 mg/kg body weight/day.

oxytetracycline dose in the artificial food	oxytetracycline dose per body weight
3 µg/ml	15

The initially cabbage-reared larvae, at third and fourth instar, were moved to an artificial food spiked with oxytetracycline. The food was replaced daily, after three days the larvae were dissected and their gut bacteria identified. A group of larvae was kept to establish a long-term colony feeding on artificial food, but high pupae mortality and no egg-laying halted the progress of the experiment. It is possible that the pupae mortality was caused by the antibiotic present in the artificial food that either had a direct toxic effect on the larvae pre-pupation, or indirectly affected the pupae by changing the composition of the larval gut microbiota. Only few adults emerged from the surviving pupae. The moths did not produce any eggs as oviposition is dependent on the host cruciferous plant [25]. In *Plutella xylostella* females oviposition is induced by non-volatile indole glucosinolates produced by cruciferous plants.

3.3.2.2 Identification of bacteria from the guts of P. xylostella larvae feeding on oxytetracycline

Even though the experiment could not continue as planned to include subsequent generations of larvae hatched on artificial food, the strains from the *P. xylostella* larvae feeding on oxytetracycline were identified. Only two different bacterial species were identified: *Enterobacter amnigenus* and *Sanguibacter keddieii*. *E. amnigenus* and bacteria from the genus Sanguibacter were also identified in the previous experiment, when diamondback moth larvae were feeding on artificial food containing ciprofloxacin, which suggests that the culturable fraction of the gut microbiome is relatively stable. Surprisingly only two bacterial species were isolated from the *P. xylostella* guts, which can contain over 300 different bacterial strains [147]. Diamondback moth microbiome is dominated by Enterobacteriales, which are readily cultured by the methods employed in our study and we expected to culture a higher diversity of species.

3.3.2.3 Antibiotic resistance in the gut bacteria of P. xylostella larvae

The bacterial strains isolated from *P. xylostella* feeding on artificial food containing oxytetracycline were assayed for antibiotic resistance by broth microdilution [132]. As before for most antibiotics tested, there was no difference between the gut-isolated strain, exposed to oxytetracycline, and the test strain (Table 3-5). Only in two cases there were differences in antibiotic susceptibility between the gut-isolated strains and type strains: *S. keddieei* gut-isolated strain was less resistant to chloramphenicol and ciprofloxacin than the type strain. As discussed before such differences are surprising, but not unexpected. However, we expected the gut-isolated strains to be more oxytetracycline resistant than the type strains, as the gut-isolated strains were exposed to oxytetracycline, but we have seen no differences between the isolates.

It is possible that no differences in antibiotic susceptibility between the gut-isolated strains and the type strains were detected because the bacteria were not exposed to the antibiotics in the insect gut for long enough. It is also possible that rearing the larvae initially on cabbage introduces bacterial strains or chemical compounds that protect the microbiome from the antibiotic in the artificial food. Ideally, the insects

should feed on the same type of food throughout their life. Additionally the two experiments described above also demonstrate that the type strains are not a sufficient control for antibiotic resistance and a more accurate method should be used instead.

Table 3.5: Antibiotic susceptibility profiles of the gut bacteria from P. xylostella feeding on artificial food with oxytetracycline. The antibiotic susceptibility of gut-isolated strains and the type strains was assessed in a broth microdilution assay. Differences in antibiotic-resistance levels between the strains have been highlighted in green when the gut-isolated strain was more resistant than the type strain and in red when the type strain was more resistant than the gut-isolated strain. $p < 0.05$.

	Minimal inhibitory concentrations of antibiotics in broth dilution assay [µg/mL]			
	Sanguibacter keddieii		*Enterobacter amnigenus*	
	gut strain	type strain	gut strain	type strain
ampicillin	2	2	>64	>64
chloramphenicol	1	2	16	16
ciprofloxacin	1	>64	<0.01	<0.01
kanamycin	2	2	1	2
rifampicin	0.02	0.02	2	2
oxytetracycline	0.02	0.02	0.02	0.02

To investigate further the response of the insect gut microbiomes to antibiotics, we addressed the issue of insufficient antibiotic exposure in the gut by switching to a different insect species. Plutella is not the only lepidopteran that can be reared on artificial diets and other species can be permanently fed artificial food.

3.3.3 *Galleria mellonella* feeding on artificial food with oxytetracycline

3.3.3.1 Greater wax moth (Galleria mellonella)

Another lepidopteran host used in this study is the greater wax moth (*Galleria mellonella*). *G. mellonella* is a larger, easier to dissect insect (with fifth instar larvae reaching up to 30 mm long), and can be reared on artificial diet over a number of generations. Greater wax moths are pests of beehives, and are also commonly used in immunity research, bacterial virulence and antibiotic efficacy studies.

In contrast to *Plutella xylostella*, the gut microbiota of *G. mellonella* is simple, containing mainly one bacterial species. *Enterococcus faecalis* (syn. *Streptococcus faecalis*) has been identified in the guts of *G. mellonella* using culture-dependent methods [149]. Culture-independent methods confirmed Enterococcus species are present in the larval guts, but identified the dominant strain as *Enterococcus munditii* [150]. The strain has no role in insect digestion [151] but together with the host provides colonisation resistance against invading pathogens [152].

3.3.3.2 Continuation of the experiment designed for P. xylostella

We decided to continue the investigation into the impact of antibiotics on the insect gut microbiota, taking into consideration the points discussed above. To continue the experiment over more than one generation of insects, a lepidopteran host able to sustain growth on artificial food was chosen. Greater wax moths (*Galleria mellonella*) are sometimes reared on honeycomb or beehive debris, but more commonly feed on bran- and wheat germ-based artificial food (Figure 3-2). The insects can reproduce while on artificial food diet. Additionally instead of assessing antibiotic resistance by comparing the antibiotic susceptibility profiles of the gut isolated strains and the type strains, we used targeted PCR amplification of all known tetracycline resistance genes.

Figure 3.2: G. mellonella larvae feeding on artificial food in a Petri dish.

3.3.3.3 Feeding procedure

For the purpose of this experiment, a *G. mellonella* colony was set up with groups feeding on artificial food without antibiotics and with different concentrations of oxytetracycline. The doses chosen were 1 mg/100 g food, 10 mg/100 g food, 100 mg/ 100 g food, 400 mg/100 g food and 1000 mg/100 g food. The concentrations of antibiotics were chosen to span a range of doses. Estimating that 20 larvae fed on 25 g artificial food for a week, the lowest dose (1 mg/100 g food) equals to 8 oxytetracycline, which is half of the recommended human dose. The relation between the other oxytetracycline concentrations in the artificial food and the estimated dose per kg body weight is summarized in Table 3-6. The 10 mg/100 g food dose is similar to a high human dose and the remaining doses are high enough to have a feeding deterrent or toxic effect on the larvae.

Table 3.6: Antibiotic doses used in G. mellonella feeding study were selected to reflect a range of physiologically relevant human doses. Oxytetracycline is recommended for human use at 15 mg/kg body weight/day. The lowest dose is representative of a sub-inhibitory antibiotic dose. The 10 mg/100g diet oxytetracycline dose represents a high therapeutic dose. The three highest doses are above the dose recommended for human dose and are high enough to have toxic effects on the larvae.

dose in artificial food	dose per body weight
1 mg/100 g diet	8
10 mg/100 g diet	80
100 mg/100 g diet	800
400 mg/100 g diet	3200
1000 mg/100 g diet	8000

The feeding procedure was carried out over five generations. A colony was established for the control group, not feeding on antibiotics. Moths reared on food without antibiotics laid eggs that hatched on food without antibiotics and that process was continued over five generations. Larvae were sampled from each generation. The same process was followed for the group feeding on artificial food with oxytetracycline at 1 mg/100 g food. Groups feeding on higher oxytetracycline concentrations sometimes experienced high mortality at early larval stage and as pupae, and it was not always possible to collect larvae from each generation (Table 3-7). For example, the third

generation of larvae feeding on 10 mg oxytetracycline/100 g food died at an early stage, so no larvae were sampled and no adults were collected for mating and oviposition. We moved eggs from the control group not exposed to antibiotics to the artificial food with 10 mg oxytetracycline/100 g food, but they also died at an early stage. We repeated the process for the fifth generation of insects and they managed to grow to fifth instar when the larvae were sampled.

Table 3.7: G. mellonella larvae collected from the feeding procedure. The + indicates at least 10 larvae were collected and the - indicates it was not possible to collect suitably large larvae and no larvae were collected at all. In some cases the larvae in an natibiotic-treated group died and not only no larvae could be collected, but also no larve reached maturity and laid eggs. In the interest of continuing the experiment, in such cases eggs laid by the adults from the control (no drug) group were moved to the food spiked with oxytetracycline.

Oxytetracycline dose	Subsequent generations of G. mellonella larvae feeding on artificial food with oxytetracycline				
	1st generation	2nd generation	3rd generation	4th generation	5th generation
no drug	+	+	+	+	+
1 mg OTC/100 g diet	+	+	+	+	+
10 mg OTC/100 g diet	+	+	-	-	+
100 mg OTC/100 g diet	+	-	+	-	+
400 mg OTC/100 g diet	+	-	+	-	+
1000 mg OTC/100 g diet	-	+	-	-	+

The samples were stored in a freezer before dissections, to avoid inconsistencies between dissections performed at different times. The entire guts were dissected under sterile conditions and pooled into groups of three. From other experiments it was predicted a single gut does not contain enough bacteria for the downstream processing, such as PCR amplification of the tetracycline resistance genes. The samples were plated on LB agar and MacConkey agar under aerobic conditions, and RCA media under anaerobic conditions.

3.3.3.4 Isolation of gut bacteria

Only three different isolates were cultured from the *G. mellonella* guts: two strains of *Enterobacteria* from the initial stock insects obtained from the Insectary before the start of the experiment, and *Bacillus subtilis* strain isolated from the fourth generation of insects feeding on diet with 1 mg OTC/100 g food. No bacteria were cultured from any other sample. When plated on oxytetracycline selective media, the Enterobacteria were oxytetracycline-sensitive and the *B. subtilis* was oxytetracycline-resistant. The presence of oxytetracycline-sensitive Enterobacteria were predicted from previous studies. *Galleria mellonella* is a monoxenic insect associated with Enterococcus species [149, 150]. When assayed for antibiotic susceptibility, the strain is normally resistant to β-lactam antibiotics and susceptible to tetracyclines [152]. When the gut microbiome is disturbed with antibiotics, colonisation resistance of *G. mellonella* gut is compromised and it can be invaded by non-native bacterial strains. An example of such strain is *B. subtilis* isolated from the guts of larvae feeding on 1 mg OTC/100 g food. The isolate is not a member of typical *G. mellonella* microbiome. It is also oxytetracycline-resistant, demonstrating how after exposure to a low dose of an antibiotic native antibiotic-susceptible microbiota can be replaced by an invading antibiotic-resistant strain.

3.3.3.5 Identification of tet resistance elements from the G. mellonella guts

After plating, genomic DNA was isolated from the larval gut contents and subjected to diagnostic PCRs for tetracycline-resistance elements. The same genes confer resistance to both tetracycline and oxytetracycline [153], and our assays probed a wide variety of them (Table 3-8). The microbiome was probed for the presence of all genes, coding for a range of ribosome protection proteins and efflux pumps, surveyed by Tian et al. [37] and additional novel tetracycline resistance genes discovered after the study was published. These genes, tetX and tetX2, code for tetracycline destructases, flavoenzymes that inactivate tetracyclines by an oxidative mechanism [154].

Table 3.8: An overview of tet-resistance genes surveyed in the guts of G. mellonella larvae.

gene name	gene product type	Reference
tetA	efflux pump	Fan et al., 2007 [125]
tetB	efflux pump	Fan et al., 2007 [125]
tetC	efflux pump	Fan et al., 2007 [125]
tetD	efflux pump	Fan et al., 2007 [125]
tetE	efflux pump	Fan et al., 2007 [125]
tetG	efflux pump	Fan et al., 2007 [125]
tetH	efflux pump	Fan et al., 2007 [125]
tetJ	efflux pump	Aminov et al., 2002, 2004 [126, 127]
tetK	efflux pump	Chopra and Roberts, 2001 [153]
tetL	efflux pump	Tian et al., 2012 [37]
tetY	efflux pump	Aminov et al., 2002, 2004 [126, 127]
tetZ	efflux pump	Aminov et al., 2002, 2004 [126, 127]
tet30	efflux pump	Aminov et al., 2002, 2004 [126, 127]
tetBP	ribosomal protection protein	Aminov et al., 2001, 2004 [126, 127]
tetM	ribosomal protection protein	Aminov et al., 2001, 2004 [126, 127]
tetO	ribosomal protection protein	Aminov et al., 2001, 2004 [126, 127]
tetQ	ribosomal protection protein	Aminov et al., 2001, 2004 [126, 127]
tetS	ribosomal protection protein	Aminov et al., 2001, 2004 [126, 127]
tetT	ribosomal protection protein	Aminov et al., 2001, 2004 [126, 127]
tetW	ribosomal protection protein	Aminov et al., 2001, 2004 [126, 127]
tet32	ribosomal protection protein	Aminov et al., 2001, 2004 [126, 127]
tet34	efflux pump	Szczepanowski et al., 2009 [128]
tetX	tetracycline destructase	Bartha et al., 2011 [129]
tetX2	tetracycline destructase	Bartha et al., 2011 [129]

A number of different genes were identified (Table 3-9). The stock larvae had a tetM gene, which codes for a ribosome protection gene. The tetM ribosome protection protein complexes with 70S ribosome, directly blocking the tetracycline binding site [155]. It is hypothesised the tetM ribosome protection protein dislodges tetracycline from tetracycline binding site to confer resistance. A variety of genes was present among the larvae not feeding on antibiotics: tetB, tetC, tet30, tetD and tetL. All these genes code for efflux pumps. The genes tetB, tetC, tetD and tet30 belong to group 1 of

tetracycline efflux proteins, which are found predominantly in Gram-negative bacteria and belong to major facilitator superfamily (MFS) tetracycline transporter family. The efflux proteins exchange a proton for a tetracycline-cation complex against a concentration gradient [156]. The gene tetL belongs to group 2 of tetracycline efflux proteins, found primarily in Gram-positive species. The proteins confer resistance to tetracycline and chlortetracycline. This dimeric efflux protein exchanges a proton for tetracycline-divalent cation complex [157].

Table 3.9: An overview of tetracycline resistance elements from the guts of Galleria mellonella larvae feeding on artificial food containing oxytetracycline. The groups of larvae for which no samples were collected due to mortality at an early stage have been shaded out. A sample where no results were obtained due to DNA degradation was marked with (*).

Oxytetracycline dose	Subsequent generations of G. mellonella larvae feeding on artificial food with oxytetracycline				
	1st generation	2nd generation	3rd generation	4th generation	5th generation
no drug	tetB tetC	tetB tetC	tetB tetC tet30	tetB tetD	tetB tetD tetL
1 mg OTC/100 g diet	tetB	tetB tetD	tetB tetD	tetB tetD	tetB tetD
10 mg OTC/100 g diet	tetB tetD	tetB tetD			tetB tet30
100 mg OTC/100 g diet	tetB		*		-
400 mg OTC/100 g diet	tetD		-		-
1000 mg OTC/100 g diet		-			-

These results indicate that the gene pool is diverse and dynamic, even in the absence of selective pressure. When the selective pressure is present, fewer genes can be observed in the population. Larvae fed a 1 mg OTC/100 g food, only have two tetracycline resistance genes: tetB and tetD. Larvae fed 10 mg OTC/100 g diet also have tetB and tetD gene, with an additional tet30 gene detected in the fifth generation. Only the tetB gene was detected in samples from larvae feeding on 100 OTC mg/100 g diet.

Similarly only the tetD gene was detected in samples from 400 mg OTC/100 g diet group. No tetracycline resistance genes were detected in the group feeding on the highest concentration of oxytetracycline, 1 g/100 g diet.

3.3.3.6 Metagenomic analysis of the G. mellonella gut contents

The genomic DNA samples from *G. mellonella* guts were prepared for a metagenomic analysis. Out of 61 gut samples prepared, 47 had detectable bacterial DNA. Out of those only 25 have met the criteria for the minimum DNA concentration required for the amplification with sequencing primers (over 30 ng/µl and the presence of DNA fragments over 1 kb). The primers used were standard primers used in 16S sequencing [124].

A low DNA concentration recovered from *G. mellonella* larvae guts supports the possibility that the larval guts did not contain a dense bacterial community. Out of 25 samples that have met the sequencing criteria only two successfully went through amplification with the sequencing primers. These two samples were antibiotic-free stock larvae collected before the beginning of the experiment and the first generation of larvae feeding on 400 mg OTC/100 g food. The other samples failed the amplification most likely due to a poor quality of DNA, such as sheared or otherwise damaged DNA.

Nearly 20,000 valid reads were recovered from each sample, with the antibiotic-free stock larvae reads assigned to 2,673 operational taxonomic units (OTUs) and the reads from larvae fed 400 mg OTC/100 g food assigned to 4,310 OTUs. Valid seqencing reads are all sequences that passed the bioinformatic control steps used to identify artifacts of the sequencing protocol. They are the reads of the sequences in the initial sample and exclude any read that is likely to be a by-product of the sample preparation methods or the sequencing protocol (for example two constructs merged together or a sequence containing an untrimmed label). Using EzTaxon database each OTU was identified down to species level by finding the closest related type strain. Some of the strains are environmental isolates lacking a valid name in the current systematic nomenclature. In such cases, for the purpose of clarity of the figures, the strain name assigned from EzTaxon was displayed in figures, even though it is not a valid species

name.

3.3.3.7 Species identified from the guts of G. mellonella

Both samples were analysed for alpha-diversity, also known as species richness, which indicates the biodiversity of a single community (in our case, each sample containing DNA from three larval guts). The species richness can be described with the use of a rarefaction curve (Figure 3-3). A rarefaction curve is plotted as the number of new operational taxonomic units discovered as more sequences are being read. It allows comparison of species richness from differently sized communities. Even though a similar number of reads was recovered from both communities, the oxytetracycline-fed larvae had guts yielded nearly two times more different operational taxonomic units, suggesting the gut communities from antibiotic-fed larvae were more diverse than in the antibiotic-free larvae. This result supports the hypothesis that oxytetracycline disrupts the simple native gut microbiota, which provides colonisation resistance, and allows establishment of a more diverse microbial community.

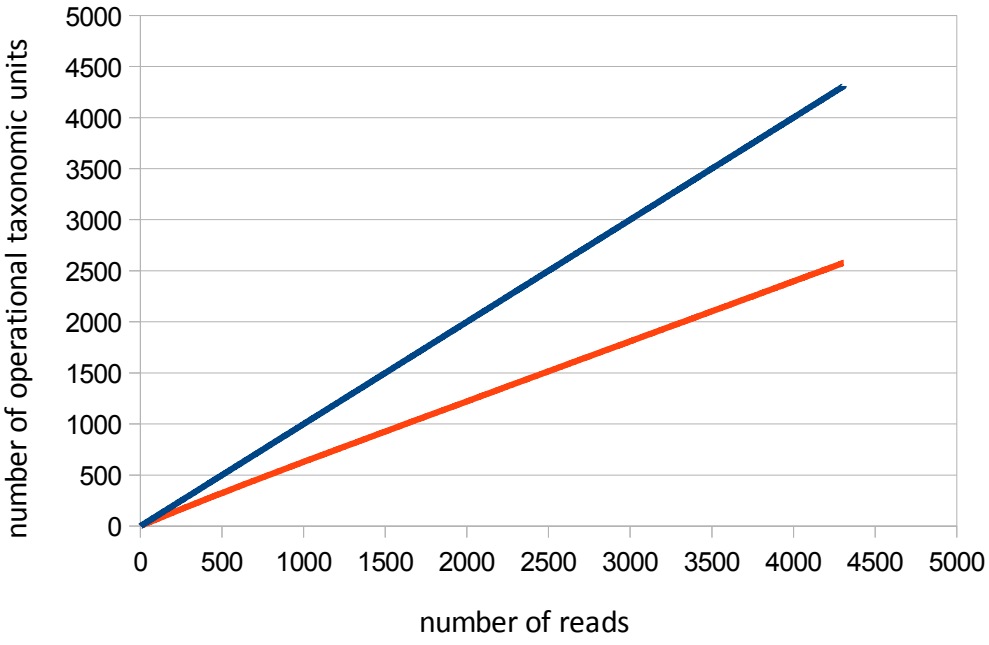


Figure 3.3: Rarefaction curves of metagenomic samples from G. mellonella guts. Species richness in the guts of G. mellonella larvae feeding on artificial food with and without antibiotics. The rarefaction curve shows the rate of increase in the number of species that are discovered as more reads are being sequenced. More different bacterial strains were identified in the guts of G. mellonella feeding on artificial food with oxytetracycline than in the guts of larvae not exposed to the antibiotic.

99% reads from the antibiotic-free sample belonged to just one phylum, the Firmicutes, and it was the only identifiable phylum of bacteria present in the sample (see Figure 3-4a). By "identifiable" I mean that some reads were assigned to reads from unidentified or unclassified bacteria, often from large metagenomic studies, where only short fragments of their genomes were sequenced. The remaining reads were eukaryotic, plant DNA from the *G. mellonella* food, and unmatched sequences, which had less than 97% similarity to any sequence in the EzTaxon-e database. On the other hand, reads from the oxytetracycline-fed larvae guts were more diverse, with 95% bacterial reads and 3% archaeal reads (see Figure 3-4b). The remaining 2% reads belonged to Eukaryota and unmatched sequences. Within the bacterial reads, not only Firmicutes (81% total reads) were present, but also Proteobacteria (4%), Thermotogae (3%) and Bacteroides (1%). For both *G. mellonella* gut samples the phylum Firmicutes was dominated by the Enterococcaceae family. This finding supports previous culture-dependent studies that have found only Enterococcus species in *G. mellonella* gut [152]. In previous studies predominantly *Enterococcus faecalis* (syn, *Streptococcus faecalis*) was isolated, but it was later identified to be more likely a strain of *Enterococcus faecium* (syn. *Streptococcus faecium*) [158]. Previous culture-independent studies described a gut community composed of Enterococcus species, dominated by *Enterococcus mundtii* [150]. In our study we found a diverse set of *Enterococcus* species. The gut microbiome from the oxytetracycline-free sample was dominated by *Enterococcus mundtii*, and the remaining strains were: strain EU465963_s, *Enterococcus dispar*, strain Enterococcus_uc, strain Enterococcus_uc_s. All remaining strains were present below 1.00% abundance. The gut community of oxytetracycline-fed larvae was dominated by strain EU465963_s, with strain Enterococcus_uc and strain Enterococcus_uc_s also present. The remaining strains were present below 1.00% abundance. *E. mundtii* was detected at 0.03%. Some of the strains isolated lack valid phylogenetic names. For example strain EU465963_s is a candidate Enterococcus species isolated from African elephant faeces [159]. The strain has not been cultured and only partial sequence of the 16S gene is available.

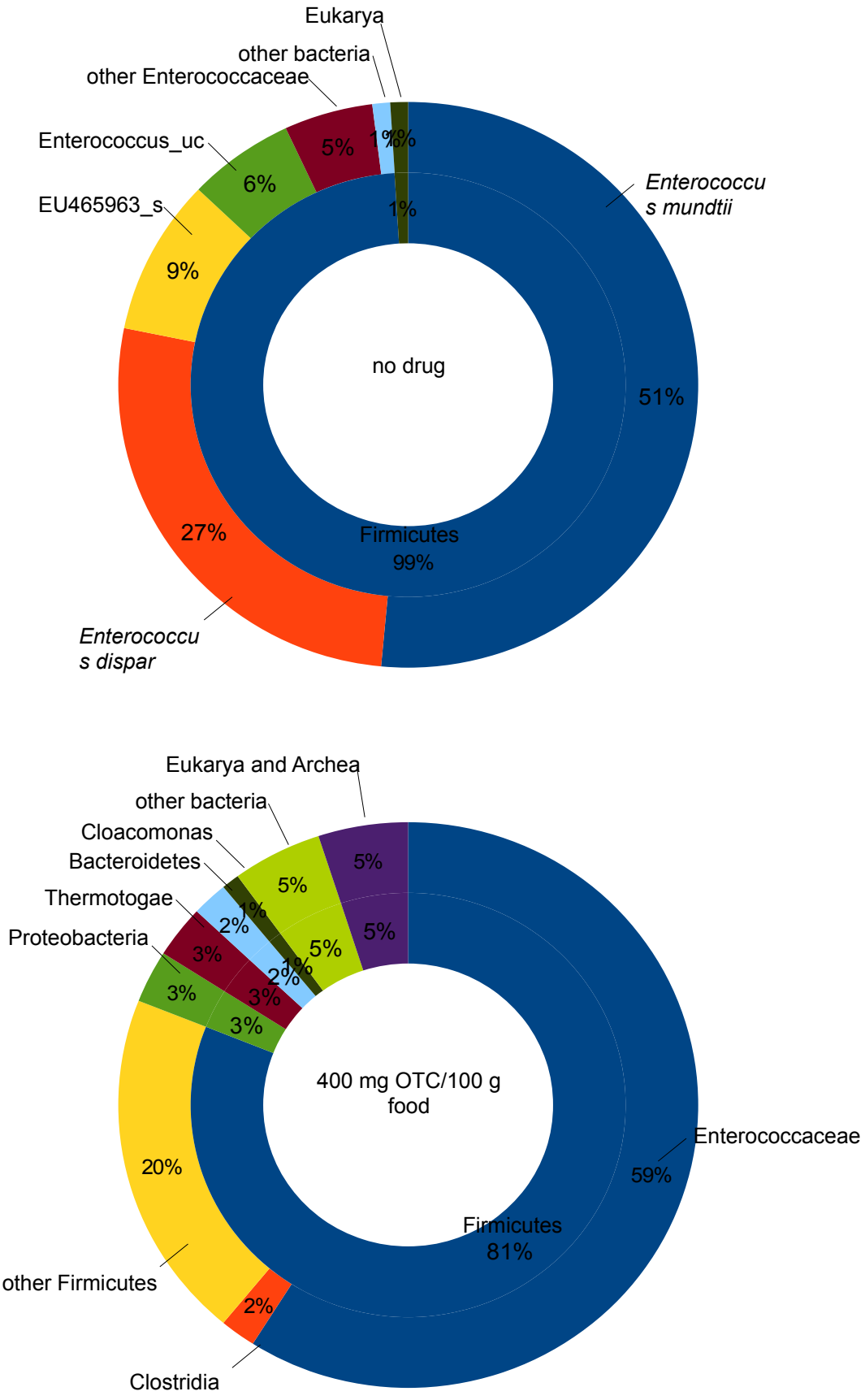


Figure 3.4: The composition of microbial communities identified in the guts of G. mellonella larvae

The bacterial diversity discovered in the guts of *Galleria mellonella* feeding on artificial food with and without oxytetracycline is higher than the diversity described before. It is possible that the insects sampled in the previous studies were not monoxenic. The guts might have contained a variety of closely related Enterococcus strains that are difficult to identify apart, without modern molecular biology techniques and sufficient sampling depth. Even with sensitive PCR methods, if not enough reads are sequenced, the microbial community might appear as monoxenic.

3.4 Discussion

The purpose of this experiment was to test whether insects feeding on compounds with antibacterial activity acquire antibiotic-resistant gut microbiota. We identified two bacterial strains from the guts of *Plutella xylostella* feeding on ciprofloxacin, but they were not more ciprofloxacin-resistant than the type strains. Similarly, two strains were isolated from the guts of *P. xylostella* feeding on oxytetracycline and again the gut-isolated strains were not more oxytetracycline resistant than the corresponding type strains. We continued the experiment over five generations using another lepidopteran host, *Galleria mellonella*, feeding on artificial food with oxytetracycline. We identified a tetracycline-resistant strain of *Bacillus subtilis* in the guts of larvae feeding on a low dose of oxytetracycline.

3.4.1 *P. xylostella* feeding on artificial food with ciprofloxacin or oxytetracycline

3.4.1.1 Comparison of bacteria cultured

In two separate experiments with *Plutella xylostella* gut bacteria, three different strains have been identified: *Enterobacter amnigenus*, shared between the microbiota from ciprofloxacin-fed larvae and oxytetracycline-fed larvae, *Bacillus subtilis* from ciprofloxacin-fed larvae, and *Sanguibacter keddideii* from oxytetracycline-fed larvae. In the light of previous investigations into *P. xylostella* gut composition [147], we have cultured only about 1% gut microbiome. It is possible that our culture methods favoured the growth of certain bacteria and their growth inhibited the growth of

slower growing cells.

3.4.1.2 The lack of ciprofloxacin and oxytetracycline resistance in the guts of P. xylostella larvae feeding on these antibiotics

Bacteria isolated from the guts of *P. xylostella* feeding on either ciprofloxacin or oxytetracycline were assayed for antibiotic susceptibility and their resistance profiles were compared to type strains obtained from a culture collection. In many cases no differences in the resistance levels between type strains and gut isolated strains were detected. We hypothesised that it is one of the most likely scenarios after the gut community was exposed to antibiotics. In a complex community like the insect gut, there is a possibility of detoxification by either the host or a member of the microbial community. It is also possible not all members of the microbial community are exposed to toxic concentrations of the antibiotic, for example by forming a biofilm.

It was predicted that some of the strains would acquire ciprofloxacin and oxytetracycline resistance in response to these antibiotics in their hosts' diets, but such a response was not observed. No differences in oxytetracycline-resistance levels were observed in oxytetracycline-fed larvae. The *B. subtilis* strain from the guts of *P. xylostella* feeding on ciprofloxacin was less ciprofloxacin-resistant than the type strain, in spite of the fact that the gut isolated strain was exposed to ciprofloxacin and the type strain was not. The *B. subtilis* type strain ATCC 6051 was originally deposited in 1930 as *B. subtilis* Marburg [160] and ciprofloxacin was not approved for clinical use until 1987 [161].

3.4.1.3 Resistance to other antibiotics in the guts of P. xylostella feeding on ciprofloxacin and oxytetracycline

It was hypothesised that antibiotic containing diets would induce multi-drug resistance. This was true for some strains, such as ampicillin- and chloramphenicol-resistant *B. subtilis* from ciprofloxacin-fed larvae. In most cases no resistance difference between gut-isolated strain and type strain was identified, even though the antibiotic concentrations in the food were physiologically relevant. The 10 mg/mL ciprofloxacin dose roughly equals to 50 mg/kg body weight/day, which is the top limit of

ciprofloxacin dose recommended for human use (25 – 50 mg/kg body weight/ day) [123]. The 3 mg/mL dose of oxytetracycline in artificial food is roughly equal to 15 mg antibiotic/kg body weight/day, which is the recommended human dose of oxytetracycline. One possible explanation of this finding is that the antibiotics underwent chemical transformations in the insect gut. Both ciprofloxacin and oxytetracycline are most stable at acidic pH and lepidopteran guts normally have basic pH. Oxytetracycline is stable at room temperature and acidic pH [162], but concentrated solutions (over 5% w/v) at neutral or basic pH degrade within one to seven days [163]. All solutions used were less concentrated than 5% (w/v), but to confirm the stability of the antibiotics in the insect gut, the gut contents and faeces should be assayed for antibiotic concentration.

Another possibility is that the antibiotics in the insect food induced transcriptomic changes leading to antibiotic tolerance or resistance, but not cell death. The use of veterinary fluoroquinolone antibiotic enrofloxacin on dairy farms has been shown to be associated with reduced antibiotic susceptibly in dairy calves [164]. The animals were treated for diarrhoea or respiratory disease with a range of veterinary antibiotics, but only the enrofloxacin use induced significant levels of resistance to other antibiotics, such as fluoroquinolones nalidixic acid and ciprofloxacin, and a cephalosphorin ceftriaxone. Ciprofloxacin is known to induce recombination of DNA via either RecBCD- or RecFOR-dependent mechanism [165]. The mechanism depends on double-strand DNA breaks and stimulates the recombination between both identical and divergent DNA sequences. The resulting increased genetic variation can include the acquisition, evolution and spread of antibiotic resistance elements.

3.4.2 *G. mellonella* feeding on artificial food with oxytetracycline

3.4.2.1 Bacteria cultured from the guts of G. mellonella

Only three strains were cultured from two samples of *Galleria mellonella* guts, even though nearly 200 guts were sampled. The control sample collected before the experiment commenced contained two Enterococcus strains, which constitute typical wax moth microbiota [166]. The strains were oxytetracycline-sensitive. A tetracycline-

resistant *B. subtilis* strain was identified from the guts of fourth generation larvae feeding on oxytetracycline at 1 mg/100 g food. This dose of oxytetracycline is roughly equal to half of the recommended human dose. The presence of tetracycline-resistant *B. subtilis* indicates that sub-inhibitory antibiotic concentrations selected for antibiotic resistant phenotypes. This supports the hypothesis that antimicrobial compounds in the insect gut lead to the acquisition of resistance, but a single isolate is not sufficient to prove this hypothesis.

No bacteria were recovered from most wax moth guts. Only three isolates were recovered from the total of 195 guts dissected. Such low recovery was not due to storage conditions, as two of the isolates were grown from the oldest samples (the stock insects from the JIC Insectary collected when the experiment was set up). Sample storage conditions can have an impact on the quality of the data collected. However for insect specimens different storage conditions such as freezing, ethanol, DMSO and CTAB, have little to no effect on the structure of bacterial community within the insect gut [167]. Equally surface sterilization in ethanol was shown to have no impact on the structure of the bacterial community recovered [167]. In the light of these findings it is unlikely that the storage conditions or sample preparation were responsible for the low number of bacteria cultivated from the wax moth guts.

Not only were few bacterial species cultured from the *G. mellonella* guts, but also little bacterial DNA was recovered from these samples. It is unlikely that the protocol used for the isolation of bacterial strains and the DNA extraction was not robust enough or too selective to suit the gut samples. Similarly to the sample storage conditions, different validated genomic DNA extraction protocols allow extraction of different amounts and quality of DNA, but ultimately the bacterial communities sequenced are undistinguishable [168]. In practice that means different methods of DNA isolation for sequencing do not have enough bias to produce different results. In our study both the few bacterial species cultured from the insect guts and the low concentrations of DNA recovered from the gut samples, point to low abundance of bacteria in the guts, not to inefficient protocols that failed to detect the presence of bacteria.

3.4.2.2 Metagenomic analysis

The metagenomic analysis of *G. mellonella* gut contents adds an extra dimension to what was already known about the composition of its gut microbiota. Previously described as having extremely simple gut community composed of one member species [150, 166], antibiotic-free *G. mellonella* harbours a community dominated by a subset of closely-related *Enterococcus* species. The community is dominated by *Enterococcus mundtii*. *G. mellonella*-derived *Enterococcus* species have been previously described as antibiotic-producers preventing colonisation by other species [151, 169] and our results confirm low abundance of other bacteria consistent with this hypothesis. However, it is not possible to hypothesise further about other potential roles of Enterococcaceae in *G. mellonella*.

3.4.2.3 Survey of tet resistance genes in G. mellonella gut

A variety of tetracycline resistance genes was identified from antibiotic-free *G. mellonella* larvae. Interestingly, the bacteria cultivated from the guts of insects before the antibiotic treatment were tetracycline-sensitive, even though tetracycline resistance gene tetM was detected in the sample. The stock insects brought to the Insectary to set up a colony had tetM resistance gene coding for a ribosomal protection protein, but after feeding on artificial food, the only detectable tet resistance genes were efflux pumps.

These results suggest a shift in microbiota after the colony was moved, even though the artificial food was prepared according to the same recipe. The initially-collected insects, collected before the start of the experiment, harboured only tetM ribosomal protection protein. After rearing the insects on artificial food for five generations, we only detected tet genes which are efflux proteins. After feeding the insects oxytetracycline, only a sub-set of these tet efflux proteins was detected. The shift in microbiota from harbouring only the tetM gene to hosting a range of efflux pumps seems to be caused by both the artificial food, which selected for a variety of efflux pump genes and the oxytetracycline in the artificial food selected for a narrower range of efflux pumps: tetB and tetD.

No resistance genes were identified in the guts of larvae feeding on the highest

concentrations of oxytetracycline. The high concentration of antibiotic has probably led to total loss of microbiota from the gut. However, high concentrations of DNA were recovered from the samples and the metagenomic analysis confirmed the presence of bacteria in the guts of larvae exposed to high levels of oxytetracycline in their food.

3.4.3 Limitations of the study

3.4.3.1 The use of type strains as experimental controls

A limitation of the study was the use of the bacterial type strains used as controls. Type strains are not a sufficient control for antibiotic susceptibility, because they are not a true negative control which would have a baseline antibiotic susceptibility. A type strain is normally the first isolate of a certain species deposited in a culture collection. Any other isolate of the species is closely related, but there is no conserved set of characteristics for the species. Bacterial isolates of the same species can have different sets of characteristics, such as antibiotic resistance genes.

The microbiota composition, not only their resistance levels, changes during the feeding experiment and the control strains used should reflect this change. Using the type strains has the advantage that if a new strain is acquired in the antibiotic-treated sample, a control type strain can be obtained easily from a culture collection. When the use of type strains is omitted it is more difficult to make comparisons between strains when a new strain is acquired.

3.4.3.2 Low numbers of bacterial strains recovered from the insect guts

The *Plutella* experiments only surveyed the culturable strains, missing the diversity represented by the unculturable strains. We tried to account for that missing diversity when the study was re-designed using *G. mellonella*. The *Galleria* experiments surveyed the unculturable strains by a metagenomic analysis and by the amplification of tetracycline resistance genes, but their functionality could not have been tested due to the poor recovery of culturable strains from the larval guts. Ideally we would like to compare the results from culture-dependent and –independent methods.

To increase the number of strains cultured from the insect guts and to obtain enough

DNA for downstream analyses the gut samples were pooled in the *G. mellonella* experiment. The protocol was developed for another experiment (see Chapter 5, Replacement of native gut microbiota) where we managed to culture multiple isolates from each sample. In the current study the sample pooling helped reach DNA concentrations required for PCR and metagenomic analyses, but did not aid recovering more bacterial strains from the guts on solid agar media.

3.4.3.3 Comments about artificial food

Notably some groups of *G. mellonella* larvae experienced higher mortality when feeding on the artificial food. There are two possible issues leading to this: mortality associated with the food itself and mortality associated with the addition of antibiotics. The first problem is normally caused by sub-optimal composition of the diet and the mortality occurs in all groups. That was not the case for *Plutella xylostella* or *Galleria mellonella*. The artificial food was composed to optimise insect growth and health, even though such diets do not facilitate easy handling of the insects. For example, larvae on low-protein diets cease silk production making it is easier to clean the food containers and finding the pupae ready for eclosure (emergence of the adult form). However such a low-protein diet also leads to fragile pupae, which break easily when handled.

There was mortality in the groups of *G. mellonella* larvae feeding on artificial food with high concentrations of oxytetracycline, but not in *P. xylostella* feeding on ciprofloxacin or oxytetracycline. Mortality associated with antibiotics in the artificial food is often due to the loss of obligate bacterial symbionts. However, *G. mellonella* can be grown axenically, and the larvae are healthy and able to digest their diets as efficiently as non-axenic cultures [170]. It is more likely that the mortality in the groups of wax moth larvae exposed to high concentrations of oxytetracycline was due to a toxic dose of the antibiotic in the diet. The highest doses were selected as part of a wide range of concentrations, spreading from sub-inhibitory concentration to above a toxic dose, in an attempt to capture the impact of antibiotics on the insect gut microbiota at different physiological concentrations.

3.4.4 Summary and conclusions

The aim of this chapter was to test whether the microbiomes of insects feeding on artificial food containing antibiotics acquire antibiotic resistance. In the first set of experiments *Plutella xylostella* feeding on ciprofloxacin contained gut bacteria that were more susceptible to ciprofloxacin than the corresponding type strain. We observed increased resistance to other antibiotics, such as ampicillin, chloramphenicol and tetracycline. To further test our hypothesis the experiment was re-designed to include oxytetracycline. *P. xylostella* larvae feeding on artificial food with oxytetracycline, had no increased oxytetracycline resistance levels and the gut bacteria were more susceptible to chloramphenicol and ciprofloxacin than the type strains obtained from a culture collection. It was not possible to expose the insects to antibiotics over a number of generations due to pupae mortality and no oviposition in adults. Instead we adopted a different lepidopteran host: greater wax moth *Galleria mellonella*. We identified oxytetracycline-sensitive bacterial strains in the guts of larvae collected before the experiment started and an oxytetracycline-resistant strain from larvae feeding on sub-inhibitory doses of the antibiotic. A survey of tetracycline resistance genes present in the larval guts identified a number of different efflux pumps in the guts of insects feeding on artificial food without antibiotics and a smaller sub-set of these efflux pumps in the guts of insects feeding on artificial food containing oxytetracycline.

The results of *P. xylostella* experiments do not demonstrate increased antibiotic resistance in response to the exposure to ciprofloxacin and oxytetracycline in the insect diet. The *G. mellonella* experiment demonstrated a bottom line diversity of tetracycline resistance genes in the larval gut and persistence of some of these elements upon exposure to oxytetracycline. The experiments described show that exposure of bacteria to an antibiotic in the insect gut does not lead to rapid acquisition of resistance to this particular antibiotic, unlike the exposure of bacteria to antibiotics on solid or liquid media. The effects of antibiotics on insect gut microbiomes are more nuanced, as demonstrated here and elsewhere. The dynamic resistome of the agriculture-associated insects' gut in the absence of a selective pressure, in the form of high concentration of clinically relevant antibiotics, can contribute to the dissemination of

resistance genes throughout environments.

4 Investigating antibiotic resistance in the insect gut bacteria: the search for novel plant-derived antibacterials

Parts of the work described in this chapter were performed by undergraduate students under my supervision. Ryan Hull dissected death's head hawkmoth larvae, identified the gut bacteria, and performed preliminary antibiotic susceptibility and plant extract susceptibility assays. George Newell dissected beet armyworm larvae, identified the gut bacteria, performed antibiotic susceptibility testing and preliminary plant extract purification.

I would like to thank Prof. Sarah O'Connor and members of her group: Dr Richard Payne and Dr Evangelos Tatsis for their advice on Catharanthus roseus.

All data in this chapter was generated by me and I produced all figures and tables on my own.

4.1 Abstract

In this chapter we investigated six different insects feeding on medicinal or toxic plants, and the antibiotic resistance of their gut bacteria. We wanted to further explore the phenomenon of antimicrobial resistance that can be found in the gypsy moth larvae in the absence of clinically relevant levels of antibiotics [121]. There were two aims to these experiments: firstly to determine if antibiotic resistance is a common phenomenon in the insect gut, and secondly to test whether the elevated levels of antibiotic resistance in insect gut microbiota can aid identification of antibacterial compounds from the insect food-plants. We selected plant/insect pairs based on the medicinal or toxic properties of the plant, and identified a number of different bacterial strains from the insects' guts, some of them typically associated with the human microbiota. After culturing the gut bacteria we tested the antibiotic susceptibility of these strains and compared it to the susceptibility of type strains obtained from culture collections. We also tested the susceptibility of both the gut-isolated strains and type strains to the plant extracts, and used the differences as a guide for fractionation of the plant extracts. Five out of six extracts tested had antibacterial properties. In some cases

type strains were more resistant to the plant extract than the gut-isolated strains that have been exposed to the plant extract. We used mass spectroscopy to identify some of the metabolites present in the plant extract fractions with antibiotic activity. We successfully identified the most abundant compounds from Madagascar periwinkle extracts and confirmed their antibacterial properties. To summarize, in this chapter we have shown that antibiotic-resistant bacteria can be found in the guts of insects feeding on medicinal and toxic plants and that metabolites with antibacterial properties can be identified through assay-guided fractionation of plant extracts. Overall, our approach was successful for identifying antibiotic resistance in the guts of a variety of insects and for fractionating the plant extracts, but the identification of plant metabolites remained the main bottleneck in discovery of novel compounds with antibacterial properties.

4.2 Introduction

4.2.1 Antibiotic discovery void

The antibiotic discovery gap encouraged the search for novel compounds in unusual places. A wide variety of microbiomes have been screened: soil [171], marine [172] and even human gut microbiome [173]. Attention has turned towards microbes considered unculturable. The methods of bacterial culture have been optimised for strains that cannot be cultured using standard protocols [174] and bioinformatic techniques allow analysis of strains that cannot be cultured by available protocols [175].

Even though such approaches are a recent development, they have already led to the discovery of novel compounds with antibiotic properties. Teixobactin is an example of an antibiotic discovered in a screen of bacteria that cannot be grown under standard laboratory conditions [87]. The screen was conducted by diluting soil samples and flowing the suspensions through a microchannel device. The protocol was set up so that single bacterial cells were separated before the device was placed back in the soil to allow diffusion of nutrients and growth of bacteria in their native environment. Over 10,000 isolates have been screened through this method and strain candidate *Eleftheria terrae* has been shown to produce a potent antibiotic [87]. The antibiotic

was named teixobactin. Its mechanism of action requires binding precursors of cell wall components: peptidoglycan and teichoic acid, and therefore inhibiting cell wall synthesis. Interestingly teixobactin-resistant mutants cannot be readily obtained *in vitro* with *Staphylococcus aureus* or *Mycobacterium tuberculosis*. The lack of resistance does not indicate that resistance is not going to appear in clinical settings, but it is likely going to appear later and less frequently.

4.2.2 Phytochemicals as novel antimicrobials

Surprisingly, a chemical space that remains underexploited is plant-produced compounds. Even though plants evolve slower than bacteria, they have larger genomes and produce an extensive variety of chemical scaffolds. Plants are thought to have over 100,000 secondary metabolites, many of which have antibacterial activity [176]. Phytochemicals have been developed as extremely successful medicines, including antimalarial drugs quinine [10] and artemisinin [12], yet there are no commercially-available plant-derived antibiotics.

An interesting approach in the search for novel plant-derived medicines is bioprospecting. Bioprospecting can be defined as a systematic search and development of biological resources, especially genetic and biochemical ones, particularly utilizing indigenous knowledge. The key element of bioprospecting is that the approach needs to be systematic. For example, it was discovered that medicinal plants from related genra are used in the same therapeutic areas in different parts of the world [177]. Plants from Nepal, New Zealand, and the Cape of South Africa have been arranged in a genus-level molecular phylogenetic tree. 3.6 – 14.0% plants assessed had a record of medicinal use in traditional practices and plants indicated for treatment of certain diseases grouped together between different regions. The medicinal plants also grouped together with plants used to produce pharmaceutical drugs used in conventional medicine. Such clustering is probably caused by independent discoveries of medicinal properties by the indigenous cultures rather than knowledge transfer between cultures. Such clustering also allows identification of good candidate genera for natural products for drug discovery.

An example of how bioprospecting can lead to bioactive compounds is the isolation of

evocarpine and four related quinolone alkaloids from the fruits of *Evodia rutaecarpa* [178]. The fruits of *E. rutaecarpa* are known in traditional Chinese medicine as Fructus Euodiae or Wu Zhu Yu, and are used to treat infections and inflammation. The compounds were isolated from the ripe dry fruits that are used to prepare the traditional decoction. Evocarpine was the most active among the compounds tested against methicillin-susceptible and methicillin-resistant *Staphylococcus aureus*, but not *Escherichia coli* reference strain. Such studies confirm the activity of traditional medicines and provide candidate molecules for drug development.

Not all plants used in traditional medicine have a specific mode of action. The activity of some plants is a result of the placebo effect or general cytotoxicity. In many cases traditional herbal remedies lack quality control and clinical data on their efficacy and safety, or the data are of sub-standard quality [179]. The chemical composition of plant formulations is often extremely variable and even standardized formulations are frequently understudied. In some clinical studies, traditional remedies fail to show a significant benefit. For example, in a large clinical study of traditional Chinese medicine remedies for childhood bronchial asthma there was no statistically significant difference in many clinical indicators between the different remedies and the placebo group [180].

Additionally, toxicity of plant preparations can be easily underestimated. Even the oldest records of traditional Chinese medicine specify a category of toxic plants [181]. The category encompasses a third of herbs described and these plants are only to be used for a short period of time. Interestingly, even though the toxicity of plant preparations for humans is a significant issue, hardly any plant is completely free from herbivory.

A number of insects feed on plants and their products, including the plants with medicinal properties. It is not uncommon for insect gut bacteria to be resistant to antimicrobial or toxic components of their foods or to contribute to the detoxification of such compounds. Royal jelly is a honey bee secretion used for nutrition of queens and larvae and has a potent antimicrobial effect [182]. While core honey bee gut bacteria are resistant to the antibacterial activity of royal jelly, the corresponding environmental strains, non-core species and control strains not associated with bee

colonies are susceptible [183]. In a disc diffusion assay, six out of eight core isolates were fully resistant to royal jelly, the other two isolates (*Neisseriaceae* sp. and *Lactobacillus* sp. A) were mildly susceptible and the corresponding environmental isolates were fully susceptible. For example *Lactobacillus kunkeei* gut-isolated strain was fully resistant while the flower-isolated strain was susceptible. This finding suggests that honey bee gut bacteria are specialized to thrive in the gut environment.

In some cases the gut bacteria are responsible for detoxification of the plant food in the insect gut before digestion. For example apple maggot flies (*Rhagoletis pomonella*) harbour in their guts *Pantoea agglomerans*, a bacterial strain capable of detoxifying phloridzin, a potent insecticidal compound present in apple leaves [184]. The flies feed on phloridzin-rich leachate present on the apple leaves and die if the toxin is not utilized by *P. agglomerans*. Additionally, the bacteria-processed leachate contains more amino acids, making it more nutritious for the flies. Both the honey bee and the apple maggot fly examples illustrate how the relationship between insects and their food is mediated by the insect gut microbiota.

4.2.3 Food with antibacterial activity can lead to antibiotic resistance in the insect gut

The insect diet exerts a strong selective pressure on the gut bacteria and can potentially lead to antibiotic resistance. It was demonstrated in the previous chapter that diet can exert a selective pressure on the gut bacteria. Bacteria resistant to ampicillin, chloramphenicol and tetracycline were found in the guts of diamondback moth larvae feeding on artificial food containing ciprofloxacin and oxytetracycline resistant bacteria were found in the guts of greater wax moth larvae feeding on oxytetracycline-containing food. Surprisingly a variety of efflux pumps conferring resistance to tetracyclines were also present in the guts of insects not exposed to antibiotics for over at least six generations.

Similarly, a range of antibiotic resistance genes was identified in gypsy moth (*Lymantria dispar*) gut [121]. The gypsy moth larvae, feeding on a variety of trees (larch, white oak, willow and aspen) in the wild, were collected and their cultured gut bacteria were resistant to many common antibiotics: carbenicillin, ceftazidime, chloramphenicol,

gentamycin, erythromycin, kanamycin, nalidixic acid, rifampicin, streptomycin, tetracycline and vancomycin. Functional metagenomic analysis of the cultured isolates was conducted to identify novel antibiotic resistance elements. Libraries of the isolates were prepared as for metagenomic sequencing analyses, but prior to sequencing they were screened by plating on antibiotic-containing media to identify genes conferring resistance to these antibiotics. The analysis revealed three types of genes responsible for the resistant phenotypes: 1) a multidrug resistance protein of the resistance-nodulation-cell division superfamily, 2) an AraC/XylS family transcriptional regulator, and 3) a novel extended-spectrum β-lactamase.

These novel resistance elements from *Lymantia dispar* and tet-resistance genes from antibiotic-free *Galleria melonella* are of clinical relevance. The presence of these genes in insect gut bacteria confers antibiotic resistance phenotypes and can readily spread to the environments inhabited by the insects. More importantly the antibiotic resistance elements present in the insect guts in the absence of clinically relevant levels of antibiotics suggests that the genes can be maintained in response to other environmental stimuli. We hypothesise that the stimuli responsible for the antibiotic resistance are components of the insect food with antibacterial activity.

4.2.4 Aims of this chapter

Previous work has shown that unexpected antibiotic resistance can be found in the guts of insects feeding on plants. The aims of this chapter were two-fold. Firstly to see whether the phenomenon of unexpected resistance to antibiotics found in gypsy moth gut bacteria feeding on the leaves of various trees could be found in other plant-insect pairs, i.e. is this a general phenomenon? Secondly to test the hypothesis that insect gut bacteria can be utilized in assay-guided fractionation of plant extracts leading to the identification of plant fractions with antibacterial activities. We proposed to compare antibiotic susceptibility profiles and plant extract susceptibility of gut-isolated bacterial strains and corresponding type strains and use the differences in the susceptibility to plant extracts between the two different isolates to identify plant extracts with the most promising antibiotic activity.

4.3 Results

To test the hypothesis that antibacterial activity of medicinal plants can be identified with the use of insect gut bacteria, we assayed gut bacteria from a variety of insects and their susceptibility to the food plant extracts. This involved the selection of six plant-insect pairs and an examination of the antibiotic susceptibility of their gut bacteria.

4.3.1 Giant stick insect (*Diapherodes gigantea*) feeding on eucalyptus (*Eucalyptus dalrympleana*)

4.3.1.1 Diapherodes gigantea

One of the first plant/insects pairs we chose to investigate was a giant stick insect feeding on eucalyptus. Eucalyptus is a plant with known medicinal properties [185] and a number of eucalyptus trees grow near the JIC, supplying food for the insects and material for plant extract preparation. The stick insects were already kept in the JIC Insectary, and they are large and easy to dissect. A single specimen provides sufficient amount of gut contents for protocol development. Together the medicinal properties of the plant and the convenience of the insect make this plant/insect pair an ideal starting point for this part of the project.

D. gigantea are insects of the order Phasmatodea, native to the Caribbean. The insects do not have larval stages, and instead they hatch as nymphs and moult five times before reaching adulthood. They are characterised by sexual dimorphism, with larger lime-green females reaching up 15 to 20 cm length and smaller brown males reaching 10-15 cm length (Figure 4-1). Both sexes have underdeveloped wings, that cannot sustain flight, but the males can make long jumps.

Figure 4.1: Giant stick insect (Diapherodes gigantea) feeding on eucalyptus (Eucalyptus dalrympleana). Female stick insect is shown in the top panel and male stick insect is shown in the bottom panel. Photo by Andrew Davies, JIC, Norwich.

4.3.1.2 Gut dissections

We obtained two female adult stick insects from the JIC Insectary. The insects died of old age and did not have to be euthanized. Before the dissection the insects were stored in a freezer to avoid decomposition. The digestive tracts of the insects were dissected aseptically by making a laceration along the body of the insect and picking

out the gut, which resembles a large tube filling most of the body cavity. The gut can be easily identified as it contains partially digested green and brown plant material with large undigested seeds.

The gut contents were serially diluted, plated on solid media and incubated in aerobic conditions. We initially incubated the gut bacteria in both aerobic and anaerobic conditions, but we have not obtained any obligatorily anaerobic bacteria. For every new species of insects we initially cultivated the gut bacteria with and without oxygen, but in every case there were no bacterial strains that would grow exclusively in anaerobic conditions. Insects' guts are normally anoxic [187] and the low oxygen levels, especially in the midgut, are maintained by endogenous chemical processes, suggesting that the bacteria isolated from the insect guts are facultatively anaerobic. A possible explanation for this is that the insect gut bacteria are often environmental strains that are adapted both to the outside environment and the insect gut, or strains specific to the insect gut that need to withstand the aerobic conditions during vertical transfer from the mother to the egg.

4.3.1.3 Identification of the gut bacteria

Three bacterial species were cultivated from the stick insect guts and identified by 16S PCR using alkaline PEG reagent. Briefly, the PCR conditions facilitate the lysis of bacterial cells and the release of genomic DNA. The 16S gene is amplified with universal primers that are specific for bacteria but not unique for any particular group of bacteria. The 16S gene codes for the 16S ribosomal RNA which is a structural component of the prokaryotic ribosome. It is composed of a series of single-strand loops and double-strand stems. The double-strand stems contain complementary RNA strands. The loop regions of the RNA are free to mutate and correspond to the variable regions of the gene. They allow distinguishing between closely related bacteria. The stem regions of the RNA are much more conserved and that regions of the gene evolve much slower than the variable regions.

We identified the three bacterial isolates from the *D. gigantea* guts: *Bacillus amyloliquefaciens*, *Microbacterium oxydans* and *Sphingobacterium multivorum*. *B. amyloliquefaciens* and *M. oxydans* are environmental strains, first isolated from soil

and air respectively. *S. mulitvorum*, on the other hand, was first isolated from the human spleen. From our observations, insect gut microbiota often contain a mixture of environmental isolates and strains considered to be human pathogens.

To investigate the antibiotic resistance in the guts of the stick insects we obtained bacterial type strains, matching the strains isolated from the stick insect guts, from the Centre de Resources Biologiques de l'Institut Pasteur in Paris (CRBIP) culture collection. Both the gut-isolated strains and type strains were assayed for antibiotic susceptibility by the broth microdilution method. It was hypothesised that in response to antimicrobial compounds in eucalyptus, the gut isolated strains would be more antibiotic resistant than the type strains.

4.3.1.4 Antibiotic susceptibility testing

The gut-isolated strains and the type strains were tested in a broth microdilution assay for their susceptibility to ampicillin, chloramphenicol, ciprofloxacin, kanamycin, rifampicin and tetracycline. This panel of antibiotics was chosen based on their varied mechanisms of action. Ampicillin inhibits the final stage of cell wall synthesis leading to cell lysis. Chloramphenicol prevents protein synthesis by inhibiting the peptidyl transferase activity of the ribosome. Ciprofloxacin is a gyrase inhibitor and kills cells by creating breaks in DNA. Kanamycin inhibits protein synthesis by interfering with the 30S ribosomal subunit. Rifampicin disrupts RNA synthesis by inhibiting RNA polymerase. Finally, tetracycline prevents protein synthesis by blocking the attachment of the aminoacyl-tRNAs to the ribosome.

In most cases there was no difference in antibiotic susceptibility between the type strains and gut-isolated strains (Table 4-1). *B. amyloliquefaciens* gut-isolated strain was more resistant to ciprofloxacin and tetracycline than the type strain. *M. oxydans* from the stick insect gut was more resistant to ciprofloxacin, but less resistant to tetracycline when compared to the type strain. Gut-isolated *S. multivorum* was more susceptible to tetracycline than the type strain, unsurprisingly as the type strain is a clinical isolate.

Table 4.1: Antibiotic susceptibility profiles of the gut bacteria from D. gigantea and corresponding type strains. MICs for six different antibiotics were assessed by a broth dilution assay. Differences in antibiotic-resistance levels between the strains have been highlighted in green when the gut-isolated strain was more resistant than the type strain and in red when the type strain was more resistant than the gut-isolated strain. $p < 0.005$.

	Minimal inhibitory concentrations of antibiotics in broth dilution assay [µg/mL]					
	Bacillus amyliliquefaciens		*Microbacterium oxydans*		*Sphingobacterium multivorum*	
	gut strain	type strain	gut strain	type strain	gut strain	type strain
ampicillin	>64	>64	>64	>64	>64	>64
chloramphenicol	2	2	1	1	1	1
ciprofloxacin	0.25	<0.01	0.03	<0.01	0.25	0.25
kanamycin	1	1	>64	>64	>64	>64
rifampicin	1	1	1	1	1	1
oxytetracycline	1	0.03	2	>64	0.25	32

4.3.1.5 16S metagenomic analysis

Sample preparation

Only a small proportion of bacteria can be cultured under laboratory conditions. It is often repeated that 99% bacteria are unculturable. To assess what proportion of bacterial strains is recovered from the insect gut by culture-dependent methods, we compared the bacteria isolated from the insects' digestive tracts by plating and by sequencing of the whole gut communities. Our metagenomic analysis was a study of all 16S genes recovered directly from the stick insects' gut contents.

To conduct this study another two stick insects were dissected as described before. Bacteria from their guts were cultivated and identified. Additionally bacterial genomic DNA (gDNA) was isolated from the samples using a kit optimised for the use with soil bacteria. Initially we used a protocol in which bacterial cell wall was enzymatically digested, but the yields and quality of gDNA were low, probably due to enzymatic degradation of the genetic material during the lengthy digestion of thick bacterial cell walls. Instead we opted for MP Bio Kit for Soil which utilizes bead-beating in a tissue lyser to break open cells. The bead-beating can be performed at a low temperature or the samples can be cooled down in between the beating steps. The yield of gDNA

greatly improved with the use of the kit.

Sequencing

After the gDNA extraction the gut samples were submitted for 16S sequencing with Chun Lab (Seoul, Korea). The sequencing lab performed quality controls, such as measuring the amount of DNA in samples and assessing their quality, and the metagenomic library construction before sequencing on the MiSeq Nano platform. The initial bioinformatics analysis, such as trimming of the initial reads, contig assembly, filtering low quality reads and chimeric sequences, was also performed by the sequencing lab. The data were read and processed in CLcommunity software.

Before the metagenomic analysis results are discussed it is important to mention the difference between the terms operational taxonomic units and bacterial species. Species can be defined as a group of organisms consisting of similar individuals capable of exchanging genes and producing fertile offspring. Within this definition the term does not apply to bacteria. A more rigorous definition is the operational taxonomic unit, which refers to a group of sequences with 97% similarity. Here we understand bacterial species as a group of strains with 97% identity between 16S genes and we use the terms species and operational taxonomic units interchangeably.

Species richness

The samples were analysed for species richness, the measure of diversity of bacterial strains present in the insect guts. Even though the samples contain different number of valid reads, they can be compared with the use of the rarefaction curves. A rarefaction curve plots the number of new operational taxonomic units discovered (which can treated as new bacterial species identified in each sample) against the number of reads sequenced. This type of plot shows not only which sample contained more different bacterial strains as the same number of sequences was being analysed, but also if the communities were sampled at a sufficient depth. When the sequencing was deep enough (the sufficient number of sequences was read), no new operational taxonomic units are being discovered, i.e. every strain in the sample has been identified.

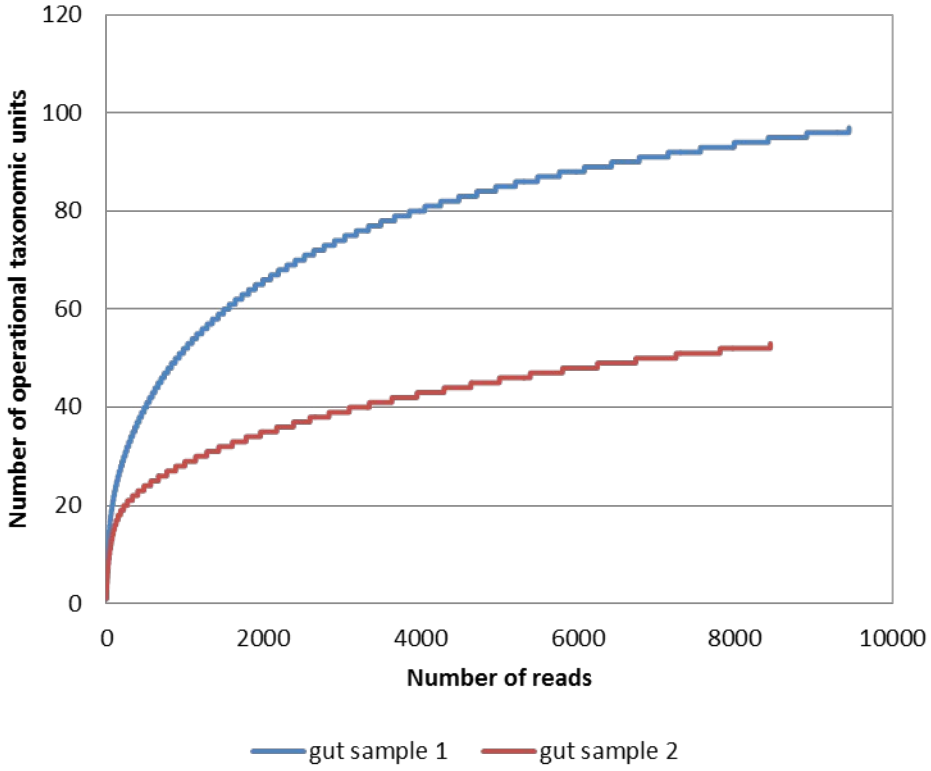


Figure 4.2: Rarefaction curves of metagenomic samples from two D. gigantea guts. A rarefaction curve shows the rate of increase in the number of species that are discovered as more reads are being sequenced. These data indicate that one of the samples had larger species diversity as more OTUs were discovered from it and that both guts were sampled sufficiently to discover most strains present in them.

The two samples, originating from two separate stick insects, contained 9450 and 8450 valid reads, yielding 97 and 53 operational taxonomic units (OTUs) respectively. The rarefaction curve reached a plateau indicating that the bacterial diversity in the guts has been sufficiently sampled (Figure 4-2). Out of the sample with 97 different OTUs, only 12 were present at above 1% abundance (Figure 4-3). The most abundant strain was *Serratia marcescens*, which constituted 33% bacterial reads retrieved from the *G. gigantea* guts. The other gut sample contained 53 OTUs and five of them were present above 1% abundance. Again, *S. marcescens* was the dominating strain in the gut community with 91% abundance. *S. marcescens* is a common pathogen of insects but it can be also found in healthy insects [188].

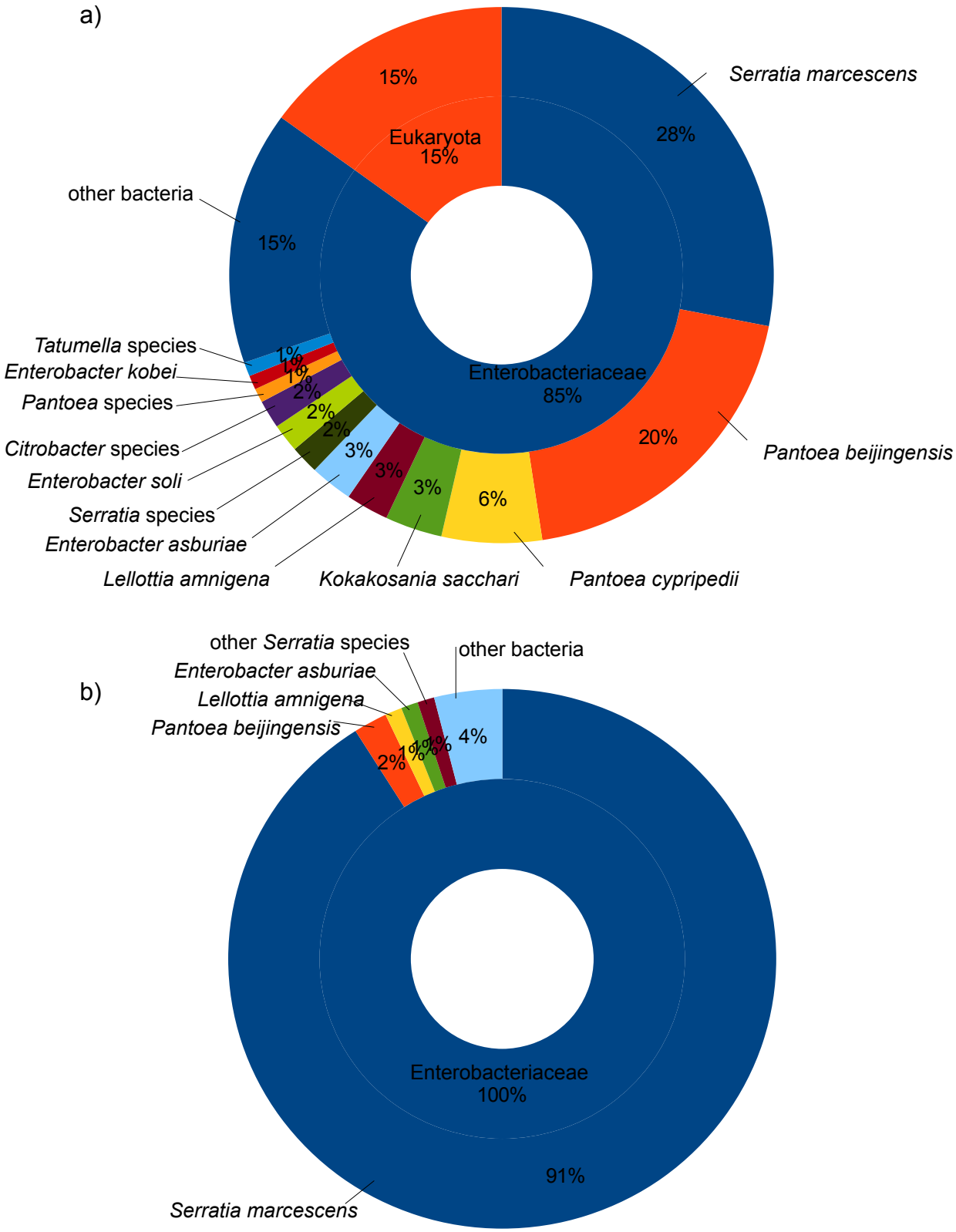


Figure 4.3: The composition of D. gigantea gut community by genus (inner ring) and species (outer ring) level. The composition of two gut communities was shown separately. The first sample (a) had contaminant plant DNA and higher species diversity. The second community (b) was dominated by S. marcescens. Bacteria with less than 1% abundance were pooled together into the "other bacteria" category.

S. marcescens was the dominant species, constituting 33% and 91% each gut dissected (Figure 4-3). The only bacterial order found in the guts was Enterobacteriales. Only one of the *D. gigantea* guts contained plant material (15%), which indicates that either the samples were insufficiently homogenized before DNA isolation or one of the insects ceased feeding earlier and its gut was enriched in bacteria. Other strains shared by the two gut communities are: *Pantoea beijingensis*, *Lellottia amnigena*, and *Enterobacter asburiae*.

Culture-dependent methods

Because the metagenomic analysis was performed on a new set of insect guts, we cultured and identified strains from these samples as described before. A set of strains was identified: *Serratia marcescens*, *Microbacterium paraoxydans*, *Kocuria rhizophila* and *Pectobacterium cypripedi*. The older samples, used for the assessment of antibiotic resistance in the insect gut, contained a different set of strains: *Bacillus amyloliquefaciens*, *Microbacterium oxydans* and *Sphingobacterium multivorum*. It is unusual no strains are shared between the two sets of *D. gigantea* guts. The only closely related strains are the two *Microbacterium* species. There are two possible explanations for this observation: 1) the two sets of stick insects had very different gut microbiomes, or 2) we have not plated enough cells to sample the true diversity in the insect guts. Most likely the differences observed are due to the combination of both these factors.

Comparison of culture-dependent and –independent methods

Surprisingly initially only *Serratia marcescens* and *Pantoea cypripedii* were found both in the cultured strains and the metagenomic dataset. To investigate if 16S sequences belonging to the strains we cultured from the insect guts are present in the metagenomic dataset, we searched the datasets for sequences with 97% similarity to the sequences from the cultured strains. We used the CLcommunity software to browse the metagenomic datasets from the stick insect guts. A BLAST search for sequences matching the cultured strains can be performed on the metagenomic data from the stick insects' guts. When the datasets were queried for sequences matching the 16S gene from *M. paraoxydans* and *K. rhizophila*, sequences with ≥97% identity were returned. This indicates that either these strains were present in the stick insect

gut but were misidentified, or a close relative was present in the gut community and the algorithm used to align the 16S sequences cannot distinguish between closely related sequences.

Such discrepancy is a result of differences between the databases used for species identification in each method. For identification of cultured strains the GeneBank database was used and the metagenomic database was based on TaxonEZ. TaxonEZ is carefully curated database and its main purpose is to allow for identification of bacterial strains down to species level. GeneBank is a larger, less curated database that allows identification of the most related strains without the focus on nomenclature and phylogeny.

Additionally the algorithm used is blastn which is optimised for similar, not identical sequences. The algorithm permits gaps in sequence alignments and scores these alignments to assign a sequence with the best overall similarity. We chose to use blastn because of its robustness, sacrificing the accuracy of the match.

Metagenomics - summary

In our comparison of culture-dependent and –independent methods we have shown that we can culture less than 3% strains from the *D. gigantea* guts. We were able to culture the most abundant strain, *Serratia marcescens* and some less abundant strains. We have also found discrepancies in the bioinformatic analyses of the sequences generated by the culture-dependent and –independent methods.

4.3.1.6 Eucalyptus extract preparation

To test the antibacterial properties of the eucalyptus leaves, an extract was prepared by infusing dried eucalyptus leaves in methanol for three days, replacing the solvent daily. The antibacterial activity of the extract was assayed by the disc diffusion method. Paper discs were infiltrated with the eucalyptus extracts and dried to evaporate the methanol. The discs were placed on bacterial lawns of the gut-isolated strains and type strains of *Bacillus amyloliquefaciens*, *Microbacterium oxydans* and *Sphingobacterium multivorum*. It was hypothesised that the type strains would be more susceptible to the plant extracts as they have not been exposed to them before.

It was observed that sometimes the type strain was more resistant to the eucalyptus extract than the gut–isolated strain (Table 4-2). No differences in plant extract susceptibility were observed for *B. amyloliquefaciens*, both the gut-isolated strain and type strain were fully resistant to the eucalyptus extract. As predicted gut-isolated *S. multivorum* strain was more resistant to the eucalyptus extract than the type strain that had not been exposed it. Surprisingly, the opposite was observed for *M. oxydans*. The gut-isolated strain was more eucalyptus extract susceptible than the type strain that has not been previously exposed to it. *M. oxydans* has shown unexpected ciprofloxacin resistance in the antibiotic susceptibility testing and it is possible that the strain is generally more resistant to a number of compounds or conditions.

Table 4.2: The susceptibility of the gut bacteria from D. gigantea and corresponding type strains to eucalyptus leaf extract. The values were obtained in a disc diffusion assay. The discs carried 10 µg dried eucalyptus extract. The differences in plant extract susceptibility between the strains were highlighted with green when the gut-isolated strain was more resistant than the type strain and in red when the type strain was more resistant than the gut-isolated strain. The assay was repeated three times.

	Diameter of hinhibition zones [mm]					
	Bacillus amyliliquefaciens		*Microbacterium oxydans*		*Sphingobacterium multivorum*	
	gut strain	type strain	gut strain	type strain	gut strain	type strain
crude	0	0	7	4	10	17
HPLC fraction	0	0	12	10	7	14

It should be noted that to compare the antibacterial activity of extracts from different plants, the extracts were standardized. Each extract was dried in an evaporator until a dry pellet was obtained. Sometimes large amounts of waxy material were present in the samples and the pellets would not appear dry. In such cases the pellet was evaporated until no more reduction in volume occurred. The pellets were then weighted and re-suspended in methanol at 100 µg/mL. These re-suspended extracts were used to infuse paper discs before assaying on the bacteria from the insect guts and the corresponding type strains. This procedure was carried out to standardize the concentrations of plant extracts and compare their antibacterial properties.

4.3.1.7 Eucalyptus extract fractionation

Preliminary fractionation

After we established the eucalyptus extract has an antibacterial activity, we attempted to purify an active fraction of the extract. Initially the crude extract was centrifuged to remove insoluble matter from the solution and that crude extract was directly injected onto the HPLC with an analytical C18 column. Only small volumes of non-concentrated sample can be analysed with such approach. Multiple repetitions of the injection were required to obtain enough HPLC fractions for antibiotic activity testing. We have discovered that the active fraction was very tightly bound to the column matrix and the column needed to be washed with large volumes of methanol to remove it.

Normal-phase HPLC fractionation

Extremely high affinity of the active fraction to the C18 matrix indicates that the components are highly hydrophobic. A more suitable method of separation in such case is normal phase HPLC. The difference between normal phase and reverse phase HPLC is the polarity of the column matrix (stationary phase) and the solvents (mobile phase). In the older type of chromatography, the normal phase, the stationary phase is polar and the mobile phase is non-polar. For example a separation on a silica column might be performed using a gradient of hexane and acetonitrile. In reverse phase chromatography the stationary phase is non-polar and the mobile phase is polar. For example a fractionation can be performed on a C18 column (a matrix made of long hydrocarbon chains) using a gradient of water and methanol.

Similar to previous fractionation with reverse phase chromatography, the compounds responsible for the activity were very tightly bound to the column matrix. The column required washing with large amounts of solvent to release the activity. In this case however, the compound was too hydrophilic for the separation on normal phase HPLC.

Introduction of sample preparation methods before fractionation

It was surprising that the active fraction contained a compound that would be too hydrophobic and too hydrophilic at the same time. However these two fractionations demonstrated that the crude extracts have many components and further processing of the extract before HPLC fractionation is advisable.

We developed two methods for processing the extracts before HPLC fractionation (Figure 4-4). Firstly, to remove any waxy substances that could interfere with the fractionations, we de-fatted the concentrated methanol extract using an equal volume of petroleum ether. To separate the most hydrophobic compounds the resulting methanol extract was partitioned with equal parts of chloroform and water. The methanol/water partition was dried and resuspended in methanol before fractionation.

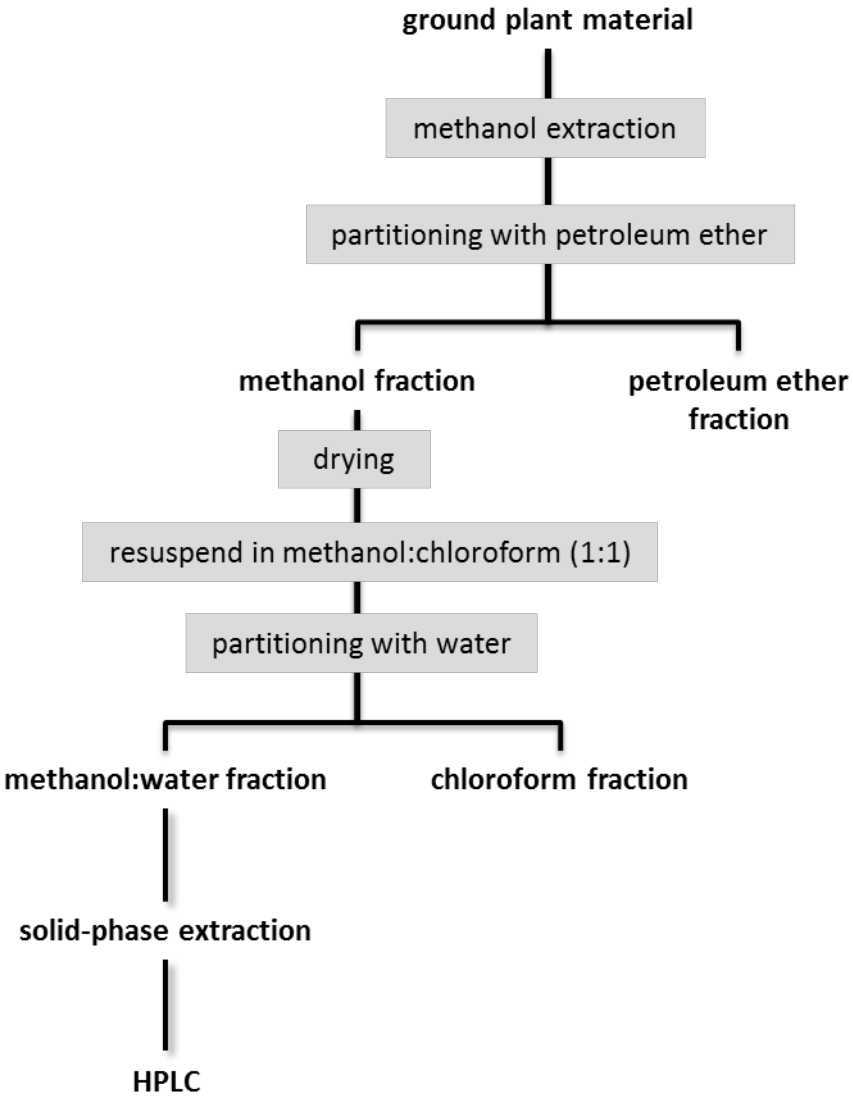


Figure 4.4: A schematic of plant extract fractionation.

Secondly, we added a solid-phase extraction (SPE) process. SPE is a type of sample preparation technique where the sample is applied to a matrix (solid phase) and is washed with different solvents to separate compounds from the sample based on their affinities to these solvents. SPE is a popular way of filtering samples before HPLC

fractionation to remove compounds that could permanently bind the HPLC matrix. In our case SPE was used to allow a two-dimensional fractionation: one of the dimensions being separation based on polarity, using C18 matrix, and the second dimension being ion-exchange SPE which separates compounds based on their charge.

We also tried size exclusion chromatography, which separates molecules based on their size but this type of separation was not accurate enough. The samples obtained contained a large mixture of different compounds and the separation was unreliable.

Final fractionations

We eventually settled on a mixture of methods described above. After establishing antimicrobial activity of the extract, it was concentrated and de-fatted with petroleum ether. The aqueous partition was further purified using weak anion exchange solid phase extraction cartridges. The active methanol fraction was fractionated on HPLC using C18 matrix. However separation of the peaks was not possible with this matrix as the sample was too hydrophilic (Figure 4-5). The compounds present in the sample did not bind to the C18 matrix and all eluted at the same time, at the beginning of the gradient.

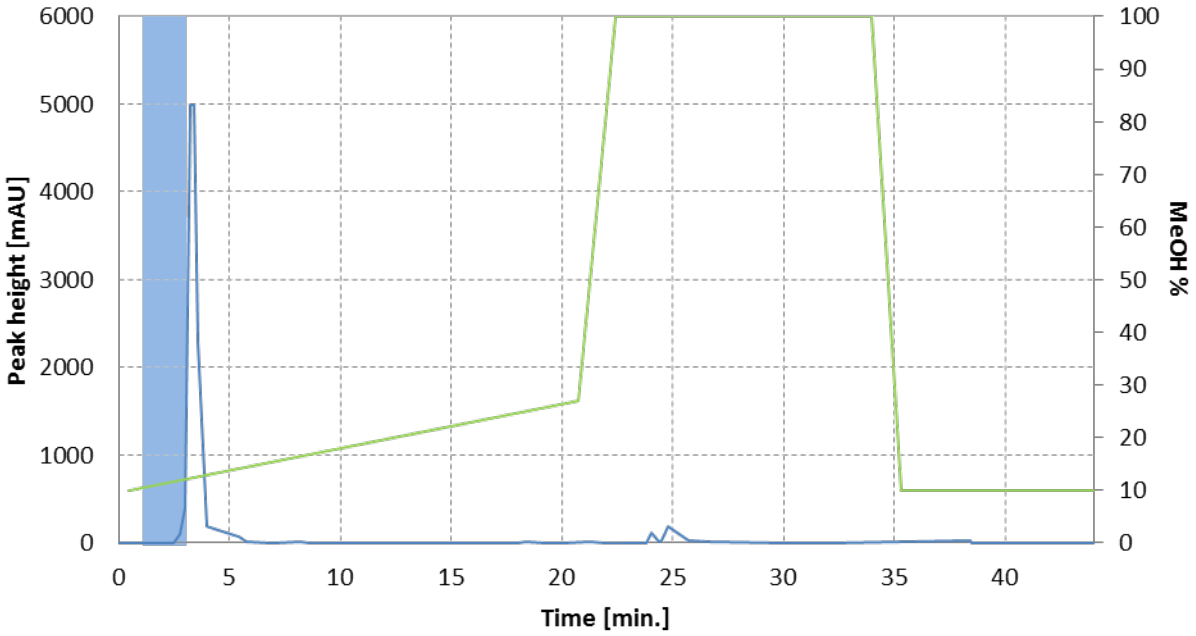


Figure 4.5: HPLC chromatogram of the eucalyptus extract. The sample was fractionated using Shimadzu LCMS-2020 (single-quad), semi-preparative C18 column and 10 – 100% methanol gradient. The UV/vis detector scanned 190-800 nm wavelengths. The extracted wavelengths are 254 nm, detecting UV-active compounds. All fractions were assayed for antibacterial activity in a disc diffusion assay and the active fraction was highlighted in blue.

The presence of a mixture of compounds of different sizes was confirmed in mass-spectrometry analysis. Between 20 and 25 different masses were identified for the most abundant ions in the sample, ranging between 264.8250 m/z and 630.9556 m/z (Table 4-3). Two of the masses were identified as formic acid and trifluoroacetic acid, which are markers added to chromatographic solvents. Not only were we not able to separate the compounds, but it is also not possible to determine the structure of these compounds with their masses only.

Table 4.3: A list of most abundant ions in the eucalyptus extract fraction with antibacterial activity detected by LCMS-IT-ToF Mass Spectometer (IT-ToF, Shimadzu) using analytical C18 column and 10 -100% acetonitrile gradient against 0.1% formic acid in water. The ions were analysed against common contaminant ions such as formic acid. The samples were compared with others that did not have an antibiotic activity and the ions shared between them were not considered to have antibiotic properties.

ion m/z ratio	retention time [min.]	compound name	comments
458.9500	4.467		
520.7500	4.594		
344.0000	7.233		
248.8000	4.706		present in samples without antibacterial activity
226.8250	6.200	TFA	
330.9000	6.73		
112.9417	5.917	formic acid dimer	
250.8583	7.600		
384.7500	4.461		
444.9000	6.113		
518.9250	6.711		
731.3000	4.967		
296.8583	6.994		
755.4083	5.100		present in samples without antibacterial activity

The further purification and identification of the compounds from the active fraction of the eucalyptus extract could not be continued. We cannot determine the structures of these compounds with nuclear magnetic resonance (NMR), as sufficient amount of

pure material cannot be obtained using the current methods. We cannot further separate the compounds with our current methods without the knowledge of their structures that would allow us to select method that would separate them.

4.3.2 Diamondback moth (*Plutella xylostella*) feeding on cabbage (*Brassica rapa*)

4.3.2.1 Plutella xylostella

The next plant/insect pair was diamondback moth feeding on cabbage. A colony of *P. xylostella* was already set up in the JIC Insectary and we used these larvae for some of our previous work (see previous chapter). There are no data on the medicinal properties of cabbage, but it was selected from other brassicas based on their anecdotal medicinal properties in Polish folklore. Cabbage and cabbage juice are believed to have health benefits and are sometimes used as a treatment for intestinal parasites.

Diamondback moths, of order Lepidoptera, are globally distributed, economically-important pests of cruciferous crops. As mentioned in the previous chapter, they are small moths with a wingspan of 15 mm and a body length of 6 mm (Figure 4-6), and the larvae feed on bottom leaf surfaces during four larval instars.

Figure 4.6: Diamondback moth (top panel) and larva (bottom panel). Photo by Russ Ottens, University of Georgia, Bugwood.org.

P. xylostella larvae are a notorious pest, due to a lack of natural enemies and developing resistance to all conventional insecticides, including the Bt toxin [146]. Few effective natural enemies of diamondback moths are known, and mildly successful parasitoids are not capable of long migrations that the moths are noted for. A wide range insecticide resistance has been described in *P. xylostella*, including carbamate (most kill insects by reversibly inactivating acetylcholinesterase), organophosphate (which kill by irreversible inactivation of acetylcholinesterase), pyrethroid (which

depolarize axonal membranes), abamectin (which causes hyperpolarization of nerve cells), benzoylphenylurea (which inhibit chitin synthesis) and dichlorodiphenyltrichloroethane – DDT (which depolarizes neurons). Additionally, extensive insecticide use often kills the parasitoids of diamondback moths, further impeding the biological control efforts. Another biological control is Cry toxin-producing *Bacillus thuringiensis*. In the alkaline insect gut the toxin inserts into the cell membranes, forming pores. Even before the introduction of transgenic Bt crops, *P. xylostella* exhibited resistance to a wide repertoire of Bt toxins, including detoxification enzymes and systems for toxin excretion (see review by Pardo-Lopez et al., 2013 [189]).

The resilience of diamondback moths allows them to feed on unpalatable or toxic plants without the health costs. The gut bacteria live in a hostile niche in comparison with other insects with less harsh diets. The bacteria are exposed to the compounds making the plants unpalatable to other insects and we hypothesised high levels of resistance can be seen in the *P. xylostella* gut bacteria.

4.3.2.2 Gut dissections

Five final instar *P. xylostella* larvae feeding on cabbage were collected and starved for two hours. Such procedure allows the insect guts to be enriched in bacteria as the food already present in the gut is being digested. The guts were dissected under aseptic conditions and immediately plated out on agar media.

The gut contents were plated as described for the stick insects. The gut bacteria were cultured and identified by 16S sequencing. The strains identified were *Sanguibacter keddieii* and *Raoultella terrigena*. One of the strains, *S. keddieii*, had been isolated in a separate experiment from *P. xylostella* feeding on artificial food containing oxytetracycline (Chapter 3), which indicates that a proportion of the microbiota is stable and persistent in the conditions observed. *S. keddieii* is a rare bacterial species, previously isolated from the blood of otherwise heathy cows [190]. *R. terrigena* was previously classified as *Klebsiella terrigena* and is normally isolated from soil and water [191].

S. keddieii and *R. terrigena* type strains were obtained from Centre de Resources Biologiques de l'Institut Pasteur (Paris) culture collection and both the type strains and

gut-isolated strains were assayed for antimicrobial susceptibility (Table 4-4). In nearly half of the tests performed there were differences in antibiotic susceptibility between the gut-isolated strain and the type strain. The *R. terrigena* type strain was less resistant to ampicillin than the gut-isolated strain. *S. keddiei* type strain was more resistant to ampicillin, chloramphenicol, ciprofloxacin and kanamycin than the gut-isolated strain. The differences in antibiotic resistance levels of *S. keddiei* were unusual and higher than the other strains tested.

Table 4.4: Antibiotic susceptibility profiles of the gut bacteria from P. xylostella and corresponding type strains. MICs for six different antibiotics were assessed by a broth dilution assay. Differences in antibiotic-resistance levels between the strains have been highlighted in green when the gut-isolated strain was more resistant than the type strain and in red when the type strain was more resistant than the gut-isolated strain. $p < 0.001$.

	Minimal inhibitory concentrations of antibiotics in broth dilution assay [µg/mL]			
	Sanguibacter keddieii		*Raoultella terrigena*	
	gut strain	type strain	gut strain	type strain
ampicillin	16	>64	>64	16
chloramphenicol	1	2	1	1
ciprofloxacin	<0.01	0.03	<0.01	<0.01
kanamycin	2	>64	1	1
rifampicin	1	1	2	2
tetracycline	1	1	1	1

To follow up the antibiotic susceptibility testing, we assayed the susceptibility of the bacterial strains to the cabbage extract. Cabbage extract was prepared by macerating cabbage leaves in methanol and concentrating the extract by evaporation. The extract and tested for antibacterial activity by the disc diffusion assay. The extract did not inhibit the growth of any of the *P. xylostella* gut or type strains (Table 4-5). Since only two strains were isolated from the guts of diamondback moth larvae, we tested additional strains: an indicator strain *Escherichia coli* ATCC 25922, and strains isolated from the stick insect gut: *Bacillus amyloliquefaciens*, *Microbacterium oxydans* and *Sphingobacterium multivorum*, with their corresponding type strains. No antibacterial activity was observed against any of these strains.

Table 4.5: The susceptibility of the gut bacteria from P. xylostella and corresponding type strains to cabbage leaf extract. The values were obtained in a disc diffusion assay. The paper discs were infiltrated with up to 20 µg dried cabbage extract. The experiment was repeated three times.

	Diameter of inhibition zones [mm]			
	Sanguibacter keddieii		*Raoultella terrigena*	
	gut strain	type strain	gut strain	type strain
crude	0	0	0	0

After no antibacterial activity was detected in the cabbage extract, no further work was carried out. We decided to focus on other plant/insect pairs, even though it would be interesting to further investigate why some plants have antibacterial activity and others do not.

4.3.3 Cinnabar moth (*Tyria jacobaeae*) feeding on ragwort (*Jacobaea vulgaris*)

4.3.3.1 Cinnabar moth

The goal of this project was to investigate both medicinal and toxic plants. Our choice of a toxic plant was ragwort (*Jacobaea vulgaris*), which is poisonous to some animals, but it also is a food source for some insects, such as cinnabar moth larvae (*Tyria jacobeae*). Conveniently ragwort plants are abundant on the playing fields around JIC and we could collect large amounts of plants with cinnabar moths feeding on them.

Cinnabar moth belongs to the order Lepidoptera and feed specifically on plants of the genus *Senecio* (ragworts and groundsels). The black and orange-yellow striped larvae reach up to 35 mm and the black and red adults have a wingspan of 32 – 42 mm and body length up to 20 mm (Figure 4-7).

Ragwort is a plant of ecological importance in the UK, being a food source for a variety of insect species. However, ragwort is poisonous to horses and cattle and *T. jacobaeae* larvae are sometimes used as biological control [192]. Ragwort is hepatotoxic due to a variety of alkaloids present in the plants. The poisonous compounds from ragwort are sometimes believed to have evolved as chemical defence against herbivores. However, some herbivores sequester a sub-group of ragwort alkaloids, pyrrolizidine alkaloids,

and use them as a predation deterrent themselves [193]. *Tyria jacobeae* is capable of sequestering one of the alkaloids, senecionine. Additionally the larvae modify the compound into N-oxide. The modified senecionine N-oxide is nontoxic and stored by the insect, but can be oxidized and regain its toxicity in the guts of the potential insectivore.

Figure 4.7: Cinnabar moth larvae (top panel) and adults (bottom panel) feeding on ragwort. Photo by Eric Coombs, Oregon Department of Agriculture, Bugwood.org.

4.3.3.2 Gut bacteria

Five bacterial strains were isolated from the guts of cinnabar moth larvae feeding on ragwort leaves: *Burkholderia fungorum*, *Bacillus licheniformis*, *Kocuria rhizophila*, *Staphylococcus epidermidis* and *Staphylococcus warneri*. *Staphylococcus* species are common members of human skin microbiota, but it is not uncommon for insect gut microbiota to include such isolates. As described in the previous chapter, *E. faecalis*, bacterial species most commonly found in human microbiota, is a member of *G. mellonella* gut microbial community. It is unlikely these species are a contamination introduced during gut dissections as the procedure was carried out in sterile conditions following appropriate dissection procedures.

S. epidermidis and *S. warneri* are the only isolates in the guts of cinnabar moth larvae that are normally associated with human microbiota. All the remaining bacterial isolates are environmental strains: *B. fungorum* was first isolated from a fungus and both *B. licheniformis* and *K. rhizophila* are soil bacteria.

4.3.3.3 Antibiotic resistance

Type strains matching the gut isolated species were obtained from CRBIP and DSMZ culture collections and all isolates were assayed for antibiotic susceptibility. In many cases the type strain was more resistant to antibiotics than the gut-isolated strain (Table 4-6). The *B. fungorum* type strain was more resistant than the gut-isolated strain to ciprofloxacin and kanamycin. The *B. licheniformis* type strain was more resistant to three antibiotics than the gut-isolated *B. licheniformis*: ampicillin, chloramphenicol and oxytetracycline. There were no differences in antibiotic susceptibility between *K. rhizophila* gut isolated strain and the type strain. The *S. epidermidis* gut-isolated strain was more resistant to ampicillin than the type strain and *S. warneri* gut-isolated strain was more resistant to ciprofloxacin than the type strain. Additionally, *S. warneri* type strain was more resistant to rifampicin and oxytetracycline than the gut strain. The large number of cases where the type strain was more antibiotic-resistant than the gut-isolated strain was unexpected, but as observed with eucalyptus extract they are not always an indication of the absence of antibacterial activity in the plant extract.

Table 4.6: Antibiotic susceptibility profiles of the gut bacteria from the cinnabar moth larvae and corresponding type strains. MICs for six different antibiotics were assessed by a broth dilution assay. Differences in antibiotic-resistance levels between the strains have been highlighted in green when the gut-isolated strain was more resistant than the type strain and in red when the type strain was more resistant than the gut-isolated strain. $p < 0.001$.

	Minimal inhibitory concentrations of antibiotics in broth dilution assay [µg/mL]											
	Burkholderia fungorum		*Bacillus licheniformis*		*Kocuria rhizophila*		*Staphylococcus epidermidis*		*Staphylococcus warnerii*	
	gut stran	type strain	gut strain	type strain	gut strain	type strain	gut strain	type strain	gut strain	type strain
ampicillin	>64	>64	2	>64	16	16	>64	16	16	16
chloramphenicol	0.03	0.03	0.125	2	2	2	2	2	2	2
ciprofloxacin	<0.01	0.02	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.25	<0.01
kanamycin	2	16	2	2	2	2	1	1	2	2
rifampicin	2	2	2	2	2	2	2	2	0.03	2
oxytetracycline	0.03	0.03	<0.01	0.03	0.03	0.03	0.03	0.03	<0.01	0.03

4.3.3.4 Ragwort extract

Ragwort extract was prepared by soaking the dried, ground leaves in methanol, concentrating the initial infusion and partitioning it to remove most hydrophobic compounds. The extract was tested for antibiotic activity in a disc diffusion assay against gut-isolated strains from the guts of cinnabar moth larvae and the matching type strains (Table 4-7). There were no differences in ragwort extract susceptibility between the gut-isolated strain and type strain of *B. licheniformis*. Four gut-isolated strains were more resistant to the ragwort extract than the corresponding type strains: *B. fungorum*, *S. epidermidis* and *S. warneri*. *K. rhizophila* gut-isolated strain was more susceptible to the ragwort extract than the type strain. Surprisingly, the strains were equally antibiotic-susceptible, making it difficult to hypothesise what caused increased resistance to the plant extract.

Even though three out of five type strains tested were more resistant to the plant extract than their corresponding gut strain, the ragwort extract exhibited a high level of antibiotic activity and the extract was investigated further. The partitioned ragwort extract was fractionated on weak anion exchange SPE and HPLC (Figure 4-8). There were a number of different peaks present in the chromatogram, indicating the extract was a mixture of different compounds. It was not practical to purify different plant extracts at the same time, as the process is very labour-intensive and the fractionation of other extracts was prioritised.

Table 4.7: The susceptibility of the gut bacteria from the cinnabar moth guts and corresponding type strains to ragwort leaf extract. The values were obtained in a disc diffusion assay. The discs were infiltrated with 10 µg dried ragwort extract. The differences in plant extract susceptibility between the strains were highlighted with green when the gut-isolated strain was more resistant than the type strain and in red when the type strain was more resistant than the gut-isolated strain. The assay was repeated three times.

	Diameter of inhibition zones [mm]											
	Burkholderia fungorum		*Bacillus licheniformis*		*Kocuria rhizophila*		*Staphylococcus epidermidis*		*Staphylococcus warnerii*	
	gut stran	type strain	gut strain	type strain	gut strain	type strain	gut strain	type strain	gut strain	type strain
crude	23	18	27	27	23	25	23	21	18	16

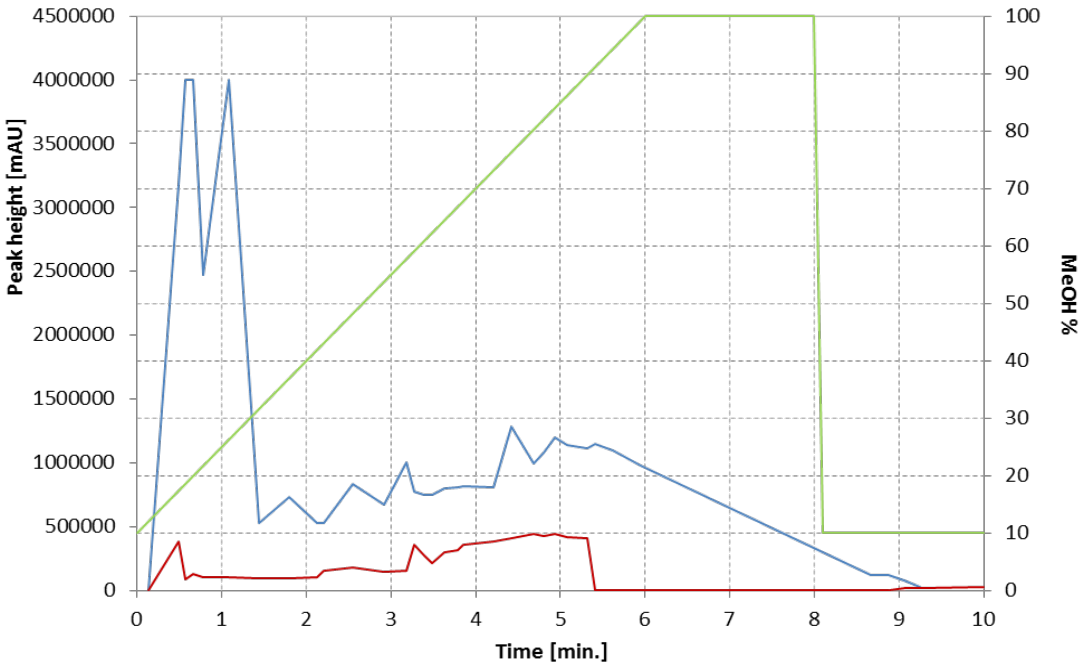


Figure 4.8: HPLC chromatogram of the ragwort extract.The sample was fractionated using Shimadzu LCMS-2020 (single-quad), semi-preparative C18 column and 10 – 100% methanol gradient. The UV/vis detector scanned 190-800 nm wavelengths. The extracted wavelengths are 254 nm, detecting UV-active compounds.

Even though we have not fractionated the ragwort extract, it was subjected to MS analysis. Mass spectroscopy is less labour-intensive than the fractionation procedure and it was performed on the ragwort samples to detect the presence of known ragwort metabolites. The most abundant ions in the ragwort extract are listed in Table 4-8. We did not detect any of the usual contaminant ions such as formic acid, which is consistent with the lack of extract fractionation and minimal sample preparation before the mass spectroscopy. We have not detected the presence of the most abundant pyrrolizidine alkaloids and their N-oxides, which are the compounds responsible for the toxicity of ragwort to cattle. These alkaloids are: jacobine (m/z 351), jaconine (m/z 387), jacozine (m/z 349), otosenine (m/z 381), retrorsine (m/z 351), seneciphilline (m/z 333), senecionine (m/z 335), and senkirkine (m/z 366), acetylerucifoline (m/z 392), erucifoline (m/z 350), integerrimine (m/z 335), jacoline (m/z 369), riddelline (m/z 350), senecivernine (m/z 335), spartioidine (m/z 333), and usaramine (m/z 351). The N-oxides have m/z ratios n+16. The low levels of these metabolites suggest that the pyrrolizidine alkaloids were either not extracted from the plant material or not selected for in the assay-guided fractionation, and are not responsible for the antibacterial activity of the ragwort extract.

Table 4.8: The most abundant ions present in the ragwort extract. The most abundant ions present in the ragwort extractdetected by LCMS-IT-ToF Mass Spectometer (IT-ToF, Shimadzu) using analytical C18 column and 10 -100% acetonitrile gradient against 0.1% formic acid in water.

Ion m/z	retention time [min.]
231.0846	0.966
255.0473	1.013
525.3544	0.192
436.4304	4.851
357.1159	0.779
214.9145	1.352
214.9158	0.547
185.0451	0.848
229.0695	0.963
371.0949	0.804

Cinnabar moth larvae feeding on ragwort was an interesting plant/insect pair to investigate. Many of the bacterial strains isolated from the insect guts were less antibiotic-resistant and less resistant to the ragwort extract than their corresponding type strains. Nonetheless the ragwort extract exhibited a high level of antibiotic activity and contained a number of compounds which could be investigated for antibacterial activity.

4.3.4 Rosemary beetle (*Chrysolina americana*) feeding on lavender (*Lavendula angustifolia*)

4.3.4.1 Rosemary beetle

Another plant/insect pair investigated was rosemary beetle and lavender. Lavender was chosen as an example of typically medicinal plant. *L. angustifolia*'s former known species name, *Lavendula officinalis*, refers to its medicinal uses. As with cinnabar moth and ragwort, the lavender plants grow around the JIC and rosemary beetles can be found feeding on them. Additionally large amounts of plant material for extractions can be obtained from gardening centres.

Figure 4.9: Rosemary beetles feeding on lavender. Photo by Katarzyna Ignasiak, JIC, Norwich.

Rosemary beetles are small beetles in the order Coleoptera. The larvae, reaching up to 8 mm long, are grey with darker stripes along their bodies and the adult beetles are metallic with purple and green stripes and reach 5 – 8 mm body length (Figure 4-9). The *C. americana* beetles cannot fly. The insects are native to Eastern Europe and the Mediterranean region, but have been found in the UK since 1990s.

The beetles are adapted to feeding on plants in the family Lamiaceae, including rosemary, lavender and thyme. In our study the insects were feeding on lavender (*Lavendula angustifolia*), an evergreen shrub, previously known as *Lavendula officinalis* due to its medicinal properties.

4.3.4.2 Rosemary beetle gut bacteria

Adult beetles were collected from the lavender plants growing outside the Chatt building on the JIC site. The beetles are small and have a rigid exoskeleton making dissections difficult. Instead the insects were surface-sterilized and ground up. The whole-insect suspensions were plated on agar media for culturing gut bacteria. Five different bacterial strains were isolated from surface-sterilized rosemary beetles: *Microbacterium foliorum*, *Microbacterium gubbeenense*, *Pantoea agglomerans*, *Rhodococcus erythropolis* and *Staphylococcus epidermidis*.

The bacteria that we refer to as gut bacteria are for rosemary beetle all bacteria that can be isolated from the entire insect. The rosemary beetle microbiota is probably dominated by the gut bacteria. As discovered for other insects, some of the rosemary beetle bacteria are environmental isolates while other strains are members of human microbiota. In case of the rosemary beetle microbiota, three strains were of environmental origin: *M. foliorum* was first isolated from the surface of leaves, *M. gubbeenense* from cheese and *R. erythrypolis* from soil. The two remaining bacterial strains were human-associated: both *P. agglomerans* and *S. epidemidis* can be found on human skin.

4.3.4.3 Antibiotic susceptibility testing

Matching type strains were obtained from the CRBIP and DSMZ culture collections and antibiotic susceptibility of all isolates was assessed in a broth microdilution assay (Table 4-9). In comparison with other insect gut microbiomes there were few differences between the strains isolated from the insect guts and the matching type strains. There were no differences in antibiotic susceptibility to ampicillin, chloramphenicol, ciprofloxacin, kanamycin, rifampicin or oxytetracycline between the type strains and gut-isolated strains of *M. foliorum*, *M. gubbeenense*, *P. agglomerans* and *R.*

erythropolis. The *S. epidermidis* type strain was more susceptible to ciprofloxacin and kanamycin than the gut strain. Even though not many differences in the antibiotic susceptibility between the strains have been detected, in two cases when this was the case the gut-isolated strain was more resistant than the type strain, indicating it was under selective pressure. The increased antibiotic resistance of the rosemary beetle gut bacteria was encouraging for further investigation of the antibacterial activity of the lavender extract.

4.3.4.4 Lavender extract

The rosemary beetles feed on lavender leaves, but the plants harvested for the preparation of the lavender extract also had flowers. Out of convenience both leaf and flower lavender extracts were prepared and assayed for antibiotic properties. The strains were tested in a disc diffusion assay for susceptibility to lavender extract (Table 4-10). The leaf extract was antibacterial against two of the bacterial strains used and the flower extract was active against all five strains. Both extracts exhibited only a mild level of activity. In most cases there were no differences in the susceptibility to lavender extracts. The *S. epidermidis* gut strain was more resistant to lavender flower extract than the type strain and the *M. foliorum* type strain was more resistant to lavender leaf extract than the gut-isolated strain. It was hypothesised that the gut-isolated strains would be more resistant to the plant extracts that it was exposed to before. The difference between *M. foliorum* gut-isolated strain and type strain is opposite to what was predicted.

Only a mild level of antibacterial activity was detected in the rosemary extracts and the flower extract was more active than the leaf extract. Only the flower extract was investigated further. The lavender flower extract was subjected to purification with SPE and HPLC. Low level antibacterial activity was detected in five HPLC fractions ranging between 17 – 50% methanol (Figure 4-10). The low level of activity hindered the assay-guided fractionation as separating the peaks in HPLC lowered the individual antibiotic activity of each peak below detection level. An identification of the most active components of the extract was not possible without a large-scale increase in the HPLC efficiency or improving the sensitivity of the assay.

Table 4.9: Antibiotic susceptibility profiles of the gut bacteria from the rosemary beetles and corresponding type strains. MICs for six different antibiotics were assessed by a broth dilution assay. Differences in antibiotic-resistance levels between the strains have been highlighted in green when the gut-isolated strain was more resistant than the type strain and in red when the type strain was more resistant than the gut-isolated strain. $p < 0.005$.

	Minimal inhibitory concentrations of antibiotics in broth dilution assay [µg/mL]											
	Microbacterium foliorum		*Microbacterium gubbense*		*Pantoea agglomerans*		*Rhodococcus erythrypolis*		*Staphylococcus epidermidis*	
	gut strain	type strain	gut strain	type strain	gut strain	type strain	gut strain	type strain	gut strain	type strain
ampicillin	2	2	16	16	>64	>64	>64	>64	>64	>64
chloramphenicol	2	2	2	2	2	2	2	2	2	2
ciprofloxacin	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.25	0.03
kanamycin	2	2	2	2	2	2	2	2	>64	2
rifampicin	2	2	2	2	2	2	2	2	2	2
oxytetracycline	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03

Table 4.10: The susceptibility of the gut bacteria from the rosemary beetle guts and corresponding type strains to lavender extract. The values were obtained in a disc diffusion assay. The discs were infiltrated with 10 µg dried ragwort extract. The differences in plant extract susceptibility between the strains were highlighted with green when the gut-isolated strain was more resistant than the type strain and in red when the type strain was more resistant than the gut-isolated strain. The assay was repeated three times.

		Diameter of inhibition zones [mm]											
		Microbacterium foliorum		*Microbacterium gubbense*		*Pantoea agglomerans*		*Rhodococcus erythrypolis*		*Staphylococcus epidermidis*			
		strain gut	strain type	strain gut	type strain	gut strain	type strain	gut strain	strain type	gut strain	strain type		
flower extract	10	10	10	10	12	12	10	10	10	10			
leaf extract	7	6	6	6	0	0	0	0	0	0			

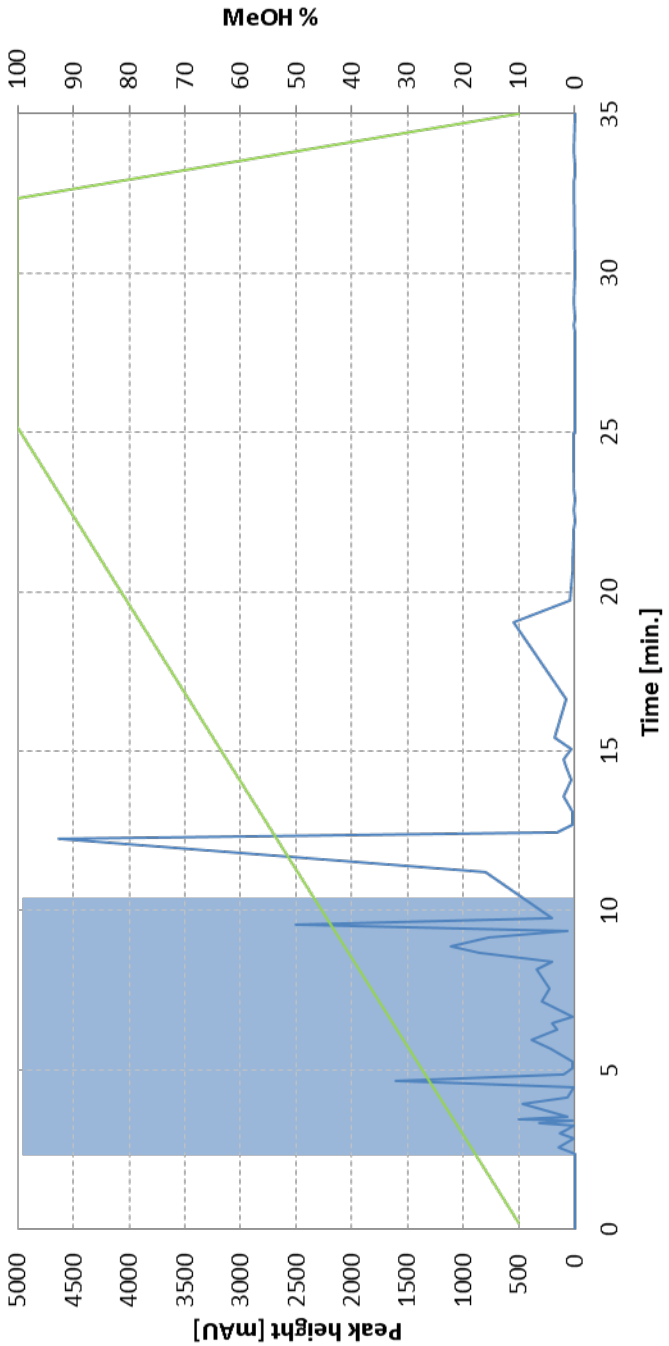


Figure 4.10: HPLC chromatogram of the lavender extract. The sample was fractionated using Shimadzu LCMS-2020 (single-quad), semi-preparative C18 column and 10 – 100% methanol gradient. The UV/vis detector scanned 190-800 nm wavelengths. The extracted wavelengths are 254 nm, detecting UV-active compounds. The fraction with antibacterial activity was highlighted in blue.

Our experiments do not confirm the strong medicinal properties of lavender extracts. Even though the antibiotic resistance differences between the gut bacteria isolated from the rosemary beetles and the type strains were promising, we have not detected

strong antibiotic activity in the lavender extracts. The low level of antibiotic activity in the lavender extracts and their active fractions hindered the experiment progress and prevented the identification of the active components.

4.3.5 Death's head hawkmoth (*Acherontia atropos*) feeding on potato (*Solanum tuberosum*)

4.3.5.1 Death's head hawkmoth

Death's head hawkmoth larvae feeding on potato were investigated as another example of an insect feeding on a toxic plant. We were particularly interested in the potential antibiotic activity of the potato leaves, which are toxic and grown in abundance as a waste product of potato crops.

Death's head hawkmoth belongs to order Lepidoptera and is primarily found in Europe. The larvae are yellow-green with diagonal yellow and purple stripes on the sides and a tail horn (Figure 4-11). The fourth instar reaches 120 – 130 mm. The brown and yellow moths have a wingspan of 90 – 130 mm and body length of 40 – 60 mm. The most striking feature of the adults is a pattern on the thorax resembling human skull, linked to folklore association of the moths with supernatural and evil forces. When disturbed the moths produce a short squeaks by drawing air in and out of their pharynx [194].

The insects are polyphagous, feeding on plants in the families Solanaceae (nightshades), Verbenaceae (verbena family), Oleaceae (olive, ash and jasmine), and Bignoniaceae (bigonias, most noted for ornamental flowers). *A. atropos* can also parasitize beehives without being detected and attacked by the bees due to chemical camouflage [195]. The hawkmoths produce four fatty acids, two unsaturated ones (9-hexadecenoic acid and 9-octadecenoic acid) and two saturated ones (hexadecanoic acid and octadecanoic acid), which prevent detection by bees. The hawkmoth fatty acids belong to a group of bees' signals to distinguish nest mates from invaders, but the exact mechanism of recognition is not known. In our study the hawkmoth larvae were feeding on potato leaves (*Solanum tuberosum*), which *A. atropos* is a mild pest of.

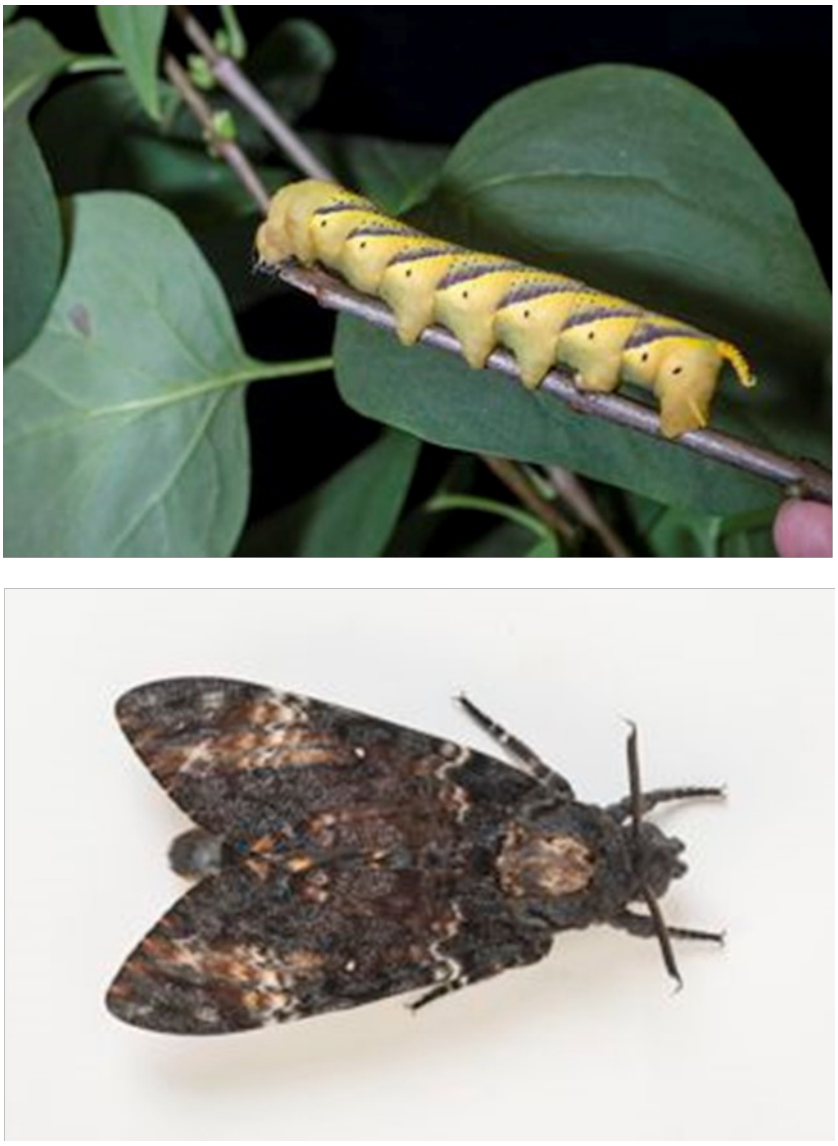


Figure 4.11: Death's head hawkmoth larva (top panel) and adult (bottom panel). Photo by Andrew Davies, JIC, Norwich.

4.3.5.2 Gut bacteria

Death's head hawkmoths were brought into the JIC Insectary. The larvae were reared feeding exclusively on potato plants. Ten of the larvae were collected and dissected. Their gut contents were plated. Three different bacterial strains were isolated from the guts of death's head hawkmoths: *Enterobacter asburiae*, *Pseudomonas putida* and *Roultella terrigena*. *E. asburiae* is a species normally isolated from clinical specimens, while the two other isolates, *P. putida* and *R. terrigena*, are environmental bacteria commonly isolated from soil and water. *Raoultella terrigena* was isolated in a previous experiment from the guts of diamondback moth larvae.

4.3.5.3 Antibiotic susceptibility testing

After obtaining type strains matching each death's head hawkmoth gut isolate, the strains were assayed for antibiotic susceptibility. There were differences in antibiotic susceptibility in the majority of tests performed and most of the type strains were more antibiotic-resistant than their matching gut-isolated strain (Table 4-11). The *E. asburiae* type strain was more resistant than the gut isolated strain to all antibiotics tested: ampicillin, chloramphenicol, ciprofloxacin, kanamycin, rifampicin and tetracycline. The *P. putida* type strain was more resistant to chloramphenicol, kanamycin, rifampicin and tetracycline. *R. terrigena* type strain was more resistant than the gut strain to chloramphenicol and more susceptible than the gut strain to kanamycin and tetracycline.

Table 4.11: Antibiotic susceptibility profiles of the gut bacteria from the death 痴 head hawkmoth larvae and corresponding type strains. MICs for six different antibiotics were assessed by a broth dilution assay. Differences in antibiotic-resistance levels between the strains have been highlighted in green when the gut-isolated strain was more resistant than the type strain and in red when the type strain was more resistant than the gut-isolated strain. $p < 0.005$.

	Minimal inhibitory concentrations of antibiotics in broth dilution assay [µg/mL]					
	Enterobacter asburiae		*Pseudomonas putida*		*Raoultella terrigena*	
	gut strain	type strain	gut strain	type strain	gut strain	type strain
ampicillin	8	>64	>64	>64	>64	>64
chloramphenicol	1	4	2	>64	1	2
ciprofloxacin	<0.125	0.5	<0.125	<0.125	<0.125	<0.125
kanamycin	8	16	4	8	16	8
rifampicin	4	16	4	8	8	8
oxytetracycline	1	2	0.25	2	1	0.5

4.3.5.4 Potato leaf extract

Potato leaf extract was prepared from dried potato leaves and assayed for antibacterial activity on the panel of bacteria isolated from the hawkmoth larvae guts and the matching type strains (Table 4-12). There were no differences in potato leaf extract susceptibility between the gut isolates and type strains of *E. asburiae* and *P. putida*. *R.*

terrigena gut-isolated strain was more susceptible to the potato leaf extract than the type strain, contrary to what was expected. The strains had some unexpected antibiotic resistance differences. The type strain was more chloramphenicol-resistant than the gut-isolated strain, opposite to the predicted resistance profile. It is possible that both the chloramphenicol resistance and the increased potato leaf extract resistance are due to the same mechanism. Because of the matching differences in antibiotic susceptibility and the plant extract susceptibility, the antibacterial activity in the potato leaf extract was investigated further, even though the activity levels were low in comparison with other extracts.

Table 4.12: The susceptibility of the gut bacteria from the death's head hawkmoth larvae guts and corresponding type strains to the potato leaf extract. The values were obtained in a disc diffusion assay. The discs were infiltrated with 10 µg dried ragwort extract. The differences in plant extract susceptibility between the strains were highlighted with green when the gut-isolated strain was more resistant than the type strain and in red when the type strain was more resistant than the gut-isolated strain. The assay was repeated three times.

	Diameter of inhibition zones [mm]					
	Enterobacter asburiae		*Pseudomonas putida*		*Raoultella terrigena*	
	gut strain	type strain	gut strain	type strain	gut strain	type strain
crude	7	7	6	6	10	7

The active methanol partition of potato leaf extract was subjected to fractionation on weak anion exchange SPE and HPLC. As observed before with eucalyptus extracts, the antibacterial activity eluted at the beginning of the gradient (Figure 4-12). This suggests that the antibacterial activity is either due to a common phytochemical present in both plant species, or the activity is caused by an artefact of the fractionation procedure, such as accumulation of formic acid. The fractionation of the different plant extracts followed the same workflow and it is possible that it was biased towards a certain class of chemicals.

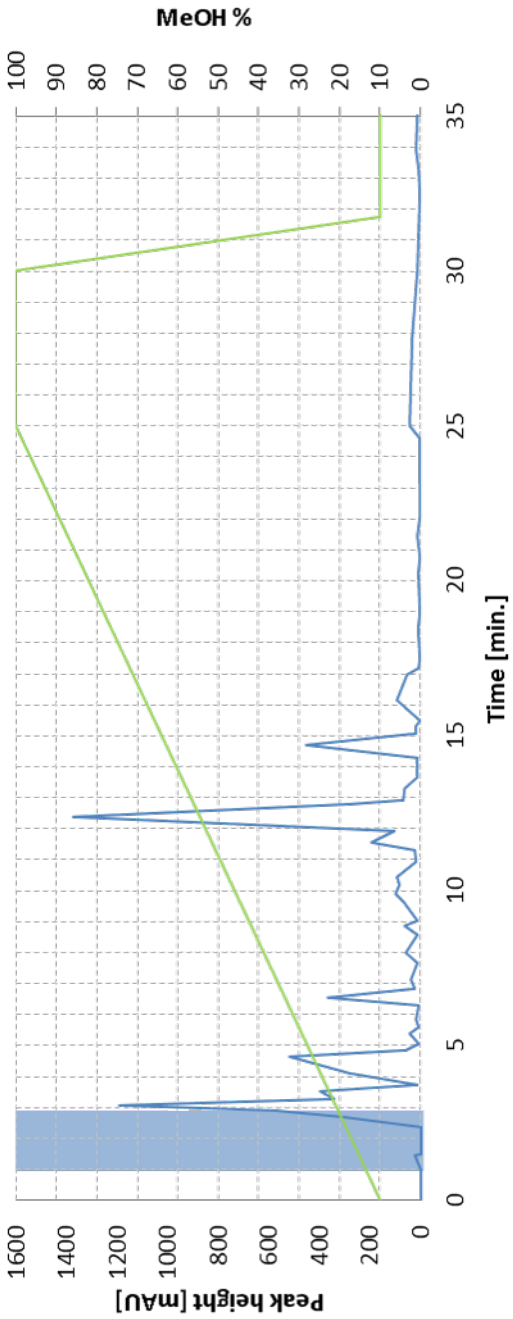


Figure 4.12: HPLC chromatogram of the potato leaf extract. The sample was fractionated using Shimadzu LCMS-2020 (single-quad), semi-preparative C18 column and 10 – 100% methanol gradient. The UV/vis detector scanned 190-800 nm wavelengths. The extracted wavelength is 254 nm, detecting UV-active compounds. The fraction with antibacterial activity was highlighted in blue.

Because the fractionation of the potato leaf extract resembled the fractionation of the eucalyptus extract, it was decided to pursue purification of just one of them. Antibacterial activity of the eucalyptus extract was higher than the activity of the potato leaf extract, and eucalyptus extract was selected for further purifications (see section 4.3.1.7).

4.3.6 Beet armyworm (*Spodoptera exigua*) feeding on Madagascar periwinkle (*Catharanthus roseus*)

4.3.6.1 Beet armyworm

We decided to investigate the antibiotic properties of Madagascar periwinkle (*Catharanthus roseus*) as the plant is known to contain alkaloids with medicinal properties. Sarah O'Connor's group in JIC Biological Chemistry Department specializes in *C. roseus* metabolites and we decided to use their expertise.

Beet armyworm is an important agricultural pest in the order Lepidoptera, native to Asia but present worldwide. The larvae are 15 – 20 mm long, dull green with darker stripes along the body (Figure 4-13). The adults are grey-brown moths with a wingspan of 26 – 32 mm. The insects are normally killed by frost and overwinter in warmer areas or greenhouses, but invade colder areas annually.

S. exigua are pests of vegetable, field, and flower crops, including alpha alpha, asparagus, aubergine, bean, beet, broccoli, cabbage, cauliflower, celery, chickpea, corn, cotton, cowpea, lettuce, onion, pea, peanut, pepper, potato, radish, safflower, sorghum, soybean, spinach, sugar beet, sweet potato, tobacco, tomato, and turnip. In our study armyworm larvae were feeding on Madagascar periwinkle (*Catharantus roseus*), a plant endemic to Madagascar.

4.3.6.2 Catharanthus roseus

C. roseus is a plant commonly used in traditional medicine throughout the world. Ethnopharmacological uses of Madagascar periwinkle range from diabetes, hypertension, malaria, dengue fever, dysentery, insect bites, skin infections, diarrhoea, leukaemia, eye irritation, indigestion, toothache, sore throat, lung congestion and as a sedative and tranquilizer [196].

Figure 4.13: Beet armyworm larva and moth. Beet armyworm larva (top panel) and moth (bottom panel). Photo by John C. French Sr., Retired, Universities: Auburn, Georgia, Clemson and Missouri, Bugwood.org.

However the plant is probably best known for the terpenoid indole alkaloids it produces which are used in cancer therapy. Vinblastine and vincristine are used in the treatment of leukaemia and Hodgkin's lymphoma. Both vinblastine and vincristine decrease the white blood cell count. Their mechanism of action depends on inhibiting microtubule assembly by binding tubulin, therefore preventing the separation of

chromosomes during metaphase and leading to cell death [197].

The production of vinca alkaloids used in cancer therapy is dependent on the Madagascar periwinkle crops, even though their semi-synthetic production is possible. The precursors to terpenoid indole alkaloids can be synthesised in yeast [198], but their production is not economically viable. The use of terpenoid indole alkaloids in cancer therapy and the dependence of the supply chain on the *Catharantus roseus* crops makes Madagascar periwinkle one of the most economically important plants.

4.3.6.3 Antibiotic resistance in beet armyworm gut bacteria

Four bacterial strains were isolated from beet armyworm guts: *Bacillus aquimaris*, *Bacillus vietnamensis*, *Microbacterium paraoxydans* and *Rhizobium pusense*. *B. aquimaris*, *B. vietnamensis* and *R. pusense* are environmental isolates, and *M. paraoxydans* is a clinical isolate. As discussed before it is not unusual for an insect gut to contain both environmental strains and isolates of clinical importance.

Type strains matching the strains isolated from the beet armyworm guts were obtained from the DSMZ culture collection and all of the strains were assayed for antibiotic efficacy (Table 4-13). Differences in antibiotic susceptibility were detected in over a half of the tests performed. *B. aquimaris* type strain was more susceptible to chloramphenicol and kanamycin, but more resistant to rifampicin than the gut-isolated strain. *B. vietnamensis* type strain was more resistant to five out of six antibiotics tested: ampicillin, chloramphenicol, ciprofloxacin, kanamycin and rifampicin. *M. paraoxydans* type strain was more susceptible to ampicillin and more resistant to ciprofloxacin than the gut-isolated strain. *R. pusense* gut-isolated strain was more resistant than the type strain to ciprofloxacin, kanamycin and tetracycline. Differences between antibiotic resistance levels were identified between the type strains and the gut-isolated strains. In six cases the gut-isolated strains were more resistant and in seven cases the type strains were more resistant.

Table 4.13: Antibiotic susceptibility profiles of the gut bacteria from the beet armyworm larvae and corresponding type strains. MICs for six different antibiotics were assessed by a broth dilution assay. Differences in antibiotic-resistance levels between the strains have been highlighted in green when the gut-isolated strain was more resistant than the type strain and in red when the type strain was more resistant than the gut-isolated strain. P < 0.01.

	Minimal inhibitory concentrations of antibiotics in broth dilution assay [µg/mL]											
	Bacillus aquamaris		*Bacillus vietnamiensis*		*Microbacterium paraoxydans*		*Rhizobium pusense*					
	gut strain	type strain	gut strain	type strain	gut strain	type strain	gut strain	type strain				
ampicillin	>64	>64	32	>64	>64	32	>64	>64				
chloramphenicol	8	2	4	32	32	32	32	32				
ciprofloxacin	0.5	0.5	0.5	1	1	4	32	0.5				
kanamycin	>64	32	32	>64	>64	>64	>64	32				
rifampicin	4	16	4	16	16	16	16	16				
oxytetracycline	8	8	8	8	16	16	32	8				

4.3.6.4 Madagascar periwinkle extract

After establishing the antibiotic resistance profiles of the gut bacteria from the beet armyworm, we investigated the antibiotic activity of the periwinkle extract. Some

metabolites are more abundant in the aerial parts of the plant and others in the roots and it was decided to investigate the extracts of both, even though the insects do not feed on the roots. The extracts were prepared by soaking the dried ground plant material in methanol. They were tested for antibacterial activity against gut-isolated strains and type strains (Table 4-14).

There were no differences in Madagascar periwinkle leaf extract susceptibility between the gut strains and type strains of the two *Bacillus* isolates: *B. aquimaris* and *B. vietnamensis*. The *R. pusense* gut isolate was more resistant than the type strain to both the leaf and root extract. Contrary to what was expected, in a number of cases the type strain was more resistant to the *C. roseus* extract than the gut-isolated strain, even though the gut isolate was exposed to the plant extract and the type strain was not. *M. paraoxydans* type strain was more resistant to both the leaf and root extract than the gut strain and both *B. aquimaris* and *B. vietnamensis* type strains were fully resistant to the periwinkle extract while the gut-isolated strains were susceptible. All three type strains that were unusually resistant to the periwinkle extract, were also antibiotic-resistant opposite to how they were predicted to be in comparison to the gut isolates.

Table 4.14: The susceptibility of the gut bacteria from the beet armyworm larvae guts and corresponding type strains to the Madagascar periwinkle extract. The values were obtained in a disc diffusion assay. The discs carried 10 µg dried eucalyptus extract. The differences in plant extract susceptibility between the strains were highlighted with green when the gut-isolated strain was more resistant than the type strain and in red when the type strain was more resistant than the gut-isolated strain. The assay was repeated three times.

	Diameter of inhibition zones [mm]									
	Bacillus aquamaris		*Bacillus vietnamiensis*		*Microbacterium paraoxydans*		*Rhizobium pusense*		leaf extract	flower extract
	strain gut	strain type	strain gut	type strain	gut strain	type strain	gut strain	strain type		
	6	6	6	6	8	6	6	7	6	16
	6	6	6	6	28	20	17	20	0	0
	6	6	6	6	28	20	17	20	17	0
	6	6	6	6	28	20	17	20	0	0

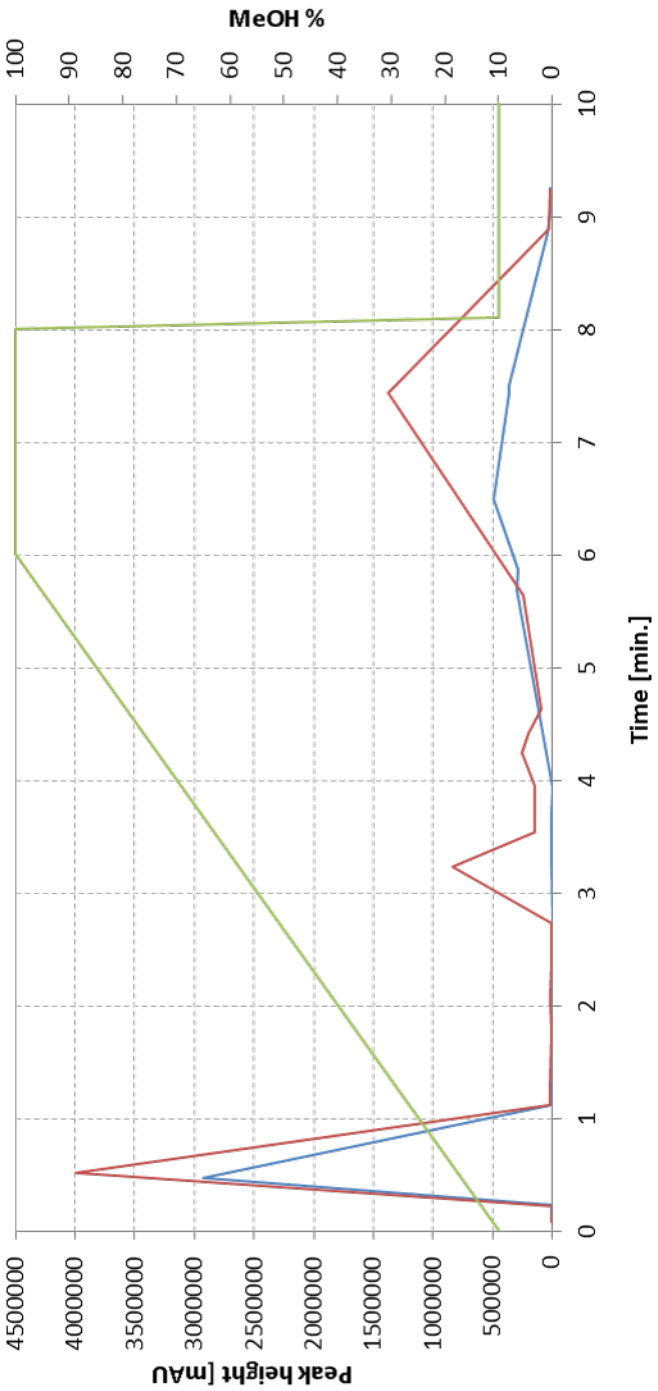


Figure 4.14: HPLC chromatogram of the Madagascar periwinkle extract. The sample was fractionated using Shimadzu LCMS-2020 (single-quad), semi-preparative C18 column and 10 – 100% methanol gradient. The UV/vis detector scanned 190-800 nm wavelengths. The extracted wavelengths are 254 nm (red), detecting UV-active compounds, and 354 nm (blue) detecting conjugated double bonds.

4.3.6.5 Extract fractionation

The root extract had higher antibiotic activity than the leaf extract and it was selected

for further purification. As mentioned before the HPLC fractionation is a labour-intensive process and only few extracts have been processed that way. Some of the downstream analyses, such as mass spectroscopy, are less time-consuming and can be performed for numerous samples. That is why some plant extracts have not been fractionated, but we obtained preliminary data on their most abundant metabolites.

The root extract was partitioned with petroleum ether to remove fats that would interfere with further purification. The partitioned extract was further purified on SPE. The fractions were similar to each other and the antibacterial activity was not specific to a particular fraction. For periwinkle extract, the SPE fractionation was not accurate enough and instead the crude de-fatted extract was fractionated on HPLC (Figure 4-14). As with other plant extracts described above the active fraction eluted at the beginning of the methanol gradient with fractions containing less than 15% methanol, suggesting the active components of the extract did not interact with the C18 matrix of the HPLC column. The HPLC fraction with the antibiotic activity was analysed by mass spectroscopy to determine which metabolites were present.

The root extract sample fractionated on HPLC was subjected to LC-MS analysis to assess how many different compounds make up the fraction with antibiotic activity. The most abundant ions are listed in Table 4-15. Some of the ions were identified by running their m/z ratios against the annotated metabolites in the Medicinal Plants Consortium database (http://metnetdb.org/mpmr_public/). The most abundant ion detected was serpentine. The other abundant ions were matched to metabolites with unknown structures. The identifiers of these metabolites are shown in Table 4-15, but the database does not provide any more information about these compounds. Present in the root extract were also loganic acid, serpentine and catharanthine. The structures of the metabolites with known structures are shown in Figure 4-15.

Table 4.15: A list of most abundant ions present in the periwinkle root extract fraction with antibacterial activity, detected by LCMS-IT-ToF Mass Spectometer (IT-ToF, Shimadzu) using analytical C18 column and 10 -100% acetonitrile gradient against 0.1% formic acid in water. The ions were matched with some known metabolites from C. roseus metabolomics database based on their m/z ratios. For ions without a good match in the database, the closest match was listed.

Ion m/z	retention time [min.]	compound name
349.1700	7.755	serpentine
397.2112	6.928	MDC-Cat-HPLC-E-POS-F1-397.2-6.93
315.0734	6.429	MDC-Cat-HPLC-E-POS-F1-315.119-6.43
396.2036	10.808	MDC-Cat-HPLC-E-POS-F1-396.184-10.81
350.1733	5.658	MDC-Cat-HPLC-E-POS-F1-350.172-5.66
377.0871	2.360	loganic acid
349.1539	2.893	serpentine
337.1836	7.153	catharanthine
398.2159	10.314	MDC-Cat-HPLC-E-POS-F1-398.145-10.31
397.2108	6.928	MDC-Cat-HPLC-E-POS-F1-397.2-6.93

The non-fractionated leaf extract was also subjected to mass spectroscopy. The most abundant ion identified was vindoline, followed by loganic acid, and serpentine (Table 4-16). As before, a number of ions were matched to metabolites with an unknown structure. The structures of the known metabolites are shown in Figure 4-15. Some of the metabolites were shared between the leaf and root extracts. Both serpentine and loganic acid were found in the root extract and leaf extract.

We confirmed the presence of vindoline in the leaf extract using commercially-available vindoline as LC-MS standard. However, at the time of the experiment the other compounds were not available and we were not able to confirm their presence in the samples.

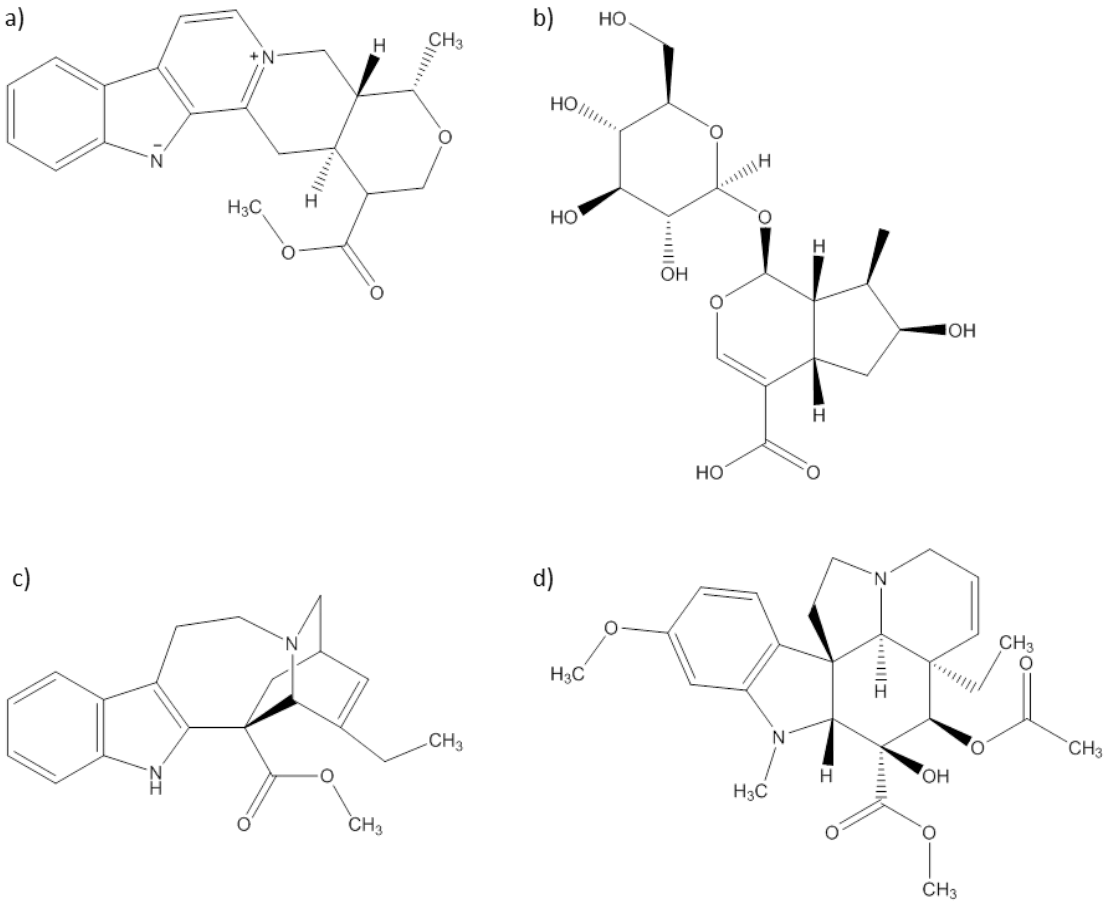


Figure 4.15: Chemical structures of the identified metabolites from C. roseus extract with antibacterial activity. The active fraction from the root extract contained serpentine (a), loganic acid (b) and catharanthine (c). The leaf sample contained vindoline (d) as the most abundant metabolite, followed by loganic acid (b) and serpentine (a).

Neither of the extracts contained a single metabolite and it is difficult to hypothesise which one of the metabolites identified was responsible for the antibiotic activity. Instead we established minimal inhibitory concentrations (MICs) for the commercially available compounds detected in the fractions (Table 4-17). The differences between the metabolites from the periwinkle extracts and tested compounds are shown in Figure 4-16. Vindoline had the lowest MIC, with 150-600 µg/mL. Previous data also discovered vindoline to be the most active terpenoid indole alkaloid, but the MIC was found to be 1000 µg/mL [199]. The other compounds tested, ajmalicine, catharanthinine and loganin, had MICs between 300-1250 µg/mL. The MIC values are not as low as hoped for, as successful antibiotics have MICs lower with at least an order of magnitude. Interestingly, the lowest MIC values were obtained for permeable *E. coli* strain, suggesting the compounds kill cells more effectively when they can efflux into cells easily.

Table 4.16: Most abundant ions present in the periwinkle leaf extract. A list of most abundant ions present in the periwinkle leaf extract, detected by LCMS-IT-ToF Mass Spectometer (IT-ToF, Shimadzu) using analytical C18 column and 10 -100% acetonitrile gradient against 0.1% formic acid in water. The extract only has a mild antibacterial activity. The ions were matched with some known metabolites from C. roseus metabolomics database based on their m/z ratios. For ions without a good match in the database, the closest match was listed.

Ion m/z	retention time [min.]	compound name
457.2317	0.829	vindoline
397.2108	2.193	MDC-Cat-HPLC-E-POS-F1-397.2-6.93
377.0871	2.360	loganic acid
349.1539	2.893	serpentine
521.06	1.884	MDC-Cat-HPLC-E-POS-F1-521.204-2.05
458.2358	0.829	MDC-Cat-HPLC-E-POS-F1-458.207-9.83
439.219	0.84	MDC-Cat-HPLC-E-POS-F1-439.202-9.5
520.9128	0.400	MDC-Cat-HPLC-E-POS-F1-520.217-9.48
656.8851	0.200	MDC-Cat-HPLC-E-POS-F1-656.345-13.73
397.2112	5.554	MDC-Cat-HPLC-E-POS-F1-397.2-6.93

Table 4.17: Minimal inhibitory concentrations of the main metabolites from C. roseus extracts. Where the metabolite was not commercially available we tested the closest metabolite or precursor that was available.

strain	Minimal inhibitory concentration [mg/mL]			
	vindoline	loganin	ajmalicine	catharanthine
Escherichia coli	0.3	0.6	0.6	0.6
Escherichia coli (permeable)	0.15	0.3	0.6	0.3
Mycobacterium smegmatis	0.6	0.6	0.6	0.6
Pseudomonas aeruginosa	0.3	0.6	0.6	0.6
Staphylococcus aureus	0.3	0.6	0.6	0.6
Bacillus vietnamensis	0.3	0.6	0.6	1.25
Microbacterium paraoxydans	0.3	0.6	0.6	0.6
Rhizobium pusense	0.3	0.6	0.6	0.6

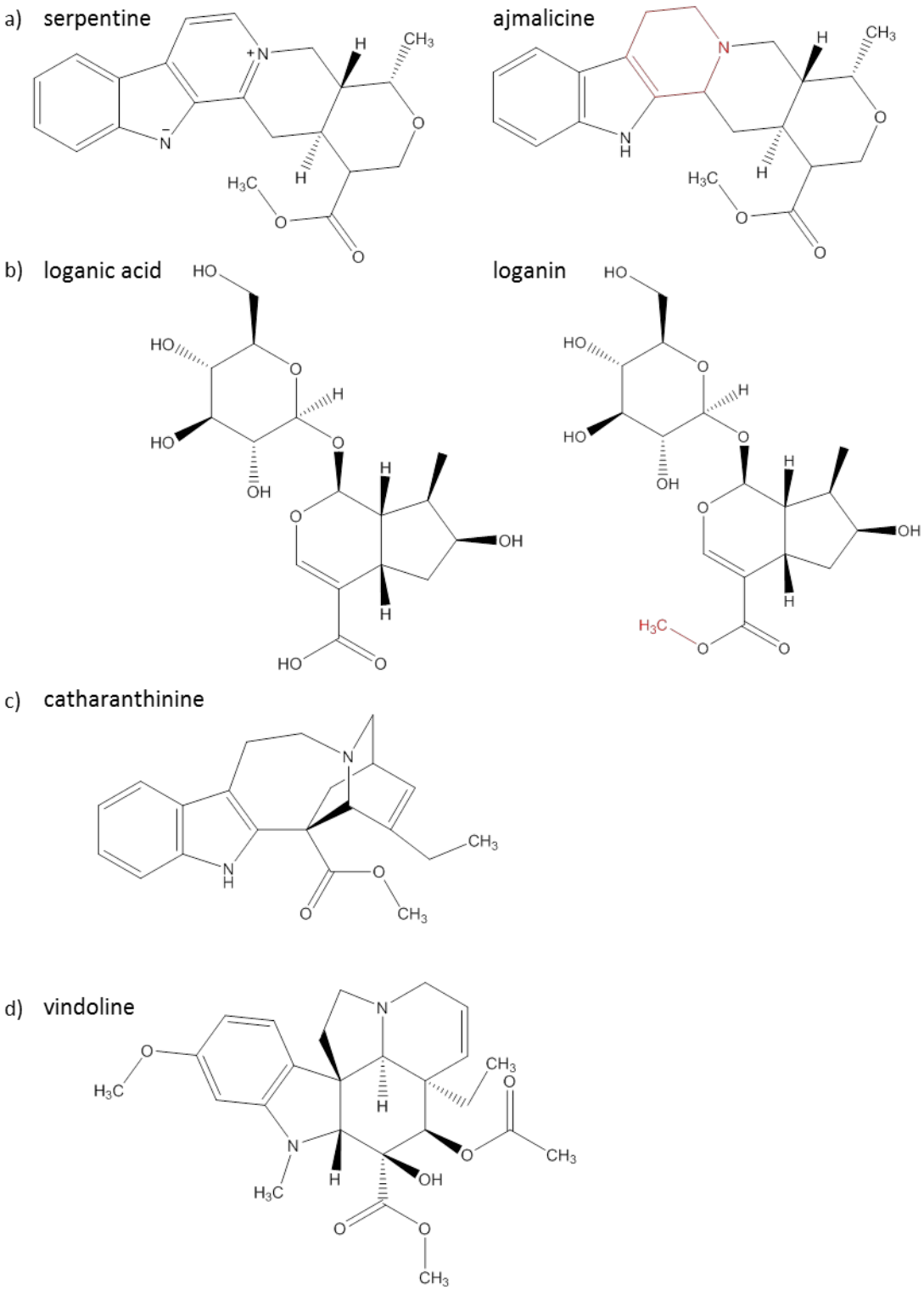


Figure 4.16: Differences between the metabolites identified in the periwinkle extract and the commercially-available compounds assayed for minimal inhibitory concentrations. (a) Serpentine and ajmalicine, b) loganic acid and loganin, c) catharanthine and d) vindoline.

For the periwinkle extract we managed to identify some of the components contributing to the extracts' antibiotic activity. Even though these compounds are not

as active as currently used antibiotics, they might have interesting modes of action and should be investigated further. These experiments show that it is possible to identify components of plant extracts with antibacterial activity using the approach of utilizing the insect gut bacteria for activity-guided fractionation.

4.4 Discussion and Conclusions

4.4.1 Giant stick insect feeding on eucalyptus

4.4.1.1 Gut microbiota

The bacteria from the stick insect guts were cultured twice, from two pairs of insects. Among the genera isolated there was only one that was shared between the two pairs, genus *Microbacterium* represented by *M. oxydans* and *M. paraoxydans*. Such different microbiota recovered from insects from the same colony suggests a high degree of intra-species variability due to differences between individuals. The differences in microbiome composition are not a result of different diets, as the insects were feeding on eucalyptus leaves from the same tree.

It is important to note that the stick insects were the only insects collected after they died, rather than euthanised after collection. The stick insects died of old age and their gut microbiota was probably affected by it. A correlation between middle and old age and gut microbiota has been demonstrated in mice [200]. Old age and frailty associated with it, is linked with the increased abundance of certain bacterial taxa. Functional profile of the gut microbiome also changed in older mice: the biosynthesis of vitamins B12 and B7 decreased, bacterial DNA repair was impaired, creatine degradation was increased and the utilisation of monosaccharides was higher than oligosaccharides, leading to the deterioration of the gut homeostasis. It is possible that similar changes can be observed in the stick insect gut when it is compared to younger specimens.

Equally few bacterial species are shared between our study and previous findings. A study focusing on the characterisation of order Phasmatodea (stick insects) gut anatomy and microbiology implemented culture-dependent methods to find four

microbial species in the gut of *D. gigantea* feeding on *Eucalyptus citriodora* [186]. In the crop there was one bacterial and one fungal species: *Serratia marcescens* and *Cryptococcus ramirezgomezianus*, the midgut was colonised exclusively by *Enterobacter cloacea*, and the hindgut was the most species-rich with three bacterial strains: *E. cloacea*, *Erwinia persicina*, *S. marcescens*. Another study identified *S. marcescens* and *Spiroplasma* species in *D. gigantea* guts via targeted culture-independent methods [201]. The authors did not speculate about the role of *S. marcescens*, but *Spiroplasma* species are a common commensal endosymbiont, maternally transmitted, and in some insects responsible for impaired reproduction by inducing the selective elimination of male progeny.

Our metagenomic analysis of the *D. gigantea* gut community confirms the abundance of *S. marcescens* in the digestive tract. However from genomic data alone it is not possible to propose a role for *S. marcescens*, which is both a human pathogen, common in hospital-acquired infections [202], and an insect pathogen sometimes utilized as a model species in immunity studies [188].

4.4.1.2 Antibiotic resistance

As discussed in the previous chapter, we hypothesised that antibiotic resistance in the insect gut is induced by antibacterial compounds in the insect food. As a way of testing this hypothesis we compared antibiotic susceptibility profiles of strains isolated from the insect gut and type strains from a culture collection. For *D. gigantea* gut bacteria this hypothesis was correct apart from in one case, when *M. oxydans* type strain was more tetracycline resistant than the gut-isolated strain. Such discrepancies can be explained by the pleiotropic effects of bacterial responses to external stimuli, when an increase or decrease in resistance to an antibiotic can be a result of seemingly unrelated gene action.

4.4.1.3 Eucalyptus extract

We only identified a mild antibacterial activity in the eucalyptus leaf extract. Antibiotic activity in eucalyptus extracts is normally described in the context of the eucalyptus essential oil. Eucalyptus oil (from *E. globulus*) is known to have an antibacterial activity,

but it is relatively low compared to other essential oils [203]. Essential oils are notorious for their high content of reactive and unstable compounds. However, our plant extract processing methodology focused on and selected for hydrophilic compounds, as hydrophobic oily substances interfered with preliminary HPLC fractionations.

The take-home message from our exploration of the stick insect and eucalyptus plant/insect pair is that it is easy to identify antibiotic resistance in the insect gut and to identify extracts with antibiotic activity. It is much more difficult to separate the compounds responsible for that activity and identify them.

4.4.2 *P. xylostella* feeding on cabbage

4.4.2.1 Gut microbiota

Only two strains have been isolated from the diamondback moth larvae by culture-dependent methods: *S. keddieii* and *R. terrigena* in our experiments. A previous study identified on average 208 operational taxonomic units from seven *P. xyllostella* larvae midguts [147]. Surprisingly only 25 operational taxonomic units had frequency higher than 0.05%, demonstrating that the majority of isolates present in the guts of diamondback moth larvae were extremely rare. The authors do not discuss the composition of the *P. xylostella* microbiomes on the species level, but their dataset can be retrieved through Sequence Read Archive (http://www.ncbi.nlm.nih.gov/Traces/sra/). An analysis of the dataset revealed no sequences with high degree of similarity to either *S. keddieii* or *R. terrigena*, but the rarefaction curves of the dataset did not reach plateau, indicating that the sequencing depth was insufficient to discover all sequences present in each sample. Another possible explanation is that the strains are not present at all, since the microbiota is variable or transient.

4.4.2.2 Cabbage extract

The cabbage extract was the only plant extract tested that had no antibiotic activity when tested against the bacteria from the *P. xylostella* guts and corresponding type

strains. Very few studies describe antibacterial activity in cabbage, and those that do only find weak activity [204], suggesting that it is rooted only in anecdotal evidence of traditional medicine.

Overall these experiments have shown that not all plant extract have antibacterial activity. They have also shown that the presence of antibiotic resistance in the gut bacteria of insects feeding on a certain plant is not always an indication of the antibacterial properties of that plant species.

4.4.3 Cinnabar moth feeding on ragwort

4.4.3.1 Gut microbiota

Out of five species of bacteria isolated from cinnabar moth guts, two belonged to genus Staphylococcus: *S. epidermidis* and *S. warneri*. Both of these isolates are typical members of human skin microbiota. It is not uncommon for bacteria considered typically human to be found in insects. *Staphylococcus* species have been described as members of the gut microbiota of Termitidae family of termites and the presence of *Staphylococcus* species was specific to that group of Australian higher termites [205]. The role of the *Staphylococcus* species in the termite gut is probably cellulose degradation [206].

4.4.3.2 Ragwort extract

Ragwort contains high concentrations of pyrrolizidine alkaloids, which are responsible for its toxicity to cattle and horses. Because of the toxicity of pyrrolizidine alkaloids to generalist herbivores, the compounds are thought to be a feeding deterrent, however it was also suggested they protect the plants against microbial attack (see review by Joosten and van Veen, 2011 [207]). In our experiments ragwort extract was one of the most antibacterial plant extracts tested against insect gut bacteria, but the pyrrolizidine alkaloids were not detected in the plant extract. There are no data about antibacterial activities of pyrrolizidine alkaloids, but they have a moderate antifungal activity against plant pathogens [208]. These data suggest that pyrrolizidine alkaloids are not the compounds responsible for the antibiotic activity in the ragwort extract.

Due to time constrains we were not able to pursue the ragwort extract further, but it is probably one of the more interesting extracts investigated. The antibacterial activity of the extract was not due to the presence of toxic pyrrolizidine alkaloids and it would be interesting to identify these compounds since their antibacterial activity was relatively high. Our experiments demonstrate that not only medicinal but also toxic plants can be a source of antibacterial compounds.

4.4.4 Rosemary beetle feeding on lavender

4.4.4.1 Gut microbiota

The rosemary beetle microbiome was another example of an insect gut community with members typically associated with human microbiota: *S. epidermidis* and *P. agglomerans*. As discussed before it is not uncommon for insects to harbour such bacteria in their guts. *P. agglomerans* is a prominent member of the desert locust *Schistocerca gregaria* [209] and produces compounds that lead to the swarming behaviour of the locusts and are necessary for the synthesis of an aggregation/cohesion pheromone. It is also know to detoxify some phytochemicals as discussed earlier in this chapter.

4.4.4.2 Antibiotic resistance

There were only two differences in the antibiotic susceptibility profiles of the gut isolates and corresponding type strains, which was the least of all microbiomes tested. It indicates that either the lavender leaves that the beetles were feeding on were not toxic enough for the gut bacteria to develop resistance, or that a member of the microbiome detoxified the extract.

4.4.4.3 Lavender extract

After HPLC fractionation each collected fraction was assayed for antibiotic activity. Unusually many fractions were active against the bacteria tested. Individually each fraction was weakly active and together they contributed to the mild antibiotic activity of the plant extract. Weakly active lavender extract is consistent with mild evolutionary

pressure on the rosemary beetle gut bacteria to develop resistance mechanisms.

These data are not consistent with the antimicrobial activity of lavender essential oil. Lavender essential oil is a potent antimicrobial, active against both bacteria and fungi, but there is no consensus on which component of the essential oil is responsible for the activity [210]. Even though the scientific evidence indicates lavender has antibacterial properties, problems around standardization of the oil and the purification of the active components hamper the development of lavender preparations into a therapeutically useful agent.

4.4.5 Death's head hawkmoth feeding on potato leaves

4.4.5.1 Gut microbiota

Death's head hawkmoth gut community was a mixture of environmental strains and strains associated with human microbiota, similar to other insect species described in this chapter. Surprisingly, the death's head hawkmoth larvae harboured strains that were most different from the type strains when the antibiotic susceptibility profiles were compared.

The *E. asburiae* type strain is a clinical isolate and it was the only type strain tested that was more resistant than the gut strain to every antibiotic in the panel. This result suggests the *A. atropos* gut is not a niche with an extremely selective environment, as the gut isolated *E. asburiae* was not as antibiotic-resistant as the type strain.

4.4.5.2 Potato leaf extract

The potato leaf extract was weakly antibiotic. Potato leaves, similarly to ragwort, are toxic to many herbivores, but they lack the strong antibacterial activity of ragwort extract. Potato leaves have not been previously investigated for antibacterial activity, as other potato waste products have been. Potato tubers unsuitable for sale have been investigated as a source of antibacterial activity [211], and had a mild level of antibiotic properties which have been attributed to phenolic compounds in the extracts.

In summary, potato waste products are an attractive source to investigate potential bioactivity. However we have shown the level of antibiotic activity is relatively low and

we have not pursued the purification of the active fraction of the extract.

4.4.6 Beet armyworm feeding on Madagascar periwinkle

4.4.6.1 Gut microbiota

There is little similarity between the bacterial species isolated from beet armyworm larvae in our experiments and previously reported studies. Previous studies identified *Ochrobactrum sp*. and *Myroides odoratus* as the main culturable strains from beet armyworm guts [212]. *Rhizobium pusense*, isolated in our experiments, is related to *Ochrobactrum* strains. It is possible that these strains are consistently present in the beet armyworm guts.

The beet armyworm microbiota was shown to be involved in the production of N-acylamino acids [212], which can be isolated from insects' oral secretions and have been indicated in eliciting plant defences. One of such N-acylamino acids, microbially-produced volicitin (N-(17-hydroxylinolenoyl)-L-glutamine) induces damaged leaves to produce volatile compounds attracting parasitic wasps that infect the armyworm larvae [213].

Two species of *Bacillus* have been isolated from the beet armyworm guts. Bacilli are abundant in soil and commonly isolated from the insect gut. It has been proposed that *Bacillus* species opportunistically invade insect guts [214]. The transition from soil to the nutrient-rich insect gut facilitates filamentous growth.

4.4.6.2 Madagascar periwinkle extract

Madagascar periwinkle is probably the most widely studied plant described in this chapter. *C. roseus* produces terpenoid indole alkaloids used in cancer therapy, but its antibacterial activity is less documented. Extracts of leaves, stems, flowers and roots have been demonstrated to have antibacterial activity [215] and some of the main alkaloids present in Madagascar periwinkle have been investigated for antibiotic activity [199]. The most active alkaloid from *C. roseus* was vindoline, but the MIC established for it was much higher than in our study.

Additionally, Madagascar periwinkle seeds contain antibacterial proteins CRCI and

CRCII (*Catharanthus roseus* cystatin I and II) [216]. These proteins are thiol protease inhibitors and exhibit antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*, but were ineffective against *Bacillus subtilis*. At 25 µg/mL the zones of inhibition were 11-14 mm in diameter. The authors hypothesise that the cystatins are at least partially responsible for the medicinal properties of *C. roseus* in traditional preparations.

Our investigation is the first attempt to link the antibacterial properties of the Madagascar periwinkle extract to the metabolites present in the plant. We have discovered that the main indole alkaloids have mild antibiotic activity against a wide range of bacteria. Vindoline had the highest antibacterial activity, especially when tested against bacteria with permeable cell membrane. In general the compounds had MIC values an order of magnitude higher than most commonly used antibiotics, but they would be worth investigating further to determine their targets and modes of action. Indole alkaloids from Madagascar periwinkle are already well-studied and that body of knowledge makes them attractive leads in antibiotic discovery.

4.4.7 General comments about bacterial species isolated from the insect guts

We isolated a variety of bacterial strains from the guts of the insects investigated. A majority of the bacterial isolates were common soil and water strains, but we also identified a number of strains with clinical significance in human health. Such composition of insect gut microbiomes is not unusual and has been described before (for example Staphylococcus species in termites [206] and Enterococcus species in greater wax moths [150]). As insects are sometimes vectors of human disease, the presence of pathogens in the healthy insect microbiota is an important factor to further investigate.

Surprisingly we have not identified any obligatorily anaerobic bacteria in the insect guts. The oxygen levels in the herbivorous insects' guts are low [187], especially in the midgut and hindgut. The oxygen level depends partially on the insect food and increases when the insects feed on artificial food. The presence of oxygen in the guts of herbivorous insects can lead to the formation of reactive oxygen species from the

ingested plant material. Additionally the autoxidation of plant phenolics is greatly reduced by the lack of oxygen, even in the highly alkaline guts of some insects. The depletion of oxygen from the insect guts is abolished by boiling, suggesting either endogenous insect enzymes or microbial activity are responsible. It is possible that both processes are involved and both the host insect and the native microbiota together sustain the gut as a suitable niche.

4.4.8 General comments about antibiotic resistance

When the antibiotic resistance profiles of the gut-isolated strains and the type strains are compared, there are three possible outcomes. Firstly, no differences between the antibiotic resistance levels can be detected. Secondly, the gut-isolated strain can be more resistant than the type strain. Finally, the type strain can be more antibiotic-resistant than the gut strain. Initially it was hypothesised no differences and higher resistance of the gut-isolated strains would be the most common outcomes of the antibiotic susceptibility testing. We expected the gut strains to develop antibiotic resistance in response to the strong selective pressures in the insect gut.

We discovered that equally often as no difference in antibiotic resistance or the gut-isolated strain being more antibiotic-resistant, the type strain had a higher level of resistance to the antibiotics tested. We expected to see a few differences like that, but the high prevalence of this outcome of the antibiotic susceptibility tests was unexpected. It is possible the elevated antibiotic resistance of some type strains is due to their origin as clinical isolates. Bacteria first cultured in hospital settings are likely to have been exposed to antibiotics and to have elevated levels of antibiotic resistance or tolerance. However, this is only one possibility and it does not explain all the unexpected differences in antibiotic resistance levels between the type strains and the gut-isolated strains.

In some cases a type strain was more resistant than the gut-isolated strain to one antibiotic and less resistant than other, which can be explained by the fitness cost of maintaining multiple resistance genes. Mutations resulting in antibiotic resistance are commonly associated with a reduction in growth rate, but that is not always the case (see review MacLean et al., 2010 [136]).

Notably, the type strains from culture collections were used as a comparison for antibiotic resistance. For bacteria and other fast-evolving organisms the type strains are deposited in culture collections in aid of taxonomic identification, not as a standard specimen of the species. A more systematic, but much more time consuming, approach to creating the antibiotic resistance profiles would be to combine culture-dependent and -independent methods. The gut resistome can be probed with targeted molecular biology methods, such as PCR and qPCR of known resistance genes, and a number of isolates of the same species, not just the type strain, can be assayed in a broth microdilution assay for resistance to a panel of antibiotic. Such an approach would place each insect gut-isolated strain in a wider ecological context, but ultimately would not aid the identification of antibacterial activity in the plant extracts, which was one of the goals of the chapter.

4.4.9 General comments about the bacterial susceptibility to plant extracts

Medicinal and toxic plants were chosen for assessment of antibacterial activity in this study, based on anecdotal evidence and, where available, scientific literature. Interestingly, there has never been a systematic study to assess whether plants used in traditional medicine are more likely to contain valuable bioactive compounds [177]. The use of certain plants in traditional medicine is not indicative of their efficacy. Such data can only be provided when standardized and chemically characterized plant preparations are tested *in vitro* and in animal models, and their efficacy is confirmed in clinical studies [179].

Even when a plant with confirmed medicinal activity is chosen, often the role of origin and variety is underestimated. *E. dalrympleana* was chosen because the stick insects in JIC Insectary were feeding on this species of eucalyptus. However, *E. dalrympleana* is a species of eucalyptus grown for timber and there is no indication of its medicinal properties. It is not normally used for essential oil production. It is likely that higher levels of antibiotic activity would be detected from a different species of eucalyptus. Similarly, different Madagascar periwinkle cultivars have different indole alkaloid contents [217] and probably display different levels of antibiotic activity.

It was hypothesised that the type strains would be more susceptible to the plant extracts than the strains from the insect gut that have been exposed to the constituents of the extracts. Surprisingly it is not always true. Most of the type strains that exhibited unexpected plant extract susceptibility also had some unusual differences in antibiotic resistance levels. For example, *Burkholderia fungorum* identified in the guts of the cinnabar moth larvae feeding on ragwort was more susceptible to ciprofloxacin and kanamycin than the type strain. Apart from the elevated antibiotic resistance, the *B. fungorum* type strain was also more resistant to ragwort leaf extract. Similarly, *Bacillus vietnamensis* type strain was more resistant then the *B. vietnamensis* isolated from the guts of beet armyworms to ampicillin, chloramphenicol, ciprofloxacin, kanamycin and rifampicin, as well as periwinkle root extract. It is possible that the unexpected antibiotic resistance and plant extract susceptibility are correlated or linked, but it is impossible to hypothesise more on the nature of this phenomenon only form the data collected.

4.4.10 General comments about the fractionation

Equally important to the choice of source plant was the choice of fractionation method. In one of our initial methods the crude extract was fractionated on normal phase HPLC, but the performance was unsatisfactory as the waxy components were binding too tightly to the matrix, requiring long wash cycles. To improve the HPLC efficiency, the plant extracts were partitioned with organic solvents and SPE before fractionation. SPE is not as efficient in separating compounds as HPLC, but the variety of matrices is larger. Two- or three-dimensional fractionation is possible, for example using ion exchange matrices, size exclusion and silica- or polymer-based matrices that separate compounds based on their hydrophobicity. In the case of plant extracts tested, size-exclusion chromatography produced peaks that were too broad and ion-exchange matrices introduced formic acid and ammonium hydroxide, which change sample pH and prevent the growth of bacteria. Eventually all experiments including ion exchange SPE included pH controls to avoid false positive results.

The HPLC fractionation was performed on a C18 matrix, which is normally used to separate mixtures of mostly hydrophobic compounds. Many plant extract components

with antibacterial activity overlapped eluting early in the gradient, suggesting that a different matrix would be more suitable (such as silica matrices). Additionally multiple injections had to be made to obtain enough fractionated plant extract for antibacterial activity testing. The efficiency of HPLC decreases the more extract is loaded onto the column. Multiple small injections are favoured if the efficiency of the fractionation is required, making preparative HPLC a slow technique.

The phytochemicals with antibacterial activity separated in the fractionations were often sticky when dried on a paper disc or evaporated to a viscous mass in tubes. They also eluted early in water/methanol HPLC gradients, indicating that the compounds were hydrophilic, and were difficult to separate into single peaks, suggesting that they might have been similar to each other. It is possible that the compounds were polyphenols or heavily glycosylated compounds, as these groups of compounds are hydrophilic and can exist as close variants of similar molecules.

4.4.11 Summary

Using six different plant-insect pairs we have shown that insect guts contain both environmental bacterial strains and strains typically associated with the human body. In general we found only a small number of culturable bacterial strains (two – six) from the insects' guts, but metagenomic analysis of stick insect gut showed that a larger number of species are likely to be present (20 – 50). We demonstrated that bacteria from the insect guts are antibiotic-resistant (compared to their type-strains from culture collections), as had been predicted from literature observations. However we also found that some species showed no difference in susceptibility to antibiotics between the gut-isolated and the type strains; this was not unexpected. More surprisingly we found several instances where the type strain was more resistant to antibiotics than the gut-isolated strain. In some cases this can be rationalised by the fact that the origin of the type strain was a clinical isolate, but in other cases such rationalisation was not possible. Taken together these data suggest that comparing antibiotic susceptibilities in gut-isolated and type strain is not necessarily a reliable comparison and that the differences in the antibiotic resistance between gut-isolated strains and type strains cannot be used as a reliable indication of antibacterial activity

in the food plant.

We confirmed the extracts of several of the plant species used have antibiotic activity, but that some plants, such as cabbage, lack obvious antibacterial activity. The susceptibility of the bacterial strains to the plant extracts can be used as a guide for identifying plant extract fractions with antibiotic activity. It was possible to fractionate the extracts and to identify fractions of plant extracts with antibacterial activity, but it was likely that they contained multiple compounds and that further separation was challenging. However in the case of Madagascar periwinkle we were able to identify the compounds in both the root and leaf extract with antibiotic activity and we established the minimal inhibitory concentrations of these and related compounds. Even though these metabolites only exhibit a mild antibacterial activity they are potentially interesting lead compounds to follow.

5 Developing *Galleria mellonella* (Greater wax moth) larvae into a laboratory model: acute toxicity trials, antibiotic susceptibility testing and replacement of native gut bacteria with human faecal microbiota

I would like to thank Dr Lindsay Hall and Dr Melissa Lawson for their advice and technical assistance in the gut community replacement experiments.

All data in this chapter was generated by me and I produced all figures and tables on my own.

5.1 Abstract

The aim of this chapter was to assess how suitable *Galleria mellonella* is for acute systemic toxicity testing, antibiotic efficacy screening and as a simple model of the human gut. Developing such tests allows bridging a gap between *in vitro* tests and experiments in murine models, which is important as *in* vitro tests alone do not supply sufficient data and the *in vivo* tests are expensive and are not statistically robust. However only limited data are available on how the larvae perform in laboratory tests and how the results compare to data obtained in mice. We developed an acute toxicity test and compared the insect LD_{50} values to values available in safety datasheets. For antibiotic efficacy tests we used established protocols to compare the dosage recommended for human use to doses effective in the wax moth larvae. Additionally we obtained preliminary data on the prospect of replacing native *G. mellonella* gut microbiota with human gut bacteria. Our results showed that greater wax moth larvae are suitable for such studies. The insects are a useful resource for obtaining better preliminary results before testing hypotheses in mammalian models, reducing the need of extensive use of laboratory mammals.

5.2 Introduction

5.2.1 Animal testing

The Organisation for Economic Co-operation and Development (OECD) and especially European Union (EU) support minimal use of laboratory animals in research, a trend strongly backed by consumers. The European Citizens' Initiative "STOP VIVISECTION", a EU-wide petition to completely abolish the use of laboratory animals, was one of few Citizens' Initiatives to have gained enough signatures to be considered by the European Commission [218]. The initiative supports the elimination of all animal testing by 2020, quoting ethical objections, statistical data indicating that animal models are not capable of predicting human response and obstruction in the development of new, more efficient methods [petition no longer available online]. The European Commission rejected the Initiative citing existing animal welfare directives that favour alternative testing approaches and ban the marketing of cosmetics and products tested on animals [219]. The Commission stressed that a complete ban on laboratory animal use in Europe would be premature for the techniques currently validated and would drive biomedical research out of Europe. A remark was made about the Directive 2010/63/EU, which describes the three R's of animal research - the requirement to replace, reduce and refine the use of animals wherever possible (replacing for non-animal methods, reducing the numbers of animals, refining the method to minimise pain, suffering or distress experienced by the animal, or to increase their welfare) [224].

An example of how concerns about animal testing have been addressed is the implementation of The Globally Harmonized System of Classification and Labelling of Chemicals (GHS). The main goal for implementing GHS was to reduce the need for animal testing by avoiding duplicative testing.

5.2.2 Acute toxicity testing

Acute toxicity data are required under The Globally Harmonized System of Classification and Labelling of Chemicals (GHS). Currently the recommended tests for

novel substances are three *in vivo* procedures in rats or mice [220]. The OECD reviews the testing guidelines to provide up-to-date and evidence-based advice. Three currently used acute toxicity tests are: Test No. 420: Acute Oral Toxicity - Fixed Dose Procedure [221], Test No. 423: Acute Oral toxicity - Acute Toxic Class Method [222], and Test No. 425: Acute Oral Toxicity: Up-and-Down Procedure [223].

GHS classification of hazards distinguishes six classes for acute toxicity ranging from GHS class 1 (most toxic, at or below 5 mg/kg body weight) to GHS class 5 (toxic at 2000 mg/kg body weight) and unclassified (not toxic above 2000 mg kg body weight). Testing above 2000 mg/kg body weight is discouraged due to solubility issues and unnecessary suffering of the test animals (see review by Hartung, 2008 [224]). High concentrations of test compounds in animal tissues are often not physiologically relevant and the detrimental effects can be due to tissues being oversaturated, not the toxicity of the compound to the target tissue.

The European Union Reference Laboratory for Alternatives to Animal Testing (EURL-ECVAM) publishes recommendations for achieving the three R objective, while maintaining or improving human and environmental protection. A document on acute systemic toxicity was published recently [225]. Instead of proposing a new test as an alternative to current OECD guidelines, it outlined reasons to remove the need for such testing, and stressed the inadequate understanding of pathways leading to systemic toxicity. The document recommended exploring chemoinformatic methods and *in vitro* procedures for specific toxicity mechanisms and a transition from *in vivo* experiments towards pathway-based assessment of toxicological risks.

5.2.3 Acute toxicity testing in alternative organisms

Apart from *in vitro* approaches to test specific pathways leading to toxicity, a number of alternative *in vivo* tests have been developed to test systemic toxicity. Pathway conservation in metazoans allows extrapolation of the results between different species [226]. However, genetic, proteomic and physiological differences between species have to be well-defined and quantified to permit an adequate extrapolation of pathway effects, dose-response values and kinetic parameters from a test to *in vivo* human response.

Non-murine *in vivo* tests often adopt insect species, as their use in research is not covered by the Directive 2010/63/EU [219]. European honey bee (*Apis mellifera*) larvae are used to assess the environmental risk of chemicals (mostly pesticides) for the brood (eggs, larvae and pupae) [227]. The test is based on a single-dose feeding procedure, translating well into the most likely route of exposure of the test chemical (oral exposure) and assessing if the chemical acts as a feeding deterrent. Another test utilizes silkworm (*Bombyx mori*), which can be fed leaves spiked with toxic compounds [40]. The test is reproducible and robust, but silkworms are highly susceptible to infection and have a non-standardized diet (mulberry leaves). A more systematic approach is the use of greater wax moth (*Galleria mellonella*) which permits both feeding studies and injections of the test compound directly into the body cavity.

5.2.4 Acute toxicity testing in *Galleria mellonella*

Galleria mellonella is normally used in feeding studies assessing the toxicity of environmentally hazardous chemicals. For example the effects of dietary transgenic pollen were assessed in *G. mellonella* [228]. The pollen from Bt-toxin (Cry1A(b) and Cry1F) producing plants caused mortality to *G. mellonella* larvae, but Cry1F was much more effective than Cry 1A(b), without affecting honey bee larvae. Bt toxins can be used for *G. mellonella* control in beehives, as transgenic pollen is collected by the bees and brought back to the hive parasitized by wax moth larvae. Similarly the effects of an insecticide azadirachtin and its fragments were assessed on galleria larvae [229]. While the fragments were able to kill larvae in feeding studies at concentrations under 0.15 mg/g, azadirachtin was only lethal by injection. Neem tree products, from which azadirachtin was isolated, are used for insect control. This study highlights how wax moth larvae can be used in toxicity assays in two ways through feeding studies and via an injection.

Only recently *G. mellonella* larvae were used in *de novo* toxicity testing. The toxicity of ionic liquids has been assayed [41]. Ionic liquids are low temperature molten salts which are used as alternative to volatile organic solvents. They are commonly labelled “ecologically friendly”, even though the class is diverse and exhibits a wide range of toxicities. In the study the systemic toxicity was correlated to the length of alkyl chains

of the 1-alkyl-3-methylimidazolium ionic liquids tested. The salts were toxic under 100 µg/g, apart from the shortest two-carbon alkyl chain salt (1-ethyl-3- methylimidazolium chloride) which had a LD_{50} of nearly 8000 µg/g, presenting a negligible toxicity. Interestingly, even though their rising popularity as an alternative to volatile organic compounds, 1-alkyl-3-methylimidazolium ionic liquids lack toxicity data.

5.2.5 Antibiotic efficacy testing in *Galleria mellonella*

Toxicity testing in *Galleria mellonella* is an extrapolation from antibiotic efficacy studies, which are an increasingly popular procedure in microbiology [230]. Antibiotic efficacy studies establish a dose of an antibiotic necessary to clear a bacterial infection. One of necessary controls in antibiotic efficacy testing is to ensure that the antibiotic itself is not toxic to the insects. Apart from establishing a safe dose for efficacy testing a LD_{50} dose can be measured.

The greater wax moth larvae have been used for virulence and antimicrobial efficacy studies. They were first used to assess antibiotic efficacy against *Acinetobacter baumannii* [231]. Cefotaxime, tetracycline, gentamicin and meropenem were assayed against a systemic *A. baumannii* infection. Gentamicin and meropenem, which *A. baumannii* is susceptible to, significantly prolonged the survival of infected larvae, while the survival of untreated larvae and larvae treated with cefotaxime and tetracycline, which the bacteria are resistant to, was less than 25% in 5 days. The model quickly gained interest with a number of academic groups using it as a standard testing model. *G. mellonella* larvae have been used to investigate emerging pathogens [232, 233] and novel treatments for persisting pathogens [234]. Most studies up to date were collated by Cook and McArthur [230].

There are many benefits to using Galleria larvae in antibiotic efficacy studies. Many larvae can be used in each experiment making pharmacokinetic and pharmacodynamic data easy to obtain. Pharmacokinetic data obtained in *G. mellonella*, such as antibiotic clearance time, elimination half-time of the drug and maximum drug concentration, directly correlates to human data [233]. Numerous studies confirm that microbial pathogenicity and virulence determinants are the same in humans, mice and wax moths [231, 234]. The insects can be bred quickly (at 37°C the full life cycle lasts about

6 weeks) at low cost and without the need for specialized equipment. Galleria larvae are large, reaching 250-300 mm length at fifth instar, enough for an intraperitoneal injection of the test compound. Additionally, the insect immune system is functionally and structurally similar to the mammalian innate immune system [240], as discussed in the Introduction (see Chapter 1).

In virulence and antibiotic efficacy testing *G. mellonella* is an valuable system, that allows rapid testing using an accessible method at low cost. The wax moth larvae cannot fully replace mammals in pre-clinical trials, but they can improve the quality of data collected and lessen the financial burden by providing more reliable and robust data.

5.2.6 Replacement of gut microbiota

G. mellonella is used in place of mice and rats in virulence and antibiotic efficacy studies because bacteria behave similarly in insect models and more complex mammalian models. Interestingly, the wax moth gut microbiome is dominated by Enterococcus species, which are often associated with human gut microbiome. *Enterococcus faecalis* (syn. *Streptococcus faecalis*) was identified in the guts of *G. mellonella* [149, 235], to be later re-classified as *Enterococcus faecium* [158]. More recent studies indicate that a variety of related Enterococcus strains co-exist in the wax moth gut [150] (see also Chapter 3). However the Enterococcus species are non-obligate symbionts and it is possible to grow Galleria with little or no gut bacteria [170]. It is possible to surface-sterilize eggs and hatch larvae on sterile food, as well as clear the digestive tract of bacteria using antibiotics.

Based on the inessential presence of Enterococcus species in *G. mellonella* gut and their association with human microbiota, we hypothesised that the native gut community can be replaced with a human-derived community. Development of wax moth larvae into a model of the human gut would allow performing preliminary studies before testing hypotheses in mice or humans, increasing the number of samples per experiment, as well as conducting long-term studies that would be prohibitively expensive in mice.

Mice with non-native microbiota are used in research on the role of microbiota on

human diseases, such as cancer, diabetes, inflammatory bowel disease, and obesity [236]. The use of humanized gnotobiotic mice allows investigation of microbiota perturbations in human-like system. The mice can be created by inoculating germ-free mice, or mice treated with a cocktail of antibiotics, with a human microbiota sample [237]. Additionally the animals can carry mutations simulating human physiology or a particular disease. All phyla and over 80% of genus-level taxa from the human faecal samples are recovered in the guts of humanized gnotobiotic mice, but often repeated inoculations are required. The mice are the best model for a number of studies, but they are expensive, especially when the germ-free mice are used as special housing is required.

Development of *Galleria mellonella* into an analogous model would provide a cheaper alternative to testing in mice and comply with the three R's of laboratory animal use: replacement, reduction and refinement. Testing hypotheses in a simpler humanized animal model cannot fully replace experiments in mammals, but would minimize the need of using big cohorts of mice by providing a more statistically robust model suitable for larger and longer studies.

5.2.7 Aims and objectives

Initially the objective of this study was to assess antibiotic efficacy testing against four different bacterial strains and demonstrate whether LD_{50} values obtained in greater wax moth larvae correlate with current toxicological data obtained in mammalian models. The objective of the antibiotic efficacy experiments was to confirm that antibiotics treat bacterial infections in *G. mellonella* at the same efficacy as recommended for humans. The aim of the toxicity screens was to develop an *in vivo* acute toxicity assay in galleria. With the success of antibiotic efficacy and acute toxicity testing the scope of this chapter was extended to include the replacement of native gut microbiota with a community derived from the human gut. The aim of the native gut microbiota replacement experiments was to assess if the gut microbiome in *G. mellonella* can be easily replaced with other community, with an ultimate goal of developing the wax moths as a model for human gut.

5.3 Results

5.3.1 Antibiotic efficacy testing

5.3.1.1 Bacteria and antibiotics tested

The efficacy of four antibiotics: ampicillin, ciprofloxacin, tetracycline and rifampicin, in *Galleria mellonella* larvae was assessed against four bacterial pathogens: Gram-positive *Mycobacterium smegmatis* and *Staphylococcus aureus*, and Gram-negative *Escherichia* coli and *Pseudomonas aerugionosa*. For each bacterial strain there was at least one antibiotic that is indicated in the treatment of an infection caused by that strain, and at least one that is predicted not to clear the infection to confirm that the antibiotic action, not the insect immunity, is responsible for the recovery.

Initially the appropriate infective dose of bacteria was determined for each bacterial species. Groups of five larvae were injected as shown in Figure 5-1 with bacterial cultures at a quantified dose. Mortality was recorded daily for five days. An infective dose was one that caused immune response observed as darkening of the cuticle and 60-80% lethality within 48 h, but not 100% lethality within 24 h. This was determined to be $5x10^6$ cfu in 10 µl for *E. coli*, *M. smegmatis* and *S. aureus*, and $5x10^4$ cfu in 10 µl for *P. aerugionosa*.

For antibiotic efficacy testing the larvae were injected with the infective dose of bacteria and incubated for 2 h to allow the bacterial cells to multiply and excrete virulence factors. A 2 hour incubation period is a standard treatment in antibiotic efficacy testing [234, 238, 239]. After the incubation the larvae were treated with the lowest dose of antibiotic by injection at 5 mg/kg body weight. The ability of the antibiotic to cure the larva from the bacterial infection was scored daily for five days. The larvae survived when the antibiotic successfully cleared the infection. When high mortality due to an infection was observed, new larvae were injected with an infective dose of bacteria as before and treated with a higher dose of an antibiotic: 25, 50 and 200 mg/kg body weight. Therapeutic dose of an antibiotic was determined when the antibiotic rescued the mortality caused by the bacterial infection. When even the highest dose of the antibiotic did not clear the infection.

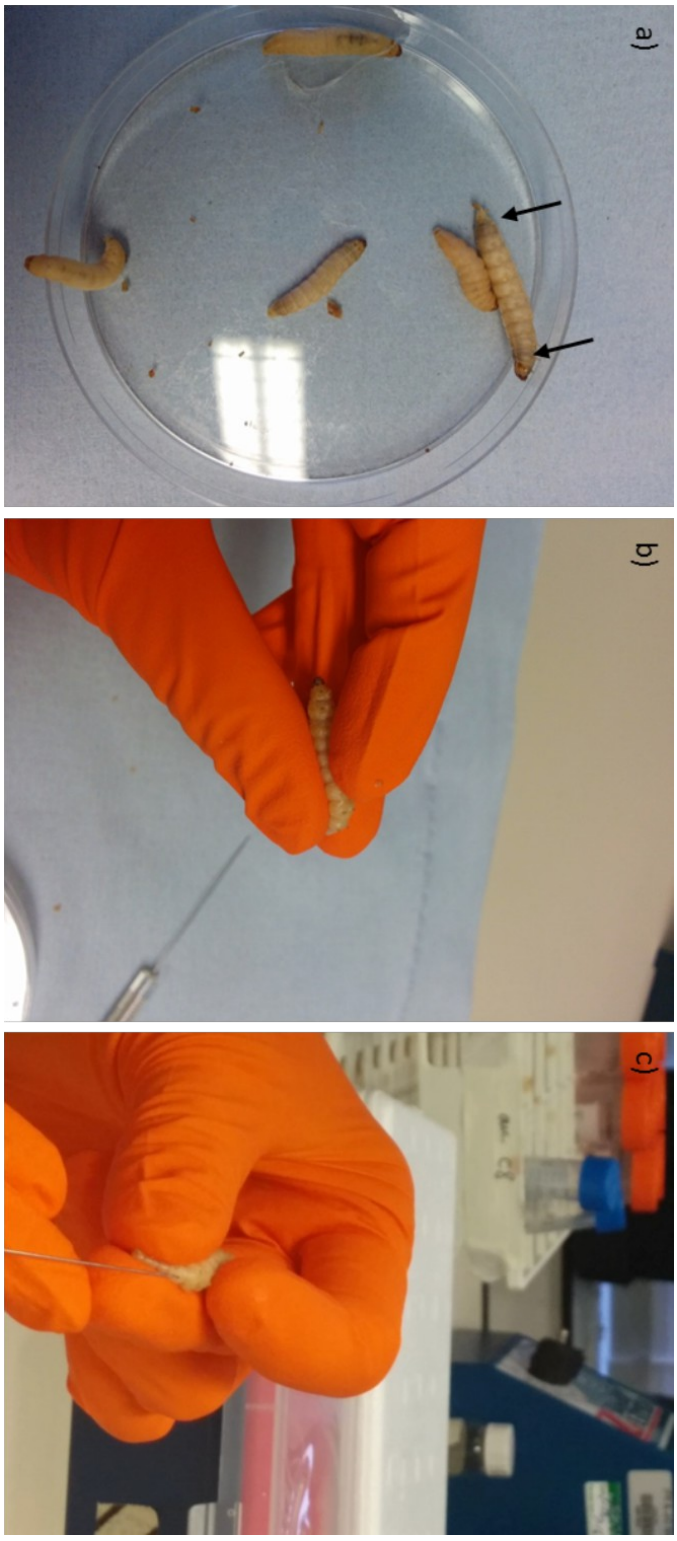


Figure 5.1: Intraperitoneal injection procedure in G. mellonella. a) Fifth instar larvae ready for injections. Larvae with signs of immune response, demonstrated by melanisation (highlighted by arrows), should be discarded. b) Preparation of the larva for an injection: the larva is held between thumb and middle finger and stabilized with index finger. c) The needle is inserted into the last left proleg of the larva.

5.3.1.2 Results of the testing

The results of antibiotic efficacy testing in *G. mellonella* larvae are summarized in Table 5-1. There are four possible outcomes of the antibiotic testing: 1) predicted dose of an antibiotic clears a bacterial infection, 2) a dose different from the predicted dose clears an infection, 3) antibiotic predicted to be ineffective is ineffective, 4) antibiotic predicted to be ineffective clears the bacterial infection. In most cases the antibiotics performed as predicted, either treating an infection within the dose predicted or being ineffective against a resistant strain.

It was found that ciprofloxacin, tetracycline and rifampicin performed as expected against *E. coli* infections. Both ciprofloxacin and tetracycline worked within the ranges prescribed for humans (10-15 mg/kg body weight/day and 25-50 mg/kg body weight/day respectively). Rifampicin was, as predicted, not effective as Enterobacteriaceae are intrinsically resistant to rifampicin. Ampicillin was expected to clear the infection at 50-200 mg/kg body weight, but it failed to treat the infection in Galleria.

Pseudomonas aerugionosa was resistant to all antibiotics used. *P. aeruginosa* is intrinsically resistant to ampicillin, tetracycline and rifampicin, and these antibiotics were tested as a negative control. The strain was expected to be sensitive to ciprofloxacin, which is active against Gram-negative pathogens, but when tested in the wax moth larvae *P. aeruginosa* was resistant to ciprofloxacin.

Mycobacterium smegmatis was also resistant to all antibiotics used. Mycobacteria are intrinsically resistant to a range of antibiotics and ampicillin, ciprofloxacin, and tetracycline were predicted to produce a resistant phenotype. Surprisingly rifampicin did not work, even though it is a standard treatment against mycobacterial infections.

Table 5.1: Predicted and determined antibiotic susceptibility profiles of bacteria in the antibiotic efficacy test.

	Therapeutic dose [mg/kg body weight]							
	ampicillin		ciprofloxacin		tetracycline		rifampicin	
	predicted	test result	predicted	test result	predicted	test result	predicted	test result
Escherichia coli	50 - 200	resistant	10 – 15	25	25 – 50	25	resistant	resistant
Microbacterium smegmatis	resistant	resistant	resistant	resistant	resistant	resistant	10 – 20	resistant
Pseudomonas aeruginosa	resistant	resistant	10 – 15	resistant	resistant	resistant	resistant	resistant
Staphylococcus aureus	50 - 200	200	10 – 15	200	resistant	50	10 – 20	25

All antibiotics used were effective against *Staphylococcus aureus*. Ampicillin worked at the high end of the spectrum normally prescribed for human use: 50-200 mg/kg body weight/day. Ciprofloxacin only cleared the infection at a concentration nearly 20 times higher than the dose recommended for human use. Tetracycline was predicted to be ineffective but it cleared the *S. aureus* infection at 50 mg/kg/body weight. The tetracycline susceptibility could have been caused by the loss of tetracycline resistance determinant from the *S. aureus* strain tested. Rifampicin, which is a standard treatment for methicillin-resistant *S. aureus* (MRSA), was effective at a low concentration.

These antibiotic efficacy studies were based on existing literature [232]. *G. mellonella* larvae have been previously used to study bacterial virulence and susceptibility to antibiotics and our study confirmed that the larvae are a suitable host for antibiotic efficacy studies. Additionally we determined that the antibiotic therapeutic dose established in *G. mellonella* matches the doses recommended for use in people.

5.3.2 Acute toxicity testing

5.3.2.1 Compounds tested

17 compounds were provided by Inspiralis Ltd to test for their toxicity in *G. mellonella* larvae. All compounds were made up in 50% DMSO, except where indicated in Table 5-2. The compounds were tested blind – they were supplied in numbered tubes and only after the test procedure was completed were the numbers linked to compound names. The aim of this approach was to avoid bias, i.e. assigning lower toxic doses to known toxic compounds and higher ones to safer compounds.

Insects (5-10 larvae) were injected with 10 µl compound into the hindmost proleg. Intraperitoneal injection was used in the procedures to strictly control the amount of toxic compound or bacterial pathogen that the larvae were exposed to. Alternative approaches, not applied in this study, use feeding procedures [240] or contact exposure [241]. The quantification of exposure to a compound is less precise for such procedures, but it is sometimes a more appropriate method when a route of exposure is known. For example pesticide toxicity in insects testing normally employs feeding studies [229] as it is the normal route of exposure that the study tries to mimic.

Insects were injected with low doses (5 mg/kg body weight) of the compounds first and the mortality was recorded daily for five days. When no mortality was observed new groups of larvae were injected with compounds at 50 mg/kg body weight and the mortality was recorded daily for five days again. When mortality was observed in 60% or more of the larvae, the compound was re-tested at that same concentration to confirm the toxicity. When mortality below 60% was observed, the compounds were tested subsequently at 125, 300 and 2000 mg/kg body weight. Each compound was assigned an LD_{50} value and a GHS class (Table 5-2) and the values were compared to toxicity data available in the MSDS pages.

No compounds were tested above 2000 mg/kg body weight in line with OECD guidelines [242]. Such high compound concentrations introduce solubility issues and are discouraged in the guidelines as unnecessary and unethical. Generally, compounds with no indication of toxicity at 2000 mg/kg body weight are considered non-toxic.

Table 5.2: The identity and toxicity of compounds in the trial. LD50 values determined in the test were compared to values available in material safety data sheets provided with the compounds. Blank space indicates the data are not available. GHS class refers to international toxicity classification, where the most toxic compounds are designated 1 and the least toxic compounds are designated 5.

	GHS class		LD_{50} [mg/kg body weight]					
	G. mellonella	*G. mellonella*	rat		mouse			
	intraperitoneal	intraperitoneal	oral	intraperitoneal	oral	intraperitoneal		
ciprofloxacin	≥4	≥300	>2,000		>2,000			
etoposide	3	100	1,784	58	215			
novobiocin	3	100	3,500		962			
amsacrine	2	40	100		243			
norfloxacine	≥4	≥300	4,000		4,000	1,064		
carbenicillin	≥4	≥300	10,000	10,000	12,000	7,600		
chloramphenicol	≥4	≥300	2,500		2,300			
kanamycin	≥4	≥300	4,000	3,200	17,500	1,353		
sodium chloride	3	100	3,000		4,000			
tetracycline	3	100	6,443	318	2,759	368		
glutamic acid	3	100	30,000					
doxurubicin	2	5.5		16	698	1.2		
glucose	3	125	25,800					
50% dimethyl sulfoxide	3	100	14.500		7,920			
chloroqune	3	125	623		500			
streptomycin	≥4	≥300	430		430			
adenosine triphosphate	3	125	>2,000		>2,000			

5.3.2.2 Comparison of G. mellonella results and available toxicity data

For most compounds the toxicity determined in this experiment correlates well with toxicity reported in the MSDS pages available for each of the compounds. The compounds with lowest LD_{50}s in mammals were the most toxic for galleria larvae (Table 5-2). The most toxic compounds had the lowest LD_{50} values: the value for doxorubicin fell within the range established by experiments in mammals (5.5 mg/kg in galleria versus 1.2 and 16 mg/kg in mice and rats respectively) and for amsacrine the value was lower (40 mg/kg via intraperitoneal injection versus 100 mg/kg in rats and 243 mg/kg in mice via oral exposure). Similar close correlation between LD_{50} values established in *G. mellonella* and LD_{50} values from MSDS pages was also observed for etoposide, ciprofloxacin, norfloxacin, carbenicillin, chloramphenicol, kanamycin and streptomycin. Kanamycin did not cause larval mortality, however, when the larvae pupated, the cocoons and pupas were malformed and fragile. This effect was not observed with any other compound. The values obtained for novobiocin and chloroquine were four to ten times smaller than the mammalian LD_{50}, but the only data available are from oral exposure, making the values difficult to compare. A number of values obtained correlate badly with LD_{50} values available from the MSDS pages. Sodium chloride, tetracycline, glutamic acid, glucose, 50% DMSO and ATP were toxic to *G. mellonella* at 100-125 mg/kg body weight, even though the mammalian LD_{50}s are over 2000 mg/kg (apart from intraperitoneal tetracycline injections, which are toxic at 318 mg/kg body weight in rats). It was hypothesised that the mortality in larvae injected with these compounds was due to DMSO toxicity.

Table 5.3: LD50 values determined in the test were compared to values available in the literature. Blank space indicates the data are not available in the literature.

	GHS class	LD_{50} [mg/kg body weight]					
	G. mellonella	*G. mellonella*	rat		mouse		
	intraperitoneal	intraperitoneal	oral	intraperitoneal	oral	intraperitoneal
ciprofloxacin	>5	>2,000	>2,000		>2,000	
sodium chloride	>5	>2,000	3,000		4,000	
tetracycline	>5	>2,000	6,443	318	2,759	368
glucose	>5	>2,000	25,800			
streptomycin	3	300	430		430	

5.3.2.3 DMSO toxicity issues

To test whether some of the mortality observed should be attributed to DMSO toxicity, a second screen was conducted. Five compounds were re-tested above their determined *G. mellonella* toxic dose using PBS buffer, rather than DMSO, as an

injection medium: ciprofloxacin, sodium chloride, tetracycline, glucose and streptomycin. Each compound was tested at 300 and 2000 mg/kg body weight as it is already known from the previous experiment that these compounds are not toxic below these concentrations. Groups of five larvae were injected with each dose. There was no mortality in the groups injected with ciprofloxacin, sodium chloride, tetracycline or glucose (Table 5-3), but all larvae injected with streptomycin at 2000 mg/kg body weight died. The values obtained correlate better with the mammalian LD_{50} values than the initial screen, confirming some of the mortality observed in the test was associated with DMSO toxicity.

5.3.2.4 Negative controls used in the screening

At each stage of the testing three control groups, containing five to ten larvae each, were used: the first group was untreated, the second had the cuticle pierced with a sterilized Hamilton needle, and the third group was injected with 10 µl injection media (50% DMSO or PBS buffer). The untreated larvae were used to monitor the general health of the *G. mellonella* colony and any background mortality, for example due to viral infections. Mortality in the group of larvae with a pierced cuticle would indicate that the injection procedure was not performed correctly and was harmful to the larvae. The mortality in the group injected with the pure injection media shows either toxicity of the medium or incorrect volume injected. Apart from the DMSO toxicity discussed above, there was no mortality in these control groups, suggesting the *G. mellonella* colony was healthy and the injection procedure did not inflict excessive damage on the insects.

5.3.3 Native gut microbiota replacement

5.3.3.1 Hypothesis

We hypothesised that native gut microbiota of *G. mellonella* could be replaced with other bacteria, for example human gut bacteria, enabling a number of studies of the interactions between members of human microbiota and the impact of antibiotics on the gut microbiome. A feeding experiment was conducted to test this hypothesis. *G. mellonella* larvae were feeding on artificial food mixed with human faecal slurry from a

healthy newborn baby, and larvae in the control group were feeding on just artificial food. The faecal slurry was rich in Bifidobacteria and we hypothesised Bifidobacteria would partially or fully colonize *G. mellonella* gastrointestinal tract. Insects were collected before the start of the feeding procedure to determine the baseline composition of the gut bacteria, and on day three, four and seven of the experiment. Three dissected larval guts were pooled for each sample to obtain enough bacterial cells and DNA for downstream analysis. Unlike previous experiments where no bacteria were found in *G. mellonella* guts (see Chapter 3), bacteria were present in all samples.

5.3.3.2 Bacterial counts in the larval guts

Before the beginning the treatment, the amount of bacteria cultured from the guts varied between less than 10 and nearly 50,000 cfu/mg gut contents (Figure 5-2). Throughout the experiment there was no difference in the amount of bacteria isolated from treated and untreated groups. The amount of colony forming units varied between samples, reaching as little as 1 cfu/mg gut contents in control group on day four of the treatment and as much as 84,000 cfu/mg in the treated group on day seven.

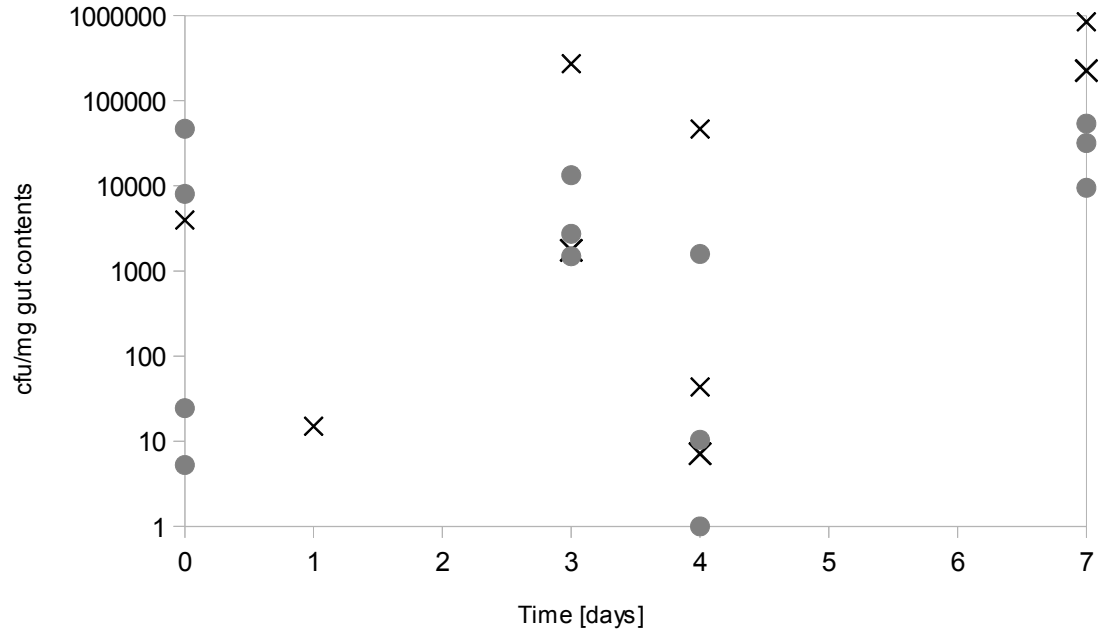


Figure 5.2: Bacterial counts in the guts of G. mellonella feeding on faecal slurry. Samples from the larvae feeding on the faecal slurry are marked with crosses and the samples from control groups are marked with circels. 12 larvae (pooled into four groups of three) were dissected prior to the feeding experiments. The remaining larvae were divided into two groups and fed on autoclaved artificial media with or without faecal slurry. On day three, four and seven each group was sampled by dissecting the guts of nine larvae (pooled into three groups of three). Two isolates were identified in all samples: Enterococcus gallinarum and Enterococcus xiangfangensis. Staphylococcus saprophyticus was identified as one of the strains present in control group on day four.

5.3.3.3 Composition of the gut communities

The bacteria cultured from the larval guts were identified by 16S sequencing. In both groups the two isolates were present: *Enterococcus gallinarum* and *Enterococcus xiangfangensis*. 58.3% of the isolates from the treated group were *E. gallinarum* and 41.7% were *E. xiangfangensis*. In the control group, feeding on just the artificial food without the faecal slurry, 50.0% isolates were *E. gallinarum*, 45.0% were *E. xiangfangensis* and the remaining 5% were represented by *Staphylococcus saprophyticus*. All three strains belong to the order Firmicutes and can be isolated from animal and human microbiota. Apart from the presence of *S. saprophyticus*, there was no difference between the slurry fed group and control group. The microbiota from insects treated with faecal slurry was not enriched in Bifidobacteria from the faecal

slurry.

The experiment was not continued in this project. Instead, it was used to design a more extensive study developing *G. mellonella* larvae into a model of the baby gut. Among the questions investigated are: the difference between species isolated from the gut and faecal samples, whether it is possible to replace native *G. mellonella* microbiome and with which strains.

5.4 Discussion

5.4.1 Acute toxicity can be assessed in greater wax moth larvae

Acute toxicity of 17 compounds has been assessed in *G. mellonella* larvae. There was good correlation between the LD_{50} values obtained in the test and reported LD_{50}s from safety documentation supplied with the compounds. Such correlation suggests *G. mellonella* larvae could be used in *de novo* acute toxicity testing providing statistically robust preliminary data before testing in mammalian models. *G. mellonella* larvae can be used as a cheap and quick way of testing compounds, however they are not free of limitations.

The first limitation of the larvae is the limitation that applies to all other toxicity testing systems: the toxicity cannot be tested in humans and it is not known how experimental LD_{50} values correlate to human values or even if the mechanism of toxicity is the same. There are numerous mechanisms of systemic toxicity and they are poorly understood. In some cases the cause of toxicity is alike in different systems. For example, doxurubicin is a DNA intercalator and poisons different organisms at low doses. However, differences in insect and mammal physiology can lead to opposing responses to compounds. Insecticidal Bt toxin is only toxic to certain groups of insects based on the alkaline conditions in their guts, while remaining insoluble and harmless in acidic mammalian digestive tract [25].

The second limitation is specific to the use of DMSO as an injection medium in the procedure. Due to DMSO toxicity the initial test lost its sensitivity for accurately estimating LD_{50} values for mildly- or non-toxic compounds (GHS class 3 and above). 50% DMSO was lethal to wax moths above 100 mg/kg body weight when used as an

injection medium. The test could not correctly assign LD_{50} to mildly toxic compounds (for example antibiotics ciprofloxacin and chloramphenicol) and non-toxic compounds (glutamic acid, glucose, sodium chloride) because of the side effects of the DMSO injection. Decreasing the amount of DMSO per injection, maintains the solubility of the compounds tested without compromising the test procedure. Alternatively the solvent effects can be subtracted from the compound effects using statistical methods. Restricting background mortality (mortality in untreated control groups) not only lowers the experimental noise but also aligns better with the guidelines on the use of laboratory animals.

Overall our experiments have shown that Galleria larvae can be reliably used in acute toxicity testing, providing more data cheaper and quicker than traditional testing systems. Testing in *G. mellonella* is unlikely to fully replace toxicity testing in mammals, but it is a convenient step between *in vitro* tests and testing in mammals, adding more complexity to the former and statistical robustness to the latter.

5.4.2 Recommended human doses of antibiotics cure bacterial infections in *G. mellonella* larvae

Our experiment confirmed antibiotic efficacy testing can be done in *G. mellonella* and the therapeutic doses recommended for human use can be translated to doses in the wax moth larvae. In most cases the exact dose recommended for clinical treatment of a systemic infection can be calculated for larval body weight and can clear an infection. Such close correlation is possible because often the mechanisms of microbial virulence are not host-specific. Previous studies have shown that fungal pathogen *Candida albicans* uses the same repertoire of effectors, involved in fungal virulence and yeast-to-hypha transition, against insects and mammals [243]. Similarly, the bacterial pathogen *Pseudomonas aeruginosa* employs a similar set of virulence genes to overcome the immune system of wax moth larvae and mice [244] and the larvae can be used to identify virulence factors required for an infection in mammals.

Antibiotic efficacy testing in *G. mellonella* does not have the same limitations as toxicity testing. Unlike toxicity mechanisms, the mechanistic basis of antibiotic action is the same in insects and mammals. Even though insects are only distantly related to

mammals and lack some crucial immune responses, *G. mellonella* larvae are suitable for antibiotic efficacy testing, generating statistically robust data and potentially replacing other organisms used.

5.4.3 Replacement of native gut bacteria needs more work

We have not succeeded in replacing the native *G. mellonella* microbiome. There was no difference in the amount of bacteria in the guts of larvae feeding on artificial food and artificial food with faecal slurry. The microbiome of the slurry-treated larvae was not enriched in bacteria from the baby gut. However, the experiment provided enough data to expand the goals and develop it into a separate project. The benefit of establishing a different microbiota in *G. mellonella* larvae would be the use of these insects in gut microbiota studies. As mentioned before there are strict restrictions on the use of vertebrates in research and cheap non-vertebrate models are desired for preliminary *in vivo* studies.

The presence in *Enterococcus* isolates in the guts of *G. mellonella* larvae has been described before [166], but it is non-essential and the larvae can be reared axenically [170]. We hypothesised that the guts could be colonised with an alternative microbiota via feeding on material infused with the faecal slurry. The faecal slurry feeding procedure utilized in our experiment has not successfully altered the gut microbiota of *G. mellonella* larvae. It has been previously described that *G. mellonella* gut microbiota can be dominated by *Enterococcus faecalis* (syn. *Streptococcus faecalis*) [166] and the isolates have been shown to be supressing the growth of ingested bacteria through lytic activity [169]. The author identified *E. faecalis* as the only isolate present in the *G. mellonella* gut. Gut-isolated *E. faecalis* produced bacteriocin and exhibited lytic activity, unlike other *E. faecalis* isolates, and inhibited growth of other even closely related strains.

More recently it has been demonstrated that *G. mellonella* and their microbiome are both responsible for the colonisation resistance [150]. The authors showed that both the insect-produced lysozyme and the broad-spectrum bacteriocin mundticin from the *Enterococcus mundtii* in *G. mellonella* gut are required for colonisation resistance. Either factor can be removed: the lysozyme by RNAi and mundticin by mutating out

munA gene, and significantly changes the composition of the gut microbiome. Without colonisation resistance, wax moth gut communities are dominated by *Staphylococcus* and *Serratia* species and the survival of larvae is lower than when *E. mundtii* dominates the microbiome.

The strong colonisation resistance of *G. mellonella* gut community prevents non-native bacteria from inhabiting the larval gut. The replacement of native bacteria with the isolates from faecal slurry was not possible due to this phenomenon. However it is possible to produce germ-free *Galleria mellonella* larvae either by sterilizing the eggs and feeding the larvae sterile diet [170] or treating the larvae with antibiotics to clear the gastrointestinal tract [150].

5.4.4 Other non-mammalian animal models

Galleria mellonella is not the only organism that has been developed as an alternative to the traditionally used laboratory animals: mice and rats. Other examples of model are: nematode *Caenorhabditis elegans*, fruit fly *Drosophila melanogaster*, zebrafish *Danio rerio* and planktonic crustacean *Daphnia magna*. *C. elegans* can be used for whole-host phenotypic screens for antibiotic discovery (*in vivo* antibiotic screening) [67, 245]. In such tests the screening of entire chemical libraries is conducted in animals. The biggest advantage of whole-host screening is that bacterial targets behave differently *in vivo* and *in vitro* and targets that are lethal *in vitro* are not always lethal *in vitro*. Similarly novel drug targets can be identified when infection is studied in the context of animal host. Drosophila has been used for studies of genetic and biochemical basis of insect immunity (reviewed by Hoffmann, 2003 [31]) as well as bacterial virulence (reviewed by Fauvarque, 2014 [246]). The tools used for manipulating drosophila genetics allow in-depth investigation of host-pathogen interactions. Both *D. rerio* and *D. magna* have been used in systemic toxicity testing [247]. These organisms can be used both as a substitute for mammals in the determination of LD_{50}s for toxic chemicals and as bioindicators in environmental hazard assessments.

The use of alternative organisms in tests traditionally performed in mammals is sometimes encouraged without scrutinizing the methodology used. That is the case

especially when comparisons between model systems are performed and one of the organisms is recommended as superior to others. For example *G. mellonella* was recommended as a better model than plants and nematodes for *Pseudomonas aeruginosa* infection in experiments mimicking burned mouse model [244]. Burned mouse model is used to study burn wound sepsis caused by *P. aeruginosa*, when the initial non-lethal would causes suppression of immunity and the bacteria can multiply freely. The virulence of *P. aeruginosa* and antibiotic efficacy against the infection is similar for plants, nematodes, insects and mice. However, the murine immune system and overall physiology is more closely related to insect than to a plant or a nematode, making the *G. mellonella* second most suitable after mice model for studying systemic *P. aeruginosa* infection. Sometimes using an alternative testing organism can complement experiments in mammals, but they can hardly replace established testing regimens.

5.4.5 Issues with currently used murine models

OECD guidelines aim to reduce the number of animals used for testing, sacrificing statistical robustness of the tests. A minimum of three to five animals are used per dosing regimen in acute toxicity tests 420, 423, and 425 [221-223]. However based on a statistical modelling approach at least five or six animals are required to obtain statistically significant results [248]. The analysis shows the revised fixed-dose procedure (acute toxicity test 420 [221]) is as stringent as the previous procedure, but reduces the number of animals dying from the procedure. Large toxicological studies in rodents can be prohibitively expensive; obtaining acute toxicity oral route data for rats cost £1,474 per compound [249]. It should be noted that such testing would be conducted on a small sample size of related animals and might miss important differences between individuals.

Inadequate understanding of how pathways are perturbed, leading to acute systemic toxicity, restricts the development of alternative *in vitro* testing methods. A number of *in vitro* tests for assessing cytotoxicity and genotoxicity, as well as more specific cardiotoxicity, hepatotoxicity and nephrotoxicity tests are available, but there are limited data how these events lead to systemic toxicity. Regulatory toxicology depends

increasingly on predictive approaches and identifying molecular initiating events (specific receptors, enzymes, or transport proteins in key pathways) that are often conserved between species [230]. Conserved pathways in non-mammalian models, which are used increasingly often, allow understanding of modes of action and chemical risks to humans and the environment.

5.4.6 *In vivo* tests do not guarantee success with clinical trials

Regardless of the amount of data collected, establishing safety or effectiveness of a compound in an animal model does not guarantee no adverse effects in humans. Unpredictable responses in human trials are sometimes unavoidable, even with extensive pre-clinical studies in model organisms. For example monoclonal antibody TGN1412 was approved for clinical trials by Medicines & Healthcare Products Regulatory Agency based on *in vitro* data and *in vivo* studies in two monkey species, that confirmed that the antibody is specific to human CD28 and established safe doses [250]. However, in phase 1 clinical trial the volunteers, injected with $1/500^{th}$ of the dose determined safe in monkeys, developed multiple organ failure due to cytokine release syndrome. Further investigations revealed single amino acid difference at the CD28 epitope binding site between human and monkey.

In the light of such incidents the value of extensive *in vitro* and *in vivo* testing cannot be underestimated. Further development of alternative testing models should allow the investigation of mechanistic basis of the phenomena investigated, before testing in mammalian models to establish safe doses.

5.4.7 Alternative testing methods lack legal regulation and scientific validation

All procedures discussed in this chapter have been designed not to cause unnecessary suffering or mortality. Current EU legislation on the use of laboratory animals does not apply to insects, and only protects live non-human vertebrate animals and live cephalopods (octopus, squid) [219], but should be used as a guideline for good laboratory practice. The directive discourages the use of death as end-point to avoid severe suffering that precedes death and instead advocates the use of a different end-

point, which allows killing the animal in a humane manner, before it experiences severe pain. Both the toxicity testing and antibiotic efficacy testing include death as end-point, but excessive suffering can be avoided when severely affected larvae are euthanized. For example larvae with excessive darkening of the cuticle should be euthanized as the immune response is systemic and normally leads to death.

The lack of legal regulation of the use of insects as laboratory animals highlights that they are underdeveloped as a resource. Many procedures performed in insects lack validation. An exhaustive validation study would require data about reproducibility, the limit of detection, relevance of results and the mechanistic basis. Preferentially a validated procedure would utilize a genetically homogenous stock.

For example, procedures such as antibiotic efficacy testing and toxicity testing in greater wax moths keeps gaining popularity, but they have not yet been fully validated. The insects are well-characterized physiologically and transcriptomic data from healthy and immune-challenged larvae is available [251], but it lacks experimental validation and the genome of the wax moths has not been sequenced. Incomplete validation and the lack of legislation should not prevent further development of *Galleria mellonella* as a laboratory animal.

5.4.8 Summary

In summary, our experiments confirmed that antibiotic efficacy can be tested in vivo in *Galleria mellonella* larvae. We established that the doses recommended for use in people are effective in systemic infections in the larvae and that the acute toxicity of compounds in wax moth larvae correlates to the toxicity in mice and rats. Additionally we attempted to establish human-derived microbiota in the larvae. Even though there was no difference in the microbiome composition between the treated and control larvae, the experiment supplied enough data to expand it beyond the scope of this chapter. *G. mellonella* is an organism that can be easily adopted in various tests. It cannot fully replace mammalian models, but it is much cheaper and can provide the statistical robustness current models lack.

6 General discussion

6.1 Antibiotic resistance is prevalent in the insect gut

Our observations have confirmed that antibiotic resistance can be readily found in bacteria resident in the insect gut. For all insect species investigated at least one bacterial strain isolated from the insect gut was more antibiotic-resistant than a matching type strain. Additionally the survey of tetracycline resistance genes in the guts of *Galleria mellonella* larvae feeding on artificial food with and without antibiotics revealed a number of tetracycline efflux pumps even in the absence of clinically-relevant levels of antibiotics.

We hypothesised that antibiotic resistance in the insect gut arises through direct exposure to a compound with antibiotic activity, as can be observed *in vitro*. However we discovered that there is no simple correlation between the presence of antibiotic activity in the insect food and antibiotic resistance in the insect gut microbiome. For *Plutella xylostella* feeding on artificial food with oxytetracycline, we found no gut-isolated bacterial strain that was more antibiotic resistant than the type strain, indicating that fully antibiotic-susceptible bacteria can be found in the guts of insects feeding on antibiotics. We also observed that antibiotic-resistant bacteria can be found in the insect gut in the absence of antibiotics, for example in *Enterococcus* species with tet-resistance genes, isolated from the guts of *G. mellonella* larvae feeding on artificial food without any oxytetracycline.

We also investigated the guts of *P. xylostella* larvae feeding on artificial food containing either ciprofloxacin or oxytetracycline for the presence of multi-antibiotic resistance. The bacteria isolated from guts exposed to ciprofloxacin were more resistant than the type strains to ampicillin, chloramphenicol and tetracycline. Such increased antibiotic-resistance levels were not observed in the bacteria isolated from the guts of larvae feeding on oxytetracycline. These results demonstrate how multi-antibiotic resistance is not always maintained in bacteria even during antibiotic exposure. Multi-antibiotic resistance is a trait with high fitness cost and maintaining it is often a disadvantage in highly competitive environments [139].

We have demonstrated that the impact of antibiotics on the insect gut microbiota is diverse. In many cases bacteria exposed to a compound with antibiotic activity acquired resistance to this compound. In some cases the bacteria did not acquire resistance to the antibiotic or plant extract and instead were less resistant than the type strain they were compared to. Additionally the composition of the gut microbiota can change in response to antibiotic exposure.

Our initial hypothesis that, in response to antibiotics in the insect diet, the gut-isolated strains would either acquire antibiotic resistance or remain antibiotic-susceptible at a similar level to the type strains holds true for *in vitro* experiments. However even a simple gut community is not a simple mixture of a few independently-living bacterial strains. Gut microbiomes inhabit different ecological niches and are normally composed of a number of bacterial isolates, which interact within and between their populations, with other strains and with the host organism. The effect of an antibiotic on such complex system is not well understood, but is more complex than originally hypothesised.

6.2 Food-plants have a diverse impact on the insect gut microbiota

Not only antibiotics, but also the insect food-plants, have a diverse effect on gut microbiota. We only partially investigated the differences between gut microbiota of insects feeding on artificial food and the food-plant they normally feed on. We isolated different bacterial strains from the guts of *Plutella xylostella* feeding on artificial food and on cabbage, but have not investigated these differences further.

We hypothesised that bacterial strains from the guts of insects feeding on plants would be more resistant to the plant extracts when compared to the type strains. However that was not always the case. In many instances we discovered that the type strains were more resistant to plant extracts, compared to the gut-isolated strains. This was unexpected as the strains from the insect gut were exposed to the components of the plant extracts and the type strains were not. One possible explanation is that the toxic compounds from the plant extracts are either rapidly metabolised or otherwise detoxified by the insect or the bacteria and the majority of the gut community is not exposed these toxins. If that was true the differences in antibiotic and plant extract

resistance would be due to natural variation between the isolates and not the exposure to phytotoxins in the insect gut.

From our experiments it is obvious that plant foods have an impact on gut microbiota. The effect of plant foods is often considered beneficial in humans, but the exact mechanisms are not known. For example it is not known how a variety of “health foods” influences the gut microbiota and the host. Many of the plant foods are rich in compounds with antioxidant or medicinal properties, such as the compounds we investigated for antibacterial activity.

6.3 The antibiotic resistance in the insect gut can aid identification of phytochemicals with antibiotic properties

Nearly all of the plant extracts tested had antibacterial activity. Out of six plant extracts tested only the cabbage extract had no antibacterial activity. It might be a general property of many plants to produce compounds with antibacterial activity. However it is difficult to assess if these compounds are utilised by the plants as defence compounds against bacteria or if they have a different role in host metabolism and the antibiotic activity is a side-effect of their native activity.

Through our approach we identified fractions with antibacterial activity in eucalyptus, ragwort, potato and Madagascar periwinkle extracts. We also identified a number of metabolites in the periwinkle leaf and root extracts and confirmed their antibacterial properties. This demonstrates that compounds with antibacterial properties can be identified through fractionating plant extracts based on their activity against bacteria from the guts of insects feeding on these plants.

6.4 Other approaches to antibiotic discovery from plants

It is difficult to judge if our approach of assay-guided fractionation with the use of insect gut bacteria is better than screening plants or their metabolites at random. There have been some attempts of high-throughput screening of plants for antibiotic discovery [99], but they never yielded any compounds that reached clinical trial stage. The outcomes of such screens have not been published, making it impossible to

compare them to other drug discovery approaches.

An approach that has resulted in a discovery of a novel compound is bioprospecting, as seen with the discovery of an antimalarial artemisinin [118]. Artemisinin was isolated from *Artemisia annua*, known in traditional Chinese medicine as qing hao. The documented use of this herb to treat malarial fevers dates back to 4th century. The compound was first isolated by Chinese scientists attempting to identify the active component of the traditional infusion. Its structure was later determined by American scientists. Artemisinin is now a part of standard antimalarial treatment, showing how approaches based on traditional medicine have been the most successful in antimicrobial discovery.

6.5 Drawbacks and pitfalls

An important factor in our struggle to discover plant extract fractions with antibacterial activity was the disc diffusion assay lacking sensitivity. We needed large amounts of a fraction to infuse into the discs and later analyse on MS for the most abundant metabolites. Such large amounts are difficult to obtain using HPLC, which as a technique is most efficient at analytical quantities.

The disc diffusion assay is a cell-killing assay. A positive result indicates a compound gets into bacterial cells and kills them. There are benefits to using a cell-killing assay, for example the efficacy of the tested compound is validated. However such assays do not identify the target responsible for the cell death and it is not possible to determine if there is a specific mechanism of action or just general toxicity. An approach that could have aided the identification of the active components of the plant extracts is prioritizing extracts with a narrow antibacterial spectrum over extracts with a broad-spectrum activity. Such an approach can only identify compounds active against a small group of organisms, but in this way extracts with general cytotoxic properties are avoided.

Finding specific phytochemicals responsible for the antibacterial activity of the plant extract was probably the most difficult task. Even with artemisinin the original hot-water extractions did not yield an active compound and only extraction with ethyl ether led to a fraction with antimalarial activity [118]. The structure of the compound

was not determined until seven years later. In our case the main issue was obtaining enough pure fractions for further testing and analysis.

Our plan to use the insect gut bacteria in assay-guided fractionation added extra considerations to the already challenging plant extract fractionation. We hoped the insect gut bacteria would aid the isolation of active compounds, but we have underestimated the complexity of the insect gut community and its interaction with the host and the food plant.

6.6 Insects are useful models for more complex organisms

Even though insects are complex animals, they are smaller and simpler than for example mammals. Insects do not lack the intricacy of interactions common to higher organisms, but they are often less numerous and complex. In many cases these interactions are evolutionarily more ancient. The presence and relative simplicity of these interactions makes insects an attractive model to study. It is easier to dissect the interactions in a simpler model, bridging the gap between *in vitro* studies and studies *in vivo* in mammals.

The composition of the insect gut communities is simpler than mammals, but their immune systems are functionally similar, meaning many interactions are conserved. We were surprised to learn about the similarities between insects and mammals, even though the evolutionary distance between them. One of the most striking outcomes of this project is the usefulness of *Galleria mellonella* in a wide range of applications. The *G. mellonella* larvae have been used in antibiotic efficacy testing, compound toxicity studies and to test if dietary antibiotics induce antibiotic resistance in the gut microbiota. Additionally the larvae were assessed as a model for baby gut. The results obtained in *Galleria* were much closer than we expected to the values reported in rodents.

6.7 Current ongoing work

We are currently further investigating the possibility of replacing native *G. mellonella* gut microbiota with human-derived bacteria. The overall aim of the project is to develop the wax moth larvae as a model for the human gut. The rationale is to develop

a cheaper and easier to use alternative to mice and rats used to study human gut microbiota.

The scope of the project has been expanded from the original experiment described in Chapter 5. The aims of the project include the establishment of human gut microbiota in *G. mellonella* larvae by clearing the gastro-intestinal tract with antibiotics and monitoring the stability of the new microbiome over the insects' life-time and generations. Furthermore we would like to investigate the impact of antibiotics and probiotics on the human-derived microbiota and the transfer of antibiotic resistance genes between the members of the community.

6.8 Recommendations for future work

A number of different areas for further investigation stem from the work presented in the previous chapters. Firstly, we discovered two plant extracts that should be followed up. Apart from some known phytochemicals, such as vindoline, we identified a number of unknown metabolites with high antibacterial activity in the periwinkle and ragwort extracts. Ragwort is particularly promising, because its antibacterial activity was unusually high and ragwort plants are weeds, making obtaining large amounts of plant material easy. After confirming the activity of the plant extract fractions, large amounts of plant extracts can be prepared and purified. The purification of a compound with a known mass is easier than assay guided purification, because we can use MS-coupled HPLC to pool the fraction containing the compound. We can later purify the compound using for example preparative thin layer chromatography. The structure of the pure compounds can be identified by nuclear magnetic resonance.

Secondly, the compounds with an antibacterial activity from Madagascar periwinkle: vindoline, serpentine, loganic acid and catharanthine, can be used as leads in antibiotic development. The molecular target of these compounds can be identified by screening them against libraries of bacteria and identifying mutations conferring resistance. Additionally, because the Madagascar periwinkle compounds are investigated in cancer research, homolog compounds exist and can be used in medicinal chemistry approach. Modifications of the compounds can modulate their properties, optimising them for use as antibacterials.

Another interesting prospect to investigate further is the development of *Galleria mellonella* into a laboratory model. Insects are a convenient, but underdeveloped, resource. The genome of the wax moth larvae has not been sequenced yet and genetically homogenous stocks are not available for use in laboratory research. The benefit of obtaining a full genome sequence is not only the insight into the insect biology, but also the potential to adapt the genetic tools developed in fruit flies. Genetically modified wax moth larvae could further aid or replace research in mice.

There are some interesting subjects that we mentioned, important in the wider context. For example, we only investigated medicinal and toxic plants, but it would be interesting to compare their antibacterial properties to the properties of other plants. It is possible that plants that are agricultural waste products could be used as a source of antibiotics. Additionally it would be interesting to assess how traditional medicine can aid the discovery of plant-derived antibiotics. A robust high-throughput assay of antibiotic properties of plant extracts could aid an assessment of which plants are worth investigating and what governs which plants are and which ones are not.

Finally, we described antibiotic resistance in the insect gut and how antibiotics have a diverse impact on the gut community. It would be interesting to know the exact mechanisms responsible for the variety of responses we see.

6.9 Concluding remarks

When I have started working with *Plutella xylostella* feeding on artificial food containing antibiotics I planned for the experiment to quickly confirm that bacteria in the insect gut acquire resistance after exposure to antibiotics, just as can be observed *in vitro*. However both the *Plutella* experiments and the *Galleria mellonella* experiments have been a “can of worms”! These experiments have shown me the complexity of insects and their microbiomes, as well as the diverse impacts of antibiotics on a bacterial community and the host organism. Have I had more time to investigate this part of the project further, I would like to link the mechanisms of antibiotic action in bacteria, their interaction with the host and macro-responses that can be observed in humans in response to antibiotic treatment.

My biggest hope for the next part of the project, the investigation into the plant/insect

pairs, was to discover a novel antibiotic. I have identified a number of known Madagascar periwinkle metabolites with a mild antibacterial activity, but I was disappointed not to identify a novel chemical scaffold. This probably illustrates an important point in antibiotic discovery: with the antibiotic resistance crisis we should focus on developing any antibiotic rather than a novel or scientifically-interesting drug. There are interesting prospects for the periwinkle metabolites, for example discovering their target or mechanism of action and that should be the main focus of further investigations. Madagascar periwinkle metabolites are not the only compounds I would like to explore more. The ragwort extract has compounds with antibacterial activity, but they are not the pyrrolizidine alkaloids responsible for ragwort's toxicity. If I had more time, I would like to further investigate their antibiotic properties, toxicity and chemical identity.

Finally, the experiments involving the development of *Galleria mellonella* into a laboratory model for antibiotic efficacy and toxicity testing have been a success. The close correlation of results obtained in *G. mellonella* and values published for rats and mice exceeded my expectations.

Overall the project went in a very different direction to what I expected four years ago. I hoped I would not have to suffer the company of insects, but in hindsight learning about insects was one of the most enjoyable parts of the project. I thought plant extracts would be less of a black magic after four years. They still are, but now I have a better understanding why they are so difficult to work with. My only regret is that now that I know more about microbiology, I am asked for my opinion on family members' theories of why bacteria have not killed us all and taken over the world!

7 References

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